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**Inflorescence Development in
Allium ampeloprasum var. *babingtonii*
(Babington's Leek)**

By Sam Harding

**A thesis submitted to the University of Wales in accordance with the
requirements of the Doctor of Philosophy in the Faculty of Science**

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Inflorescence of *Allium ampeloprasum* var. *babingtonii* (Babington's Leek) taken at Worcester, Summer 2004

"Mine eyes smell onions: I shall weep anon."
All's Well that Ends Well,
William Shakespeare.

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Nomenclature for this report will follow Mathew (1996) with the non-bulbiliferous variant of *Allium ampeloprasum* referred to as *A. ampeloprasum* var. *ampeloprasum* and abbreviating *Allium ampeloprasum* var. *babingtonii* to var. *babingtonii*.

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In memoriam

†Veronica and Michael Harding†

With love to MST, AT, MT, DSH, TH and PH

Abstract

Within the horticultural industry, clonal propagation is desirable allowing for the maintenance of true lines, with more uniform cropping and flowering characteristics. Clonal propagation through tissue culture can be expensive, requiring equipment and facilities not always available to the breeder, whilst more traditional methods of clonal propagation may be slow, producing limited numbers.

Many *Alliums* produce bulbils or have the ability to produce bulbils if appropriate conditions prevail. *Allium ampeloprasum* var. *babingtonii* always produces both sterile florets and bulbils in the inflorescence as well as daughter bulbs and bulblets. The ability to manipulate the inflorescence towards the production of bulbils may lead to improved methods of clonal propagation. Literature suggests that bulbil production may involve reversion or partial reversion of floral primordia at critical stages in inflorescence development.

Wax embedding, sectioning and staining techniques have been used to examine bulb physiology, and allowed the construction of a developmental timetable. A protocol was developed for the maintenance of apices in tissue culture to monitor floral determination of the apex. The sampled population of *Allium ampeloprasum* L. var. *babingtonii* (Borrer) Syme was found to have both a vernalization requirement and a maturity requirement for floral competence. Vernalization for six weeks at 7° C produced 100 % flowering in plants with a minimum size of 3 cm diameter or approximately 13 g mass at the beginning of the growth season, producing ten or eleven leaves prior to expression of the floral state. Determination occurred during February; the meristem widened followed by elongation of the scape and development of the spathe. Cymes develop in a

regular pattern over the inflorescence, florets forming initially with bulbils developing at the base of the pedicels.

Gene expression in *Allium* species has been not recorded in detail, but comparisons with *Arabidopsis* and other monocotyledons such as rice (*Oryza sativa*) have provided a working model. Degenerate primers were constructed based on the rice *RLF* (Rice LEAFY homologue) gene. This was used to establish the presence of a putative homologue in *Allium ampeloprasum* var. *babingtonii* (*ABLFY*), this being expressed in floral meristems but not vegetative meristems.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
2-iP	N6-(2-isopentenyl) adenine
A	Adenine
B5	Gamborg's culture medium
BA	benzyladenine, a cytokinin
BAP	6-benzylaminopurine
BDS	Gamborg's culture medium modified by Dunstan and Short (1977a)
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BDS	Gamborg's B5 medium modified by Dunstan and Short
2C	DNA content of a normal somatic cell in G1 phase of the cell cycle
C	Cytosine
Chloramine B	N-chlorobenzenesulfanamide sodium salt
COPS	Controller(s) of phase switching factors
d.f.	degrees of freedom
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DEPC	diethyl pyrocarbonate
DTT	dithiothreitol
EDTA	disodium ethylene diamine tetra acetate
Fichlor	Sodium dichloro isocyanurate
FLIP	Floral initiation process
G	Guanine
GA	Gibberellins
GA ₃	Gibberellic acid
IAA	Indoleacetic acid
IBA	indole-3-butyric acid
KAH	Kaurenoic acid hydrolylase
KIN	Kinetin
LAR's	Leaf area ratios
LD	Long day
LDP	Long day plant
<i>LFY</i>	<i>LEAFY</i> gene
LS	Longitudinal section
MS	Murashige and Skoog culture medium
Mt	Metric tonne
NAA	1-naphthaleneacetic acid, an auxin
NBT	nitroblue tetrazolium chloride
PBA	6-benzyl-9-tetrahydropyran adenine
PBS	phosphate buffered saline solution
Picloram	4-amino-3, 5, 6-trichloropicolinic acid
PFD	Photon flux density
PVP	polyvinylpyrrolidone
RAPD	Randomly amplified polymorphic DNA
RNA	Ribonucleic acid
SD	Short day
SDP	Short day plant

SDW	Sterile distilled water
SEM	Scanning electron microscope
Sp.	Species
Spp.	Species (pl.)
T	Thymine
TS	Transverse section
UCW	University College Worcester

Summary of botanical nomenclature used in this thesis

Bract	a modified leaf subtending a flower or inflorescence
Bracteole	a small bract above the bract but below the calyx
Bulb	underground organ of perennation and vegetative reproduction containing stored assimilates
Bulbil	small bulb formed aboveground in the inflorescence
Bulblet	small bulb formed belowground on rhizomes attached to the parent bulb
Cyme	a sympodial inflorescence growing by means of lateral branches each with a flower at its apex
Daughter bulb	produced by the parent bulb from two axillary vegetative buds during floral development
Exserted	protruding (e.g. anthers beyond corolla)
Floret	a flower in a large or composite inflorescence
Inflorescence	a shoot bearing one to many flowers and no leaves
Pedicel	the stalk of a single flower in an inflorescence
Peduncle	the stalk of a whole inflorescence (see also scape)
Rhizome	underground stem producing shoots
Scape	flower stalk growing from ground level (as in herbaceous plants)
Sympodial	growth where the main axis is not formed by continuous growth from the apex but by the growth of lateral buds near the apex
Tepal	perianth with little or no difference between the calyx (sepals) and corolla (petals)

In this thesis, this nomenclature is applied to: -

Var. *babingtonii* - producing both florets and bulbils, reproductively sterile (i.e. no seeds) reproducing clonally through production of bulbils, bulblets and daughter bulbs

Var. *ampeloprasum* – producing fertile florets leading to seed production

Var. *bulbiferum* – producing both florets and bulbils, as with var. *babingtonii*, but with smaller bulbils

Inflorescence development in

***Allium ampeloprasum* var. *babingtonii* (Borrer) Syme**

(Babington's Leek)

1.0 Introduction to important members of the Genus *Allium*, their physiology, phenology, and flowering processes

In this thesis, various parameters of the floral response in the wild leek *Allium ampeloprasum* var. *babingtonii* are presented in a series of “experimental” chapters. In Chapter One, flowering behaviour of this species and closely related species is presented under nine sub-headings – taxonomy, horticultural importance, reproductive strategy, habitat and phenology are presented in the first five sections. Vegetative and floral development is presented in the next two sections and the Introduction ends with a section on floral genes in higher plants, followed by the aims of the work.

Allium ampeloprasum var. *babingtonii* is a wild leek largely found on roadside hedges, coastal cliffs, scrub, streamsides, waste ground and dune grassland in Cornwall (French *et al.*, 1999). It is also grown in a small number of commercial nurseries in Great Britain (Appendix 1), largely as a vegetable curiosity (Ross, 2000 personal communication).

Although it produces an inflorescence, it has not been recorded as producing seed. Instead, it reproduces clonally (Treu 1999) by the production of bulblets on rhizomes

from the parent bulb, by division of the parent bulb into two daughter bulbs following flowering, and by the production of bulbils in the inflorescence (Figure 1).

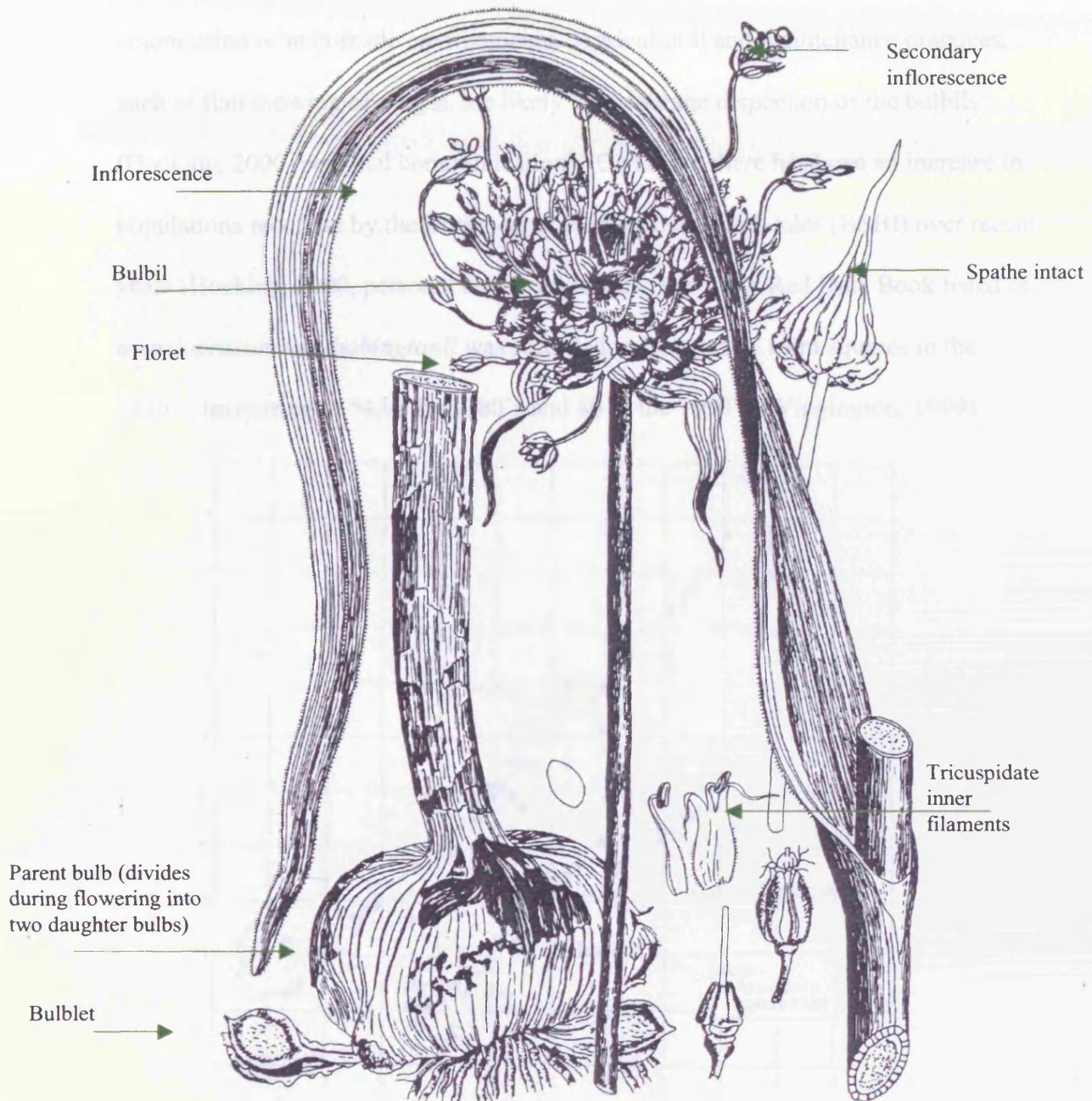


Figure 1: *Allium ampeloprasum* L. var. *babingtonii* (Borrer) Syme del. H. Reynolds (based on the illustration in English Botany, Suppl. 4: t. 2906 (1847) (Adapted from the Botanical Society of the British Isles, 1987).

Var. babingtonii has strong associations with cultivation practices and may have been cultivated at one time itself. Stearn (1987) suggested that as bulbils provide the advantage of food storage, but the disadvantage of limited dispersal, this favours colonisation of man-made environments. Agricultural and maintenance practices, such as flail mowing of verges, are likely to encourage dispersion of the bulbils (Hocking, 2000, personal communication). Certainly, there has been an increase in populations recorded by the Botanical Society of the British Isles (BSBI) over recent years (Hocking, 2000, personal communication). Formerly Red Data Book listed, *A. ampeloprasum* var. *babingtonii* was recorded in just eleven 1 km squares in the 1970's, increasing to 54 in the 1980's and 80 in the 1990's (Wiggington, 1999).

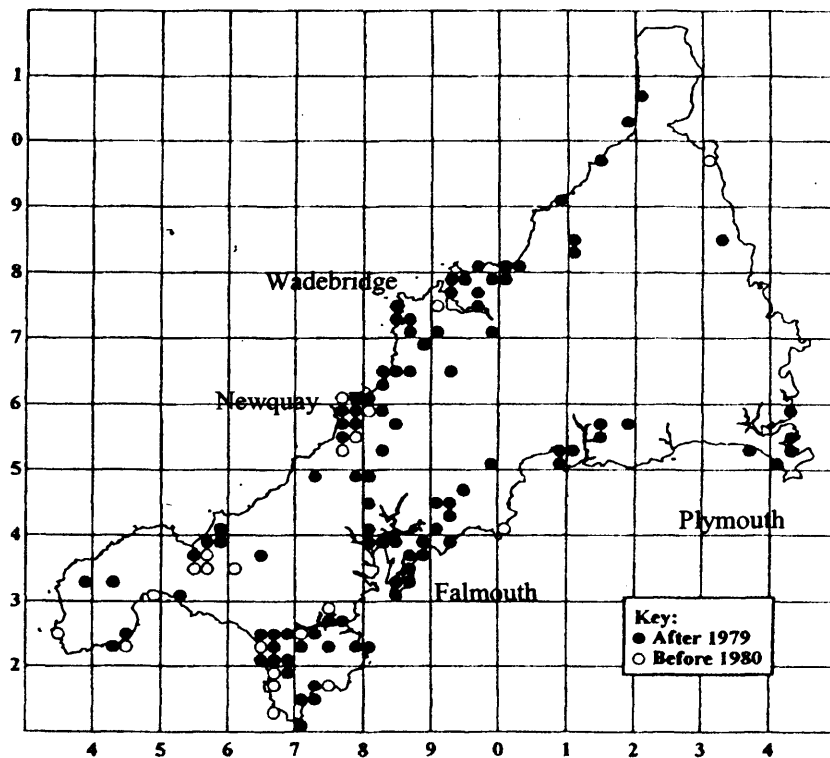


Figure 2: Map of Cornwall showing location of populations of *Allium ampeloprasum* var. *babingtonii* (Borrer) Syme (adapted from French *et al.*, 1999).

The Cornish populations are discrete (Figure 2), usually small (numbered in tens of plants), though some populations number hundreds or thousands of plants, and comprise the majority of known populations. However, populations have also been recorded in most of the main islands of Scilly, W. Ireland and the Is. of Aran (Wiggington, 1999; Treu, 1999). Its large size, vigorous spring growth and prolific reproduction via bulbils make it well able to compete with rank wayside and ruderal species (Wiggington, 1999). *Allium ampeloprasum* spp. (wild leeks) are robust perennials, found in western and southern Europe, including the Mediterranean Islands, from Iberia and the Balearic Islands to Turkey, Iraq and the Caucasus, typically flowering in July and August, reproducing vegetatively and by the production of seed (Wiggington, 1999).

Table 1: Some varieties of *Allium ampeloprasum* (Brewster, 1994¹; Wiggington, 1999²; Heukels, 2000³; Fritsch and Friesen, 2002⁴)

Variety	Characteristics	Location
<i>A. ampeloprasum</i> var. <i>ampeloprasum</i> ²	Abundant in 1625, but declining rapidly, with only hundreds of plants being recorded recently; compact umbels with no bulbils; seed producing	A very few sites on the coasts of England and Wales, Steep Holm and Flat Holm in the Bristol Channel and near South Stack, Anglesey
<i>A. ampeloprasum</i> var. <i>bulbiliferum</i> ^{2 3}	Dense globose umbels with small bulbils (6-8mm)	Channel Islands and Northern France
<i>A. ampeloprasum</i> var. <i>kurrat</i> ; <i>A. Kurrat</i> Schweinf ex Krause (<i>kurrat</i>) ^{1 4}	Does not normally produce bulbils; flowers Spring-early Summer; may have bulbils; fertile seed; can be crossed with leek	Grown in Egypt and adjacent areas
<i>A. ampeloprasum</i> var. <i>holmense</i> (Mill.) Aschers. Et Graebn. (great headed garlic), ^{1 4}	No bulbils usually, cloves of 2 sizes; flowers in Spring but seeds are sterile	Cultivated through Greece and Egypt, through southwest Asia to India, California
<i>A. ampeloprasum</i> var. <i>porrum</i> (L.) Gay (leek) ^{1 4}	Bulbs normally absent; flowers Spring-early Summer; sometimes bulbils are produced	Important crop plant throughout Europe, north America
<i>A. ampeloprasum</i> var. <i>sectivum</i> Lued. (Pearl onion) ^{1 4}	Little pseudostem; bulbils, fertile seed; large numbers of daughter bulbils	Atlantic and temperate Europe

Three morphologically distinct varieties are recognised in Britain; Var. *babingtonii* (bulbils 8 – 15mm length); var. *ampeloprasum*, a seed producing variant considered to be very rare (Wade *et al.*, 1994) growing on rocky sea cliffs in Wales and Anglesey (Roberts and Day, 1987) as well as in Cornwall (Mathew, 1996); and var. *bulbiferum* (Syme) (syn. var. *bulbilliferum* Lloyd), a bulbil producing form endemic in the Channel Islands and Ile d'Yeu, N. France (Mathew, 1996), and also found in W. France (Heukels, 2000; Wiggington, 1999), producing bulbils that are noticeably smaller than those of var. *babingtonii* (6 – 8mm) (Heukels, 2000) (Table 1). Cultivated varieties include the leek (*A. ampeloprasum* var. *porrum* L. Gay), widely grown as a crop throughout Europe, the kurrat, great-headed garlic and the pearl onion (Brewster, 1994) (Table 1).

Opinions differ on the status of *A. ampeloprasum*, some treating it as a probable ancient introduction, possibly in association with early culinary practices, whilst others treat all varieties as being native (Wiggington, 1999). Clapham *et al.* (1987) point out that all British *Alliums* apart from *A. schoenoprasum* and *A. ursinum* appear to have been introduced by man. However, it is widely accepted that *A. ampeloprasum* var. *porrum* (leek) and *A. ampeloprasum* var. *kurrat* (kurrat) are derived from the wild *A. ampeloprasum*, although they could also have arisen as a result of selection from one of the closely related species (Mathew, 1996).

1.1 Taxonomic classification of *A. ampeloprasum* var. *babingtonii*

Classification in *Alliums* can be difficult as morphological differentiation may be weak, while other disciplines provide only limited information (Mathew, 1996).

Early taxonomists placed *Alliums* in the *Liliaceae*, reflecting the superior ovary that is a characteristic of this family, the *Amaryllidaceae* being reserved for petaloid monocotyledons (*Liliales*) with inferior ovaries. John Hutchinson (1884-1972), suggesting that the importance of ovary position had been overstressed, remodelled the *genera* in 1934, and included *Alliums* in the *Amaryllidaceae*, reflecting the umbellate inflorescence characteristic of that family (Hanelt, 1990; Heywood, 1978; Stearn, 1978). Since the *Alliums* have characteristics of both *Liliaceae* and *Amaryllidaceae*, the families have been united by some authorities (e.g. Cronquist, 1968; Thorne, 1968) or separated further creating the *Alliaceae*, for example, Takhtajan (1959). Stearn (1978) also prefers to emphasise the differences of *Alliums* from the *Amaryllidaceae* and *Liliaceae*, and places them in the *Alliaceae*. This has subsequently been adopted by a number of authorities (e.g. Hanelt, 1990; Fritsch and Friesen, 2002).

Allium is a wide-ranging genus, with more than 750 species and approximately 60 groups at subgenera section and subsection level. There are a further 650 synonymous species names (Stearn 1992; Gregory *et al.* 1998) reflecting the complexities of precise classification.

Eduard von Regel (1815 - 1895), an early monographer of *Allium*, grouped the species into six sections (*Alliorum adhuc cognitorum Monographia*, 1875), reflecting the informal groups established by George Don (*Monograph of the Genus Allium*, 1827). More recently, Hanelt *et al.* (1992) proposed 6 subgenera, with 57 sections and subsections. Mathew, (1996) reviewed section *Allium* listing 115 species, and suggesting that it was impossible with the current state of knowledge, to

produce a meaningful infra-sectional classification. He suggested six informal groupings based on character correlations;

- *Ampeloprasum* group
- *Rotundum* Group
- *Spaerocephalon* Group
- *Guttatum* Group
- *Scabriflorum* Group
- *Filidens* Group

Early classifications of *Alliums* separated the bulbilliferous and non-bulbilliferous species (e.g. Linnaeus, 1753, *Species Plantarum*; Don 1827, *Monograph of the Genus Allium*). However, some species have flowers that are partly or wholly replaced by bulbils and, therefore cannot be defined by this characteristic and are usually regarded as conspecific. Indeed many species have the potential to produce bulbilliferous individuals, and variants have received very different taxonomic treatment, which is reflected in the names they have been given (Stearn, 1978). Stearn solves this dilemma by considering that when the bulbilliferous variant has a range that is not coincident with that of the non-bulbilliferous free-seeding one and extends outside that range, then it should be considered as a subspecies.

Allium ampeloprasum var. *babingtonii* was recorded as *A. halleri* (after Albrecht von Haller 1708 - 1777, 18th century Swiss biologist) by Charles Babington (1808 – 1895). William Borrer described it as a new species in 1847 in *English Botany* using the name *Allium babingtonii*, to commemorate Charles Babington. Later it was referred to as *Allium ampeloprasum* var. *babingtonii* (Syme) (1869), reflecting its physiological similarities with *Allium ampeloprasum* var. *porrum* (Table 2). Though other synonyms have been used historically, this classification is now largely adopted into common usage (e.g. Stace, 1991; Mathew, 1996; Treu, 1999).

Table 2: Taxonomy of *Allium ampeloprasum* var. *babingtonii* (adapted from Stace, 1989)

Kingdom	<i>Plantae</i>
Subkingdom	<i>Embryobionta</i>
Division (Phylum)	<i>Tracheophyta</i>
Subdivision	<i>Spermatophytina</i>
Class	<i>Angiospermopsida; Liliopsida</i> , (Takhtajan, 1997)
	<i>Monocotyledones</i> (Hanelt, 1990; Brewster, 1994)
Subclass	<i>Monocotyledonae</i>
	<i>Liliidae</i> (Stace, 1991; Takhtajan, 1997)
Superorder	<i>Liliiflorae</i> (Hanelt, 1990; Brewster, 1994;). <i>Liliianae</i> (Takhtajan, 1997)
Order	<i>Liliales</i> (Stace, 1991). <i>Amaryllidales</i> (Takhtajan, 1997)
	<i>Asparagales</i> (Hanelt, 1990; Brewster, 1994)
Family	<i>Liliaceae</i> (Stace, 1991)
	<i>Alliaceae</i> (Hanelt, 1990, Brewster, 1994, Takhtajan, 1997)
Subfamily	<i>Allioideae</i> (Stace, 1991, Takhtajan, 1997)
Tribe	<i>Allieae</i> (Stace, 1991, Brewster, 1994, Takhtajan, 1997)
Genus	<i>Allium</i> (Stace, 1989, Takhtajan, 1997)
Subgenus	<i>Allium</i> (Hanelt, 1990)
Section	<i>Allium</i> (Mathew, 1996)
Subsection	<i>Scordoprasum</i> (Hermann, 1939)
Species	<i>ampeloprasum</i>
Variety	<i>babingtonii</i> (flowers and bulbils) (Stace, 1991); <i>ampeloprasum</i> (no bulbils) (Stace, 1991); <i>bulbiferum</i> (bulbils and flowers) (Syme, 1869) (Stace, 1991);

Principal ranks are shown in **bold**. All ranks are taken from Stace (1989) unless shown otherwise.

Treu (1999), investigated biosystematic, cytological and molecular perspectives concluding that var. *babingtonii*, should remain conspecific with *A. ampeloprasum*, both on morphological grounds, and on the basis of random amplified polymorphic DNA (RAPD) analysis.

1.2 The horticultural importance of genus *Allium*

The genus *Allium* includes many important crop plants (Brewster, 1994), with high levels of economic and dietary significance (Hanelt, 1990) (Appendix 2). Important species include *A. sativum* (garlic – Subgenus *Allium*, Section *Allium*), *A. cepa* (onion – Subgenus *Rhizirideum*, Section *Cepa*), and the closest commercially important relative of *A. ampeloprasum* var. *babingtonii*, the leek, *A. ampeloprasum*

var. *porrum* L. (Gay) (Brewster, 1994). *A. cepa* (onion) is the world's second largest vegetable crop (measured by weight) (Brewster, 1994) and figures are steadily increasing (Table 3).

Table 3: World production of dry onion bulbs and garlic (FAOSTAT data 2004)

Year	World production onions dry tonnes	World production garlic tonnes
1991	30,074,271	7,067,506
1992	31,382,512	7,323,642
1993	32,766,633	7,425,707
1994	34,374,825	7,645,957
1995	37,839,592	8,405,369
1996	39,443,552	8,868,102
1997	39,327,411	8,857,640
1998	43,756,889	9,044,556
1999	46,370,939	9,421,363
2000	46,529,044	10,067,396
2001	46,750,117	10,121,008
2002	52,381,254	12,182,291
2003	52,068,053	12,407,368

A. ampeloprasum var. *porrum* has been cultivated from very early times, being popular in the ancient Near East around 2500 BC. It was important for both Greeks and Romans and later its use spread throughout Europe (De Clercq and Van Bockstaele, 2002). The EU production of leeks is about 25% of the annual production for bulb onion over the same area. However, because the price per unit weight of leeks is higher, the monetary value of the crop approaches that of onions (Brewster, 1994). It is cultivated on about 30,000 ha in Europe (Table 4) and is becoming increasingly popular in the USA and elsewhere (De Clercq and Van Bockstaele, 2002)

Table 4: Leek production in EU countries (Eurostat, 1999)

Country	Area (ha)	Country	Area (ha)
Belgium	5700	Ireland	<100
Denmark	400	Italy	1000
Finland	<100	Netherlands	3700
France	8300	Spain	2400
Germany	2400	Sweden	<100
Greece	1800	UK	2600

There is increasing commercial interest in the production of *Alliums* as ornamental plants and cut flowers (Davies, 1992; Cottrell, 1999; Kamenetsky and Fritsch, 2002). These include *A. giganteum*, *A. sphaerocephalon*, and *A. moly*, with sales figures increasing significantly over recent years (Cottrell, 1999). For example, the Netherlands is the world's largest producer of ornamental *Allium* bulbs, increasing by 33% from 1995/6 to 1998/9, including 40 species and cultivars and using 113 ha of land (Kamenetsky and Fritsch, 2002). Kamenetsky and Fritsch (2002) list approximately 100 ornamental *Alliums* as popular species, from approximately 300 species presented in horticultural catalogues and books, identifying 19 species as being of economic importance.

Additionally, a number of health benefits have been reported for some *Alliums*, many having been used in traditional and folk medicine since ancient times (Keusgen, 2002). Increasing interest in putative medicinal properties have been intensively investigated, for example, antibiotic, anti-fungal and anti-yeast effects, the cardiovascular effects of garlic and onion, and one of the most thoroughly investigated effects of onion, its anti-asthmatic properties (Keusgen, 2002). *Alliums* have also been implicated in treatment of metabolic diseases, showing anti-diabetic activity, anti-cancer activities and immune effects. The benefits of other *Allium* species are not yet clear and more studies are urgently needed (Keusgen, 2002).

Allium spp. contribute significantly to the world economy, as crops, ornamentals and medicines, and if recently observed trends continue, they will continue to increase in importance and production in the near future.

1.3 Apomixis and Sexual Reproduction

Production of any crop, whether edible, ornamental or medicinal, requires the marketing of plants and plant products when demanded by the proposed markets.

The ability to manipulate breeding and propagation to improve the product to meet the requirements of that market is a valuable tool necessary to meet the often stringent demands of the market place.

Improving the understanding of clonal reproduction is useful to plant breeders as it provides a potential mechanism for the production of desirable lines (Kamenetsky and Rabinowitch, 2002). Hartmann *et al.* (1997), lists the major trade benefits of clonal propagation as:

- Fixing superior genotype
- Uniformity of populations
- Facilitating propagation
- Reduced time to maturity
- Combining genotypes in a single plant
- Controlling developmental phases.

Apomixis, (the habitual reproduction by non-sexual means), may be vegetative apomixis, which replaces sexual reproduction with vegetative, as in the case of *A. ampeloprasum* var. *babingtonii*, or agamospermy where seeds are formed by pseudosexual means (Stace, 1989) e.g. some *Taraxacum* spp. (Grime *et al.*, 1990).

The majority of *Allium* species are sexual, out-breeding, non-hybridising species (Stace, 1989). Outcrossing is encouraged by protandry (Currah and Ockenden, 1978) and natural cytoplasmic sterility (Jones and Clarke, 1943) with self-pollination estimated at only 5-25% in cultivated *Alliums* (Berninger and Buret, 1967). Onion, leek and chives (*A. cepa*, *A. ampeloprasum* var. *porrum*, and *A. schoenoprasum*

respectively), all reproduce sexually from seed, though *A. ampeloprasum* var. *porrum* may produce bulbils if early flowers are removed (Brewster, 1994). Whilst the seed may be plentiful in some species, the product is genetically variable, and may take several years to produce a bulb mature enough to flower, particularly in some of the larger plants such as many of the ornamental *Alliums* (Brickell, 1992; Fritsch and Friesen, 2002).

Some *Allium* species are unable to produce viable seed, relying entirely on vegetative apomixis, e.g. most *A. sativum*, (Brewster, 1994), whilst others such as *A. vineale*, combine the two reproductive methods in varying proportions.

Kamenetsky (1993) listed five types of vegetative reproduction in the genus *Allium*:

- Vegetative replacement or renewal, where two bulbs are produced after flowering, of which the largest is the renewal bulb, which continues the parent plant;
- Vegetative increase, where bulblets are formed from buds in the axils of the foliage leaves;
- Bulblets can be produced via stolons arising from the parent bulb; Galil (1965) reported *A. ampeloprasum* producing up to 35 bulblets per season;
- Vegetative reproduction in rhizomatous species, which leads to independent plantlets as the connecting rhizome decays;
- Bulbil production, with or without the production of florets in the inflorescence.

A. ampeloprasum var. *babingtonii* reproduces using three of these types, vegetative replacement, bulblets, and the most important in terms of numbers produced, bulbils.

It has never been observed to set seed being both male- and female- sterile (Treu, 1999).

Bulbil production is generally important within the genus *Allium*, with Flora Europaea (Tutin *et al.* 1993) listing twenty species/varieties/subspecies, which produce bulbils. These sprout and develop similarly to bulbs, and this is reflected in their similar morphology (Mann, 1960).

Some *Alliums* will produce florets even though the species is an obligate apomict for example, *A. sativum* (garlic) where all cultivated forms are believed to be sterile relying entirely on vegetative reproduction (Etoh and Simon, 2002). *Allium ampeloprasum* var. *babingtonii* produces both florets and bulbils in the inflorescence in widely varying numbers with a moderate positive correlation between the number of bulbils and flower number and a weak but significant correlation between flower number and total bulbil weight (Treu, 1999). The main bulb divides into two sister bulbs after flowering. If flowering does not occur, the bulb remains as a 'round' (Treu, 1999). The bulblets are produced in small numbers, often having a tough sclerified layer and sometimes an inner layer as well. They are helmet shaped with a short, tapered (acuminate) apex, a double protective layer, and are produced in variable numbers by rhizomes emerging from the bulb basal plate (Treu, 1999).

1.3.1 Sexuality – costs and benefits

Charlesworth (1993a, 1993b) modelled the evolutionary advantages of sex and genetic recombination. He found that in the case of a steadily moving optimum environment, a large shift from sexual to asexual reproduction may result in a large reduction in mean fitness. However, conditions with a cyclical or randomly varying environment are much more stringent, therefore less favourable for the evolution of increased recombination. Conditions favourable for asexual reproduction are more likely to be those with a constant environment. This is to be expected, since the traditional view of sexual reproduction is that it allows for adaptation through variation.

Manning and Dickson (1986) showed that re-establishment of equilibrium following environmental change is usually faster in a sexual population, suggesting that asexual populations may experience a greater long-term disadvantage during changes in fitness through harmful mutations. Earlier, Manning (1976) suggested that as sex minimises the mutational load, this may be the mechanism that normally maintains sex within a population.

The suggestion that the advantage of sex is the accumulation of rare beneficial mutations by allowing simultaneous allele replacements at many loci, depends on the restrictive assumption that the fitness of a genotype is determined by fitness potential, a single intermediate variable to which all loci contribute additively, allowing alleles to accumulate in any order (Kondrashov and Kondrashov (2001)). They suggest that individual-based simulations of sexual and asexual populations show that sex may in fact retard adaptive evolution, under generic selection. When new alleles are beneficial only if they accumulate in a particular order, the sexual population may evolve two or three times slower than the asexual population, because asexual reproduction permits some overlap of successive allele replacements (Kondrashov and Kondrashov, 2001). The cost of sexuality also depends on the relative resource allocation to male and female gametes (Hoekstra and van Loo, 1986).

When Bengtsson and Cepitits (2000) studied the balance between sexual and asexual propagation in an evolutionary model where the processes are genetically determined, they concluded that a mixed reproductive system can evolve, if the difference in fitness of the propagules varies over the years. When the propagules

are very similar to each other, evolution will tend towards a state dominated by the one or the other reproductive system.

Peck *et al.* (1999) presented a model in which a sexual population produced an asexual mutant. They concluded that asexual takeover is likely in an unstructured environment, but less likely if the environment is subdivided into demes that are connected by migratory paths, being more unlikely with a greater number of demes and with less migration.

A number of mechanisms have been implicated in sterility in *Alliums*. For example, current commercial clones of garlic (*A. sativum*) are sterile (Etoh and Simon, 2002). Suggested reasons include competition for nutrients between generative and vegetative buds (Koul and Gohil, 1970b), premature degeneration of the tapetum (Novák, 1972), or infection with degenerative-like diseases (Konvicka, 1973, 1984). Etoh (1985) suggests that garlic is actually transitional from sexual to asexual reproduction, this process being accelerated by generations of agricultural selection.

Stearn (1987) suggested that the production of bulbils in *Allium* species allows the establishment of plants without some of the hazards that endanger germinating seeds, though the weight of bulbils may deprive them of the wide dispersal experienced by seeds in the wild. However, with modern agricultural practices providing the dispersal mechanism for the bulbils, this possible disadvantage over the seed bearing variants is overcome.

Allium ampeloprasum spp. show diverse reproduction methodology. Var. *babingtonii* only reproduces asexually through bulbils, bulblets and daughter bulbs, whilst var. *ampeloprasum* reproduces largely through seed (also bulblets and daughter bulbs but in relatively small numbers). The two varieties exist in discrete populations, the former largely in Cornwall, and the latter in S. Wales. Physiologically similar, they occupy similar scrubby, sandy habitats, with var. *babingtonii* increasing rapidly, and var. *ampeloprasum* decreasing and Red Book protected (Wiggington, 1999), suggesting differences in fitness linked to reproduction. Treu (1999) concluded that while the high ploidy level of var. *babingtonii* is unlikely to be a factor in sterility, the observed cytological instability might prevent normal meiotic segregation. He suggests that either a chromosome mutation could have led to meiotic failure or another factor such as level and/or duration of summer temperature may prevent seed set, as for example in the case of *Allium triocum* (Nault and Gagnon, 1993). This spring ephemeral consists of sexual and asexual ramets, both producing large numbers of inflorescences. Most of these fail to mature, and most reproduction is vegetative, daughter bulbs being produced during the reduction in apical dominance when the inflorescence is initiated. However, during years when environmental conditions prolong the growing season, seed production increases significantly and this is thought to be related to the accumulation of resources (Nault and Gagnon, 1993).

Single clones that are geographically widespread are highly unusual, with exceptions having very restricted ecological ranges (Ellestrand and Roose, 1987). Treu (1999) considered that var. *babingtonii* has a wide ecological niche, successfully occupying varied habitats, the disadvantages of asexuality such as the accumulation of

deleterious mutations possibly buffered by the multiple alleles present in polyploids.

He suggests that within a sexual population, an asexual mutant could occur, spreading rapidly due to short-term benefits (such as maintenance of favourable combinations of genes), until a point is reached where it is unable to adapt to a change in environmental conditions or is unable to deal with deleterious mutations.

Many weeds are commonly asexual or self-fertilizing, and Halliday (1993) suggested that an environment with cleared areas of ground, limiting competition, favours the production of large numbers of genetically uniform progeny. Bierzychudek (1989) further suggested that obligate asexuals may have a more general-purpose genotype, with the often polyploid nature of asexuals providing increased ecological tolerance.

1.4 Habitat

A. ampeloprasum var. *babingtonii* is found in a wide range of habitats including streamsides, sea cliffs, open woodlands, peaty heath and roadsides (Treu *et al.* 2001), path and field borders and banks (personal observation). These range from wet (streamsides) to dry (cliff sites) and exposed sunny positions to lightly shaded (open woodland). Soil may be high in organic matter (peaty heath), or sandy (coastal scrub) (personal observation). The common features of these habitats are that any evergreen species are low growing (e.g. grasses), the size of potential competitors is limited and many plants are winter dormant.

These sites may also be poor in nutrient levels, either because the soil is shallow, where sandy soil allows leaching of nutrients, or where construction practices leave poor quality, often stony soil (for example, road and path sides), and this will tend to inhibit the growth of larger species in these sites. Further, agricultural practices such

as spraying, ploughing, mowing etc., limit the size of competitive plants, as does the disturbance caused by pedestrian and road traffic. Most of these sites could generally be described as scrub or wasteland, usually free draining, with limited competitive species. Where plants experience more severe competition they are often smaller or when lightly shaded they become paler and etiolated (personal observation).

The majority of *Alliums* grow in open, dry, sunny, arid and moderately humid climates and are often found in rocky, limestone formations (Fritsch and Friesen, 2002). De Clercq and Van Bockstaele (2002) note that close relatives the leeks (*A. ampeloprasum* var. *porrum*) will grow in any open-textured (well-drained) soil. Most species of *Allium* grow in regions of autumn-spring precipitation, with summer drought, i.e., Mediterranean and Irano-Turanian phytogeographical regions (central Turkey, south to Israel and Saudi Arabia, East to Central Asia) (Figure 3).

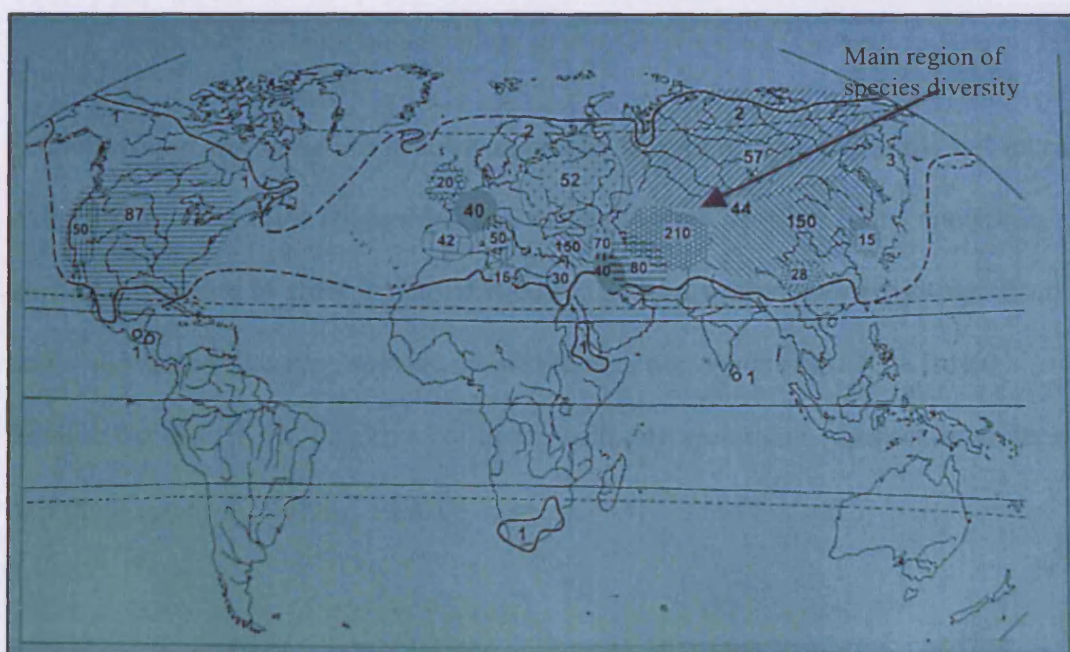


Figure 3: World distribution of wild species of the genus *Allium* L. The numbers on the map indicate the number of species found in each region (adapted from Fritsch and Friesen, 2002).

The main region of high species diversity is from the Mediterranean basin to Central Asia and Pakistan, with a second smaller centre in western North America (Fritsch and Friesen, 2002; Mathew, 1996). The Royal Botanic Gardens, Kew hold approximately 1000 accessions, comprising at least 250 species out of 750 known in the genus (Mathew, 1996).

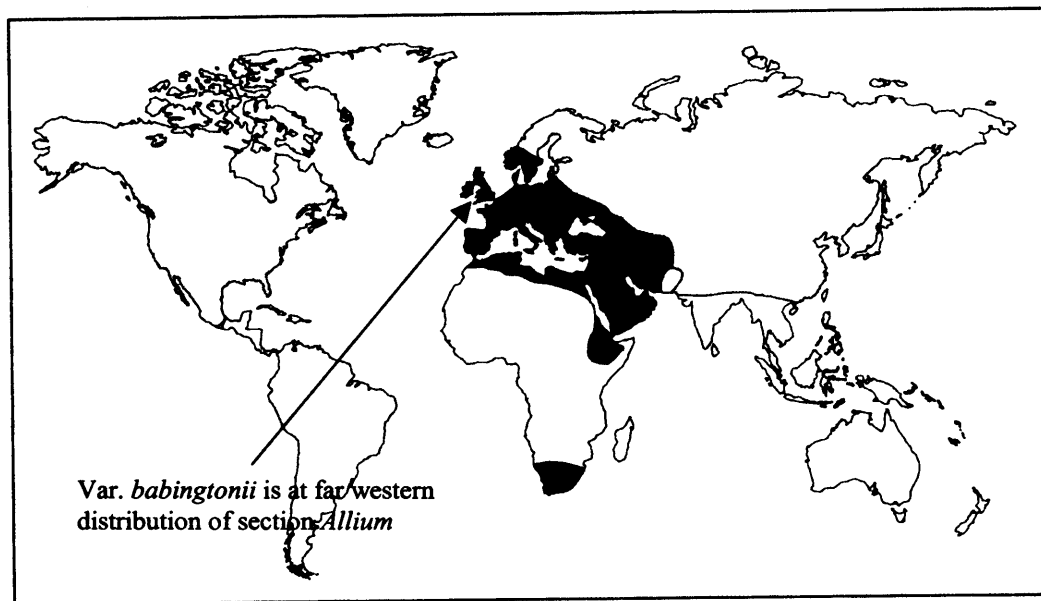


Figure 4: Distribution of *Allium* species; *Allium* section *Allium* in black (adapted from Mathew, 1996)

Section *Allium* has some 115 species, extending from Portugal eastwards to Central Asia (Figure 4) with thirty recorded in Europe (Mathew, 1996). Many species in Section *Allium* are in areas that are difficult to access, so that they are known from preserved material or type specimens, which may no longer exist. The Royal Botanic Gardens, Kew, houses a collection of living specimens representing over half these species (Mathew, 1996).

Allium spp. occur from sea level up to an altitude of 3050m above sea level, some species having a wide range within these general limits. For example, *A.*

ampeloprasum, *A. rotundum*, *A. guttatum* and *A. vineale* can be found from sea level to 2000m (Mathew, 1996). *A. ampeloprasum* is found from Portugal in the west through to the Mediterranean countries to western Iran in the east and is often associated with cultivated or abandoned fields, together with *A. vineale*, and *A. scorodoprasum*. These all increase rapidly by vegetative means, such as bulblets and bulbils (Mathew, 1996). They are poor competitors, requiring vigorous weed control in cultivation (Bosch Serrah and Currah, 2002). It is not surprising therefore, that var. *babingtonii* will flourish in areas where abiotic factors limit competition. As var. *babingtonii* produces leaves throughout the winter months, this ensures that its canopy is always exposed to receive maximum photosynthetic benefit, dying down in early summer when other species are becoming more light-competitive, and then using its stored resources in the bulb to maintain subsequent growth.

1.5 Phenology

Treu (1999) recorded that *A. ampeloprasum* var. *babingtonii* (Cornish populations) showed visible above ground sprouting in October. There was limited growth between November and February, followed by rapid growth in March and April. The inflorescence (if produced) emerged in late spring/early summer, maturing in mid-late summer. The foliage died down in mid-summer, the plant becoming dormant for a short period before growth recommenced with root development in late September, followed by above ground growth once again in October (Treu, 1999). *A. ampeloprasum* var. *babingtonii* is believed to flower in its third year, the inflorescence becoming visible above the leaves during late spring and maturing in late summer (July – August) (J. Shipton, *pers. comm.* 2000)

Similarly, *A. ampeloprasum* var. *porrum* (leek) is a biennial (perennial) species needing vernalization in order to flower. It blooms when days are long in midsummer (approximately 10 June to 20 July in Europe) though premature bolting (flower development) can be a problem with early planting (De Clercq and Van Bockstaele, 2002).

This is in many ways typical *A. ampeloprasum* phenology, reflecting its Mediterranean origins in relatively dry areas (Figure 5). However, the time of flowering is a little later than that generally found in other *A. ampeloprasum* spp. originating in semi-desert or Mediterranean conditions ('e' Figure 5), and perhaps more consistent with *Alliums* from cooler regions ('a' and 'b', Figure 5).

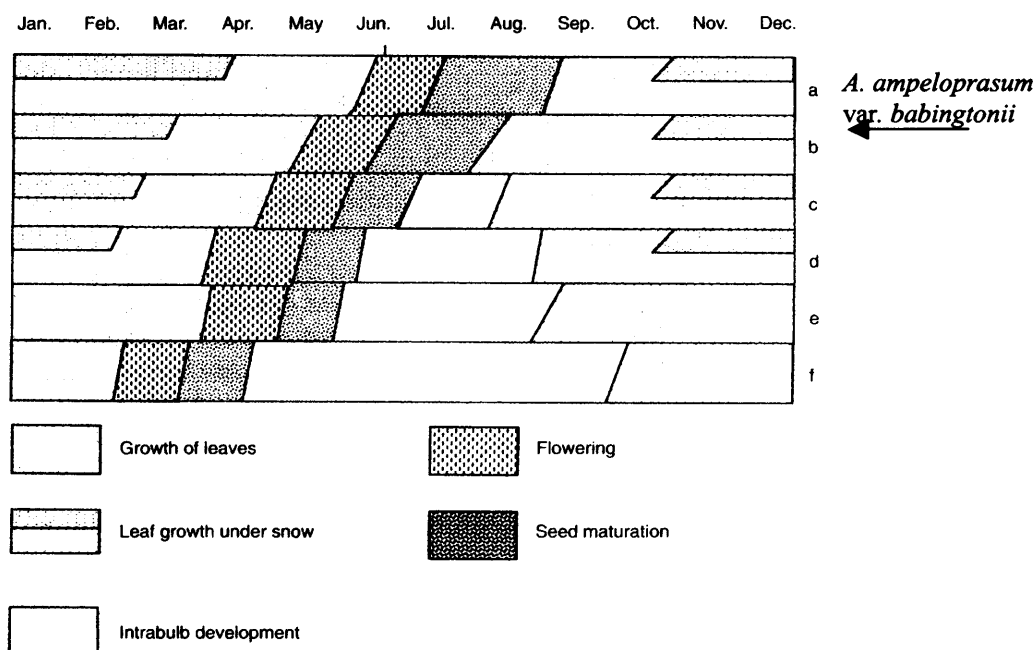


Figure 5: Annual life cycles of some *Allium* species. (a) *A. nutans* (mountains, Siberia); (b) *A. pskemense* (mountains, Kazakhstan); (c) *A. caeruleum* (steppe, Russia); (d) *A. karataviense* (semi-desert, Kazakhstan); (e) *A. ampeloprasum* (semi-desert, Mediterranean); (f) *A. rothii* (desert, Israel) showing approximate relationship of *A. ampeloprasum* var. *babingtonii* relative to these species (adapted from Kamenetsky, 1996b).

1.6 Vegetative Development

Patterns of organ formation and development vary within *Allium* species. This may depend on the environmental conditions under which it has grown, as well as on the species (DeMason, 1990). Some commercially important varieties (see 1.2) have been extensively researched, e.g. *A. cepa*, (onion) and much of the development of leeks (*Allium ampeloprasum* var. *porrum*) is similar to this (Brewster, 1994).

Therefore, these species will be used as a model for var. *babingtonii*. Mathew (1996) summarised the diagnostic characters of section *Allium*:

- Bulb ovoid to globose, often with bulblets
- Leaves sheathing stem for 1/4 to 2/3
- Leaf lamina either solid and flat to channelled in cross-section, or hollow and semi-terete to terete
- Spathe valve 1, long-beaked and caducous, or valves 2-4 persistent
- Perianth campanulate to ovoid, never stellate
- Outer 3 filaments usually simple, triangular-subulate
- Inner 3 filaments 3(5-7)-cuspidate, the median anther-bearing cusp usually longer than the lateral sterile cusps
- Ovary with distinct nectariferous pores

He further defined the characteristics of the *ampeloprasum* Group:

- Bulb tunics membranous
- Bulblets yellow-brown to brown, often small, numerous and helmet shaped
- Leaves solid, flat/canaliculated
- Spathe 1-valved with a long beak, caducous
- Anthers exserted

1.6.1 Bulb

1.6.1.1 Structure

Var. *babingtonii* forms a bulb (Treu 1999), unlike var. *porrum* (Brewster, 1994), but similar to most wild *A. ampeloprasum*. Jones and Mann (1963) record that these have well developed bulbs, usually consisting of two thick, bladeless storage leaves, surrounded by the thin bases of the foliage leaves, possibly with many cloves.

Bothmer (1974) describes the Aegean members of the *Ampeloprasum* complex in some detail, as possessing one fleshy, colourless modified leaf for storage (storage cataphyll), forming the bulk of the bulb. This nourishes the sprout in the early stages, before declining. It is surrounded by a bladeless sclerified protective leaf (Bothmer, 1974). Inside the storage leaf, a bladeless sprout leaf grows out and forms the basal sheath of the plant, with the normal foliage leaves formed inside this (Figure 6). The basal bulb plate is a modified stem, which regenerates every vegetative period, unlike the cultivated *A. Ampeloprasum* var. *porrum* or *A. kurrat*, both of which are biennial. The old bulb plate (basal plate) remains attached to the new bulb plate (basal plate) (Bothmer, 1974).

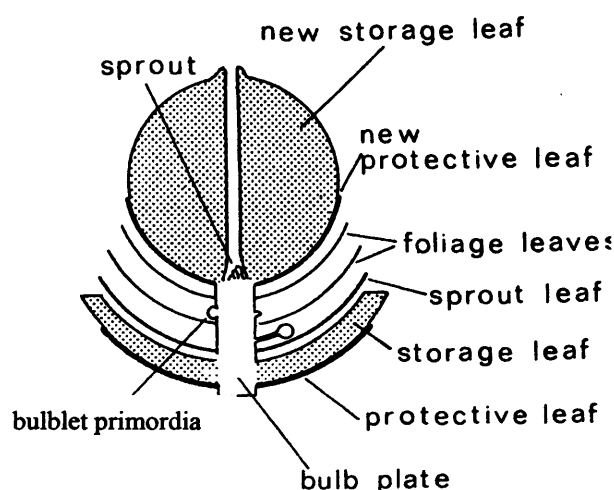


Figure 6: *Allium ampeloprasum* complex. Schematic drawing of renewal bulb structure half-way through the vegetative period (adapted from Bothmer, 1974)

Bulblets are vegetative axillary buds forming on rhizomes from the true stem, whilst bulbils are vegetative buds developing in the inflorescence. Conversely, leeks (*A. ampeloprasum* var. *porrum*) do not form a significant bulb under most temperate conditions, though under long day conditions and between temperatures of 12°C and

18°C, some food reserves can accumulate in the leaf sheath bases (Rubatzky and Yamaguchi, 1997).

The vegetative onion (*A. cepa*) axis is a rosette shoot with a short, squat stem (basal plate), lacking internodal elongation. The stem is heart-shaped, when dissected longitudinally (De Mason, 1990).

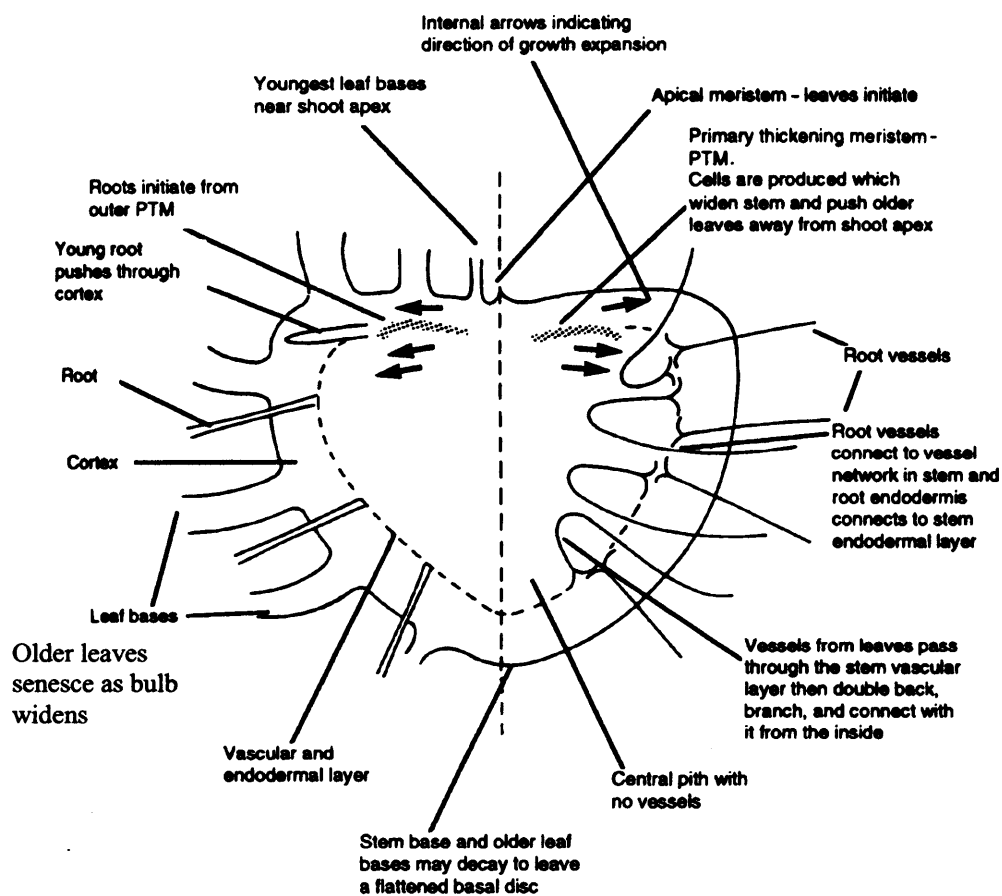


Figure 7: Diagrammatic longitudinal section through an onion (*A. cepa*) or garlic (*A. sativum*) stem (baseplate) showing the main tissues, leaf bases, root origination and regions of cell division (left side); and how the vessels of the stem, root and leaves interconnect (right side) (adapted from Brewster, 1994).

Since *A. ampeloprasum* var. *porrum* is similar to *A. cepa* (Brewster, 1994), it is likely that var. *babingtonii* has a structure closely related to those described for these two species and the wild *A. ampeloprasum* spp. described by Bothmer (1974). However, *A. ampeloprasum* is a polymorphic species, (Bothmer, 1974) and there is

considerable variation in bulb form in the *Allium* genus as a whole. The bulbs may be protected by membranous, fibrous or coriaceous tunics (Hanelt, 1990, Fritsch and Friesen, 2002). Underground storage may be bulbs, rhizomes or swollen roots. They may be true bulbs (one or two thickened prophylls) or false bulbs (thickened basal sheath plus prophylls) (Fritsch and Friesen, 2002). Suggestions for structure in *var. babingtonii* can only be tentative until experimental data has been reviewed.

1.6.1.2 Storage compounds

Fructans are the primary reserve carbohydrate (non-structural carbohydrate) in 12 – 15% of higher plants, its accumulation mechanism being distinct from that of natural starch-accumulators. The fructans are synthesised directly from sucrose, and are accumulated in the vacuoles of both photosynthetic and storage cells (Cairns, 2003).

In *Allium* spp. the simple carbohydrates and the smaller fructans are usually present in the largest proportions (Gubb and MacTavish, 2002). The non-structural carbohydrate composition of *A. cepa* is believed to be largely (but not entirely) dependant on genetic factors, with fructose showing most sensitivity to environmental factors (Kahane *et al.*, 2001). The fructans in *A. cepa* are accumulated at the onset of bulbing (Darbyshire and Henry, 1978, 1979), the degree of polymerisation (DP) varying considerably between *Allium* spp. with up to DP50 in garlic and between 3 and 15DP in *A. cepa* (Kahane *et al.*, 2001). In *A. cepa* these non-structural carbohydrates comprise 65 – 80% of the dry matter (Darbyshire and Henry, 1978), the bulb also containing 80 – 93% water according to cultivar (Gubb and MacTavish, 2002). This contrasts with the majority of bulbs which have a dry matter content of about 30% (Le Nard and De Hertog, 1993). Kahane *et al.*, (2001)

suggest that the sucrose and small fructans (DP3) are transient in the production of the larger fructans. Throughout storage (during the dormant period), the fructans are gradually hydrolysed to fructose. Subsequently, during sprouting, sucrose is synthesised (Gubb and MacTavish, 2002).

Some authorities suggest that fructans confer stress resistance to drought and cold on plant tissues (e.g. Hendry, 1992; Vijn and Smeekens, 1999), though this is controversial (Cairns, 2003). As a species originating in Mediterranean regions, it might be expected that *A. ampeloprasum* spp. are less adapted to the winter cold of the British Isles. However, var. *babingtonii* is believed to survive temperatures as low as -15°C (Shipton, *personal communication*, 2000) and is grown commercially as far north as Scotland (Royal Horticultural Society Plant Finder, 2003). The presence of fructans in *Allium* spp. may be one mechanism by which this *genus* is able to survive low temperatures.

1.6.2 Roots

Var. *babingtonii* commences root development in late September, before visible above ground growth in October (Treu, 1999 J. Shipton, *personal communication*, 2000) but little detail is known of the root growth and development in this species.

Allium roots may be annual or perennial (Fritsch and Friesen, 2002). *A. cepa* (onion) roots are homorhizic, i.e. the primary root of the seedling is short lived, and subsequent roots are produced from the stem (adventitious), without any secondary growth (De Mason, 1990). Roots are produced in rings in the true stem, with successively produced rings of roots with more primordia produced above the level

of older ones as the stem diameter gets larger (De Mason, 1990). Roots increase little in diameter once formed, they have sparse branching if any, and usually lack root hairs (Jones and Mann, 1963).

A. cepa have one of the most limited root systems among the vegetable crops (Jones and Mann, 1963). For example, Bosch Serra *et al.* (1997) found that root growth was concentrated in the top 40cm of soil with maximum average root density in the top 20cm. Growth was promoted by frequent irrigation. Jones and Mann (1963) suggested that new roots will not grow into dry soil. Therefore, if water does not reach the bulb base during the growing season, above ground growth will be limited. This is consistent with the view of Brewster (1994) that when deprived of water, growth will cease, and can be difficult to restart again. Bosch Serra and Currah, (2002) suggest that root growth may be most rapid during the period immediately before bulbing.

A. ampeloprasum var. *porrum* (leek) also have shallow root systems, forming extensive mats of interlocking roots (Smith, personal communication, 1999; Rubatzky and Yamaguchi, 1997). It is interesting to note, that though var. *babingtonii* almost always grows in well-drained soil, it is frequently fairly damp, for example, following the banks of a stream or ditch, suggesting sensitivity to moisture levels as for other *Alliums*.

The presence of contractile roots has been reported in some *Allium* spp. for example they are present in *A. sativum* and *A. ampeloprasum* var. *porrum* but not in *A. cepa*. These are common in plants with bulbs or corms, contracting so that the outer root

cortex is thrown into large folds, literally pulling the bulb down into the soil (DeMason, 1990).

1.6.3 Foliage

Leaf growth in *A. ampeloprasum* var. *babingtonii*, is visible above ground in late October, with limited growth until February, followed by rapid growth until April (Treu, 1999). Treu (1999) also recorded some vegetative characters (n = 40), collecting data in early May 1996 when growth was well advanced (Table 5). All plants were taken from population 1 (Appendix 3). The data shows considerable variation in plant size in this population, with a mean leaf length of 636mm and width of 32.5mm.

Table 5: Some vegetative characters of 40 plants of *Allium ampeloprasum* var. *babingtonii*. Data collected early May 1996 (Treu 1999).

Character	Range	Mean \pm SE
Total height (mm) (maximum achieved by any leaf)	315 - 1110	636 \pm 26
Leaf breadth (mm) (maximum breadth of basal leaf)	6 - 39	32.5 \pm 1.1
Stem (pseudostem) diameter (mm) (diameter at level of basal leaf)	4 - 22	13.6 \pm 0.6

As with bulb and root characteristics (above), leaf development in *Alliums* has many features in common. In the much-studied *A. cepa*, new leaves are formed from the ring-like meristem, inside the earlier leaf sheath, producing the leaf as a tube-like structure, which is then modified to become a linear leaf blade. Foliage leaves comprise the tubular ensheathing leaf base leaf sheath, which projects up from the true stem, and the unifacial leaf blade. Collectively, these leaf sheaths are sometimes referred to as the pseudostem (Rubatzky and Yamaguchi, 1997; De Mason, 1990) (Figure 7).

The leaves have a distichous phyllotaxy (leaves aligned in two vertical ranks), being initiated at the apical meristem with a 180° divergence angle (De Mason, 1990) (Figure 8). The bases of successive leaves become larger in diameter, resulting in the earlier-formed leaves being torn open and shed (DeMason, 1990).

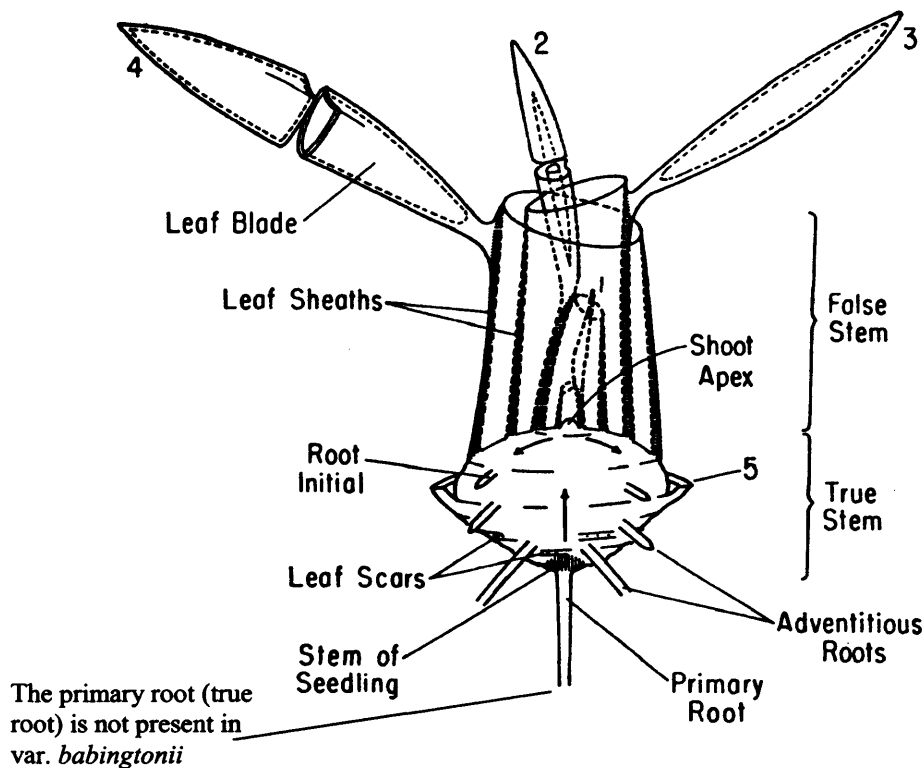


Figure 8: A diagrammatic picture of the development of the stem, leaves, and roots, in a young plant of the common onion (*A. cepa*). Each new leaf arises at the stem or shoot apex, leaf 1 being the youngest and leaf 4 the oldest of the four leaves shown. Each leaf arises as a ring of tissue which grows upwards as a tubular sheath. The leaf-blade elongates from one side of the sheath's top. As the stem grows upwards, it also broadens, as is shown by the divergent arrows in the diagram. New roots continually arise in the younger (upper) part of the stem. In this figure, the space between adjacent leaf-sheaths is much exaggerated (adapted from Jones and Mann, 1963).

Gregory (1996) placed *Allium ampeloprasum* spp. in Group II of section *Allium* (subsection *Scordoprasum* of F. Hermann, 1939). She defined the leaf characteristics of this group (Figure 9) as:

- Leaves almost flat to shallowly or distinctly V-shaped in T.S., often with abaxial keel
- Vascular bundles in two rows

- Abaxial row of large and small bundles with xylem facing adaxial surface
- Adaxial row of small inversely orientated bundles.

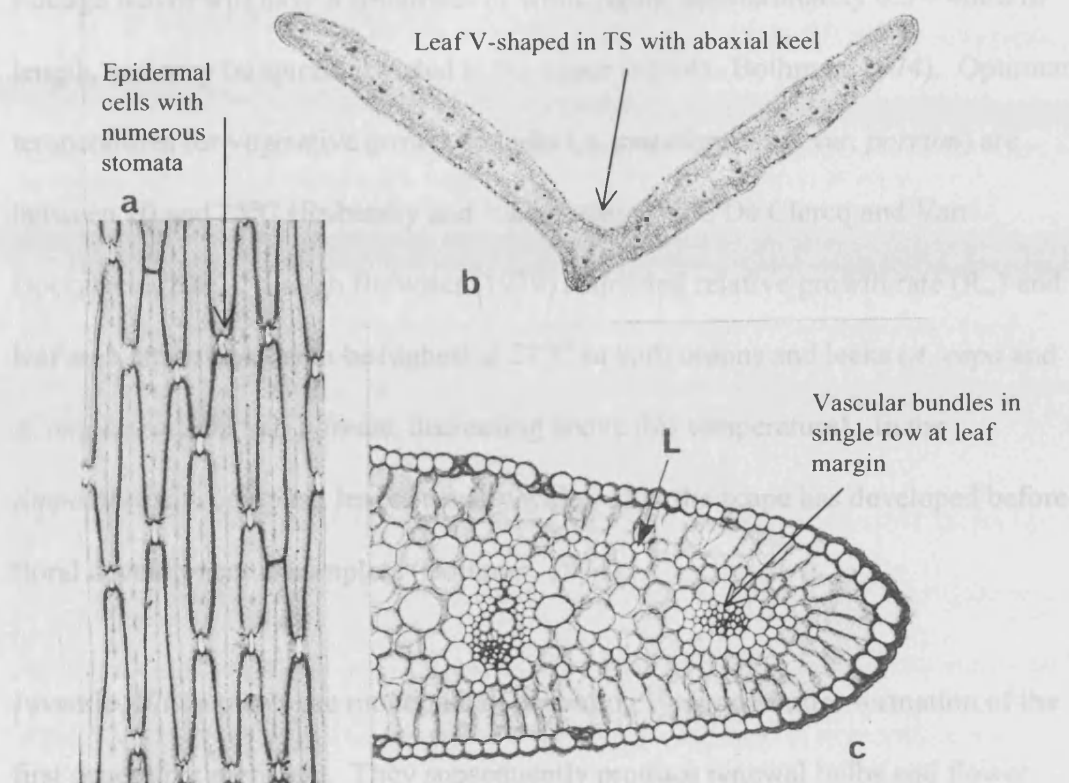


Figure 9: Group II. A, *A. ampeloprasum* leaf surface view x 150; b, c, *A. rotundum* leaf T. S., b x 25, c, margin x 150. L = laticifer (after Gregory, 1996)

Further characteristics of this group are:

- Leaf pseudo-dorsiventral
- Hairs (when present) short simple unicellular
- Cuticle bearing a central longitudinal striation
- Epidermal cells in regular files longitudinally elongated
- Stomata numerous, anomocytic (lacking morphologically differentiated subsidiary cells), in most files of cells except over ribs
- Epidermal cells and stomata may be similar on both surfaces or abaxial epidermal cells slightly smaller
- Stomata +/- equally numerous on both surfaces, sunken
- Outer walls plus cuticle usually moderately thick
- Inner walls thin to slightly thickened, radial walls thin
- Palisade tissue in one layer adaxially and abaxially (except sometimes over midrib)
- Spongy mesophyll circular to lobed, fairly large intercellular space, tending to break down between vascular bundles, forming small air canals in some species
- Laticifers, 2-3 layers below epidermis of both surfaces, mostly at inner boundary of palisade tissue, numerous
- Vascular bundles in two rows, (except sometimes near margins), abaxial row – large and small bundles with xylem facing adaxial surface (except marginal bundles); adaxial row, small bundles, inversely orientated

- Bundle sheaths parenchymatous, cells of same size as or larger than surrounding cells (Gregory, 1996)

Foliage leaves will have a colourless or white ligule, approximately 0.5 – 4mm in length, and may be spirally twisted in the upper regions (Bothmer, 1974). Optimum temperatures for vegetative growth in leeks (*A. ampeloprasum* var. *porrum*) are between 20 and 25°C (Rubatzky and Yamaguchi, 1997; De Clercq and Van Bockstaele, 2002), though Brewster (1979) estimated relative growth rate (R_w) and leaf area ratios (LARs) to be highest at 27°C in both onions and leeks (*A. cepa* and *A. ampeloprasum* var. *porrum*, decreasing above this temperature). In the *Ampeloprasum* complex, leaves usually wither after the scape has developed before floral development is complete (Bothmer, 1974).

Juvenile *Allium* plants are monopodial, becoming sympodial after formation of the first generative meristem. They subsequently produce renewal bulbs and flower every year (Kamenetsky and Rabinowitch, 2002).

1.7 Floral Development

McDaniel, Singer and Smith (1992) examined floral initiation, emphasising developmental fates rather than physiological processes (Figure 10). A developmental signal is sent to the meristem cells, usually from the leaves. This inductive activity may be continually expressed or may be latent, for example in plants where the photoperiod is the external stimulus. If the meristem is competent to respond to this signal, it will be evoked into the florally determined state. Under permissive conditions, this florally determined state is then expressed as the initiation of floral morphogenesis (McDaniel *et al.*, 1992).

Competence requirement unpublished for var. *babingtonii*
 Induction signals unpublished for var. *babingtonii*

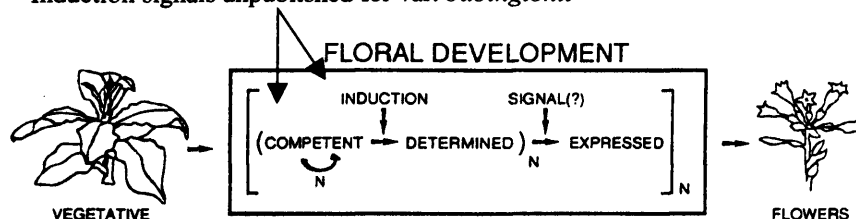


Figure 10: A diagrammatic illustration of some of the possible processes and conditions that may be associated with floral morphogenesis. Arrows indicate the normal direction of events but do not imply irreversibility. Competent cells/tissues are those that have the ability to respond to a developmental signal in a specific way. Cells and tissues can acquire different competences via either endogenous or exogenous means. Induction occurs when a developmental signal acts on competent cells/tissues to determine them for a particular developmental fate. The determined state is subsequently expressed. The 'N' indicates that the bracketed sequence may be iterated. These processes and conditions can occur throughout the plant (e.g., in roots, in leaves, in shoot apical meristems, and what happens in one part of the plant may influence processes and conditions in other parts of the plant (adapted from McDaniel *et al.*, 1992).

The processes involved in flowering are likely to be controlled by many factors both inductive and autonomous (Chaylakhyan, 1977). Bernier *et al.*, (1993) suggests that the majority of plants use environmental cues such as photoperiod, temperature and water availability to regulate the transition to flowering, whilst autonomous-flowering plants (those that do not require a particular temperature or photoperiod to flower) are usually sensitive to irradiance (Bernier *et al.*, 1993). It is likely that plants rely on chemical and biophysical systems working in conjunction (Lyndon, 1994) with different families of plants responding to differing stimuli (Lyndon, 1998). These may be positive, for example, gibberellins in long-day rosette plants and conifers, auxins, auxin analogues or ethylene in bromeliads such as pineapple, or negative, for example, removal of inhibitors such as gibberellins or cytokinins (Lyndon, 1998).

Different factors may be perceived by different parts of the plant. For example, Bernier *et al.*, (1993) suggests that all plant parts perceive temperature. However, low temperature is generally perceived by the shoot apex, whilst water availability is

perceived by the root system. These factors interact strongly, and each factor can change the threshold value for the effectiveness of the others (Bernier *et al.*, 1993).

The developmental programs that operate in the meristem direct a number of processes, for example the rate of primordia initiation, the phyllotaxy of the emerging primordia, the development of internode length as well as the determined or indeterminate state of these primordia (Huala and Sussex, 1993; Coen and Carpenter, 1993). Groups of cells on the periphery of the meristem are partitioned off to form either organ primordia or secondary meristems. The pattern of partitioning gives rise to the phyllotaxy of the developing tissues (Coen and Carpenter, 1993) i.e. it is the position of the cells rather than the identity of the cells that determines the development of the cells (Bossinger and Smyth, 1996). Following induction, two new meristem types are usually produced (Figure 11); the inflorescence meristem, and the floral meristems which often arise as small bulges on the periphery of the inflorescence meristem (Coen and Carpenter, 1993).

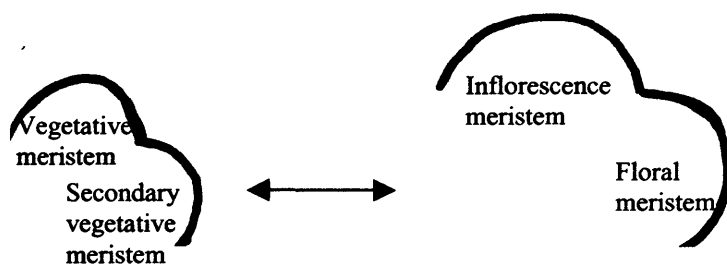


Figure 11: The change from the vegetative to flowering state based on Coen and Carpenter (1993).

Arabidopsis thaliana, a small member of the *Brassicaceae*, has been used as a model for plant development. Here the flower primordia usually arise from a group of four cells on the inflorescence flank, the radial axes of the mature flower being apparently

set by these cells (Bossinger and Smyth, 1996). Primordia respond quantitatively to floral induction signals, and their fate can be modified after primordia initiation (Hempel *et al.*, 1998).

1.7.1 Floral determination

A shoot apical meristem is florally determined if it forms a flower in conditions where vegetative growth would be predicted (McDaniel *et al.*, 1992; McDaniel, 1996). A classic way to test for floral determination is to expose a plant to an inductive treatment, excise the shoot apical meristem and culture it in non-inductive conditions. Floral formation in these conditions confirms the florally determined state, (see for example, Durdan, 1998).

No work has previously been carried out on floral determination of *A. ampeloprasum* var. *babingtonii*, though some has been carried out on its close relative *A. ampeloprasum* var. *porrum* (leek), (see for example Weibe, 1994, Wurr 1997 and Wurr *et al.*, 1999).

However, *A. cepa* (onion) has been extensively researched, providing a model for other members of the family. *A. cepa* plants are biennial, that is, they normally flower in the second season of their development, although under favourable conditions, they will flower (bolt) in the first season (Rabinowitch, 1990).

Flowering will occur, provided that the plants have passed their juvenile phase, and are then exposed to several environmental factors which will induce and mediate inflorescence development. Vernalization, the exposure to low temperatures, is likely to be of most importance of these environmental factors (Rabinowitch, 1990).

1.7.1.1 Maturity

Kamenetsky and Rabinowitch (2002), suggest that all *Allium* plants need to reach a certain physiological age (maturity) in order to be florally competent. This may be months or years depending on species, the length of the juvenile phase depending on environmental conditions as well as on genetic make up (Kamenetsky and Rabinowitch, 2002).

The precise physiological nature of this requirement is not clear. Bulb size is the major and most easily measured factor used to determine the capacity to flower in many bulbous genera, and this may relate to the size of the apical meristem (for example, in *Triteleia laxa* syn. *Brodiaea laxa*; *Tecophilaeaceae*) or to the quantity of reserves accumulated (for example *Tulipa*) (Halevy, 1990; Le Nard and De Hertogh, 1993). Brewster, (1985) measured carbohydrate levels in onions (*A. cepa*) finding that a low reserve carbohydrate content required a higher minimum leaf number for inflorescence initiation. Cv. Rijnsburger was grown at 17°C with photon flux density (PFD) of $600\mu\text{mol m}^{-2}\text{s}^{-1}$ producing high carbohydrate levels (23.1% dry mass) in the bulb, and at 25°C with PFD $200\mu\text{mol m}^{-2}\text{s}^{-1}$ producing low carbohydrate levels (13.6% dry mass) in the bulb. This produced an estimated leaf number for 50% inflorescence initiation of 6.88 and 8.19 respectively. Those plants with low carbohydrate content produced fewer inflorescences, if the light levels were very low during vernalization ($50\mu\text{mol m}^{-2}\text{s}^{-1}$ PFD). It is interesting that figures for cv. Senshyu showed more variation within the sample and more variation in vernalization requirements, therefore changes in carbohydrate levels could not be directly compared with those of cv. Rijnsburger (Brewster, 1985).

Where bulb size has been used to predict maturity in *Allium* spp., there has been great variation between species. Minimum circumference varies from 3 – 5 cms for *A. caeruleum*, *A. neapolitanum* and *A. unifolium*, 12 – 14 cms for *A. aflatuense*, *A. cristophii*, and 20 – 22 cms for *A. giganteum*. Plants with larger bulbs may take 3 – 5 years of growth before they become florally competent (De Hertogh and Zimmer, 1993).

Maturity in *Allium* spp. has also been measured by bulb mass, for example, minimum bulb mass for floral initiation in sets of 'Ailsa Craig' (*A. cepa*) was found to be 4 - 7 g fresh weight (Rabinowitch, 1990), and for *A. ampeloprasum* var. *porrum*, it has been measured at approximately 2g (Weibe, 1994).

Leaf number has been frequently used as a marker for assessing maturity in *Alliums*, (see Rabinowitch, 1990). In subgenus *Allium*, of which var. *babingtonii* is a member, inflorescence initiation has been found to occur only in growing plants, following the development of 7 – 9 leaves (Kamenetsky, 1996a). Studies with seedlings of *A. cepa* have shown that a minimum of 10 - 14 leaves is necessary before flowering can be initiated (Rabinowitch, 1990). Weibe (1994) confirmed the requirement for maturity in *A. ampeloprasum* var. *porrum* before competence is reached, this being at a minimum size of approximately 5 visible leaves in the three cultivars studied. Others have reported it to be capable of floral initiation after formation of 6 – 7 true leaves/leaf primordia (De Clercq and Van Bockstaele, 2002; Kamenetsky and Rabinowitch, 2002). Wurr *et al.*, (1999) confirmed the requirement for completion of the juvenile phase in cv. Prelina, estimating that minimum leaf

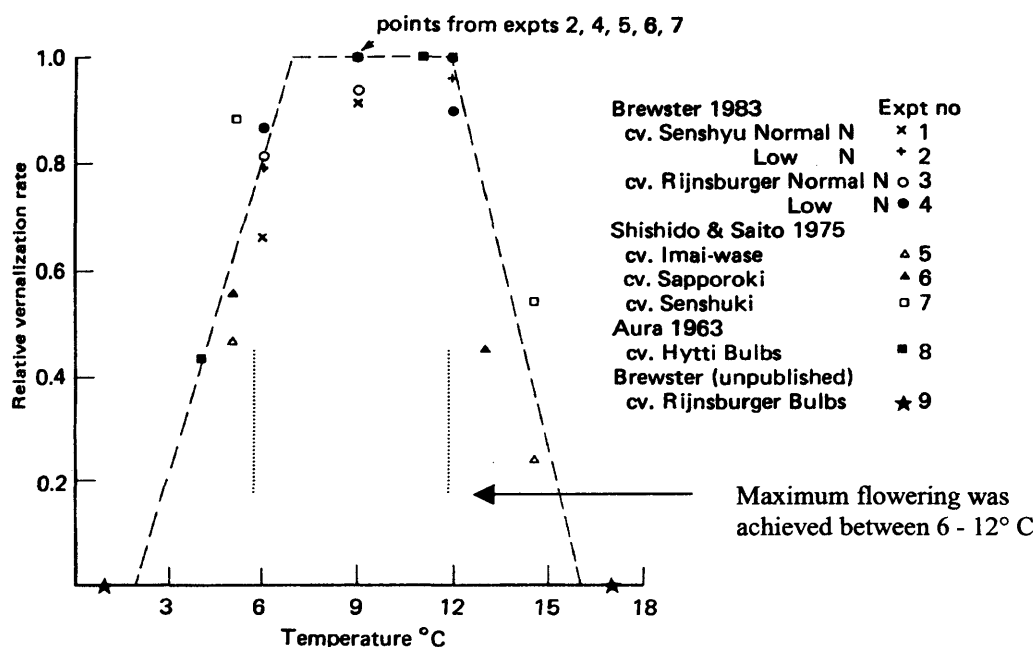
number for floral competence was probably less than the 5-true-leaves-visible stage recorded by Weibe (1994). However, as the minimum leaf number for floral competence varies with carbohydrate levels in *A. cepa* (Brewster, 1985, above), leaf count as a predictor of maturity may also be variable in other *Allium* spp. and environmental conditions.

The interplay between photoperiod, fluence rate and temperatures is complex. For example, in *A. cepa* a low fluence rate ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) combined with high temperatures (25°C) required longer to initiate inflorescences than plants raised at higher fluence rate of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the lower temperature of 17°C (Brewster, 1985). Initiation time for *A. cepa* at 9°C was accelerated by long photoperiods; initiation was as rapid at 12°C as at 9°C but was slower at 6°C (Rabinowitch, 1990). Also in *A. cepa*, levels of nitrogen fertiliser may influence floral development (Rabinowitch, 1990). A reduction in the nitrate concentration accelerates inflorescence initiation, particularly in photoperiods and temperatures not normally conducive to rapid initiation, but with different cultivars showed differing degrees of response (Brewster, 1983). Some *Allium* crops, e.g. Chinese chives (*A. tuberosum*) require long photoperiods for inflorescence initiation and further differentiation (Saito, 1990; Van der Meer and Hanelt, 1990; De Clercq and Van Bockstaele, 2002).

1.7.1.2 Vernalization

Vernalization is defined as a cold treatment given to seeds or plants, which induces flowering when the plant is exposed to more favourable conditions (e.g. long days

and 20°C). It is slow and quantitative, most plant species requiring 1 – 3 months of low temperatures (1-7°C), and with vernalization becoming more effective with increased duration (Dennis *et al.*, 1996). Flowering may be induced in *Alliums* by low temperatures provided bulbs are beyond their juvenile stage of development (Kamenetsky and Rabinowitch, 2002). Many reports have shown that temperatures between 5 - 15°C are likely to stimulate inflorescence development in *Alliums*, whilst lower or higher temperatures are likely to inhibit it (Rabinowitch, 1985). Brewster (1987) developed a mathematical model for the prediction of the response



to vernalization of several *A. cepa* cultivars.

Figure 12: Relative rate of vernalization vs. temperature of Japanese and European onion cvs. (Adapted from Brewster, 1987).

Data from a number of experiments were combined and scored relative to the fastest flowering rate (Figure 12). This model was used to predict flowering in the field with a high degree of accuracy. This predicts maximum flowering between

approximately 6 - 12°C, with percentage flowering reduced at higher or lower temperatures (Brewster, 1987).

Vernalization is effective, when the low temperature treatment includes the shoot apex. However, Wellensiek (1964), working with *Lunaria biennis*, proposed that all actively dividing cells may be capable of responding to vernalization. More recent work into vernalization in *Thlaspi arvense* and *Arabidopsis thaliana*, supports this view (Metzger, 1988; Burn *et al*, 1993).

A. cepa can be vernalized during dormancy as well as during active growth, for example cv. Ailsa Craig (Rabinowitch, 1990; Kamenetsky and Rabinowitch, 2002). Bertaud (1988) examined vernalization in three *A. cepa* cultivars. Those chilled before sprouting largely failed to respond, with higher rates of flowering being obtained if they were sprouted for eight weeks before chilling. Mature bulbs with green leaves failed to produce flowers when grown on at lower temperatures. Whilst mature plants initiated inflorescences more readily than unsprouted bulbs, both were slower than sprouted plants. The apex may have become dormant in the mature plants following the growing season, suggesting that dormant apices are less responsive to vernalization than active apices (Bertaud, 1988). Brewster, (1994) found that the minimum dry weight required by dry bulbs (*A. cepa*) to initiate flowering during storage was much higher than in growing plants, also suggesting changes in the level of response to vernalization of dormant and active apices. This is consistent with the view that in dormant bulbs, internal processes may continue in preparation for rooting and sprouting, with mitosis declining, but not ceasing (Gubb and McTavish, 2002).

Older plants (*A. cepa*) require less chilling (Rabinowitch, 1990; Kamenetsky and Rabinowitch, 2002). Most studies have shown that the time for initiation was minimal when temperatures were in the range of 5 - 12°C. However, it is highly variable depending not only on maturity, but also on genotype, and may even be variable within the same cultivar from different sources (Rabinowitch, 1990).

Weibe (1994) investigated the temperature effects on flowering in *A. ampeloprasum* var. *porrum* suggesting that it probably has an obligatory vernalization requirement with optimum temperature of approximately 5°C, more plants bolting with 6 weeks vernalization than with 3 weeks. The inductive temperature ranged from 0 - 18°C, with long days during vernalization delaying induction, whilst temperatures higher than 18°C caused devernization. Wurr (1997) suggested that 7°C was optimum for this variety, with a range of 2 - 25°C. Further study by Wurr *et al.*, (1999) confirmed that cv. Prelina needed a vernalization stimulus. The optimum temperature for vernalization was 7°C, though not all plants became floral suggesting some genotypic variation, with variation in expression from year to year (Wurr *et al.*, 1999).

There is widespread variation in the temperatures that induce vernalization in *A. ampeloprasum* var. *porrum* cultivars. Van Doorne *et al.* (1988) induced *A. ampeloprasum* var. *porrum* to flower in the same year of planting by vernalizing for 6 weeks at 4°C. Rubatzky and Yamaguchi (1997) suggest that many *A. ampeloprasum* var. *porrum* cultivars will bolt when grown continuously at or less than 15°C, with some bolting even at 21°C. However, in others, temperatures above

18°C may cause devernalization (Rubatzky and Yamaguchi, 1997). To my knowledge, vernalization requirements in var. *babingtonii* have not been published. This has been addressed in this thesis.

Starch mobilization is an essential process in the control of the flowering transition in some plants, for example, *A. thaliana* and *Sinapis alba* (both facultative long day plants, and facultative cold requiring) (Bernier *et al.*, 1993). *Arabidopsis thaliana* is a useful genetic model for floral induction because it has many well-characterized metabolic mutants, as well as numerous flowering mutants. Sucrose, the major sugar in leaf and apical exudates in *A. thaliana*, increases rapidly in induced plants, preceding the activation of energy-consuming processes such as mitosis. However, the promotive effect of vernalization on flowering is, in fact, unrelated to starch metabolism (Bernier *et al.*, 1993). Dennis *et al.*, (1996) suggest that the vernalization-dependent pathway is normally blocked by methylation of the promoter of a gene or genes necessary for floral induction. They propose that vernalization reduces the methylation status of the gene(s), resulting in expression of kaurenoic acid hydroxylase (KAH), a key enzyme in the gibberellic acid biosynthetic pathway necessary for the promotion of flowering. Ronemus *et al.*, (1996) suggest that DNA methylation is an essential component in the process of phase transitions and meristem determinacy, and speculate that a methylation gradient might be established during meristem growth, that directs meristem determinacy. It is suggested that flowering is normally actively repressed beginning from embryonic development, but the genetic basis for the perception and response to vernalization has not been fully explored in any plant species (Dean *et al.*, 1999).

1.7.1.3 Gibberellins (GAs)

Exogenous GAs can induce or promote flowering in many species, particularly long day (LD) plants (Evans, 1969; Evans *et al.*, 1990) and have been extensively used to promote flowering in *Lilidae*. For example, GA has been successfully used with *Zantedeschia* (Funnell *et al.* 1992; Corr & Widmer, 1991), *Gladiolus* (Misra, Tripathi and Chaturvedi, 1993), and *Spathiphyllum* (Ogawa *et al.* 1994; Shibata & Endo, 1990; Eltorky, 1993). GAs are implicated in the autonomous pathway (see Section 1.7 Floral development) and, it is suggested, promote flowering in *Arabidopsis thaliana* by functioning in combination with sucrose to activate the promoter of the meristem identity gene, *LEAFY*, (Blasquez *et al.*, 1998).

Hanks (1979) reported the use of gibberellins to advance flowering in *Tulipa* spp. in combination with vernalization, suggesting that the cold treatment has two components;

- An absolute requirement, unaffected by gibberellin, allowing for extension of internodes,
- And a secondary requirement, for optimum forcing for which gibberellin can substitute.

More recent studies with *Lolium temulentum* ‘Ceres’ have demonstrated that photoperiodic induction results from two signals (McDaniel and Hartnett, 1996):

- The first, of an unknown nature (possibly sucrose, Levy and Dean 1998b), switched the developmental fate of the shoot meristem from commitment to produce leaves to commitment to produce flowers;
- The second signal, gibberellin (GA), triggered expression of this florally determined state.

When gibberellin was applied to the leaves, it did not act directly on the apex to cause floral determination and initiation; rather, it appeared to stimulate the production of a signal in the leaves which led to floral induction (McDaniel and Hartnett, 1996). Application of GAs may bypass vernalization completely since they

act during processes such as floral evocation which occur well after the cold treatment (Levy and Dean, 1998b). Vernalization may increase the sensitivity of plants to GAs, although the GAs themselves may play no part on the vernalization process itself (Chouard, 1960; Levy and Dean 1998b). Levy and Dean (1998b), suggest that application of GAs is not sufficient to overcome a requirement for vernalization in the majority of species studied to date, and this includes most cereals and non-rosette plants. However, Brewster and Butler (1989) used gibberellic acid (GA₃) on *A. cepa* prior to chilling, increasing the flowering of some of the genotypes studied. The effects of GAs on *Alliums* are varied; for example, they have been applied to *A. cepa*, to improve cropping (bulb diameter and weight), (Sobeth and Wright 1988), and to increase seed production by approximately 30% (g/m² (Naamni, *et al.*, 1980). GAs also inhibit flowering in some short-day (SD) plants (Evans, 1969; Evans *et al.*, 1990).

Rates of application vary widely, as do methods of application, which include spraying (Naamni, *et al.*, 1980), immersion, surface application (Evans, *et al.*, 1990) and injections directly into the bulb (Brewster and Butler, 1989). The method of application will necessarily affect the amount of GA absorbed into the plant tissues and the effectiveness of the treatment. Different gibberellins will induce different responses. For example, Evans *et al.*, (1990) found variation of over 1000-fold in the effective dose of different GAs needed to initiate flowering in *L. temulentum*, while different doses of the same gibberellin may produce different responses in the same species. Working with *Lolium temulentum* (LD plant), Evans *et al.*, (1990) found the minimum dose for inflorescence initiation was 3µg applied in 10µl of 95% ethanol:water (v/v) near the base of the uppermost expanded leaf blade, as a single

application. Smaller doses produced stem elongation, with different GAs often affecting either inflorescence initiation or stem elongation, or both.

1.7.2 Inflorescence development

The morphology of the inflorescence in *Allium ampeloprasum* L. var. *babingtonii* was recorded in 1847 (English Botany, Suppl. 4: t. 2906) (Figure 1) and confirmed by Treu, (1999). However, little is known of the development of the scape and the early stages of the inflorescence, an issue which has been addressed in this thesis, with *A. cepa* and other *Allium* species being used as a model.

1.7.2.1 Environmental influences

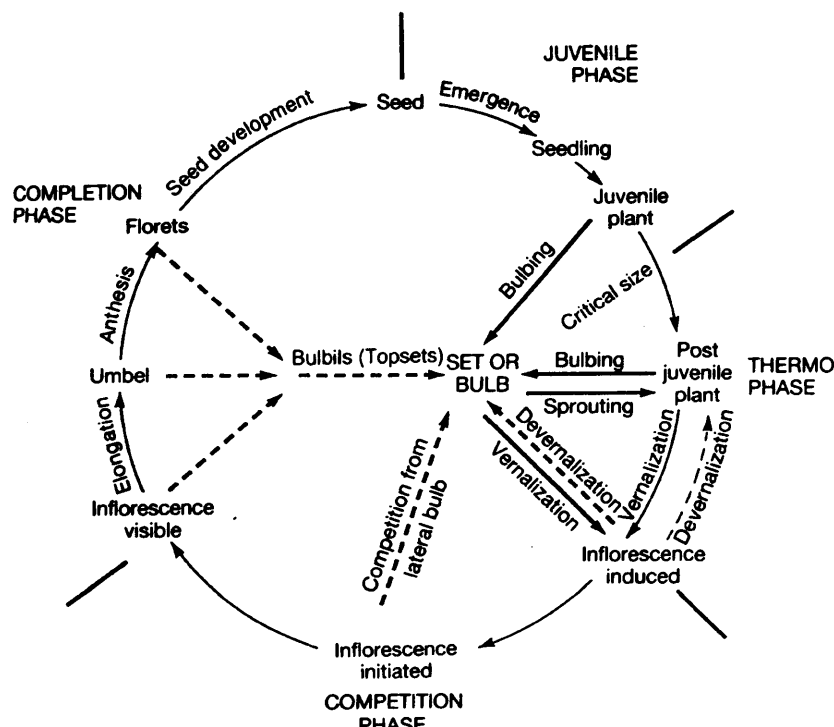


Figure 13: Stages in the life cycle of onion (*A. cepa*) plants with particular reference to flowering. Unusual or reversionary developmental processes are shown by dotted lines (Brewster, 1994).

Scape elongation and flowering in *Allium* species is largely mediated by interactions between storage and growth temperatures as well as light conditions (Brewster,

1982; Kamenetsky and Rabinowitch, 2002). Rabinowitch (1990) defines three temperature phases necessary for inflorescence production in *A. cepa* (Figure 13):

- The 'thermo-phase' where exposure to low temperature is essential for floral initiation (vernalization)
- The 'competition-phase' where growth and development of the inflorescence can be suppressed by conditions favourable to bulbing (e.g. long days and high temperatures).
- Higher temperatures and longer days favour inflorescence development.

Changes in temperature can both enhance and suppress scape development, being implicated both in devernalization, and in the formation of bulbils (Figure 13).

The effects of temperature are highly dependent on genotype as well as maturity (Rabinowitch, 1990). Temperature may be:

- Optimal - inflorescences are induced and developed
- Supraoptimal - high temperatures, in which little or no floral initiation takes place, with any that are initiated being damaged or destroyed
- Suboptimal - low temperatures at which a longer time is required for initiation, and where floral development may be suppressed or delayed (Rabinowitch, 1990).

Cool temperatures of approximately 10 - 17°C enhance scape elongation in *A. cepa* (Holdsworth and Heath, 1950; Krontal *et al.* 2000), while high temperatures of approximately 25 - 30°C suppress emergence of already initiated inflorescences (Heath and Mathur, 1944; Holdsworth and Heath, 1950; Rabinowitch, 1985; Krontal *et al.* 2000). They may abort completely when conditions are unfavourable (Bertaud, 1988; Kampen, 1970; Roberts and Struckmeyer, 1951). In long days, abortion of inflorescences occurs most often before emergence (where the inflorescence is still small at the time of transfer from chilling conditions), whereas in cool short days, abortion tends to occur after emergence (Bertaud, 1988). In contrast, Both Brewster (1994) and De Clercq and Van Bockstaele (2002) reported that there is no specific photoperiod requirement for inflorescence development in

A. ampeloprasum var. *porrum*, a characteristic which facilitates the growth of the same genotype over a wide range of latitudes. To my knowledge environmental effects on floral development var. *babingtonii* have not been published.

1.7.2.2 The scape

During the transition to flowering in *A. cepa*, rib meristematic activity increases, the flattened vegetative apical meristem becomes rounded and dome shaped as rapid longitudinal growth commences (Figure 14).

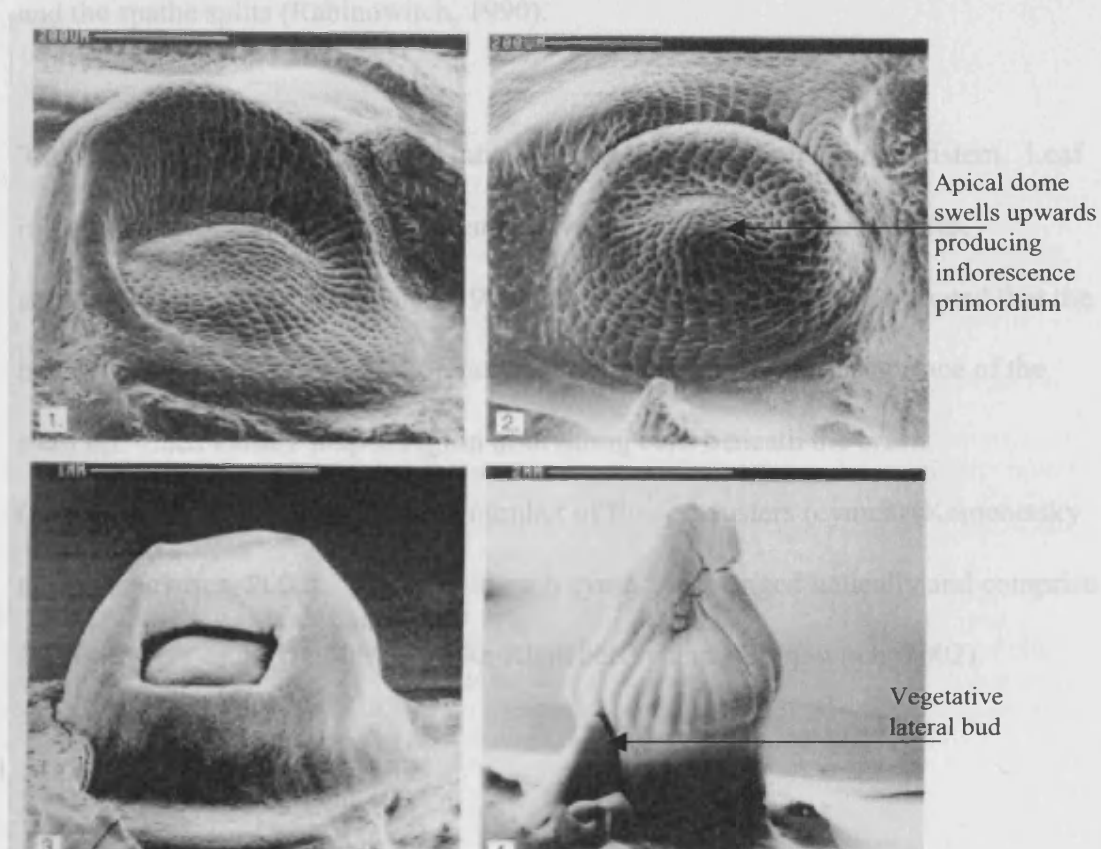


Figure 14: Scanning electron micrographs of successive stages of inflorescence differentiation at the onion (*A. cepa*) shoot apex. 1, non-floral; 2, early floral; 3, spathe begins to overgrow the apical dome; 4, the ridged spathe surrounds the apical dome and a definite stalk (scape) is visible. A vegetative lateral bud is present adjacent to the scape, which can sometimes compete with the young inflorescence (adapted from photographs courtesy of Horticultural Research International, the copyright holder).

The scape arises from the vegetative shoot apical meristem and is therefore terminal, and represents one internode, this being the only one to elongate (Brewster, 1994; De Mason, 1990). Cell division is confined to the more basal regions of the scape, with elongation occurring predominantly in the lower parts of the scape, between the last formed leaf and the spathe (Brewster, 1994; De Mason, 1990). At the base of the inflorescence, the cells are small and little tissue differentiation occurs. At the top of the inflorescence axis, the cells are larger and the greatest amount of differentiation occurs (De Mason, 1990). The scape continues to develop until flowering begins and the spathe splits (Rabinowitch, 1990).

The spathe forms in the apex, and quickly elongates, enveloping the meristem. Leaf initiation ceases and the apical meristem divides into (usually four) centres, separated by bracts (Rabinowitch, 1990). Jones and Emsweller (1936) noted that the bracts were numerous and membranous, developing over the broad surface of the stem tip. Each kidney shaped region of dividing cells beneath the bracts (Rabinowitch, 1990) gives rise to a number of flower clusters (cymes) (Kamenetsky and Rabinowitch, 2002). The buds in each cyme are arranged helically and comprise 5 - 10 flowers each (De Mason, 1990; Kamenetsky and Rabinowitch, 2002).

1.7.2.3 Floret development

Treu (1999) examined floret development in var. *babingtonii*. The tepals were pink, maturing to purple, and the florets do not fully open at any stage of inflorescence development. This may possibly be associated with the very poor development of the anthers, which were not always exerted (protruding beyond the perianth). The

number of pollen grains per anther and the percentage pollen stainability (using aceto-carmine for absolute counts, and cotton blue for pollen viability estimation) was extremely low or zero. Ovaries showed considerable abnormalities with only 36 out of a possible 240 ovules being present (four individuals), and those that were present were small or malformed (Treu, 1999). He suggested that there may be a total meiotic or pre-meiotic breakdown. The spathe splits longitudinally into two persistent valves, in contrast with other *A. ampeloprasum* spp. where it separates as a whole from the inflorescence by a horizontal split all round its base and is then cast off intact (Borrer, 1847; Stearn, 1987).

Mathew (1996) describes the inflorescence in *A. ampeloprasum* spp.:

- "Spathe 1-valved, ovate at base, abruptly narrowed to a long beak up to about 10(-13) cm long, caducous.
- Umbel normally with flowers only, but rarely with flowers and bulbils mixed, spherical, (3-) 5 - 8 (- 9) cm diameter, dense.
- Pedicels unequal, up to 4.5 cm long, smooth; bracteoles present, silvery-white, lacinate at the apex.
- Perianth broadly campanulate or subspherical; segments white, pink or deeper reddish-pink, sometimes with a darker green or purple median vein, (3.5 -) 4 - 5.5 mm long, with large sparse papillae on the outer surface, especially on the keel, the outer ones oblong-lanceolate, elliptic or elliptic-obovate, subacute, shortly mucronate, the inner ones ovate, spatulate or elliptic, obtuse, rounded or rarely truncate, equalling or shorter than the outer.
- Stamens with anthers shortly exerted or sometimes equalling the segments; filaments white or purplish, strongly arching outwards, usually ciliate at the base, the outer ones simply and narrowly triangular or with an oblong base narrowed to a triangular apex, rarely with minute lateral cusps, the inner one with the anther-bearing cusp a third to half as long as the very widely expanded undivided basal part and about half as long as the lateral cusps; lateral cusps much exceeding the segments; anthers yellow or purplish-red.
- Style usually exerted. Capsule ovoid or subglobose"

Floret development in *A. cepa* has been recorded in detail, (see, for example, Rabinowitch, 1990; Brewster, 1994). The flowers do not open in a regular pattern, so that buds and flowers at all stages of development can be present all over the head, in varying proportions in different regions of the umbel (Rabinowitch, 1990). However, within a cyme, the flowers open in a definite sequence, and there is a

tendency for flowering to start at the top centre of the umbel, and proceed downwards (Rabinowitch, 1990) (Figure 15).

From outside to inside, the floret develops three members in five whorls, outer and inner perianth, outer and inner stamens and then carpels, developing as globose projections (Brewster, 1994). Jones and Emsweller, (1936) reported that each perianth lobe with its subtended stamen arose simultaneously from a single primordium the first whorl comprising outer tepals and stamens, the second whorl comprising inner tepals and stamens. This pattern is also reported in *A. sativum* (Kamenetsky and Rabinowitch 2001). However, De Mason (1990) reports that the whorls arise outermost first, the outer tepals followed by the inner tepals, then the outer stamens followed by the inner stamens. The carpels develop as three U-shaped swellings on the surface, which grow towards the centre, where their inturned edges meet and fold to form the ovules (two in each carpel). The style arises at the apex of the fused carpels and is still elongating when the floret opens (Brewster, 1994). The ovaries include nectaries on the outer walls, (Fritsch, 1992) opening to the surface through a pore (Kamenetsky and Rabinowitch, 2002). Comparable floral characterisation is lacking in var. *babingtonii*, an issue which has been addressed in this thesis.

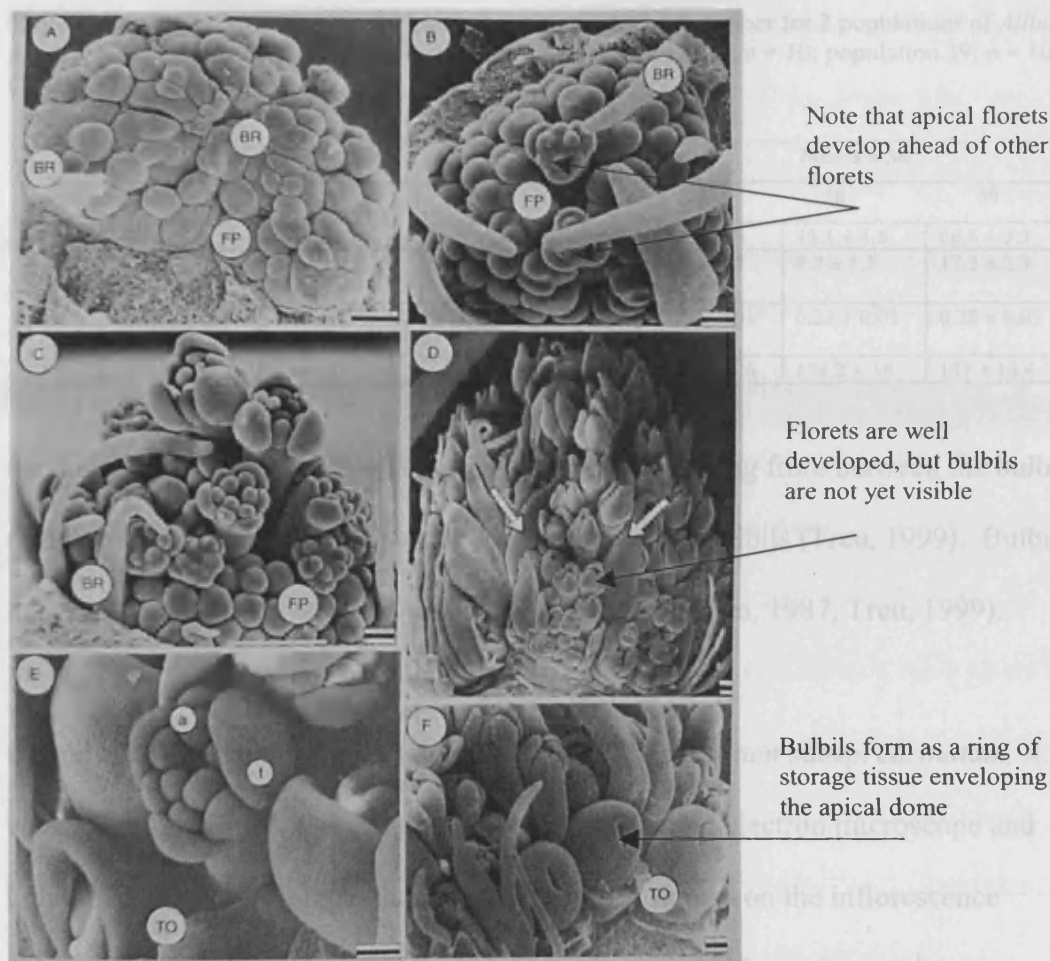


Figure 15: Scanning electron photomicrographs of *Allium* spp. Floral development. Bar = 0.1 mm. A. Flower primordia (FP) in shallot inflorescence. Four centres of differentiation are separated by the bracts (BR). Spathe and peripheral and central bracts removed. B. Early stages of garlic floral development. Floral differentiation is visible in older flower primordia (FP), while younger flowers still appear as meristematic domes. Leaflike bracts (BR) form at the periphery of the inflorescence. Spathe removed. C. the inflorescence of garlic becomes hemispherical and consists of numerous floral primordia (FP). Differentiation of FP is uneven: floral parts occur in the oldest floral primordia, while younger ones still appear as undifferentiated meristematic domes. Spathe removed. D. Floral pedicels and leaf-like bracts elongate. In individual garlic flower clusters (arrows), which are separated by leaf-like bracts, floral primordia develop unevenly in a helical order. New flower primordia continue to appear at the base of the inflorescence. E. Magnification of the basal part of garlic inflorescence. Newly developed meristems appear and rapidly differentiate to form small inflorescence bulbs (referred to as topsets - TO). In the florets, tepals (t) and anthers (a) are visible. F. Topsets (TO) in garlic inflorescence (adapted from Kamenetsky and Rabinowitch, 2002).

1.7.2.4 Bulbil development

Treu (1999) recorded the numbers of bulbils compared with the number of florets produced in var. *babingtonii* (Table 6). There was a weak positive statistical correlation between the number of florets and the number of bulbils.

Table 6: Summary statistics of bulbil number/weight and flower number for 3 populations of *Allium ampeloprasum* var. *babingtonii*. Population 1, n = 70; population 38, n = 10; population 39, n = 10 (Treu, 1999).

Population number	Range			Means \pm SE		
	1	38	39	1	38	39
Number of bulbils	24 - 110	24 - 79	49 - 76	49.5 \pm 2.0	45.1 \pm 4.8	60.6 \pm 3.3
Total bulbil weight (g) inflorescence ⁻¹	3.5 - 29.5	2.7 - 18	10- 23.9	11.7 \pm 0.7	9.7 \pm 1.5	17.3 \pm 2.3
Mean weight (g) bulbil ⁻¹ inflorescence ⁻¹	0.07 - 0.77	0.08 - 0.43	0.17- 0.45	0.24 \pm 0.01	0.22 \pm 0.03	0.28 \pm 0.03
Number of flowers	12 - 275	51 - 210	105- 204	110.1 \pm 6.6	124.2 \pm 16	147 \pm 10.4

Secondary inflorescences were sometimes produced arising from between the bulbils (range 0 – 19, mean 5.9 ± 0.6) and these also produced bulbils (Treu, 1999). Bulbils may sprout whilst still attached to the inflorescence (Stearn, 1987, Treu, 1999).

Cottrell (1999) examined bulbil development in *A. carinatum* subsp. *carinatum*, *A. vineale* and *A. paradoxum* using a low vacuum scanning electron microscope and light microscopy. Bulbils in these species arose as domes on the inflorescence meristem. They developed as a central dome, surrounded by an incomplete ring. During the early stages, the dome was taller than the ring, and developed into the apical meristem. The ring enlarged and grew up to cover the dome, enclosing it completely and forming the storage tissue (Cottrell, 1999). Early inflorescence bulbil primordia were indistinguishable from floret primordia, becoming distinguishable when approximately 0.4mm in diameter and 0.38mm in height (Cottrell, 1999). The shape of developing bulbils was also influenced by the development of adjacent structures (Cottrell, 1999), similar to the process described by Treu (1999) for var. *babingtonii*, where the pressures created by the close proximity of adjacent tissues caused malformations and distortions in development.

A. sativum L. (garlic) produces an inflorescence with sterile florets and bulbils (Kamenetsky and Rabinowitch, 2001). Although not part of the *Ampeloprasum* complex, this species has been much studied and may provide a useful comparative model for bulbil development in *A. ampeloprasum* var. *babingtonii*. Flower-stalk elongation precedes the swelling of the inflorescence meristem, the floral initials differentiating after the scape has reached 5 – 7 mm with an apex diameter of > 0.5mm (Kamenetsky and Rabinowitch, 2001). The apex subdivides into several centres of floral development, each of which gives rise to several floret primordia. Bracts develop at the periphery and in the centre of the inflorescence dividing the umbel into distinct floral clusters. Within these clusters, primordia develop unevenly in a helical pattern, florets appearing before topsets (bulbils). New flower primordia continue to arise as older ones develop (Figure 15). New undifferentiated domes form at the base of the inflorescence, which then develop into bulbils. Their size, rate of development and number show great variability and are determined by genotype. Once the spathe opens, the florets are visible to the naked eye, but the rapidly growing bulbils appear to stifle them and they quickly degenerate (Kamenetsky and Rabinowitch, 2002). In some clones, removal of the bulbils can result in normal flowering, pollination and seed production (Koul and Gohil, 1970b; Etoh and Simon, 2002). Each bulbil is able to grow into a new clonal plant (Koul and Gohil, 1972).

Sterility in *A. sativum* has been assumed to result variously from degeneration of the tapetum, degenerative-like diseases induced by organisms and/or viruses, chromosomal deletions or competition for nutrients between floral and vegetative buds (Kamenetsky and Rabinowitch, 2001). Fertility can be restored in some clones

by continuous removal of developing topsets (bulbils) (Kamenetsky and Rabinowitch, 2002).

1.7.2.5 Manipulation of bulbil production

Treu (1999) suggested that the bulbils in var. *babingtonii* may have a constricting effect on the pedicels of the florets, causing early withering. However, removal of bulbils from the inflorescence only caused florets to persist approximately 10 days longer than intact inflorescences, and seed was not produced. If the development of bulbils caused destruction of the developing florets either directly through mechanical pressures, or indirectly via diverted nutrients from florets, there should be a negative correlation between bulbil number and floret number. Treu (1999) did not find such a correlation, and suggested that the production of bulbils may be a back-up mechanism within *Allium* following loss of flowers or seed production.

Florets have been removed from several *Allium* species to promote bulbil formation. For example, Andrew (1951) concluded that clipping caused bulbil development in *A. cepa*. Cottrell (1999) studying *A. carinatum* subsp. *carinatum*, *A. vineale* and *A. paradoxum*, made the same conclusion, though results were highly variable. *Allium ampeloprasum* var. *porrum* may form bulbils (Schweisguth 1970, 1972; Rubatzky and Yamaguchi, 1997; De Clercq and Van Bockstaele, 2002), and has traditionally been treated by growers in this way to propagate desirable lines (Brewster, 1994). Koul and Gohil (1970a & b; 1972) examined the relationship between developing bulbils and flowers in *A. sativum*. They also suggested that floret sterility may be linked with diversion of nutrients into bulbils, the bulbil initials being accelerated when the sexual cycle breaks down at microsporogenesis. When bulbils were

removed from developing inflorescences, the life of the flowers was prolonged, but male gamete development proceeded no further than the production of microspore tetrads followed by microspore degeneration while still within the common wall, the same stage as when bulbils remained on the inflorescence. Female gamete development was prolonged, but again fertility was not restored. Koul, Gohil and Langer (1979) also note that in different clones of *A. sativum*, sexuality is thwarted at different states of organogeny, sporogenesis and gametogenesis.

Bulbil production in *Alliums* has been linked with both temperature and day length. Short days are implicated in some *Alliums*, when combined with high or low temperatures, whilst high temperatures alone may be sufficient in other *Allium* spp. For example, Brewster (1994) suggested that bulbil production was affected by temperature, with high storage temperatures causing reversion of floral parts to a vegetative state, if the inflorescence has reached a certain state of development. Yamada (1961), working with *A. cepa* var. *multiplicans*, found that higher temperatures increased the number of bulbils in mixed inflorescences. Aura (1963) found that bulbils were induced in *A. cepa*, when they were stored for 6 weeks at 28 – 31°C, following coldstorage at 3 – 13°C for 5 – 6 months. Bulbs stored at 21°C after coldstorage did not produce bulbils. Aura (1963) proposed that high temperatures caused a reversion of the inflorescence, returning to a vegetative state, therefore forming bulbils. It was also suggested that a more complete reversion would be obtained, if the bulbs were treated sooner with the high temperature regime, following coldstorage. Indeed, some treated onions produced an inflorescence consisting of only one large bulbil. If the treatment was delayed, the inflorescence was more likely to consist of mixed bulbils and florets. A more

advanced state of floral development requires longer treatment to cause reversion (Kamenetsky and Rabinowitch, 2002).

Roberts and Struckmeyer (1951) suggested the production of bulbils (*A. cepa* ‘Rochester Bronze’) could be attributed to a combination of short days and high temperatures (9 hours, 20°C minimum) during scape development. In contrast, Bertaud (1988) found that at 15°C in 8h days, many of the inflorescences (*A. cepa*) contained bulbils or malformed florets, and suggests that low floret numbers and malformations observed in cool short days are a physiological response to temperatures too low for adequate floral development.

The influence of plant growth regulators on bulbil production in *Alliums* has been studied. For example, Andrew (1951) suggested that some *Alliums* have the potential for bulbil production even if it was not always expressed. He sprayed clipped inflorescences of *A. cepa* with various auxins, but no increase in bulbil production was observed. However, inflorescences were treated when the spathe was almost ready to open, which may have been too late to divert development from floret to bulbil. Thomas, (1972) used benzyladenine (a cytokinin) at 10mg/l to increase the number of bulbils on some onion cultivars. Vest, Subramanya and Jackson (1977) induced bulbils in onion inflorescences by treating bulbs with PBA (6-benzyl-9-tetrahydropyran adenine) in methyl Cellosolve® before planting. Interestingly, Wang, Tan and Ji (1988) found that *A. sativum* bulbils produced abscissic acid (ABA) which was translocated to the scape, and this may be a factor in

the prolonged life of the florets observed by Koul and Gohil (1972) (*A. sativum*) and Treu (1999) (*A. ampeloprasum* var. *babingtonii*).

1.8 Genetic pathways in floral determination and development

1.8.1 Initiation of flowering

Plants have complex pathways controlling the transition to floral development (see section 1.7.1). These pathways are of two general types:

- Pathways designed to sense environmental cues, e.g. changes in daylength, temperature or soil moisture;
- Autonomous pathways that involve the state of development, e.g. many species must pass through a juvenile phase before acquiring floral competence (Aukerman and Amasino, 1998).

The examination of mutants of *Arabidopsis thaliana* that are defective or abnormal in their floral response, and the corresponding wild-type genes that complement these mutant phenotypes, have identified three different functions (Weigel and Meyerowitz, 1994):

- Meristem identity genes, responsible for the positive initial induction of the genes that specify organ identity;
- Cadastal genes, which are spatial regulators of the genes controlling organ identity;
- Organ identity genes with direct control of organ identity, presumably by activating downstream genes (Weigel and Meyerowitz, 1994).

Lyndon (1994) separates the meristem identity genes into two further groups, giving four different groups of homeotic genes that are involved in floral determination and development:

- Those allowing or accelerating the change to floral growth;
- Those causing the floral identity in inflorescence side shoots.
- Those that establish the boundaries for the action of the floral growth and identity (spatial regulators)
- Those that specify particular organs (organ identity genes);

Genes controlling flowering have been well documented in the model plant *Arabidopsis thaliana* and show complex interactions between pathways (Figure 16).

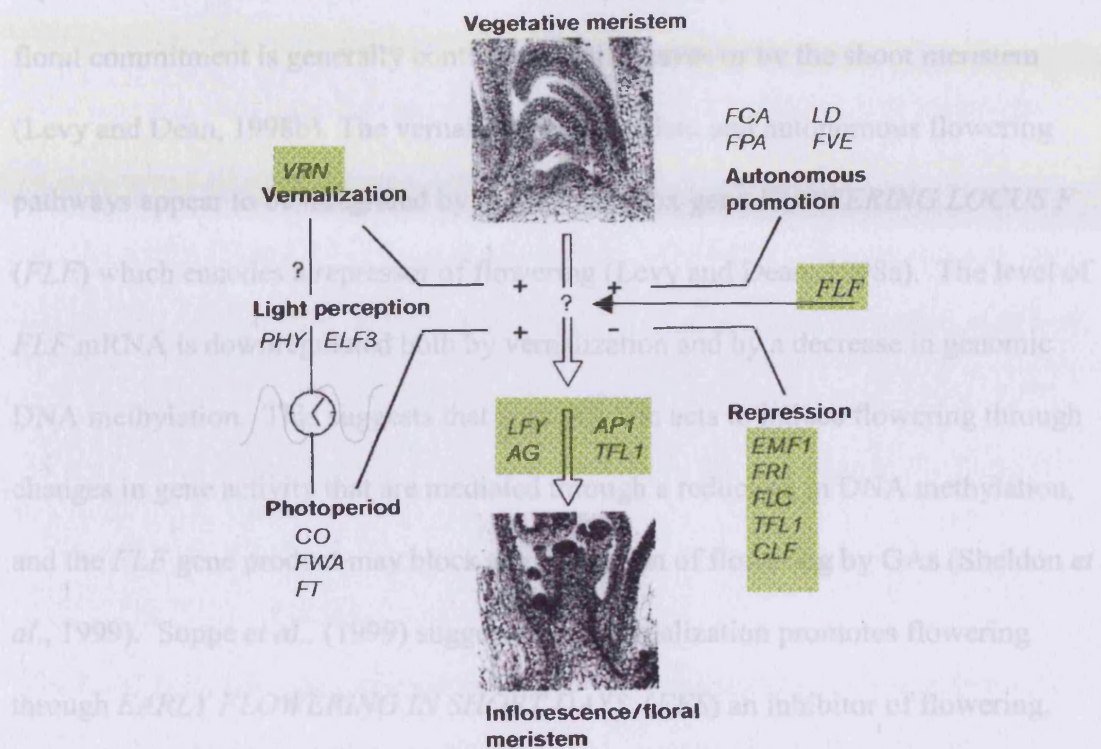


Figure 16: Some of the physiological pathways and genes controlling flowering in *Arabidopsis thaliana*. Physiological studies have identified different pathways that either promote (+) or repress (-) the transition of the apical meristem from vegetative to inflorescence/floral development. The circadian clock is implicated in the measurement of photoperiod via the perception of light. *Arabidopsis thaliana* strains in which flowering is promoted by vernalization also show strong acceleration of flowering by far red-enriched light, so vernalization and perception of light quality appear to be closely related processes. The inputs from the different pathways are somehow integrated (symbolised by a ?) and eventually lead to activation of inflorescence/floral meristem identity genes (adapted from Levy and Dean, 1998a).

Levy and Dean (1998b) suggest there are at least four pathways in *Arabidopsis thaliana*; two appear to monitor the endogenous developmental state of the plant. Firstly there is a floral repression pathway that may prevent flowering until the plant has reached a certain age or size, secondly an autonomous promotion pathway that is believed to increasingly antagonise this repression as the plant develops. The other two pathways mediate signals from the environment, and include the photoperiodic promotion pathway, and the vernalization promotion pathway.

Not all interactions have been tested directly and little is known about how the floral repressors interact with the various promotive pathways; neither is it known whether floral commitment is generally controlled by the leaves or by the shoot meristem (Levy and Dean, 1998b). The vernalization-dependent and autonomous flowering pathways appear to be integrated by the MADS¹ box gene *FLOWERING LOCUS F* (*FLF*) which encodes a repressor of flowering (Levy and Dean, 1998a). The level of *FLF* mRNA is downregulated both by vernalization and by a decrease in genomic DNA methylation. This suggests that vernalization acts to induce flowering through changes in gene activity that are mediated through a reduction in DNA methylation, and the *FLF* gene product may block the promotion of flowering by GAs (Sheldon *et al.*, 1999). Soppe *et al.*, (1999) suggested that vernalization promotes flowering through *EARLY FLOWERING IN SHORT DAYS*, (*EFS*) an inhibitor of flowering, specifically involved in the autonomous promotion pathway.

FRIGIDA (*FRI*) is another gene that represses flowering in *Arabidopsis thaliana*, and whose action is antagonised by vernalization. It confers a dominant requirement for vernalization and is the major determinant for flowering time variation (Dean *et al.*, 1999). Repression of flowering by *FRI* requires dominant alleles at the *FLOWERING LOCUS C* (*FLC*) locus, which may act antagonistically to *FCA*, *FPA* and *FVE* (Sanda and Amasino, 1996).

¹ MADS box genes were so-called because a consensus sequence was shared by homeotic genes of totally unrelated species (M = minichromosome maintenance factor in humans, A = *agamous* in *Arabidopsis thaliana*, D = *deficiens* in *Antirrhinum*, S = serum mating factor in yeast).

FLC is the best characterised of the genes required for the late flowering conferred by *FRI*. It encodes a MADS-box gene and is therefore likely to function as a transcriptional regulator (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

FCA interacts with meristem identity genes in multiple pathways, one leading to activation of *LEAFY* (*LFY*) and *APISTILLATA1* (*API*), and another one acting in parallel with *LFY* and *API* (Dean *et al.*, 1999). *CONSTANS* (*CO*) triggers flowering irrespective of daylength, and when expressed, it initiates rapid transcription of both *LFY* and *TERMINAL FLOWERING* in long days (*TFL*) (Simon *et al.*, 1996). *LFY*, *API*, *AG* (*AGAMOUS*) and *TFL1* are all important in the transition to the floral state.

1.8.2 The Floral Initiation Process (FLIP)

The floral homeotic genes encode transcriptional regulators, all of which (with the exception of *AP2*), contain a MADS box (a conserved DNA-binding domain) (Jurgens, 1997). Once *LFY* expression has been initiated by the flowering time genes (above), its continued expression is necessary for further development of the floral initiation process (Figure 17). It is implicated in formation of new flower primordia, suppression of leaf formation and internode elongation, and enhances expression of other genes such as *API* and *CAUFLOWER* (*CAL*) for continuing development (Pidkowich *et al.*, 1999).

Activation of the FLIP genes is coordinated, independent of one another and becomes progressively stronger as floral development proceeds, with the rate of FLIP activation dependent on *TFL1* activity and daylength.

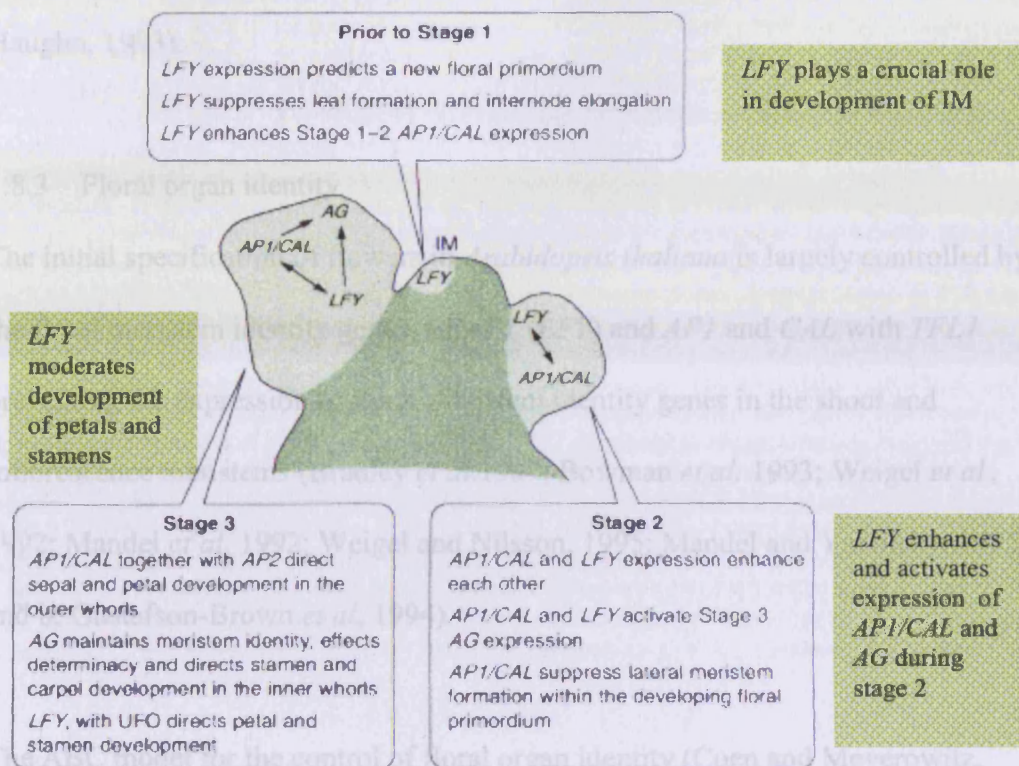


Figure 17: Functional relationship between *LFY*, *AP1/CAL* and *AG*. *LFY* is expressed before other FLIP genes. Early in floral meristem identification, other FLIP genes are activated independent of *LFY*, but their expression is relatively weak. One role of *LFY* is to enhance the activation of *AP1*, *CAL* and *AG* at this stage of development. In turn, *AP1* and *CAL* enhance expression of *LFY*. Once expressed the FLIP genes are required to direct different aspects of floral development. For example, *LFY* is required for petal and stamen development and plays a role in the activation of *AG*, while *AP1* is required for sepal and petal development and indirectly for stamen and carpel development by activating the organ identity gene *AG*. *AG* maintains meristem identity in the centre of the floral primordium as well as promoting stamen and carpel development. (IM – inflorescence meristem; UFO - UNUSUAL FLORAL ORGANS) (adapted from Pidkowich *et al.*, 1999).

Schultz and Haughn (1993) stated that *Arabidopsis thaliana* must have a mechanism that monitors developmental time, activating morphological programs associated with phase transition (inflorescence to floral states), as has been suggested for other species. This mechanism must be responsive to the environment, with the activity level of the factor(s) changing with the number of nodes produced. As critical levels are reached, morphological programs are activated. Controller(s) of Phase Switching factors (COPS), co-ordinately activates the FLIP genes, resulting in the

morphological changes associated with inflorescence phase change (Schultz and Haughn, 1993).

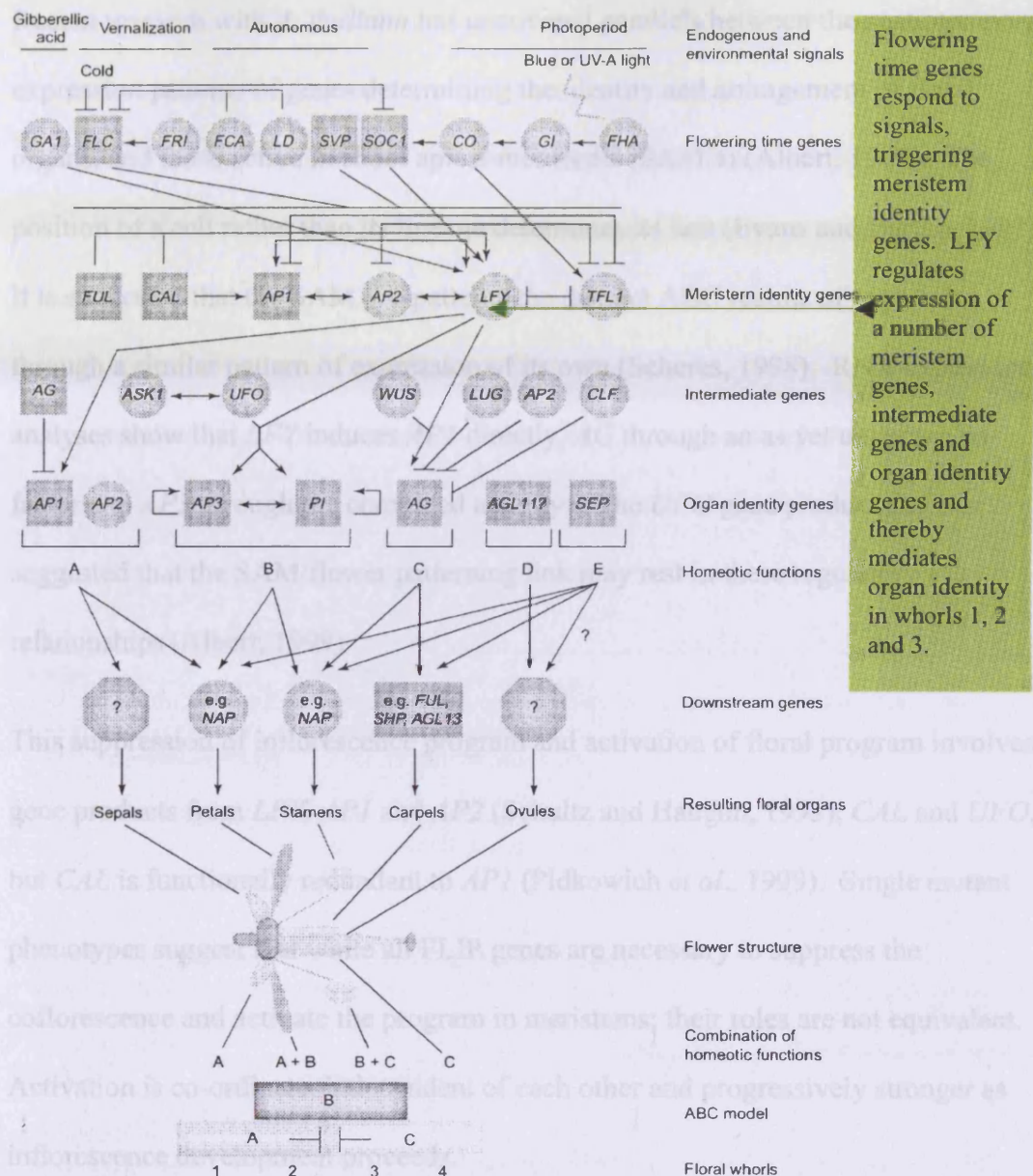
1.8.3 Floral organ identity

The initial specification of flowers in *Arabidopsis thaliana* is largely controlled by the floral meristem identity genes, *LEAFY* (*LFY*) and *API* and *CAL* with *TFL1* preventing the expression of floral meristem identity genes in the shoot and inflorescence meristems (Bradley *et al.* 1997; Bowman *et al.* 1993; Weigel *et al.*, 1992; Mandel *et al.*, 1992; Weigel and Nilsson, 1995; Mandel and Yanofsky, 1995a and b; Gustafson-Brown *et al.*, 1994)

The ABC model for the control of floral organ identity (Coen and Meyerowitz, 1991) suggests that three classes of genes with overlapping fields of expression control whorl identity. Class A genes act in whorls one and two; Class B act in whorls two and three; Class C genes act in whorls three and four. Class A and those of Class C are mutually exclusive, genes from each restricting the expression of the other. However, Class A appears to influence Class B expression (Schultz and Haughn, 1993; Jurgens, 1997) (Figure 18).

Alvarez and Smyth (1997) suggested a further class of whorl genes designated 'class D genes' that would include those found to act only on carpel development, for example, *CRABS CLAW* (*CRC*) and *SPATULA* (*SPT*).

Figure 18: Simplified, preliminary depiction of the genetic hierarchy that controls flower development in the eudicot model plant *Arabidopsis (thaliana)*. Examples of the different types of genes within each level of the hierarchy are shown. 'Gibberellic acid', 'vernalization', 'autonomous' and 'photoperiod' refer to the different promotion pathways of floral induction. 'Intermediate genes' summarizes a functionally diverse class of genes including 'cadastral genes'. MADS-box genes are shown as squares, non-MADS-box genes as circles and genes whose sequences have not been reported as octagons. Some regulatory interactions between the genes are symbolized by arrows (activation), double arrows (synergistic interaction) or barred lines (inhibition, antagonistic interaction). Not all the known genes and interactions involved in flower development are shown. In the case of the downstream genes, just one symbol is shown for each type of floral organ, although whole cascades of many direct target genes and further downstream genes are probably activated in each organ of the flower. At the bottom of the figure, a generic flower diagram is shown with the classic 'ABC model' of floral organ identity. According to this model, floral organ identity is specified by three classes of 'floral organ identity genes' providing 'homeotic functions' A, B and C, each of which is active in two adjacent whorls. A alone specifies sepals in whorl 1; the combined activities of A and B specify petals in whorl 2; B and C specify stamens in whorl 3; and C alone specifies carpels in whorl 4. The activities of A and C are mutually antagonistic, as indicated by barred lines: A prevents the activity of C in whorls 1 and 2, and C prevents the activity of A in whorls 3 and 4. Abbreviations: AG, AGAMOUS; AGL, AGAMOUS-like gene; AP, APETALA; ASK,1 ARABIDOPSIS SKP-like 1; CAL, CAULIFLOWER; CO, CONSTANS; FLC, FLOWERING LOCUS C; FRI, FRIGIDA; FUL, FRUITFULL; GI, GIGANTEA; LD, LUMINIDEPENDENS; LFY, LEAFY; LUG, LEUNIG; NAP, NAC-like, activated by AP3/PI, PISTILLATA; SEP, SEPALLATA; SHP, SHATTERPROOF; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CO1; SVP, SHORT VEGETATIVE PHASE; UFO, UNUSUAL FLORAL ORGANS; TFL1 TERMINAL FLOWER1 (adapted from Soltis *et al.*, 2002)



Recent research with *A. thaliana* has uncovered parallels between the spatiotemporal expression patterns of genes determining the identity and arrangement of floral organs, and those active in shoot apical meristems (SAM's) (Albert, 1999). The position of a cell rather than its lineage determines its fate (Evans and Barton, 1997). It is suggested that the SAM pre-patterns the distinct ABC realms of expression through a similar pattern of expression of its own (Scheres, 1998). RNA expression analyses show that *LFY* induces *AP1* directly, *AG* through an as yet unknown co-factor and *AP3* through the combined activity of the *UFO* gene product and it is suggested that the SAM/flower patterning link may rest in these regulatory relationships (Albert, 1999).

This suppression of inflorescence program and activation of floral program involves gene products from *LFY*, *AP1* and *AP2* (Schultz and Haughn, 1993), *CAL* and *UFO*, but *CAL* is functionally redundant to *AP1* (Pidkowich *et al.*, 1999). Single mutant phenotypes suggest that while all FLIP genes are necessary to suppress the coflorescence and activate the program in meristems; their roles are not equivalent. Activation is co-ordinate, independent of each other and progressively stronger as inflorescence development proceeds.

Both *LFY* and *AP1* are expressed in response to environmental conditions such as day length, and presumably also in response to the action of genes that affect the time of flowering (Schultz and Haughn, 1993). Simon *et al.*, (1996) further propose that *CO* acts within one genetic pathway to activate *LFY* and *TFL*, but that an additional pathway is required for rapid activation of *AP1*.

In *Arabidopsis*, therefore, *LFY* is a ‘master transducer’ of environmental signals to the floral developmental program. *LFY* controls the switch between vegetative and floral growth responding to both gibberellin and light stimuli, and directs or co-directs the transcription of key components of the ABC system for flower organ determination (Albert *et al.*, 2002).

In most diploid angiosperms studied to date, the *LFY* sequence exists as a single orthologous nuclear locus, though in most gymnosperms two divergent *LFY* paralogs are found, with sex-specific expression in conifers (Albert *et al.*, 2002).

Table 7: Some patterns of *LFY* expression

Gene	Expression	Reference
<i>ALF</i> ; <i>Actinidia deliciosa</i> (kiwi)	First order axillary buds (later to become floral) floral meristems	Walton <i>et al.</i> (2001)
<i>FLO</i> ; <i>Antirrhinum majus</i>	Floral meristems and bracts	Carpenter and Coen (1990); Coen <i>et al.</i> (1990)
<i>LEAFY</i> ; <i>Arabidopsis</i>	Floral meristems and organs (but also expressed at low levels in leaf primordia)	Schultz and Haughn (1991); Huala and Sussex (1992); Weigel <i>et al.</i> , (1992); Blasquez <i>et al.</i> , 1997)
<i>BOFH</i> ; (<i>Brassica oleracea</i> var. <i>botrytis</i> (cauliflower)	Curd initiation to petal formation (not in vegetative meristems)	Anthony, R. G. <i>et al.</i> , (1996); Anthony <i>et al.</i> (1993)
<i>ELF1</i> ; <i>Eucalyptus globulus</i>	Developing floral organs, leaf primordia, young leaves	} Southerton <i>et al.</i> , (1998)
<i>ELF2</i> ; <i>Eucalyptus globulus</i>	Pseudo-gene	}
<i>RFL</i> ; <i>Oryza sativa</i>	Young panicles and epidermal cells of young leaves during vegetative phase	Kyozuka <i>et al.</i> , 1998
<i>FALSIFLORA</i> <i>Lycopersicon</i> (tomato)	Floral and vegetative meristems, leaf primordia and leaves, all floral organs	Molinero-Rosales <i>et al.</i> (1999)
<i>AFL1</i> ; <i>Malus</i>	Floral buds	} Wada <i>et al.</i> , (2002)
<i>AFL2</i> ; <i>Malus</i>	Vegetative apices, floral buds and organs	}
<i>NFL1</i> ; <i>Nicotiana tabacum</i>	Vegetative and floral meristem	Kelly <i>et al.</i> , (1995); Ahearn <i>et al.</i> (2001)
<i>NEEDLY</i> ; <i>Pinus radiata</i>	Vegetative buds, male cone primordia	Mouradov <i>et al.</i> (1998); Mellerowicz <i>et al.</i> , (1998)
<i>PRFLL</i> ; <i>Pinus radiata</i>	Vegetative buds, male cone primordia	Mellerowicz <i>et al.</i> , (1998)
<i>PTLF</i> ; <i>Populus trichocarpa</i>	Developing inflorescences, leaf primordia, young leaves, esp. in apical vegetative buds near inflorescences; seedlings	Rottmann <i>et al.</i> (2000)

The link with *LFY* (or its homologues) and floral expression is clear in many plants, though this pattern of expression is not repeated in all plants investigated to date

(Table 7). *LFY* homologues have also been found in Citrus spp. (Daming *et al.*, 2001), *Lolium temulentum* (Gocal *et al.*, 2001) and *Zea mays* (corn) (Andrews *et al.*, 2000).

In spite of considerable work on the flowering and breeding of geophytes, little is known of the basic chain of processes involved in normal flowering, including gene and protein expression (Kamenetsky and Rabinowitch, 2002). Molecular markers for developmental phases are highly necessary for further work (Le Nard and De Hertogh, 2000). The importance of *LEAFY* in the change from the vegetative to the floral in a number of angiosperms, combined with the wealth of information regarding the structure and function of this gene, makes it a suitable candidate for study in this species. Nothing is known about the floral genes in *Allium ampeloprasum* var. *babingtonii*, and one aim of my work was to identify the presence of the homologue of *LFY* and to clone it, examining its expression in vegetative and floral tissues.

1.9 Aims

This literature review has focussed on floral morphogenesis in bulbs of *Allium* spp. alongside model species such as *Arabidopsis thaliana*. The commercial significance of *Alliums* has been highlighted together with a specialised review of available literature on *A. ampeloprasum* var. *babingtonii* using more fully studied *Alliums* such as *A. ampeloprasum* var. *porrum*, *A. cepa* and *A. sativum* to provide insights into possible mechanisms. Clearly, the ability of *A. ampeloprasum* var. *babingtonii* to exhibit flowers and bulbils means that shoot apical meristems of this species can exhibit different fates. It therefore emerges as a very useful model to study cell fate

during floral morphogenesis. Hence, a clear aim of the work reported in this thesis was to resolve the mechanisms by which shoot apical meristems can switch floral fate. However, in order for this species to serve as a model for understanding the switch to flower or bulbil production, various standardisation experiments were necessary, including the establishment of precise tissue culture conditions that enabled the integration of both morphological and molecular changes into a precise developmental timetable. Moreover, the development of an appropriate protocol for fixing and staining material for histological examination was a necessary technique to underpin this study.

The aims of the work presented in this thesis were:

- 1 To establish a population of *Allium ampeloprasum* var. *babingtonii* as a source of material of known age
- 2 To develop a protocol for the maintenance of *Allium ampeloprasum* var. *babingtonii* in culture
- 3 To establish a histological protocol for use with *Allium ampeloprasum* var. *babingtonii*
- 4 To construct a developmental timetable for *Allium ampeloprasum* var. *babingtonii* for vegetative and floral growth *in vivo*
- 5 To identify the nature and timing of floral determination in *Allium ampeloprasum* var. *babingtonii*
- 6 To identify and clone a homologue to the meristem identity gene *LEAFY* in *Allium ampeloprasum* var. *babingtonii* and examine its expression in the floral transition.

2.0 Sources of *Allium ampeloprasum* var. *babingtonii* and *Allium ampeloprasum* var. *ampeloprasum* plant material

2.1 Cornish populations

Treu (1999) recorded a number of populations in Cornwall, of which the largest (Population 1, Appendix 3) was near Newquay (SW 784567-771584), with approximately 5000 individuals of *Allium ampeloprasum* var. *babingtonii*. This population was mainly linear in dispersion, growing in intermittent single rows along the side of a public footpath.

The Botanical Society of the British Isles (BSBI) confirmed that a licence would not be needed to collect the plants, as it is not a scheduled species and not considered to be threatened (Treu, 2000). The Cornish Wildlife Trust further confirmed that numbers were increasingly rapidly (Hocking 2000, *personal communication*) and that no harm would be done to the population if samples were taken. Part of the footpath is owned by Holywell Bay Holiday Park, who gave permission for small numbers of samples to be taken. Ownership was unclear for other parts of the population, therefore they were not sampled. Sampling of inflorescences was accomplished in August when the inflorescence was likely to be fully developed, but not yet dehiscing (Treu, 1999).

2.1.1 August 1999 (SH99)

Mature plants were not taken, as digging was felt to damage the amenity landscape of the Holiday Park. Samples were taken as entire inflorescences from plants selected from the population, ensuring that some bulbils were taken from each site within this

population (Appendix 5). Randomization was ensured as far as possible within these limitations, by taking every 10th visible inflorescence from this largely linear population.

The scapes were brown and dry, many florets had been lost, and those that remained were withered. Some bulbils were still green, but most had formed brown, papery outer layers. There was no foliage on the plants. Each inflorescence was removed with part of the scape still attached, and labelled with sample number and location (Appendix 5). Each head was placed in a pollen bag, to contain the bulbils as they dehisced, whilst allowing reasonable air movement to deter fungal rots.

Smith (1999) recommended storage of the bulbils at a temperature as close to 0°C as possible, but avoiding freezing. They were stored in a cooled incubator, initially at 1°C, but as this produced occasional sub-zero temperatures, in response to environmental changes, this was raised to 3°C. Others were grown on in outdoor beds to provide plants of known age for developmental data. Three hundred bulbils sampled at random (SH99) were grown at University College Worcester (UCW). They were planted in 13 cm pots of John Innes No. 2 compost (Treu, 1999) during September 1999 and grown on in cold frames for protection against environmental extremes and predation. A further 500 were planted similarly during January 2000, as the sprouting rates from the September planting were poor. By May 2000, only 4% of the September planting had sprouted compared with 62% of the January planting. Non-sprouting bulbils were examined, and found to be necrotic. Contributing factors were likely to be inadequate drainage and ventilation. This could be particularly pertinent to the earlier planting, as the bulbils

were suffering poor drainage during wet and cold winter periods. Smith (*personal communication*, 2000) suggested that *Alliums* would be unlikely to thrive in conditions with restricted root growth (this was later confirmed by personal observation of *Allium ampeloprasum* var. *babingtonii* grown in containers at UCW since 1995 for other projects). Therefore, all surviving plants from the cold frames were transplanted to outdoor beds. The site had light, sandy, well-drained soil, resembling the soil from the Holywell Bay site, was sheltered, and faced southwest. Irrigation was supplied as required.

2.1.2 August 2000 (SH00)

Permission was obtained to sample a small number of mature plants that could be grown on at UCW. They were located by their scapes, and were sampled from a number of points within the site as previously. The whole plants were potted into John Innes No. 2 compost at the site to reduce disturbance and breakage to the plants. They were planted at UCW, on a site facing south-west, with sandy, well-drained soil in October, 2000 (Appendix 6). They had developing roots and shoots and they were planted with root ball intact as far as possible. The plants were spaced approximately 45 cm apart, and watered to assist in re-establishment. The inflorescences were contained in pollen bags, remaining on the plant until dehiscence.

It became apparent that the population continued extensively further West, into the Holiday Park itself. This site was mown regularly from making it difficult to estimate numbers, but was likely to be several thousand. Plants could only be located from

November to March, when foliar growth was allowed to proceed. Permission was obtained to sample these plants the following year.

2.1.3 March 2001 (SH3/01)

Growing plants were collected from within the holiday park, and grown on in beds at UCW as above (Appendix 7). Samples were not obtained from the footpath as this was closed as a result of the Foot and Mouth epidemic. Sampling was dictated by accessibility together with the need to avoid causing damage to the maintained landscape. As far as possible, they were taken from varied locations within the Holiday Park. Bulb size (maximum width and height as previously) was recorded where still present, together with maximum leaf length and the number of visible leaves.

2.1.4 November 2001 (SH 11/01)

Intact inflorescences were collected from the footpath as available, many of the inflorescences having already dehisced. Sprouting mature bulbs were taken from the campsite where accessible, as previously. These were selected for a minimum bulb diameter of 3cm for examination of floral development. Bulbs were measured across the largest part of the diameter, many of the bulbs being asymmetrical (Appendix 8).

2.1.5 March 2002 (SH 0302)

Mature plants were selected, so that floral development *in vivo* could be examined. Plants with a bulb diameter of at least 3 cm were required, to fulfil the maturity requirement established by this time. However, bulbs were necrotic and disintegrating,

therefore estimation of size was largely subjective. Plants were sampled from a number of sites from both footpath and within the campsite, as previously from as many different locations as possible, this being limited by accessibility and the need to preserve the landscape, as before. Plants were actively growing therefore they were sampled with root balls intact as far as possible, and packed in John Innes No. 1 compost for transport. Maximum leaf length was recorded in 10 intact plants but many outer leaves were showing signs of necrosis, or damage, probably from mechanical effects, pests and diseases (Appendix 9).

2.2 Nursery suppliers

Allium ampeloprasum var. *babingtonii* is also marketed by a small number of nurseries (Appendix 1) (Royal Horticultural Society, 2000). John Shipton Nurseries (Camarthen) had the largest population (20) and confirmed that his stock plants had Cornish origins. Parent plants were inspected during flowering (September 2000) to confirm identity, and 20 dormant bulbs were purchased. These were two year old bulbs, expected to flower in 2001 (Shipton, 2000, *pers. comm.*). Also purchased were 20 *Allium ampeloprasum*, var. *ampeloprasum*, for developmental comparison. This variety has been recorded in only three sites in the past twenty years, and numbers are declining (Wiggington, 1999). It has been Red Data Book Listed to protect remaining populations (French *et al.*, 1999). Therefore, sampling from the wild was not an option, and John Shipton Nurseries was the only source material for this species. Maximum bulb width, height and weight were recorded before planting (Appendix 10). Sources and types of material obtained are summarised in Table 8.

Table 8: Summary of sources of *Allium ampeloprasum* var. *babingtonii* and *Allium ampeloprasum* var. *ampeloprasum*

	Date	Material	Method	Ref.
Var. <i>babingtonii</i>	August 1999	Bulbils from footpath	Stored at 3°C/grown on	SH99
	August 2000	Bulbils from footpath	Stored at 3°C	SH00
	August 2000	Whole plants from footpath	Grown on	SH00p
	Sept. 2000	Dormant bulbs from John Shipton Nurseries	Grown on	JS00
	March 2001	Whole plants from holiday park	Grown on	SH03/01
	Nov. 2001	Whole plants from holiday park and footpath	Grown on	SH11/01
	Nov. 2001	Bulbils from holiday park and footpath	Stored at 3°C	SH11/01
	Mar. 2002	Whole plants from holiday park and footpath	Grown on	SH03/02
Var. <i>ampeloprasum</i>	Sept. 2000	Dormant bulbs from John Shipton nurseries	Grown on	JS00amp

3.0 Development of protocol for wax embedding, sectioning and staining of *Allium ampeloprasum* var. *babingtonii* samples for histological examination

Examination of changes from the vegetative to the floral phases in var. *babingtonii*, required monitoring of meristem development over a period of time representing vegetative growth, floral determination and the production of inflorescence meristems and floral meristems, as well as morphogenesis of the inflorescence itself. The activity of the terminal meristem involves cellular processes; therefore, with few exceptions, study of meristems involves the preparation of stained sections from fixed material (Steeves and Sussex, 1989).

Tissue is fixed as rapidly as possible to prevent distortion and decay as far as possible (Peacock, 1966). There are many fixatives, embedding materials and stains appropriate for different tissues. Clarke's Fluid is widely used for fixing plant material, acts rapidly and causes little shrinkage of tissues, fixing cytoplasm and nuclei (Peacock, 1966). Tissue may be embedded in blocks such as paraffin wax for sectioning, enabling sections of as little as 2µm thickness to be prepared. This also preserves the morphology and the wax is easily removed for further processing (Schwarzacher and Heslop-Harrison, 2000). Herbert (1991) fixed, wax embedded and stained sections of *Pharbitis nil*, and this protocol was modified for use with *Allium* spp. by Cottrell (1999). However, she noted difficulties with penetration of wax and brittle tissues. A protocol was developed based on Herbert (1991) and Cottrell (1999) for fixing, wax embedding and staining, suitable for *A. ampeloprasum* var. *babingtonii*. This permitted the examination of vegetative and floral material for recording developmental changes over a period of time.

Meristem development in some *Alliums* has been studied extensively, (e.g. *A. cepa* Brewster, 1994). Here the apical meristem is in the centre of the upper bulb surface where the leaves initiate, whilst another meristem – the primary thickening meristem- is separate from this and doughnut shaped around the apical meristem. This gives rise to roots and thickening of the basal plate (see section 1.6.1.1, Figure 7) with many *Alliums* (for example, *A. ampeloprasum* var. *porrum*, *A. sativum*) sharing similar developmental characteristics (Brewster, 1994). A comparable developmental pattern might therefore be expected in var. *babingtonii*.

Angiosperms generally share similar patterns of cell development in the meristem. The outermost layers (tunica) show largely anticlinally oriented cell divisions, and generally consist of one to five layers, with the majority of species having two layers (Steeves and Sussex, 1989). The number of layers may vary in the same plant in some species, or may fluctuate seasonally or in relation to primordia initiation. Below this is the corpus where planes of cell division are less regularly oriented (Steeves and Sussex (1989). The cells of the apex above the youngest leaf primordia may be small, nearly isodiametric, thin-walled, with a high nucleocytoplasmic ratio and inconspicuous vacuolation (Steeves and Sussex, 1989).

In a number of species, a group of cells at the summit of the apex, including both tunica and corpus have been recognized as being somewhat larger and are designated tunica and corpus initials. This central zone is likely to stain less densely with histological stains, both nuclear and cytoplasmic. Here cells divide infrequently, and are surrounded by a peripheral region composed of small, densely staining cells in which divisions appear to be more frequent. The Feulgen reaction (Appendix 11)

specifically identifies DNA, and this lighter staining correlates with larger, albeit thinly stained nuclei; the DNA content is the same as in other cells (Steeves and Sussex, 1989). Fainter cytoplasmic staining may be related to the highly vacuolated state of these cells, this typically being large numbers of small vacuoles giving a frothy appearance, rather than the small numbers of large vacuoles in mature or maturing cells (Steeves and Sussex, 1989).

Development of a satisfactory protocol for use with var. *babingtonii* would allow the meristem to be compared with the patterns in other angiosperms, as well as recording development from the vegetative to floral phases.

3.1 Materials and methods

3.1.1 Wax embedding

Bulbils were selected at random from SH99 (see 2.2 Table 8) as this material was readily available in large quantities, and would also provide much of the source material for later tissue culture work. All samples for treatments were selected using random numbers.

Explants were dissected out from the bulbils, taking the centre 'post', from the base to the apex. This was approximately 1-2 mm wide, 1-2 mm in depth, and the height of the bulbil. The top portion was then removed, leaving a post approximately 3-4 mm in height (Figure 19).

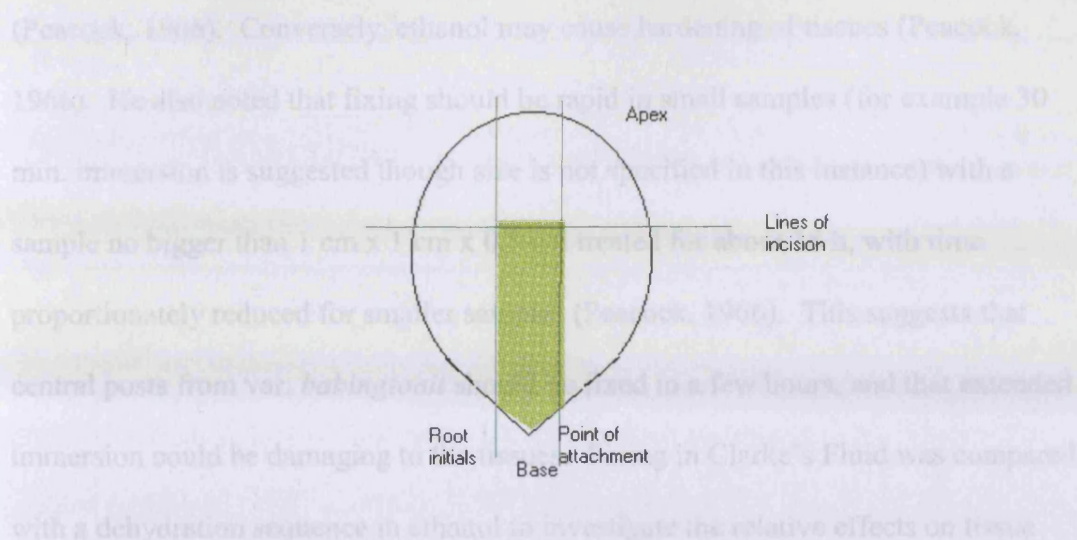


Figure 19: Excision of centre post of tissue. This section included the apical shoot, part of the basal plate, root initials and point of attachment.

The protocol for wax embedding was modified from Herbert (1991) (*Pharbitis nil*), and Cottrell (1999) (*Allium* spp.) (Table 9).

Table 9: Fixing and wax embedding protocol (Herbert, 1991; Cottrell, 1999)

Treatment	Time	
	Herbert 1991	Cottrell 1999
Fixing in Clarke's Fluid	Overnight	2 + d
100% ethanol	2 x 15 min. rinses	1 – 3 d
Ethanol: Histo-Clear 1:1 (v/v)	2 x 15 min. rinses	1 d
100% Histo-Clear	2 x 1 h	1 – 3 + d
Histo-Clear:paraffin oil (1:1 v/v)	2 h	1 d
100% paraffin oil	2 h at 60°C	7 + d
Paraffin oil:paraffin wax at 60°C	Overnight	2 d
Paraffin wax at 60°C	6 h	2 d
Blocks were cooled at -20°C	-	Yes

Cottrell (1999) noted difficulties with this method, and similar difficulties were initially experienced with var. *babingtonii*. Wax penetration into the tissues was poor, samples were brittle and the wax blocks were frequently fractured. Clarke's Fluid (3:1 v/v 100% ethanol:glacial ethanoic acid) is suggested for general work, fixing cytoplasm and nuclei without causing hardening of tissues, though glacial ethanoic acid may soften tissue, rendering samples unsuitable for embedding

(Peacock, 1966). Conversely, ethanol may cause hardening of tissues (Peacock, 1966). He also noted that fixing should be rapid in small samples (for example 30 min. immersion is suggested though size is not specified in this instance) with a sample no bigger than 1 cm x 1 cm x 0.5 cm treated for about 18 h, with time proportionately reduced for smaller samples (Peacock, 1966). This suggests that central posts from var. *babingtonii* should be fixed in a few hours, and that extended immersion could be damaging to the tissues. Fixing in Clarke's Fluid was compared with a dehydration sequence in ethanol to investigate the relative effects on tissue destruction or hardening.

Samples were allocated at random to the treatments (Table 11). The tissue was either fixed in Clarke's Fluid, (Appendix 11) for three days at room temperature in histology cassettes (Tissue-Tek) then rinsed twice in 100% ethanol, or subject to a dehydration series of 5 min. each in 30 % ethanol, 50 % ethanol, 70 % ethanol, 90 % ethanol, 100 % ethanol, then immersed for a further 60 min. in 100 % ethanol.

All samples were subsequently placed in 1:1 (v/v) 100 % ethanol:Histo-Clear (National Diagnostics HS-200), for approximately 24 h, followed by immersion in 100 % Histo-Clear for a further 24 h. Wax infiltration was initiated by placing the cassette into 1:1 (v/v) Histo-Clear:paraffin oil (Paraffin liquid BDH 294375J) for approximately 24 h, then into 100% paraffin oil for approximately 24 h, then paraffin oil:paraffin wax (Paramat BDH Gurr 36133 4C) at 60°C for 24 h, and finally, into paraffin wax at 60°C for 24 h.

Table 10: Comparison of samples fixed in Clarke's Fluid and samples dehydrated in ethanol

Fixing	Ethanol:Histo-clear	
A) 5 min. each in 30%, 50%, 70%, 90% 100% ethanol, then 60 min. in fresh ethanol	24 hours	Embedding as above
B) 3 days in Clarke's Fluid		Embedding as above

The tissue was embedded in freshly melted wax. Some samples were positioned for transverse sectioning (TS) and some for longitudinal sectioning (LS). They were cooled rapidly to -20°C (Cottrell, 1999) then stored at 4°C.

3.1.2 Sectioning

The wax blocks were sectioned using a Spencer Microtome set at 5 µm. The ribbons of sections were floated on the surface of a water bath at 42°C containing 4 ml/l subbing solution (Appendix 11). Slides were previously ethanol washed (70 % aq.), and air-dried at room temperature. Samples were positioned on the slides and air-dried horizontally at room temperature.

3.1.3 Staining

After drying for approximately 24 h, the slides were placed in slide racks and immersed in Histo-Clear for 20 min. to remove the wax (Table 12). This was followed by a hydration series with 5 min. immersion each of 100%, 70%, 50%, and 30% ethanol (aq.). They were then rinsed in distilled water.

Sections were hydrolysed by immersion in 1M HCl for 8 min. at 60°C, and then transferred to Schiff's Reagent for 1 h to stain nuclear material. This was followed

by immersion in 45 % Ethanoic Acid for 1 min., then two rinses in SO₂ water (Appendix 11) to halt the reaction and remove excess colouration.

The slides were then put through a dehydration series from 30 %, 50 %, 70 % and 100 % (aq.) ethanol in preparation for Light Green Stain (Appendix 11). Slides were immersed in Light Green Stain for 20 s, and then rinsed twice for 5 min. in 100 % ethanol. Finally, the slides were rinsed twice in Histo-Clear and allowed to air dry.

Table 11: Staining procedure for *Allium* apical sections (modified from Herbert 1991; Cottrell 1999)

Treatment	Time (min.)
Histo-Clear	20
100% ethanol	5
70% ethanol	5
50% ethanol	5
30% ethanol	5
Distilled water	5
1M Hydrochloric acid at 60°C	8
Schiff's Reagent	60
45% Ethanoic acid	1
Sulphur dioxide water	1
Sulphur dioxide water	1
30% ethanol	5
50% ethanol	5
70% ethanol	5
100% ethanol	5
Light green stain	20 seconds
100% ethanol	1
100% ethanol	1
Histo-Clear	1
Histo-Clear	1

Samples deteriorated during the staining process, with tissue missing, either partially or completely. This was most noticeable following the hydrolysis and immersion in Schiff's Reagent. The effect of hydrolysis in hydrochloric acid was assessed using differing immersion periods from 5 to 10 min.

Prepared slides were selected at random, and then assigned to a time period randomly. They were processed as above, but with different immersion times in hydrochloric acid.

Randomly selected slides were mounted as above, using pre-subbed slides. These had been immersed in subbing solution (1 % gelatine, 1 % potassium dichromate w/v aq.) and allowed to air dry (approximately 48 h at room temperature) before use.

3.2 Results

3.2.1 Wax embedding

Samples that were fixed without immersion in Clarke's Fluid, had inadequate penetration, and complete samples were not produced. Approximately 80 % of slides were missing or incomplete. Therefore, this method was discontinued. Assessment of the quality of the slides is largely subjective, therefore data are not presented.

Some of the inner layers of tissue detached from the wax during sectioning. This was thought to be due to traces of moisture forming a barrier between the wax and the tissues. Therefore, a dehydration series was added to the protocol, after the Clarke's Fluid. Samples were immersed for 5 min. each in 80 %, 90 % and 100 % ethanol (Clarke's Fluid being 75 % ethanol). Initially this made no discernible difference, and was therefore incrementally increased to 1 d at each concentration, and samples improved. Increasing the times of immersion of later stages to 2 or 3 d also increased the quality of the samples, with tissue more likely to remain intact when sectioned, and to have complete penetration of the wax to the centre of the apex. The wax was clearer, suggesting improved removal of water from the tissues. Further adjustments to the protocols were made at intervals after examination of the previous results; assessment of quality is largely subjective, the aim of the protocol being to reliably

produce slides with complete penetration of wax and intact tissues. Therefore, data are not presented. Modification was continued until intact slides could be reliably reproduced. The protocol was considered satisfactory when in excess of 80 % slides remained intact with complete penetration of wax.

Some of the blocks showed fracturing (approximately 70 %), leading to breakage during sectioning. Therefore, randomly selected blocks were allowed to cool at room temperature. These showed no fracturing of the wax, therefore rapid cooling was discontinued. Larger samples generally had less effective wax penetration. Satisfactory results were achieved when the size of the sample was <2mm width and depth, 4-7mm height following the revised protocol. However, samples needed to be large enough to allow for correct orientation during the embedding process.

3.2.2 Sectioning

Once wax penetration was complete, sectioning could proceed at 5 μ m. Particular tissues in later work were prone to brittleness, and these were very young bulblets, root tips and florets. All these tissues are notable for the lack of depth of tissue and the recent morphogenesis of the tissue. It seems likely that a protocol rigorous enough to soften the cell walls and allow complete penetration of substantial tissues such as the inflorescence receptacle, basal plate, storage layers, and enfolding layers of leaves in the apex, is also likely to weaken the structure through the action of the ethanoic acid and cause hardness of fragile tissues leading to the brittleness and crumbling experienced. Whilst it is possible to reduce immersion times of fixing and dehydration sequences, when tissues of known fragility are to be examined, most

samples involved tissues of various types, for example, the developing inflorescence or the vegetative apex and basal plate. Any methodology must necessarily be a compromise between thorough penetration and the preservation of the tissues intact.

3.2.3 Staining

Varying the times of hydrolysis in HCl had little effect on the quality of sections (Table 12). There was a high rate of survival on all slides treated for 8 min. and one slide treated for 5 min. though other slides treated for 5 min. lost all the sections, suggesting that hydrolysis times are not a factor in sample loss. Losses were 100% at 6, 7 and 10 min.

Table 12: Effects of hydrolysis times on sections

Slide no.	Hydrolysis time (min.)	No. of sections	No. of complete sections
1	10	13	0
2	5	9	0
3	10	9	0
4	8	8	8
5	5	8	0
6	7	9	0
7	8	9	9
8	7	8	0
9	6	8	0
10	5	8	7
11	6	9	0
12	10	8	0
13	7	9	0
14	8	11	10
15	6	9	0

The effectiveness of the subbing solution in adhering samples to the slides was also examined. It was increased in strength x 2, x 4, x 10 in the water bath during flotation of the tissue samples. Prepared slides were selected at random, allocated to subbing solution strength and then processed as previously. Increasing the strength of the subbing solution in the water bath had no observable effect on the survival of samples

(data not presented). However, using pre-subbed slides completely eliminated losses during the staining process. Subbing solution (Appendix 11) was warmed to approximately 30°C to ensure that it was thoroughly melted. Slides that had been washed in 70 % ethanol and air dried, were dipped in subbing solution and air dried again at room temperature. Slides were placed flat for drying to encourage even distribution of subbing solution. These slides were used in conjunction with subbing solution in the water bath at the original concentration (Appendix 11), consistently producing slides that remained 100 % intact during the staining process.

3.3 Final technique

Fixing times were increased up to 5 days depending on the tissue. Large sections including basal plate material (Figures 20-23) or inflorescences (Figure 24-25) required longer, whilst small samples especially those known to be fragile required shorter times (Figures 26-27). The dehydration sequence was included for all samples, and the wax infiltration process was increased to two days for each immersion, with three days for the final immersion in 100% wax. Blocks were allowed to cool at room temperature without chilling. Blocks were sectioned at 5µm thickness. Ribbons of sections were floated on the surface of a water bath at 42°C containing 4 ml/l subbing solution (Appendix 11) then positioned on pre-subbed slides (Appendix 11) and allowed to dry at room temperature for at least 24 h. The staining protocol was used as for Table 11 (3.1.3). Slides were made permanent by dipping them in xylene twice for 1 min. each (Hopkin & Williams Ltd GPR Grade A) in a fume cupboard, then placing a drop of DPX mountant (Fluka 44581) over the samples

before pressing down the cover slip for a few seconds. They were kept level and allowed to dry for 48 hours in a fume cupboard, before examination.

The adjustments made to this protocol ensured that a high proportion of samples could be successfully embedded, sectioned and stained for examination. Complete wax penetration could be reliably reproduced. No slides were lost during staining. Although a small number of slides still produced brittle material that tended to crumble during sectioning, the adjustments made to this protocol produced large numbers of high quality slides for histological examination (Fig. 20 – 27).

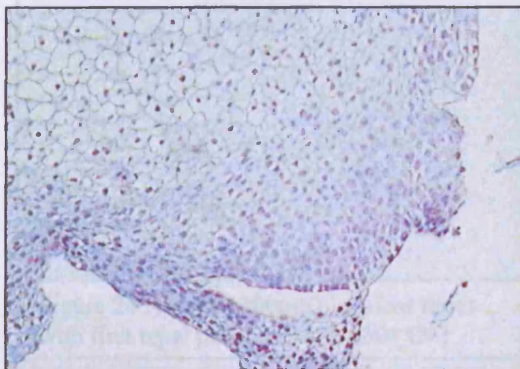


Figure 20 (height 1200 μ m): Apical dome and basal plate (SH99 1 year 24 weeks) LS

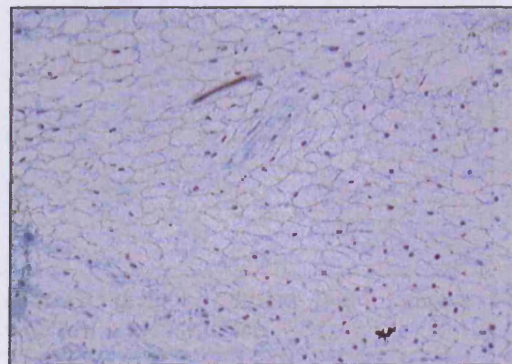


Figure 21 (height 1200 μ m): Storage tissue (SH99 1 y 4 weeks) LS

Figure 26 (height 1200 μ m): Bulbous (SH99 1 y 4 weeks)

Figure 27 (height 1200 μ m): Formation of daughter cells (ST0301 D11)

3.3.1. Observations (Table 13)

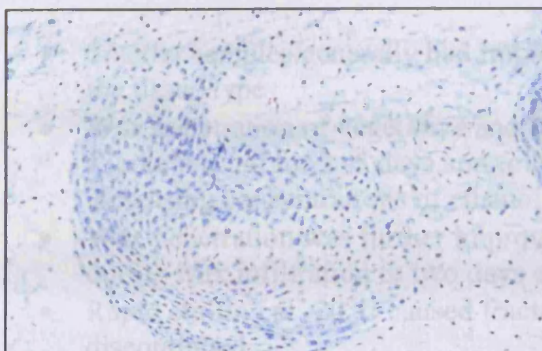


Figure 22 (height 1200 μ m): LS root formation within storage tissue (SH00) 32 weeks)

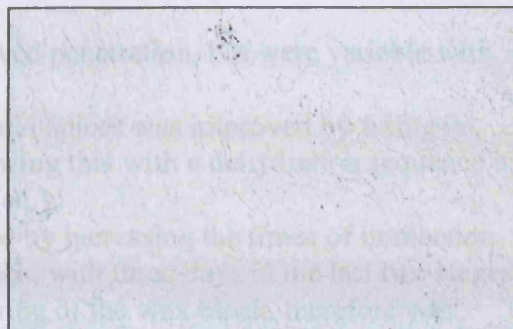


Figure 23 (height 1200 μ m): Old storage tissue showing breakdown of walls and loss of nuclear material (SH0301 D14)

Table 13: Revised timetable for living and non-living

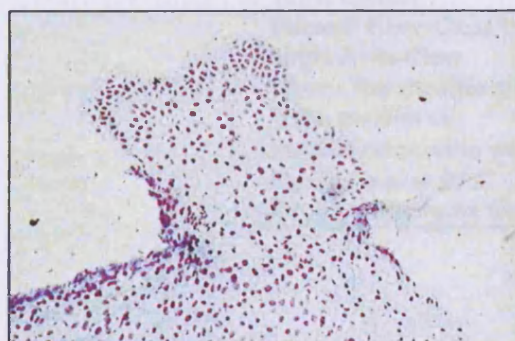


Figure 24 (height 1200 μ m): Apical floret with first tepal primordia (SH0301 D6)



Figure 25 (height 1200 μ m): Bulbil forming with bract (SH0301 D8)



Figure 26 (height 1200 μ m): Bulblet (JS00e 16 weeks)

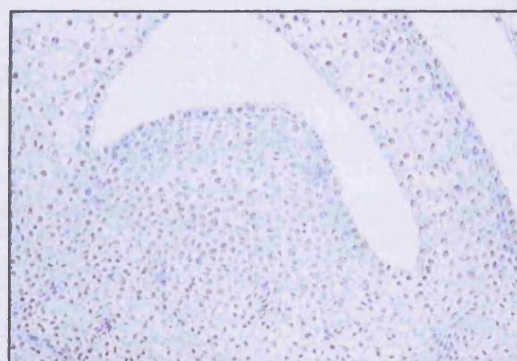


Figure 27 (height 1200 μ m): Formation of daughter bulb (ST0301 D11)

3.3.1 Conclusions (Table 13)

- Smaller samples generally had improved penetration, but were variable with the tissue type
- Wax penetration of vegetative and floral apices was improved by fixing in Clarke's Fluid for 3 -5 days and following this with a dehydration sequence in increasing concentrations of ethanol (aq.)
- Wax penetration was further improved by increasing the times of immersion during wax infiltration to two days each, with three days in the last two stages
- Rapid chilling at -20°C caused fracturing of the wax block, therefore was discontinued
- The use of pre-subbed slides eliminated losses during the staining procedure
- The staining and section procedures (Herbert, 1991) were unaltered (Table 11)

Table 13: Revised timetable for fixing and wax embedding

Treatment	Time (days)
Fixing in Clarke's Fluid	3-5
80% ethanol	1
90% ethanol	1
100% ethanol	1
Ethanol: Histo-Clear 1:1 (v/v)	2
100% Histo-Clear	2
Histo-Clear:paraffin oil (1:1 v/v)	2
100% paraffin oil	2
Paraffin oil:paraffin wax at 60°C	3
Paraffin wax at 60°C	3
Freshly melted wax for embedding	

4.0 Development of protocol for the maintenance of *Allium ampeloprasum* var. *babingtonii* apices in tissue culture

Tissue culture enables plant responses to be investigated in isolation, both from the environment, by providing a controlled sterile environment free of pathogens and biotic influences, and from the rest of the plant, permitting investigation into particular tissues or organs (Dodds and Roberts, 1995). It has been used to establish the timing of floral determination of the apical shoot (see sections 1.7.1, 1.8).

Determination is the condition where the tissue forms a flower in conditions where vegetative growth would be predicted (McDaniel *et al.*, 1992; McDaniel, 1996).

Species that flower in response to endogenous signals can be tested for determination, by separating the buds from the mature plant, which could otherwise be a source of signals to flower or to remain vegetative (Bernier *et al.*, 1993). Tissue culture is used to assay floral determination, as apices can be grown in a neutral environment, e.g. Durdan (1998) used apical shoot tips to investigate the timing of floral determination in *Pharbitis nil*. However, one limitation of this method is that some leaf primordia are likely to be included. Also, Huala and Sussex (1993) suggest that determination occurs not only in the meristem but also in other tissues of the plant.

The development of a protocol for the tissue culture of *A. ampeloprasum* var. *babingtonii* would enable the timing of floral determination to be investigated, establishing a foundation for the construction of a floral developmental timetable for this species.

Much work has been carried out looking at the behaviour of *Allium* spp. in tissue culture (Appendix 4). Most of this has been directed towards developing protocols that allow for the regeneration of plantlets from desirable gene lines, and to provide a rapid and efficient method of propagating those that do not easily produce viable seed. Further research has been directed at the culture of callus, the undifferentiated tissue being genetically unstable (Brewster, 1994) producing new lines for further study and development, for example, the examination of ploidy and the changes associated with changes in ploidy levels (Novák, 1990). It is also used to produce virus free stocks, and for the preservation of vegetatively propagated forms (Keller and Lesemann, 1997, Novák, 1990), and meristem culture is particularly useful for this as it maintains high genetic stability, making it suitable for *in vitro* germplasm conservation (Novák, 1990). Material used frequently includes parts of the basal plates, and the meristematic parts of young inflorescences (Appendix 4).

Allium spp. have been cultured as apical shoot explants (e.g. Bhojwani *et al.*, 1982/3; Dunstan and Short, 1977a and b; Rodrigues *et al.* 1997) and as entire bulbils (e.g. Havel and Novák, 1988; Novák, Havel and Doležel, 1986), as well as other tissues such as flower heads (Havel and Novák, 1988), embryos (Novák, *et al.*, 1986), individual flowers (Baumunk-Wende, 1989; Rauber and Grunewaldt, 1988) and anthers (Baumunk-Wende 1989). However, whilst the culture of entire bulbs or bulbils would avoid tissue damage and eliminate any wounding responses, it would only provide information about the developmental state of the whole plant. Alternatively, culture of the apical shoot would provide information about the developmental state of the apex in isolation.

Novák (1990) suggested that most *Allium* tissues can be cultured on MS medium (Murashige and Skoog, 1962), and others have also used this medium (e.g. Seo and Kim, 1988, Ziv *et al.*, 1983), sometimes at half strength, whilst other researchers (e.g. Bhojwani *et al.*, 1982/3) have used Gamborg's B5 (Gamborg *et al.*, 1968). Dunstan and Short (1977a, b) developed a new medium (BDS). This was based on Gamborg B5 but with increased levels of phosphate and nitrogen, agar was 8.0 g/l, pH 5.5, giving improved growth rates. The study was designed to increase the growth of onion tissue cultures and improve the regenerative capacity of the tissue. Other media used for *Allium* tissue culture include White (1963) and Nitsch and Nitsch (1969) (Novák, 1990).

Novák (1990) suggests that *Allium* cultures will grow well within a wide range of photoperiods and light intensities, and fluorescent lights at approximately 1500 lux for a 16 hr photoperiod is likely to be satisfactory, though quality and regime are also important. Baumenke-Wende (1989), Dunstan and Short (1977a and b) and Keller and Lesemann (1997) grew on at 25°; Lu *et al.* (1989) at 25±5°C; Ziv *et al.* (1983) at 25±1°C. Seo and Kim (1988) grew callus at 26°C and 60±5% relative humidity, while Hussey and Falavigna (1980) grew explants taken from basal plate tissue, at 20°C. These figures accord with Rubatzky and Yamaguchi (1997) who suggest that the optimum temperature for vegetative growth in leeks is between 20°C and 25°C.

Summary of aims:

- To compare bulbil and explant development *in vitro* determining the most appropriate tissue for maintenance in culture
- To optimise surface sterilisation methodology

- To identify a medium that supports active growth, (e.g. examining carbon source, carbon source concentration, and standard macro/micro nutrient preparations)
- To identify photoperiod and light intensity that supports active growth without promoting dormancy or bulbing.

Bulbils were used, as they were readily available in large numbers, whilst numbers of bulbs and bulblets were limited. Surface sterilisation methods were investigated using both explants from bulbils, and whole bulbils. Standard tissue culture media were compared, as were carbon sources and concentrations. *Allium* spp. have a wide range of responses to environmental cues; flowering, bulbing and dormancy may all be influenced (Brewster, 1994). Therefore, light levels and photoperiods were also examined, to determine appropriate conditions for the development of shoots in culture.

4.1 Materials and methods

4.1.1 Preparation of plant material

Inflorescences collected from Holywell Bay Holiday Park, Cornwall (SW 784567-771584), were selected using random numbers; the bulbils were removed and pooled. Individual bulbils were then selected at random. Although the variety reproduces clonally, there is some genetic mutation occurring (Treu, 1999). Therefore, it was felt important to randomise selection of the inflorescence, as well as randomising selection within the inflorescence. Inflorescences collected in 1999 (SH1999, see Chapter 2) were used during the following season, later work was with inflorescences collected in 2000 (SH2000, see Chapter 2), and similarly randomized.

The appearance of bulbils was variable; some formed brown coriaceous layers (tunic), whilst others had no apparent tunic layers; others formed partial coriaceous coverings (Figure 28). This



Figure 28: Bulbils from a single inflorescence, showing variability of tunic development (scale in mm).

layer might restrict nutrient supply and gaseous exchange, and reduce effectiveness of surface sterilisation methods. Therefore, it was removed from all bulbils prior to surface sterilisation, whether for use as explants or growth as whole bulbils in culture. Removal involved no damage to the bulbil, as the tunic was apparently necrotic and easily shed. Those with partially formed coriaceous layers were discarded, as they were continuous with living tissue and removal would damage the bulbil.

Figure 29: Flow chart depicting the excision of explant for culture

Where explants were required, the central core containing the shoot, together with subjacent basal plate material and the superjacent storage material was excised (Figure 29).

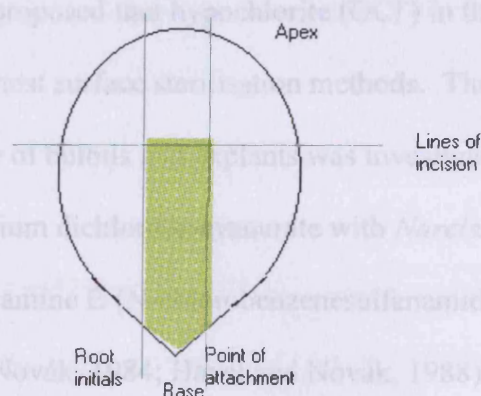


Figure 29: Excision of explant for culture

The upper surface was cut at an angle to identify orientation for correct placement in culture. Explants were placed in sterile distilled water (SDW) prior to culturing, to prevent dehydration. Where whole bulbils were used for culture, they were rinsed in SDW to reduce numbers of pathogen propagules.

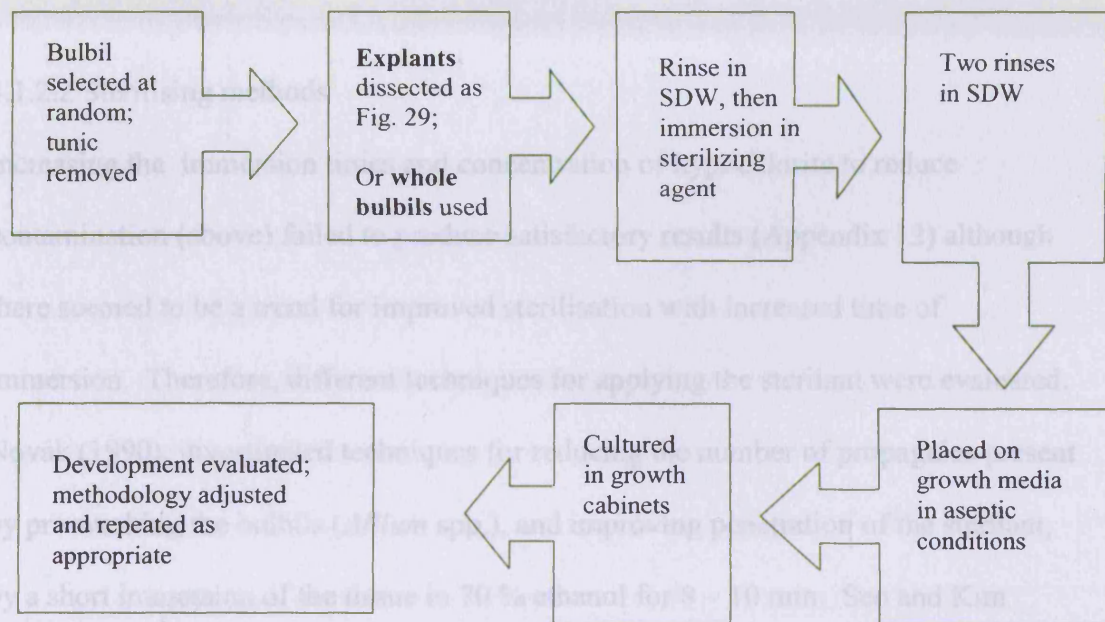


Figure 30: Flow chart depicting tissue culture methodology

4.1.2 Surface sterilisation

Dodds & Roberts (1995) proposed that hypochlorite (OCl^-) in the form of domestic bleach is appropriate for most surface sterilisation methods. The effectiveness of immersion in hypochlorite of bulbils and explants was investigated. Others have used sterilants such as sodium dichloroisocyanurate with *Narcissus* spp., (Puddephat, pers. com. 2000), or Chloramine B (N-chlorobenzenesulfanamide sodium salt) with *Allium* spp. (Doložel and Novák, 1984; Havel and Novák, 1988). The three surface sterilisation reagents were evaluated for their effectiveness at reducing contamination (Appendix 12).

4.1.2.1 Immersion period and concentrations of hypochlorite

A number of different surface sterilisation immersion periods from 1 – 180 min were investigated (Appendix 12). Domestos® was diluted to 5 % or 10 % (v/v) with SDW with detergent as a wetting agent (Appendix 12).

4.1.2.2 Sterilising methods

Increasing the immersion times and concentration of hypochlorite to reduce contamination (above) failed to produce satisfactory results (Appendix 12) although there seemed to be a trend for improved sterilisation with increased time of immersion. Therefore, different techniques for applying the sterilant were evaluated. Novák (1990), investigated techniques for reducing the number of propagules present by pre-washing the bulbils (*Allium* spp.), and improving penetration of the sterilant, by a short immersion of the tissue in 70 % ethanol for 8 – 10 min. Seo and Kim (1988) also used ethanol to clean *Allium cepa* (shallot) bulbs. Holmes (pers. com. 2000) suggested mechanical agitation to improve penetration of the sterilising fluid. These techniques were compared with hypochlorite immersion alone (5 % and 10 %) for 120 min (Appendix 12).

4.1.2.3 Comparison of sodium dichloroisocyanurate and Chloramine B as surface sterilising treatments

Doložel and Novák (1984) and Havel and Novák, (1988) used Chloramine B (N-Chlorobenzenesulfonamide sodium salt) at 5 % (v/v) (Sigma C2279) for 30 and 20 minutes respectively. Puddephat (pers. com. 2000) recommended immersion of plant tissues for 5 min. in sodium dichloroisocyanurate (Sigma D2536), 0.5g in 30 ml

SDW with Igepal CA-630 (Sigma I-3021) as wetting agent. Puddephat (pers. com. 2000) also suggested incubating the bulbils in a warm water bath, to allow spores to germinate, before treating them with sterilising agents. This would enable the agent to be applied when the propagules were more vulnerable than the resting stages. The effectiveness of Chloramine B and sodium dichloroisocyanurate both with and without the incubation period were compared (Appendix 12).

4.1.2.4 Minimal sterilisation of explants

Initial work showed lower contamination rates for explants than for bulbils (Appendix 12), suggesting that the contamination was present on surface tissues rather than internally. This was investigated, by producing explants with all surface tissue removed (Figure 31).

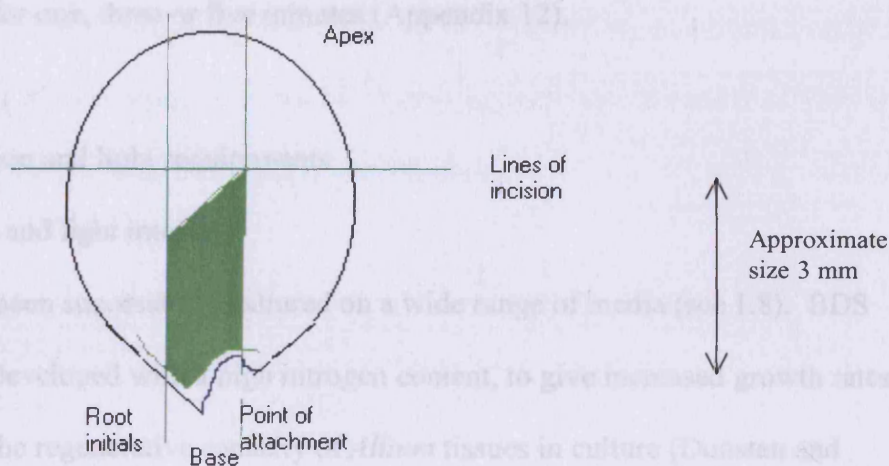


Figure 31: Excision of explants, removing all external tissues.

The point of attachment comprised desiccated material that intruded into the storage tissues of the bulbil. Penetration of sterilising fluid into these tissues was likely to be imperfect; therefore, this was completely trimmed away.

As many explants failed to thrive even when not contaminated, it was decided to also examine the possibility that surface sterilising agent had detrimental effects; for example, the high salt content of ‘Domestos®’ could have osmotic effects on such small pieces of tissue with a large cut surface area (Puddephat, pers. com. 2000).

Alliums are known to be sensitive to saline *in vivo* (Brewster, 1994), however, LeverFabergé declined to comment on the salt content of ‘Domestos®’ (personal communication, 2004). Explants prepared without exposure to hypochlorite or any other surface sterilising agent were compared with those with those immersed in hypochlorite for one, three or five minutes (Appendix 12).

4.1.3 Nutrition and light requirements

4.1.3.1 Media and light intensity

Alliums have been successfully cultured on a wide range of media (see 1.8). BDS medium was developed with a high nitrogen content, to give increased growth rates and improve the regenerative capacity of *Allium* tissues in culture (Dunstan and Short, 1977a, b). However, Brewster (1994) commented that high nitrogen levels *in vivo* may lead to an extended juvenile phase before *Alliums* can be florally determined, imposing more stringent requirements for the acquisition of floral competence (i.e. larger minimum bulb size, increased minimum leaf number). Therefore, B5 (Gamborg *et al.*, 1968) as previously used, White’s (1963) and M & S (Murashige and Skoog, 1962) media were compared (Appendix 12).

Allium species grow well at a variety of light intensities and photoperiods (Novák 1990). For example, Baumunke-Wende (1989) used 16 hr photoperiods at 1500 lux; Hussey and Falavigna (1980) used 8 and 16 hr photoperiods at 8000 lux. Therefore, low and high light intensities were compared, in relationship with the different media (Appendix 12). For comparison with earlier work, light levels were measured in lux (TES-1334 Digital Illuminance Meter) as well as $\mu\text{M m}^{-2} \text{s}^{-1}$. Explants were cultured at a fluence rate of $95.2 \mu\text{M m}^{-2} \text{s}^{-1}$, mean 4500 lux (Sanyo Growth Cabinet) or $19.5 \mu\text{M m}^{-2} \text{s}^{-1}$, mean 750 lux (Gallenkamp Cooled Incubator).

4.1.3.2 Sucrose concentration

The available carbon source (sucrose) will affect not only growth rates, but may also affect contamination rate (Dodds & Roberts, 1995). Therefore, a number of different concentrations were compared (Table 14), assessing survival and quality of growth, looking at colour and length of shoots, after 30 days.

Table 14: Summary of sucrose treatments *in vitro*

Sucrose concentration (w/v)	Molarity
1%	0.029M
2%	0.058M
3%	0.088M
4%	0.117M
6%	0.175M
8%	0.230M

Explants were prepared as in Figure 31; surface sterilisation was a rinse in sterile distilled water. The medium was Gamborg's B5 basal salts, with 0.8 % (w/v) agar. Cultures were maintained in a Gallenkamp Cooled Incubator in a continuous low fluence rate ($19.5 \mu\text{M m}^{-2} \text{s}^{-1}$) at 25°C.

4.1.3.3 Carbon source and photoperiod

Starch is not formed in *Alliums*, instead a series of oligofructans comprise the reserve carbohydrate (Fritsch & Friesen, 2002). Fructans are formed from a glucose molecule, together with 2 to 260 fructose units (Salisbury and Ross, 1992).

Throughout storage periods, the fructans are gradually hydrolysed to produce fructose, and then during sprouting, sucrose is synthesised (Gubb & MacTavish, 2002). The complex interactions between the sugars suggest considerable variation in concentration and ratio, both spatially and temporally, reflecting changes in metabolism and growth patterns. Since fructose and glucose are implicated in *Allium* metabolism as well as sucrose, the effects of these sugars as carbon sources were investigated and compared with sucrose. Some of the explants grown earlier on sucrose, developed abnormal shoots, instead of the single straight shoot associated with normal healthy growth *in vivo* in *A. ampeloprasum* var. *babingtonii*. Therefore, growth quality as well as shoot length was examined.

Concentration of sugars was also examined. Durdan (1998) working with *Pharbitis nil*, found that higher concentrations of sucrose shortened the time for floral development, particularly for carpels. In *Alliums*, accumulation of assimilates may reflect aspects of maturity necessary for floral competence (Kamenetsky and Rabinowitch, 2002). This suggests that higher concentrations may promote flowering, or be necessary to support floral material *in vitro*. Therefore, shoot development under two concentrations of each sugar were investigated, comparing 3 % (w/v) which is similar to levels in the majority of plants *in vivo* (Dodds & Roberts

1995) with 6 % (w/v) (Table 17), the level at which Durdan (1999) found significant changes in carpel determination time.

Many explants in culture developed new storage tissue and became dormant.

Alliums such as *A. cepa*, (onion) *A. cepa*, Aggregatum group, (shallots) *A. sativum* (garlic) and *A. chinense* (rakkyo), bulb in response to long photoperiods and high temperatures and may be easily switched on and off by manipulation of the photoperiod (Brewster, 1994). *Allium ampeloprasum* var. *porrum* (leek) does not form a fully developed bulb (Brewster, 1994), but some 'bulbiness' can be induced with 24 h photoperiods, although with wide variation between plants. Low soil temperatures may also favour 'bulbiness' (Dragland, 1972).

Therefore, the relationship between photoperiod and bulbing was also examined by culturing explants under 24 or 14 h photoperiods (Table 15) in combination with the different sugars at different concentrations.

Table 15: Treatments given to assess the effects of carbon source and photoperiod on development *in vitro* (sugar concentrations given as w/v and Molarity).

Carbon source	Conc.	Molarity	Photoperiod (h)	Treatment no.
Sucrose	3 %	0.088	24	1
	6 %	0.175	24	2
	3 %	0.088	14	3
	6 %	0.175	14	4
Fructose	3%	0.167	24	5
	6%	0.333	24	6
	3%	0.167	14	7
	6%	0.333	14	8
Glucose	3%	0.166	24	9
	6%	0.333	24	10
	3%	0.166	14	11
	6%	0.333	14	12

4.1.3.4 Nutritional restriction

Leaves were rarely longer than 10 cm *in vitro* even when cultured for a period of 6 months. *In vivo* for a similar period (October to March) leaves were up to 100 cm in length. Whilst not desirable *per se*, it was necessary for the plants to be metabolically active in order to develop inflorescences for examination. Since the leaves of var. *babingtonii* rarely reach more than 3 cm in width compared with 100cm in length *in vivo* (pers. obs.), the measurement of leaf length would provide a non-invasive and non-destructive indicator of development in culture.

Generally, *Alliums* are sensitive to nutritional and water shortages. Once growth has slowed or ceased it is difficult if not impossible to restart, even if nutritional and water levels are adjusted (Brewster 1994). Subculturing was carried out on a monthly basis, before any visible shrinking of the growth medium. Comparing elongation patterns in 50 ml medium as previously, with that in 100ml medium but still subculturing at monthly intervals would indicate the extent of sensitivity to nutritional and water deprivations under these conditions (Appendix 12). Gamborg's B5 basal salt mixture did not contain any added vitamins (Appendix 11), therefore the effect of additional vitamins (Gamborg's vitamin Solution, Sigma G1019) (Appendix 11) on growth was also evaluated (Appendix 12).

4.1.4 Dormancy and sprouting

4.1.4.1 Light effects on sprouting

Many seeds are dependent on light or its absence to break dormancy and allow germination to proceed (Hartmann *et al.* 1997). Possibly light may have a similar

effect on bulbil sprouting, leading to low sprouting rates in culture. This was tested by selecting 20 bulbils using random numbers (SH11/01) and sprouting half in total darkness and half in continuous light (Appendix 12).

4.1.4.2 Effect of storage period on dormancy

The effects of storage on viability and dormancy were not known, and hence were investigated by comparing sprouting in bulbils that had been stored for 3 months (SH11/01) with those that had been stored for 15 months (SH00).

4.1.4.3 Culturing effects on dormancy

Twenty bulbils were selected using random numbers from SH11/01 (chapter 2.0), and randomly divided into two groups of 10. Half were excised as previously (Figure 29) and cultured as previously. The other half were planted in compost and grown in the same growth cabinet (Appendix 12). This would allow a comparison of dormancy numbers in explants with dormancy numbers in whole plants grown in identical conditions. This data would indicate if the culturing processes (such as wounding, nutritional supplies, sub-culturing etc.,) were implicated in the dormancy response.

4.1.4.4 Photoperiod and dormancy

The effect of photoperiod was further investigated in *A. ampeloprasum* var. *babingtonii*, using plants that had been grown from explants *in vitro* for 6 months in conditions as above and sub-cultured at monthly intervals.

Plants were separated into those that were dormant (but still apparently viable) (n = 19), and those that were sprouting (n = 16). The dormant plants had developed storage tissue, and were chlorophyllous and turgid, resembling a normally developed bulbil with the leaves having died back, whilst the sprouting plants had green leaves as well as storage tissue. Half of each (selected using random numbers) was placed in a 14 h photoperiod, the others in a 24 h photoperiod. They were sub-cultured at monthly intervals, and assessed after 4 months (Appendix 12). The numbers sprouting and dormant in each photoperiod were recorded and compared with the numbers sprouting and dormant before being placed in the two photoperiods.

4.2 Results

4.2.1 Surface sterilization

Increasing the time of immersion in hypochlorite increased the proportion of clean cultures for both bulbils and explants (Appendix 12). Increasing the strength of the hypochlorite to 10 % produced cleaner cultures than using 5 %, but this was offset by the increased number of bulbils becoming necrotic (10 % in each treatment, compared with 0% becoming necrotic at 5 %). The culture of explants rather than bulbils produced the highest proportions of clean cultures (Appendix 12).

None of the techniques examined for improving penetration of the surface sterilising agent such as mechanical agitation or a preliminary rinse in ethanol, showed any significant difference in contamination levels when tested using χ^2 at $p = 0.05$ (Appendix 12) when compared with immersion in hypochlorite alone. Chloramine B and sodium dichloroisocyanurate also failed to show any significant difference when tested similarly using χ^2 at $p = 0.05$. Contamination levels with or without a

preliminary incubation period (Appendix 12) varied between 10 and 50 %; therefore, use of these sterilants was discontinued.

When the effect of hypochlorite for 1-5 min was compared with a rinse in SDW (using χ^2 at $p = 0.05$), the number of clean explants was not significantly different between the treatments (Appendix 12). A rinse in SDW was as effective as immersion in 5% hypochlorite for up to 5 minutes, provided that all external tissues were removed, providing 90% clean explants. When the proportions of sprouting explants were compared in each of these treatments (χ^2 at $p = 0.05$), there was a significant difference (Appendix 12), with the largest proportion sprouting following treatment A (rinse in SDW). This suggested that hypochlorite is detrimental to sprouting, and its use was discontinued. However, of those explants that sprouted, there was no significant difference in the length of the shoot (χ^2 at $p = 0.05$), suggesting that hypochlorite acts to prevent sprouting, but that once initiated, sprouting continues normally. The surface sterilization method adopted for future work was treatment A, removal of all external tissues from the explant (Figure 31), followed by a rinse in SDW.

4.2.2 Nutrition and light levels

4.2.2.1 Media and fluence level

Analysis of contamination showed a very significant difference (χ^2 at $p = 0.05$) between the treatments, with Treatment 1 (White's Medium, high light levels) having the highest number of contaminated samples (Table 16) and also the least number of viable cultures. There was no significant difference (χ^2 at $p = 0.05$) in numbers sprouting or shoot lengths between these treatments (Appendix 12).

Table 16: Contamination and survival numbers for explants cultured on different media and with different light levels. Assessed at 30 days; mean length is per surviving and developing explant. Viable is assessed as surviving, clean, and developing.

	High fluence levels			Low fluence levels		
	White's	M & S	B5	White's	M & S	B5
Treatment number	1	2	3	4	5	6
N =	10	8	10	10	9	10
No. clean	1	8	6	7	7	8
No. viable	0	3	4	5	5	5
Mean shoot length (mm)	-	3.0	10.4	4.8	6.6	10.8
Chlorosis	-	Present	None	Present	None	None

Shoot lengths were also ranked and examined using Two-Way Non-parametric

ANOVA, confirming that there was no significant difference ($p = 0.05$) in the shoot lengths as a result of the light level, though there was a difference in the shoot length as a result of the different media. This result was fairly close to the critical value, suggesting that there may be a small difference, which could be confirmed by more stringent testing.

4.2.2.2 Sucrose concentrations

Sucrose concentration may affect growth rates and contamination rates (Dodds & Roberts, 1995), and this was reflected in the results of the treatments (Appendix 12).

There was more contamination at sucrose levels above 4 %, though the overall percentage of contaminations was 13.3 % confirming that excision of all external tissue with a rinse of SDW before culture produces repeatable levels of clean cultures (Figure 32). Sucrose levels below 3 % resulted in the least shoot elongation, the longest shoots being on medium containing 3 % sucrose. The largest number of viable explants was on 1% sucrose, but these were also the smallest shoots.

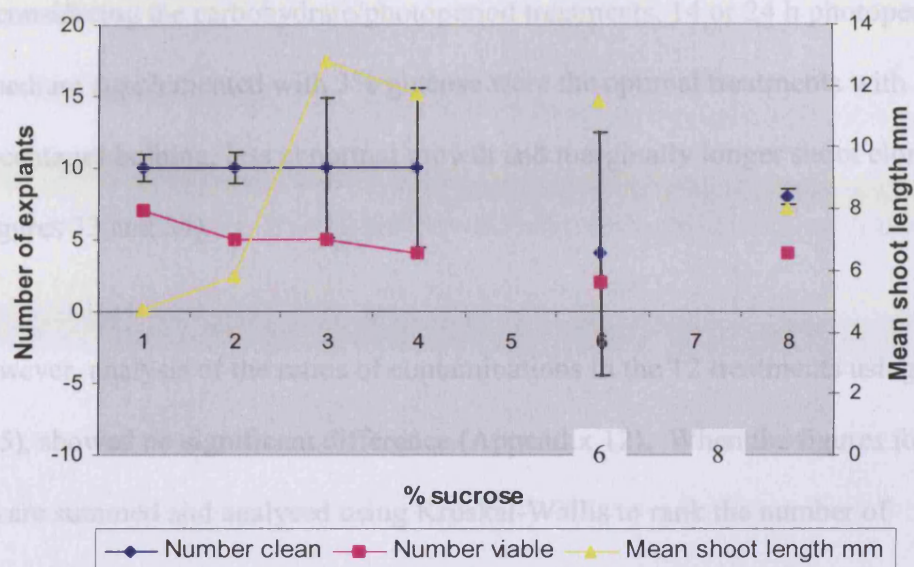


Figure 32: Comparison of contamination and shoot elongation of explants cultured on media containing differing sucrose concentrations (although $n = 10$ for each treatment, numbers producing shoots were often very small range, 0 – 6, therefore error bars may be missing or extremely large). See Appendix 12-7 for raw data).

4.2.2.3 Carbon source/photoperiod

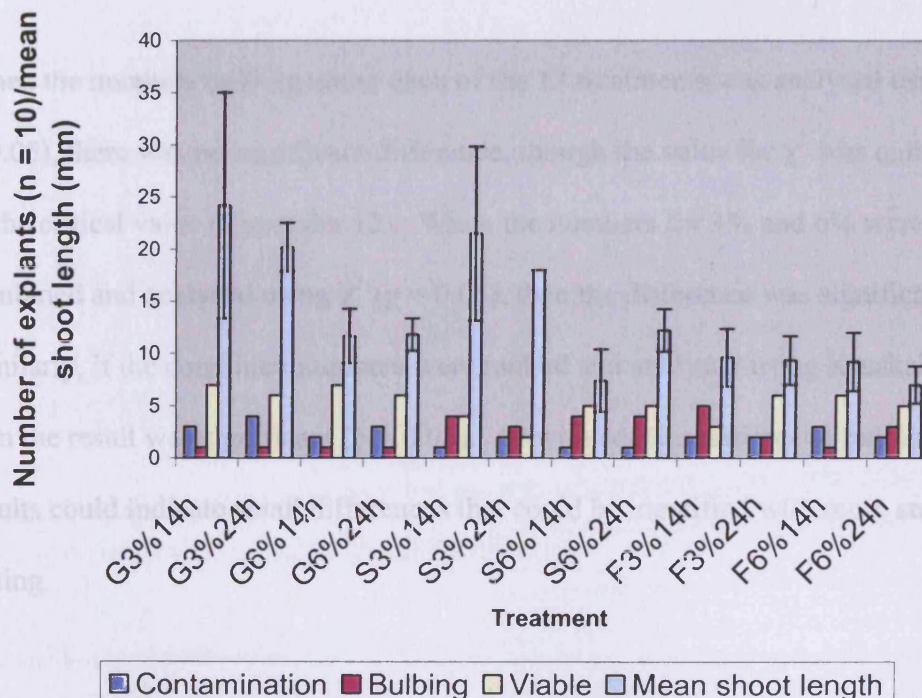


Figure 33: Summary of contamination numbers ($n = 10$ for each treatment), bulbing numbers, number of viable explants and mean shoot length per treatment. S = sucrose, F = fructose, G = glucose; 3%/6% = concentration of sugar used in growth media (w/v); 14h/24h = photoperiod. (For complete data see Appendix 12.8, Tables 12-6, 12-7, 12-8).

In considering the carbohydrate/photoperiod treatments, 14 or 24 h photoperiods and a medium supplemented with 3% glucose were the optimal treatments with lower percentages bulbing, less abnormal growth and marginally longer shoot elongation (Figures 33 and 34).

However, analysis of the ratios of contaminations in the 12 treatments using χ^2 ($p = 0.05$), showed no significant difference (Appendix 12). When the figures for 3% and 6% are summed and analysed using Kruskal-Wallis to rank the number of contaminations per carbon source, then the result was significant. This is only valid if the interactions between the sugars and the photoperiods are similar, but might suggest some small difference in the numbers of contaminations that could be identified by more stringent testing.

When the numbers bulbing under each of the 12 treatments was analysed using χ^2 ($p = 0.05$), there was no significant difference, though the value for χ^2 was quite close to the critical value (Appendix 12). When the numbers for 3% and 6% were combined and analysed using χ^2 ($p = 0.05$), then the difference was significant. Similarly, if the combined numbers were ranked and analysed using Kruskal-Wallis, then the result was significant ($p = 0.05$). As with contamination and bulbing, these results could indicate small differences that could be identified with more stringent testing.

The numbers of viable explants per treatment was also examined using χ^2 ($p = 0.05$). Viable was judged to be alive, not contaminated, and producing active, normal growth. Therefore, all those that were bulbing (becoming dormant) were discounted,

as were those that produced abnormal shoots. It was necessary to develop a protocol that supported active growth in the explants, so that floral determination and development could subsequently be examined *in vitro*. However, there was no significant difference in the proportions of viable explants given these treatments.

The mean shoot length was analysed using the Kruskal-Wallis test to compare medians ($p = 0.05$). Shoot length was used as an indication that the culture conditions were appropriate for active growth. This would be necessary for investigations into inflorescence development in this species. There was a significant difference between the medians, with treatment 1 and 2 (3% glucose at 14 and 24h photoperiods) contributing most to the significance (Appendix 12).

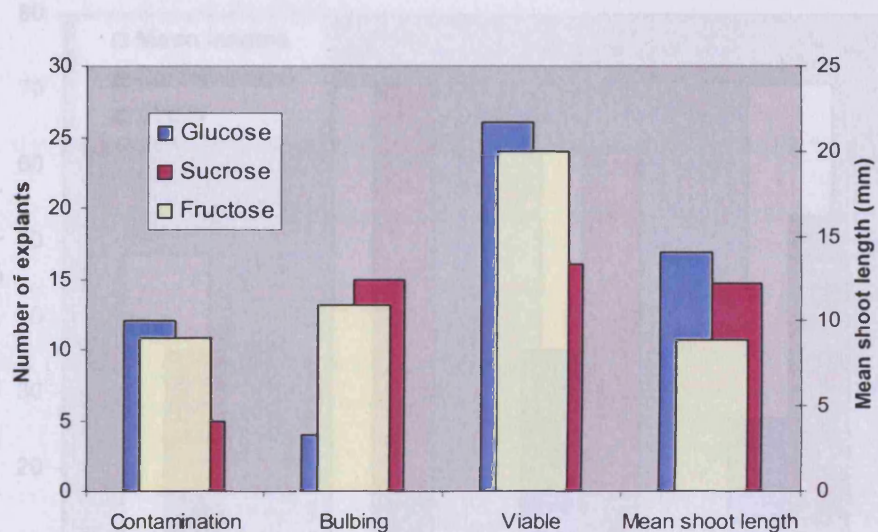


Figure 34: Summary of shoot development of summed data (3% + 6%) for each medium for explants grown with different carbon sources (Appendix 12).

The difference in shoot length was also examined using Non-parametric Two-way ANOVA (Barnard *et al.*, 2001), a version of ANOVA (ANalysis Of Variance, shoot length/carbon source) adapted for use with non-parametric data, allowing the comparison of the means of different populations, with the data for 3% and 6%

summed. This also showed a significant difference in shoot length as a result of different carbon sources, though not as a result of daylength. There was also a significant difference between the shoot length as a result of the interaction between photoperiod and carbon source ($p = 0.05$)

4.2.2.4 Nutritional restriction

The proportions of contaminated explants were compared using χ^2 ($p = 0.05$), but there was no significant difference between the treatments. The proportions of viable explants were similarly compared, but again there was no significant difference between the treatments. When the mean shoot lengths were assessed using Kruskal-Wallis ($p = 0.05$), again there was no significant difference.

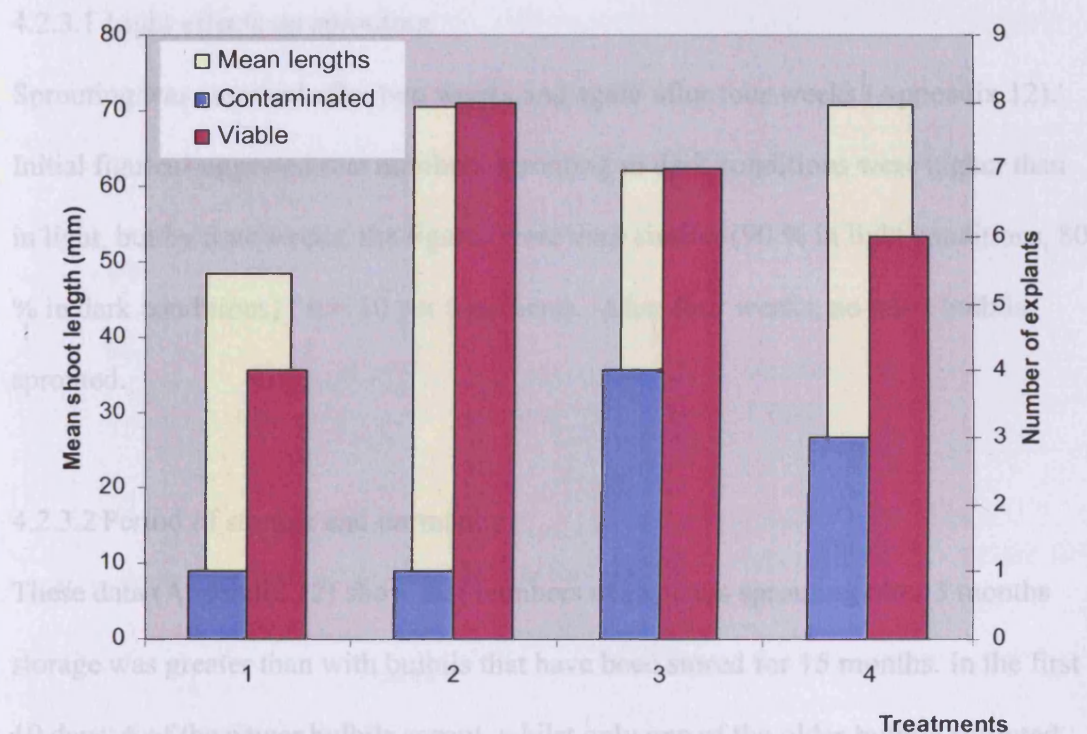


Figure 35: The effect of different media volumes and supplementary vitamins on development. Treatments: 1 = 50 ml excluding vitamins; 2 = 100ml excluding vitamins; 3 = 50ml including vitamins; 4 = 100ml including vitamins. No explants were visibly bulbing after 3 months.

Mean shoot length was longer in treatments 2 and 4 (100 ml medium) (Figure 35); There were most viable explants in treatment 2 (100 ml excluding vitamins) with least contamination in media without vitamins though none of these treatments are statistically significant (Appendix 12). Bulbing developed after 4 months in culture, with highest proportions on the media without vitamins (67 % compared with 31 % on media with vitamins) (Appendix 12) which suggested that nutritional factors may be a stimulus in the bulbing process. However, when the proportions of explants bulbing were compared using χ^2 ($p = 0.05$), there was no significant difference between the treatments.

4.2.3 Dormancy

4.2.3.1 Light effects on sprouting

Sprouting was assessed after two weeks and again after four weeks (Appendix 12). Initial figures suggested that numbers sprouting in dark conditions were higher than in light, but by four weeks, the figures were very similar (90 % in light conditions, 80 % in dark conditions) ($n = 10$ per treatment). After four weeks, no more bulbils sprouted.

4.2.3.2 Period of storage and dormancy

These data (Appendix 12) show that numbers of explants sprouting after 3 months storage was greater than with bulbils that have been stored for 15 months. In the first 19 days, 6 of the newer bulbils sprout, whilst only one of the older bulbils sprouted. The mean shoot length was 9.7 cm for newer bulbils, with only 1 cm mean sprout length produced by older bulbils. However, by 94 days, seven new bulbils have sprouted, compared with four of the older bulbils, reducing the difference.

The newer bulbils produced one dormant bulbil by 94 days, whilst five out of the six older bulbils became dormant by this time. These data are too few for meaningful analysis, but it would be likely that some deterioration of plant tissues would occur over prolonged storage periods. Since more uniform sprouting may be useful in the manipulation and examination of plant growth, newer bulbils were used wherever possible.

4.2.3.3 Culturing effects on dormancy

There was little difference in numbers sprouting *in vivo* and *in vitro*, there being six *in vivo* and five *in vitro* after 19 days, but six each from 36 days and subsequently.

However, the shoots *in vivo* were much larger and less chlorotic (Figure 36).

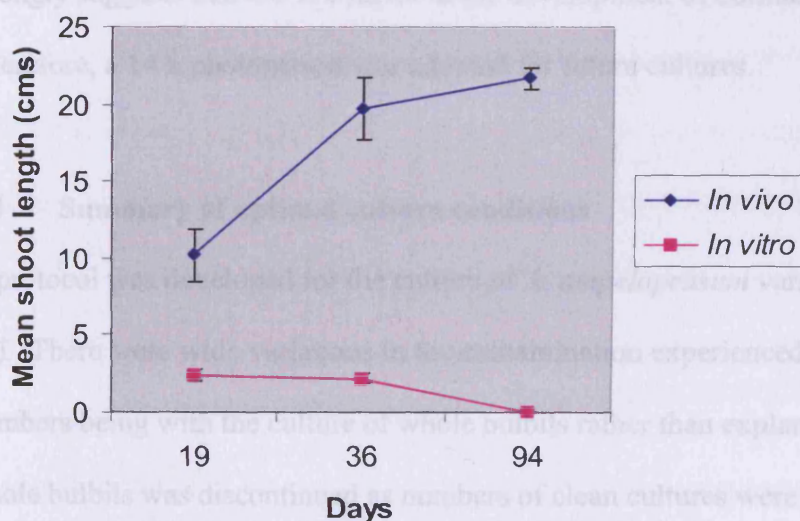


Figure 36: The relationship between mean shoot length (cm) and time *in vivo* and *in vitro* ($n = 10$ for each treatment). (NB error bars for growth *in vitro* too small for display, 0.34, 0.35 and 0 for 19 days, 36 days and 94 days respectively).

The difference in shoot lengths was examined using the Mann-Whitney test for differences in medians ($p = 0.005$). The difference was shown to be very highly significant (Appendix 12), with the mean shoot length *in vivo* increasing to 21.7 cm, whilst the mean shoot length *in vitro* reached a maximum of 2.4 cm.

Numbers bulbing in the two treatments were different; those cultured *in vitro* had all bulbed after 94 days, whilst none of those grown *in vivo* had bulbed.

4.2.3.4 Photoperiod and dormancy

Under the original growth conditions of CL, 19 plants out of a total of 35 became dormant after 6 months. None of the dormant plants re-sprouted in response to the change in photoperiod. However, of the sprouting plants in 14h photoperiod, 1 of 9 became dormant, whilst in the 24h photoperiod, 6 of 7 became dormant. This strongly suggests that CL is a factor in the development of dormancy (Appendix 12). Therefore, a 14 h photoperiod was adopted for future cultures.

4.3 Summary of optimal culture conditions

A protocol was developed for the culture of *A. ampeloprasum* var. *babingtonii* (Table 17). There were wide variations in the contamination experienced, the highest numbers being with the culture of whole bulbils rather than explants. The culture of whole bulbils was discontinued as numbers of clean cultures were not improved either by immersion in hypochlorite or by the use of sodium dichloroisocyanurate or Chloramine B, nor by attempts to improve penetration of the waxy cuticle and point of attachment using mechanical agitation or ethanol immersion (Appendix 12). Additionally, bulbils did not sprout during culture over a 3 month period, producing

roots but no leaves, compared with 62 % of bulbils sprouting *in vivo* over a similar period.

Table 17: Summary of tissue culture conditions adopted for assessment of floral determination of *A. ampeloprasum* var. *babingtonii*

Condition	
Tissue	Explants with all external tissue removed
Surface sterilization	None, immersion in SDW to protect against dehydration and propagules transferred during excision
Media	Gamborg's B5 No added vitamins 100ml volume
Carbon source	Glucose 3 % (0.088 M)
Photoperiod	14h
Temperature	25°C

Explants were consistently cleaner than whole bulbils, and when the excision procedure was adjusted to remove all external tissues, notably the region around the point of attachment (see Figure 29), clean cultures were consistently produced with very low numbers of contaminations. The use of hypochlorite was discontinued as this had an inhibitory effect on sprouting numbers. Explants were rinsed in SDW to prevent dehydration and remove pathogens transferred during excision.

Exposure to light (CL) in culture was not a factor in the sprouting numbers of bulbils, though photoperiod did affect the numbers becoming dormant after sprouting. A photoperiod of 14 h reduced the numbers of explants bulbing as did the use of newer bulbils rather than those that had been stored for prolonged periods. The shorter photoperiod also reduced the numbers of abnormal growth patterns observed. These

were fleshy and twisted, and may relate to the production of storage tissue, even though dormancy was not fully established. Bulbing was also increased when the media did not contain added vitamins, suggesting that it may also be related to nutritional supply. Comparing this with the growth patterns *in vivo*, dormancy is during summer when the daylength is at its greatest, and nutritional supply may be reduced through the lack of water available. However, dormant explants failed to re-sprout when the photoperiod was shortened. Shoots *in vivo* are much larger than *in vitro*, with those grown *in vitro* bulbing much sooner than those *in vivo*, suggesting dormancy as a response to physical trauma.

The media adopted for future use was Gamborg's B5, with no added vitamins (less contamination) 100ml in Magenta vessels. Shoot lengths were greater when the explants were cultured in 100 ml medium than in 50 ml medium, indicating sensitivity to nutritional shortages, even though culturing was at monthly intervals before any visible shrinkage of the medium. Although the tests failed to find significance in media, light levels or concentration of sucrose, the chance of a Type II error (accepting the null hypothesis when it is false) always exists, particularly when sample sizes are small. Although sample sizes were usually based on 10 or 12 explants, the reduction in useful data as a result of lack of sprouting, contamination, and bulbing, often resulted in small numbers of explants for assessment.

Explants cultured on media containing glucose (3 % w/v 0.088 M) developed longer shoots and fewer malformations than explants cultured on media containing sucrose or fructose. The difference in mean shoot length between the lower and higher concentrations of glucose, suggest inhibitory effects at the higher concentration.

Sucrose showed a similar effect (mean 21.5mm compared with 7.4mm for 14h, and 18.0mm compared with 12.2mm for 24h). This effect was not seen in fructose where the mean shoot lengths were similar (9.6mm and 9.2mm for 14h, 9.33mm and 7.0mm for 24h). These differences are likely to be related to the breakdown products of sucrose, which are glucose and fructose. Since fructose was not producing this effect at higher concentrations and glucose and sucrose are, it was probable that the glucose produced by the breakdown of sucrose was an active factor inhibiting shoot growth under these conditions. Glucose at 3% was used in all further work.

The initial temperature chosen was 25° C as being optimal for growth (Brewster, 1994), and this was not varied as higher temperatures as well as lower temperatures may cause changes to inflorescence development in susceptible members of the *Alliaceae*.

5.0 Vegetative development in *A. ampeloprasum* var. *babingtonii* from bulbil formation to floral competence

Although much is known of vegetative characteristics in commercially important *Alliums* such as *A. cepa*, *A. sativum* and *A. ampeloprasum* var. *porrum*, details of the physiology and development of *Allium ampeloprasum* var. *babingtonii*, have not been similarly investigated. However, much can be conjectured by comparison with similar species. For example, Kamenetsky & Rabinowitch, (2002), noted that all *Alliums* need to reach a certain physiological age (or critical mass) in order to flower when grown from seed. Therefore, it is likely that *Allium ampeloprasum* var. *babingtonii*, also has a maturity requirement for floral competence, and must pass through an initial juvenile (vegetative) phase. Generally, *Alliums* with large bulbs take longer to reach maturity and achieve the amount of reserves (critical mass of the bulb) necessary for floral competence (Kamenetsky and Rabinowitch, 2002). *Allium ampeloprasum* var. *porrum* can be florally determined after the production of seven true leaves, including primordia (De Clercq and Van Bockstaele, 2002), often bolting in its first year (Brewster, 1994). Since *Allium ampeloprasum* var. *babingtonii* is a large plant, with leaves reaching up to 1 metre in length, and a scape of up to 2 metres in height, it is likely to have an extended juvenile period, whilst the appropriate critical mass is attained. Observation suggests that *Allium ampeloprasum* var. *babingtonii* is likely to flower in its third year from planting as bulbils (Shipton, 2000).

The phenology of *Allium ampeloprasum* var. *babingtonii*, has been outlined on a number of occasions, (e.g. Stearn, 1978, 1987; Stace, 1991; Treu, 1999). In order to identify characteristics associated with floral development, vegetative development

must first be characterised. In the work described in this chapter, bulbils (vegetative growth produced in the inflorescence) were examined, planted and monitored through three growth seasons (a growth season for this species is considered to be from sprouting in autumn to dormancy the following summer), and characteristics such as leaf number and size, bulb size and bulblet (vegetative growth produced on rhizomes underground generated by the parent bulb) numbers were recorded to establish a developmental timetable for the vegetative phase. In particular, characteristics that would indicate floral competence were looked for. The importance of vernalization as a requirement was also investigated. Gibberellic Acid (GA₃) was applied to immature explants in culture to investigate its effects in promoting floral determination in var. *babingtonii*.

5.1 Bulbil physiology

5.1.1 Materials and methods

Mann (1960) stated that bulbil morphology in *Alliums* is similar to that of bulbs. Bulbil morphology was recorded for comparison with mature bulbs. Bulbils were harvested as part of whole inflorescences during August 1999 from Cornwall (SH99, see 2.0) and examined by dissection microscope (x 40). However, though storage tissue was well developed, the basal plate could not be seen, nor could any leaf scales. An apical shoot was apparent in some bulbils, but was not visible in most bulbils examined. This appeared to be unrelated to bulbil size (data not presented). The following year, morphological examination of bulbils was completed using material from the inflorescences of whole plants that were collected from the same population in Cornwall, which were replanted in outdoor beds in Worcester (SH00p). The bulbils were matured on the inflorescence until dehiscence (mid-October) and

examined using a dissection microscope (x 40). Bulbils were selected using random numbers, and then dissected; the stages of dissection were recorded using a Sony digital camera MVC FD85. The numbers of bulbils per inflorescence were noted. Six fresh bulbils chosen at random were dissected to the apex and recorded using a JEOL 520LV scanning electron microscopy (SEM).

5.1.2 Results



Figure 37: All the bulbils from one inflorescence (two year old grown from bulbil at Worcester, maturing in July 2001) (SH99) Scale in mm. This inflorescence has no secondary inflorescences, and is fairly small with just 17 bulbils. The remains of the floret pedicels are attached to some of the bulbils. Three have fully developed coriaceous tunics, five have partially developed tunics, nine remain green.

- C = bulbil with coriaceous tunic
- B = bulbil lacking coriaceous tunic
- P = partially developed coriaceous tunic
- Pe = pedicel of floret
- S = stalk or point of attachment to receptacle

Bulbils develop in a wide range of sizes and shapes, spherical-to-ovoid, usually 6 – 15 mm length (Fig. 37). Some develop with hard, brown, outer tunics whilst others remain green, even when left to further mature on the plant until dehiscence, or during further storage in cool conditions (approximately 3°C, for up to a year). The tunics were frequently incomplete. Papery layers were also often present, probably

the remains of the bracteole subtending the cyme. The bulbils are largely sessile, unlike the florets, though occasionally a very short pedicel of up to 1 mm length may be present (Fig. 37). Bulbils from secondary inflorescences tend to be smaller (3 – 5 mm), and usually more spherical than ovoid (Fig. 38)



Figure 38. Entire secondary inflorescence, with three bulbils and the remains of one floret. The scape is thickened and twisted, and the remains of the bracteole are visible.

S = secondary scape
F = floret
B = bulbil
Br = bracteole

(Scale bar 10 mm)

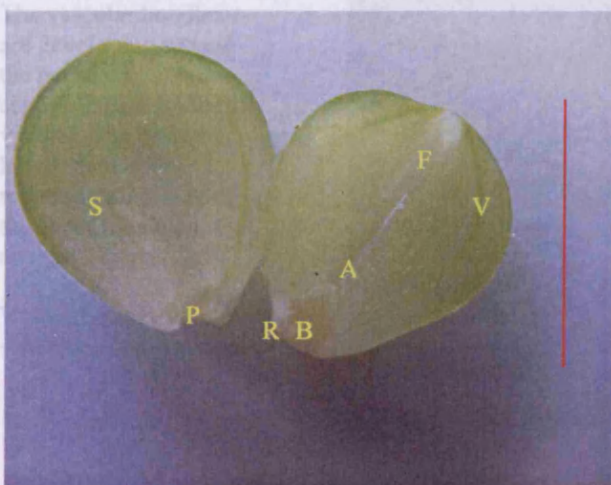


Figure 39: Bulbil bisected longitudinally. The venation is well developed in the storage tissue. The basal plate is close to the point of attachment to the inflorescence. The apical shoot has 3 layers of leaves, and is developing through the fissure. Root initials are developing but not yet emerging from the bulbil. Scale bar 10 mm

S = storage layer
V = venation
F = fissure
A = apical shoot
R = root
B = basal plate
P = point of attachment

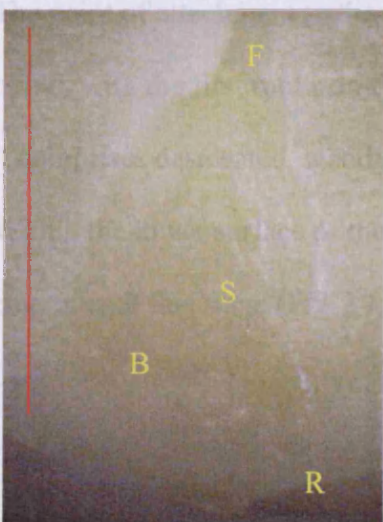


Figure 40: Median LT section through bulbil 9 x 9mm. Shoot is clearly defined, (approx 3mm length) with the central fissure extending above it. There are at least 3 leaf primordia within the shoot. The basal plate shows as darker tissue beneath the shoot with the root initial arising on the perimeter. Scale bar approx. 3mm.

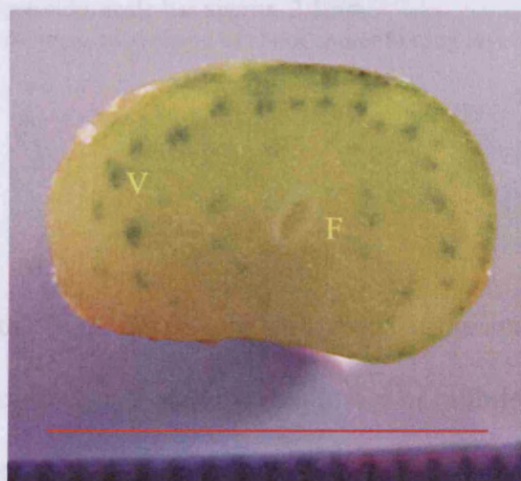
B = basal plate
S = shoot
F = fissure
R = root initial

Above the basal plate, the storage tissue is a single layer, a modified scale leaf, with a central fissure through which the shoot develops. Several leaf layers are apparent within the shoot, surrounding the apical dome. The venation in the storage tissue of both bulbils and leaves is typical of most members of the superorder Liliidae, with parallel veins running the length of the tissue, similar to the model proposed by Brewster (1994).

The storage tissue surrounds the developing shoot, a narrow fissure being formed at the centre of the storage tissue, allowing the shoot to develop (Fig. 41).

Figure 41: Bulbil bisected transversely. The vascular bundles are developing around the perimeter, and the central fissure is clearly visible. The fissure is longer than wide, reflecting the shape of the shoot (Scale bar approx. 12mm).

V = vascular bundle; F = fissure. Scale in mm.



The point of attachment to the inflorescence is typically to one side of the base of the bulbil, with the first root primordia developing on the opposite side (Fig. 40A and B). It comprises desiccated, woody material which partially penetrates into the bulbil. Within the lower surface of the basal plate, root initials are visible, but not usually emerging at this stage (Fig. 39, 40 and 41).

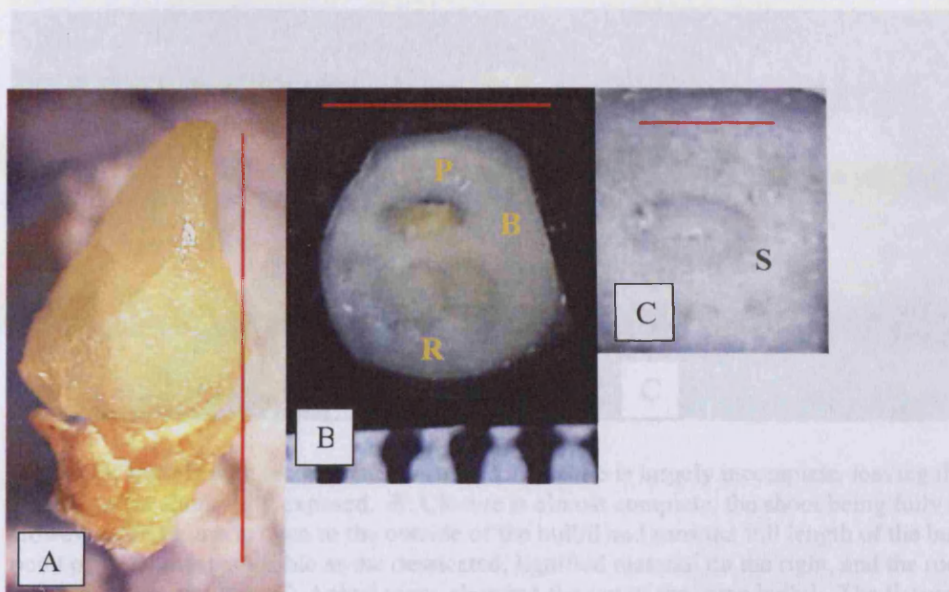


Figure 42: Internal structures.

A: Shoot with storage tissue removed, scale bar 5mm.

B: Section through basal plate, showing lignified point of attachment intruding into basal plate tissue, with three root initials arising to one side, scale bar approx. 3.5mm.

C: Section through storage layer showing oval shape of shoot and enfolding layers of leaves, scale bar approx 2mm.

B = basal plate

P = point of attachment

R = root primordia

S = shoot;

In the majority of bulbils examined, the fissure was sealed at the apex, but in a small number, this closure was incomplete (Fig. 43). A number of bulbils showed the fissure extending to the outer tissues, from apex to basal plate. Occasionally, two bulbils developed together as 'twins' sharing the same point of attachment, often in association with floret pedicels. Bulbils are also frequently misshapen as a result of mechanical pressures (Fig. 44).

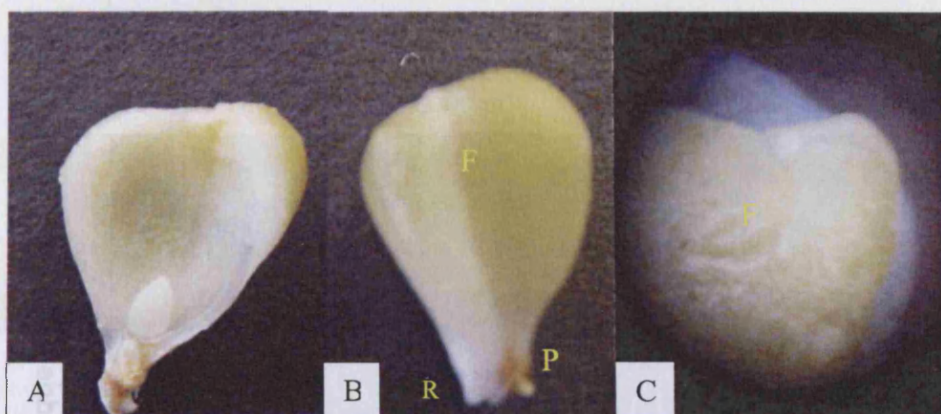


Figure 43: Bulbils with incomplete closure. **A**: Closure is largely incomplete, leaving the apparently normal apical shoot fully exposed. **B**: Closure is almost complete, the shoot being fully enclosed. However, the fissure is open to the outside of the bulbil and runs the full length of the bulbil. The point of attachment is visible as the dessicated, lignified material on the right, and the root primordia are apparent on the left. **C**: Apical view, showing the top of the same bulbil. The fissure is seen as a spiral shape, probably reflecting the morphogenesis of the bulbil. Bulbils approximately 7mm diameter.

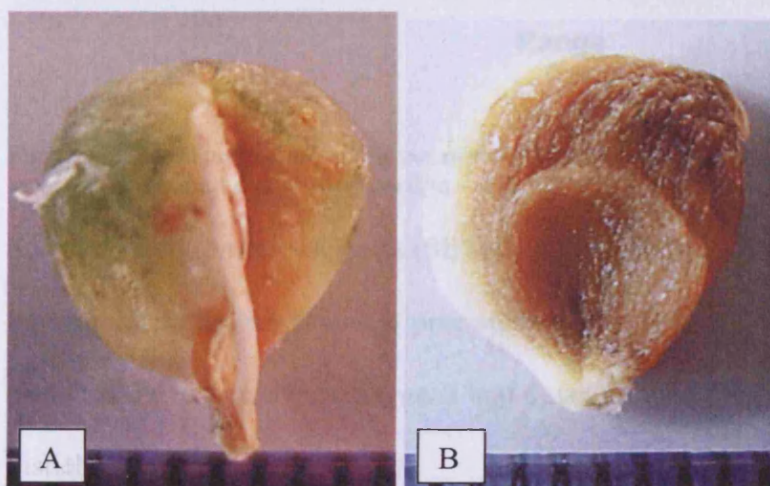


Figure 44: **A**: 'Twin' bulbils sharing the same point of attachment each with their own apical shoot internally. Also visible is the pedicel for an attached floret. **B**: Bulbil showing deformation as a result of pressure exerted by adjacent bulbil (Scale in mm).

The number of bulbils per inflorescence was widely variable (range 19 – 90) with a mean of 45.67 ± 1.95 , (Fig. 45).

3.2 First season development

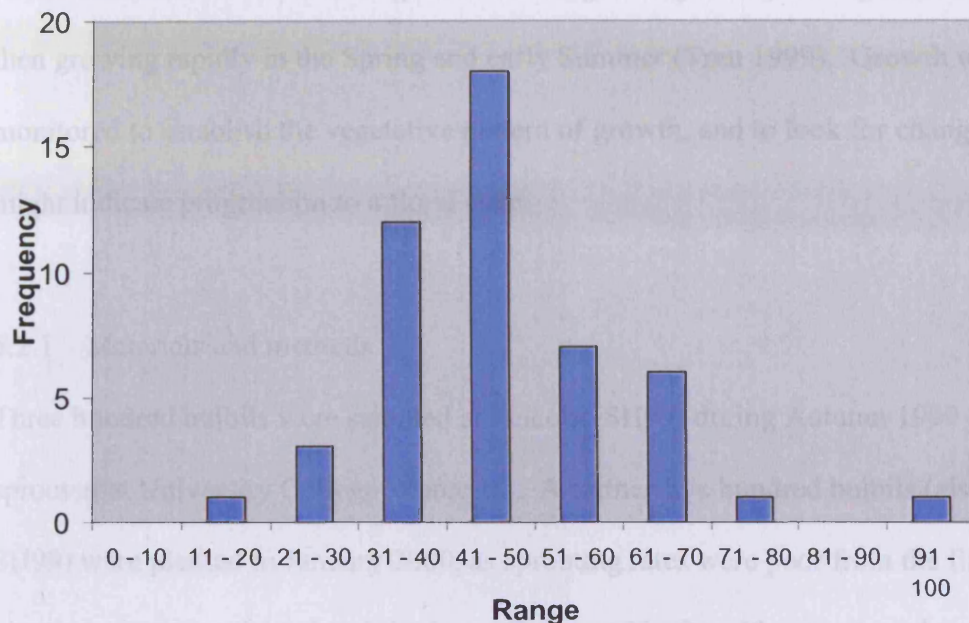


Figure 45: Mean number of bulbils per mature inflorescence (SH99). Floret numbers were not recorded as they had partially dehisced ($n = 49$).

Scanning electron micrographs (SEMs) of the bulbils showed each primordium developing within the previous primordium (Fig. 46). As the leaves developed, they enclosed the newer primordia, each leaf developing at 180° to the previous leaf in a distichous phyllotaxis.

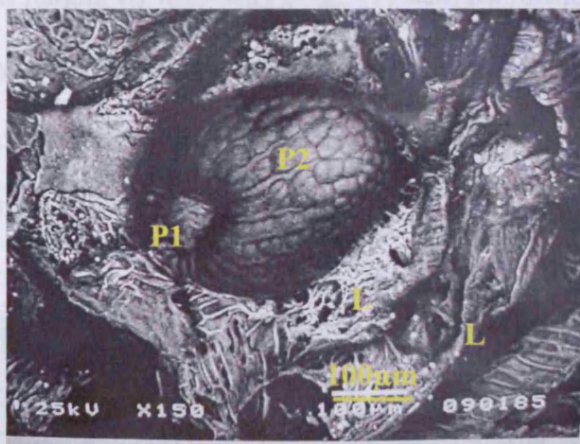


Figure 46: Scanning electron micrograph (SEM) of bulbil (SH00) dissected to show apex. Newest primordium is approximately $100\mu\text{m} \times 60\mu\text{m}$ (P1). This is almost completely enclosed by previous leaf, approximately $720\mu\text{m} \times 500\mu\text{m}$ (P2). Two further leaves (L) were removed.

5.2 First season development

The growing season is approximately October to August, the bulb becoming dormant in late summer. Shoots re-emerge in Autumn, growing slowly throughout Winter, then growing rapidly in the Spring and early Summer (Treu 1999). Growth was monitored to establish the vegetative pattern of growth, and to look for changes that might indicate progression to a floral state.

5.2.1 Materials and methods

Three hundred bulbils were sampled at random (SH99) during Autumn 1999 and sprouted at University College Worcester. A further five hundred bulbils (also SH99) were planted in January 2000, as sprouting rates were poor from the first planting. Initially planted in John Innes Compost No. 2 in 13 cm pots and grown in cold frames, these were transferred to outdoor beds at University College Worcester, in May 2000 (see 2.0). Growth was examined in May, June, July and August.

5.2.2 Results

Each sprouting bulbil produced 1 – 3 above-ground leaves approximately 15 – 20 cm in length, by mid-May (Fig. 47) with no further sprouting after this date. The asymmetrical root development noted in sprouting bulbils continues throughout the growth period, with roots arising largely, but not exclusively to one side of the original point of attachment.



Figure 47: *Allium* beds, May 2000.

Development may have been slower than expected in the wild, due to adverse growing conditions initially (poor drainage) and transplantation trauma.

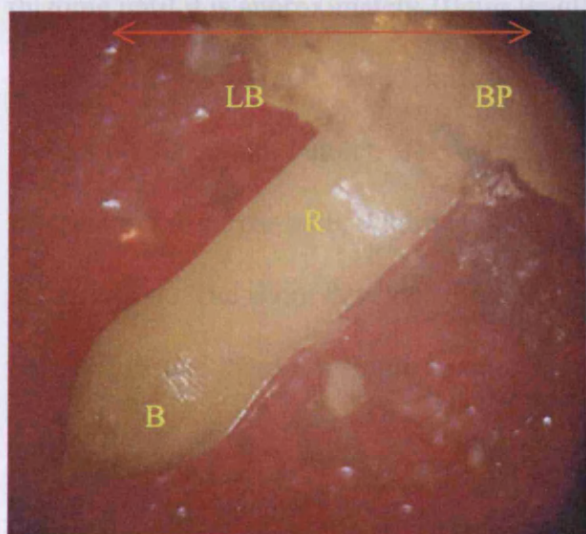


Figure 48: Axillary bud developing from basal plate (*A. ampeloprasum* var. *babingtonii*). Scale bar approx 3mm.

Bulblet shows developing venation and chlorophyll (green colouration at tip).

LB – leaf base
BP – basal plate
R – rhizome

Each plant also produced 0 – 2 single bulblets from below-ground axillary buds (Fig. 48). As the parent bulb increased in girth, and the outer leaves senesced, the bulblet became externalised. However, bulblets did not sprout *in situ*. Observations may be influenced by loss of bulblets due to predation, for example, by beetle larvae, or necrosis, for example due to wet conditions or fungal rots. In either case, numbers observed would be lower than the number actually produced by the plant.

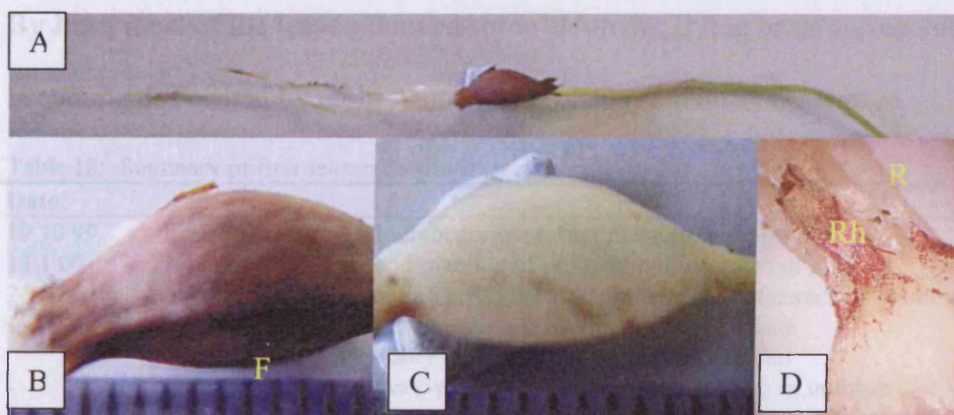


Figure 49: Developing bulblet. A: Bulblet sprouting, with roots developing. B; Bulblet with outer covering showing flat side (F) that developed adjacent to parent bulb. Scale in mm. C. Same bulblet

with outer covering removed. Bulblet is still flat sided but without hard corners typical of the bulblet helmet-shape. D: Dissected through the centre. Remains of rhizome (Rh) are visible, with root development (R) to one side of this.

Bulblets removed and planted separately sprouted similarly to bulbils, in both morphology and timing (Fig. 49). The bulblet continues to develop at the tip of the rhizome until it is approximately 10mm length. A hard outer covering (tunica) is formed. It is thicker and more robust than that developed on bulbils and is always present on the mature bulblet. The bulblet is helmet-shaped, the flat side always adjacent to the parent bulb. Ultimately, the rhizome withers. The outermost bulblet is the largest. The shoot develops through the centre fissure from the basal plate, as with bulbils and there is a single layer of storage tissue as with bulbils.

The divergence angle of the leaves is 180° from emergence to maturity, each leaf being enfolded by the previous leaf (Fig. 50).



Figure 50: Shoot (approximately 10 cm) showing 180° angle of divergence (see also Figure 17c for transverse section)

By June, most of the leaves showed some chlorosis, dying back during July, and becoming desiccated by early August. All plants remained vegetative.

Table 18: Summary of first season development

Date	Age	
19 10 99	0	All bulbils dormant, first planting into pots
13 1 00	12 weeks	Second planting of dormant bulbs (stored at 3°C)
26 5 00	30 weeks	Sprouting is 4% for first planting, 62% for second planting. No further sprouting is observed after this date Typically 0 – 2 bulblets produced per plant Leaves typically 15 – 20 cm length, 1 – 3 in number Roots produced largely to one side – but not exclusively – of the basal plate
17 6 00	34	Some chlorosis apparent in leaf tips

18 7 00	38 weeks	Leaf dieback begins from tips of leaves, moving downwards
5 8 00	40 weeks	Aboveground growth ceases, all leaves necrotic

In the first season, (Table 18), typically, only one – three leaves were produced, these reaching up to 20 cm in length, before senescing in the summer. Small numbers of bulblets were produced, usually one or two, but these did not sprout during this growth season. Morphology of the bulb showed little change, root growth was asymmetrical at sprouting, and tended to remain so during growth. Flowering was not observed, neither were any changes that might be indicative of a change to the floral state. The phenology was confirmed as that recorded by Treu (1999), with growth from Autumn to Summer, followed by a period of dormancy. Sensitivity to water-logging leading to necrosis of bulbils planted in pots was noted, and all further planting was carried out direct into the plot at UCW.

5.3 Second season development

5.3.1 Materials and methods

Plants are likely to flower in their third year (John Shipton *pers. comm.*). Floral competence is likely to be related to maturity, which is usefully quantified by leaf number or bulb size rather than time (1.7.1.1). Therefore, plants in their second season were monitored closely, to complete the vegetative developmental timetable, and to identify developmental patterns that could be used to indicate floral competence. Plants from the first season (SH99) were grown on in the same plot, and were sampled at random at monthly intervals, commencing in October 2000. The apices were excised, wax embedded, sectioned and stained (3.1). One was photographed during April when growth was likely to be fairly advanced but before the dormancy period, using the JEOL 15V scanning electron microscope (SEM).

5.3.2 Results

Leaves emerged during October (Figure 51), but their elongation was slow, reaching just 25 cm by March.



Figure 51: One year old plant sampled in October, at the beginning of the second growth season (scale in mm). Rooting continued to be asymmetrical.

Leaf elongation was rapid from March to June, reaching 60 cm before beginning to die back in June/July, a similar phenology to the previous season. The storage tissue became necrotic in March, and was non-existent in samples taken in April (Table 20). In June, the development of a new storage layer was visible, whilst outer leaves became papery, forming a protective layer. Root development continued in the asymmetrical pattern established during the first season's growth.

None of the apices dissected for examination showed any signs of floral development (Fig. 52).

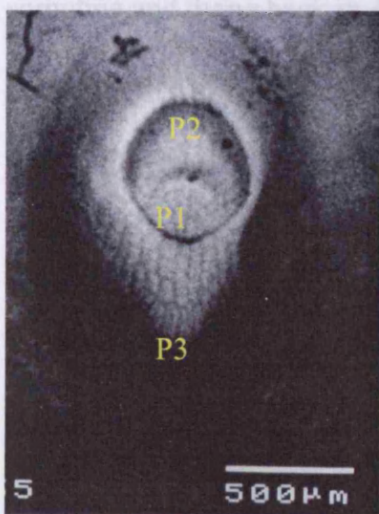


Figure 52: SEM of vegetative apex sampled 9 4 01 (SH99). The youngest primordium is approximately 350μm width at widest point (P1) with the second and third primordia clearly visible (P2 and P3). The 180° angle of divergence of the leaves is also clearly visible, each leaf developing to enfold the younger primordia.

However, from the whole population of SH99 grown in this year (approximately 300 plants), two produced

inflorescences. These were both small containing less than 20 bulbils. In each of these plants, the parent bulb divided into two daughter bulbs with their own storage tissue, before the basal plate disintegrated beneath the new structures.

Bulblets were also produced in late winter/spring, typically three or four, on all plants whether floral or vegetative. However, none of these sprouted (Table 20).

This compares with the first season's growth where one or two bulblets were produced (all plants remained vegetative), and none sprouted (Table 18).

Table 19: Summary of development of 2nd season plants (SH99). Leaf number includes primordia. Leaves were removed during dissection, primordia recorded from slides where possible. Number is shown relative to the storage tissue (st) i.e. number of leaves is shown, then the presence of storage tissue is indicated, then the leaf primordia within this. All were evaluated by dissection and production of slides, except where shown as SEM (scanning electron micrograph).

Date of sampling	Age of plant (years; weeks)	Storage tissue	Total no. of leaves (including primordia)	No. of bulblets	Leaf length (longest) cm
10.10.00	1 y	Firm	St + -		0.8
17.1.01	1y 12w	Firm	St + -		1.3
12.2.01	1y 16w	Firm	St + 10		2.6
13.3.01	1y 20w	Shrinking	St + 11	4	25
9.4.01	1y 24w				
	a) wax embed	None	11	4	38
	b) SEM	None	10	3	43
4.6.01	1y 32w	New	5 + st + 1	3	60 chlorotic
28.6.01	1y 34w	New	5 + st + -	3	Dying back, outermost completely dessicated
		0.34cm diameter			

The phenology for the second season is similar to that of the first season, the plants sprouting and dying back in Autumn and late Summer respectively (Table 19). The storage tissue was observed to decay in Spring, and a new storage layer was developed in Summer. However, the second season plants produced more and longer leaves, and more bulblets, none of which sprouted in either season. Root development continued to be asymmetrical. A small number of plants produced inflorescences, but these were small compared with those recorded by Treu (1999).

5.4 Third season development

The very small numbers of plants flowering in the first two seasons' growth, shows that time *per se* is not a controlling factor in maturity requirements for floral competence. Therefore, floral competence could not easily or accurately be identified by age, but size measured by leaf number or bulb size was more likely to provide useful data. Mature plants were purchased from John Shipton Nurseries (see 2.2). These were supplied as having completed two seasons growth, at time of purchase and planting in October 2000. Twenty *Allium ampeloprasum* var. *babingtonii* and twenty *Allium ampeloprasum* var. *ampeloprasum* were obtained, for physiological comparison (JS00 and JS00 amp). *Allium ampeloprasum* var. *ampeloprasum* is extremely similar in its phenology and physiology, but produces fertile florets rather than bulbils (see 1.0, Table 1). Examination of these two species in the probably year of inflorescence development could provide insights into bulbil production in var. *babingtonii*.

5.4.1 Materials and methods

The plants were dormant at the time of purchase, and initial height, width and weight were recorded. At monthly intervals, two var. *babingtonii* and two var. *ampeloprasum* plants were sampled using random numbers throughout the growth season from October, to July when any inflorescence would be well developed (Treu 1999). They were examined by eye and recorded photographically (Sony MVC85 digital camera). One of each variety was dissected, wax-embedded, sectioned and stained (3.3.1). The other was placed into tissue culture (see 4.3, Table 18) for evaluation of floral determination of the apex. This was grown for one month and then wax embedded and sectioned (3.3.1). Additionally, in weeks 22, 26 and 28

from planting, apices were photographed using SEM. This period is likely to include early floral development as vernalization is likely to be a requirement of floral competence in line with other *Alliums*, and full inflorescence development occurs in Summer probably in the third season (Shipton, 2000 personal communication). Leaf number, bulblet number and maximum leaf length were recorded.

5.4.2 Results

The plants followed a similar pattern to previous years, sprouting during late Autumn, growing slowly during the winter, and then growing rapidly during Spring and Summer. Leaf length reached a maximum of 54 cm in early June (Fig. 53).

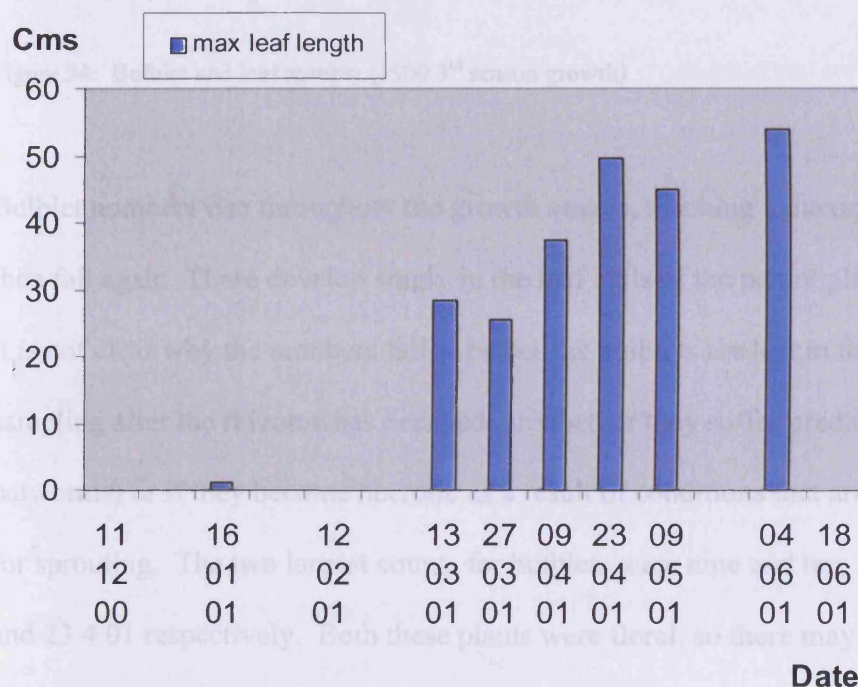


Figure 53: Maximum leaf length achieved during third season (cm) (JS00). NB n = 1 for each sample.

Bulblet and leaf number may have been affected by losses due to predation and necrosis (Fig. 54). Therefore, these figures represent a minimum extent of

elongation. Leaves may also have been affected by mechanical damage, predation or disease, so leaf length also represents a minimum level of elongation of this organ.

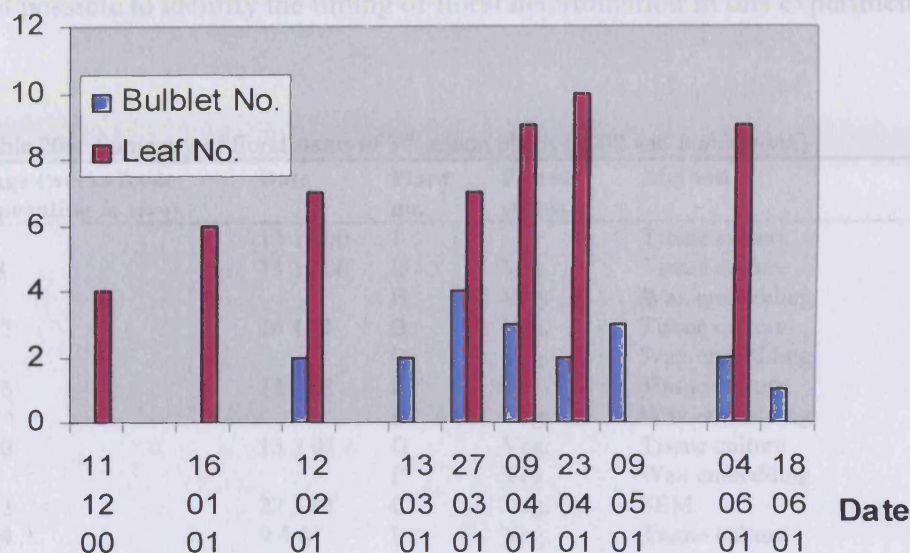


Figure 54: Bulblet and leaf number (JS00 3rd season growth)

Bulblet numbers rise throughout the growth season, reaching a maximum in March, then fall again. These develop singly in the leaf axils of the parent plant on rhizomes. It is not clear why the numbers fall, whether the bulblets are lost in the soil during sampling after the rhizome has decayed, or whether they suffer predation (e.g. from cutworms) or if they become necrotic as a result of conditions that are not favourable for sprouting. The two largest counts for bulblets were nine and ten, sampled 9 4 01 and 23 4 01 respectively. Both these plants were floral, so there may be some relationship between bulblet number and the floral state, possibly relating to the critical mass of the bulb and the requirements for floral competence.

Overall, three of 17 *A. ampeloprasum* var. *babingtonii* showed floral development (Fig. 55, Table 20), whilst only one of 16 *A. ampeloprasum* var. *ampeloprasum*

sampled showed floral development (Table 20). However, none of the samples excised and maintained in tissue culture showed floral development, therefore it was not possible to identify the timing of floral determination in this experiment.

Table 20a: Summary of floral status of 3rd season plants (JS00 var. *babingtonii*)

Age (weeks from sprouting <i>in vivo</i>)	Date	Plant no.	Floral status	Method
4	13 11 00	J	-	Tissue culture
8	13 12 00	D	Veg.	Tissue culture
		H	Veg.	Wax embedding
12	16 1 01	B	Veg.	Tissue culture
		Q	Veg.	Wax embedding
16	12 2 01	I	-	Tissue culture
		E	Veg.	Wax embedding
20	13 3 01	G	Veg.	Tissue culture
		P	Veg.	Wax embedding
22	27 3 01	C	Veg.	SEM
24	9 4 01	L	Veg.	Tissue culture
		A	Floral	Wax embedding
26	23 4 01	F	Floral	SEM
28	9 5 01	K	Floral	SEM and wax embedding (half each)
30	21 5 01	R	Veg.	Tissue culture
32	4 6 01	O	Veg.	Wax embedding
34	18 6 01	N	Veg.	Wax embedding
36	3 7 01			Sampling discontinued as dormancy was well advanced

Table 20b: Summary of floral status of 3rd season plants (JS00 var. *ampeloprasum*)

Age (weeks from sprouting <i>in vivo</i>)	Date	Plant no.	Floral status	Method
4	13 11 00	O	Veg.	Tissue culture
		L	Veg.	Wax embedding
8	11 12 00	R	-	Tissue culture
		A	Veg.	Wax embedding
12	16 1 01	F	Veg.	Tissue culture
		P	Veg.	Wax embedding
16	12 2 01	J	-	Tissue culture
		M	Veg.	Wax embedding
20	13 3 01	S	Veg.	Tissue culture
		G	Veg.	Wax embedding
24	9 4 01	I	Veg.	Tissue culture
		Q		Wax embedding
28	9 5 01	B	Veg.	Wax embedding
30	21 5 01	D	Floral	Wax embedding
32	4 6 01	H	Veg.	Wax embedding
34	18 6 01	C	Veg.	Wax embedding
36				Sampling discontinued as dormancy was well advanced

5.5 Floral emergence

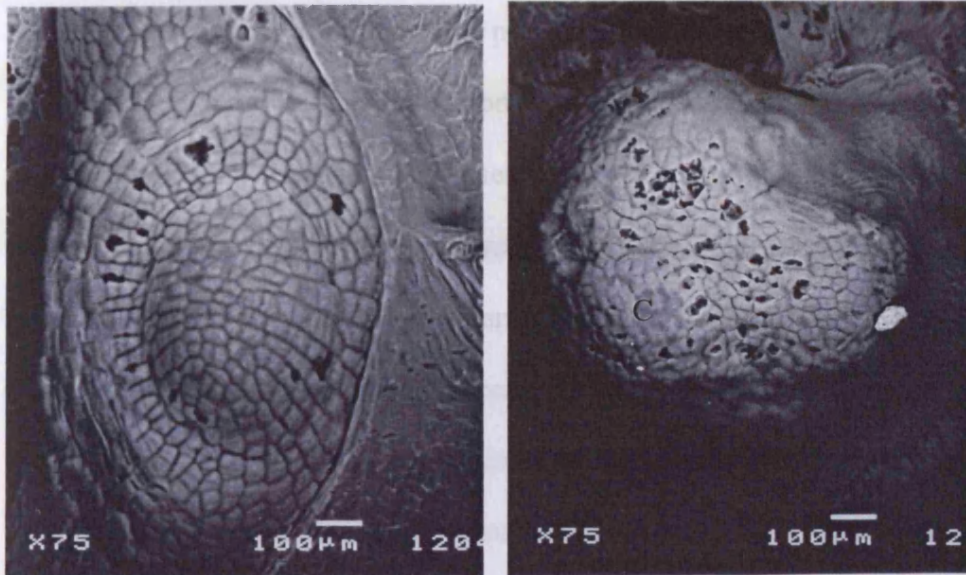
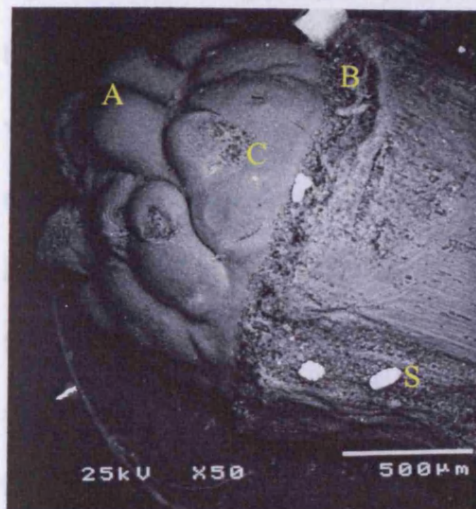


Figure 55: Floral development (JS00F – 26 weeks into 3rd season of growth). A) Apical dome of daughter bulb forming in apex of last formed leaf. B) Apical dome of inflorescence (approximately 600 μm diameter) beginning to develop areas that correspond to cymes.

Figure 56: Floral development (JS00K – 28 weeks into 3rd season of growth)



Cymes have developed into well-defined outswellings, with primordia arising. Inflorescence is approximately 1500 μm diameter at base, and approximately 1000 μm in height. Apical florets are beginning to form. The bract has been removed.

C = cyme
A = apical floret
S = scape
B = line of removed bract

It is apparent from second and third season development, that flowering is not simply a function of time of growth, but that any maturity requirement must be related to other factors, which may be interacting with time. This would be consistent with other work with *Alliums*, which have shown a minimum size requirement, measured as minimum bulb size (critical mass) or leaf number. This temperature is thought to

5.5 Floral competence

Floral competence is the ability of the plant to respond to the floral stimulus.

‘Maturity’ and duration of vernalization are considered as likely facts that interact to impose floral competence in this species (1.7.1). The hypothesis that a minimum size (critical mass) of the bulb is necessary for a floral response was tested by growing bulbs of known size *in vivo*, and examining for a correlation between bulb size and the production of an inflorescence. The hypothesis that vernalization is necessary for floral competence was tested in two ways; firstly, by subjecting unsprouted bulbils to a potentially vernalizing treatment and comparing the proportion that became floral, with the proportion that became floral in a control population. Secondly, by subjecting larger bulbs to a potentially vernalizing treatment, and comparing the proportion that became floral, with the proportion that became floral in a control population. This enabled me to test whether a vernalization requirement must interact with a requirement for maturity.

Gibberellins, particularly Gibberellic acid (GA₃), have been used to induce flowering in a number of monocots (1.7.1.3.). Therefore, the hypothesis that GA₃ would induce floral development was tested by application of GA₃ at various concentrations to apices in tissue culture and the resulting development examined for the induction of flowering in *Allium ampeloprasum* var. *babingtonii*.

5.5.1 Materials and methods

5.5.1.1 Vernalization

All bulbils collected from population 1 were stored at 3°C (see 2.0); none of them had subsequently flowered either *in vivo* or *in vitro*. This temperature is thought to be sub-optimal for vernalization (Smith, 2000) and maintains the unsprouted state,

and so was used as the control non-vernalizing treatment for unsprouted bulbils. Unsprouted bulbils (N = 160) were selected at random (SH99, stored for eight months), of which half (selected at random) were treated to a potentially vernalizing treatment of 6 weeks at 7°C (Smith 1999), whilst the other half remained at 3°C for the same period (Fig. 57). These had not sprouted since harvesting from the inflorescences, so the vernalization response could be examined in isolation from any putative maturity requirement for floral competence. Apices were excised from all bulbils in both treatments, and were cultured on 3% sucrose, 0.8% Agar Technical No. 3 (Oxoid), B5 salts, 25°C, 24h photoperiod. Explants were selected at random, (10 vernalized and 10 non-vernalized) dissected, wax-embedded, sectioned and stained (see 3.3.1) each month following for 6 months (Fig. 57).

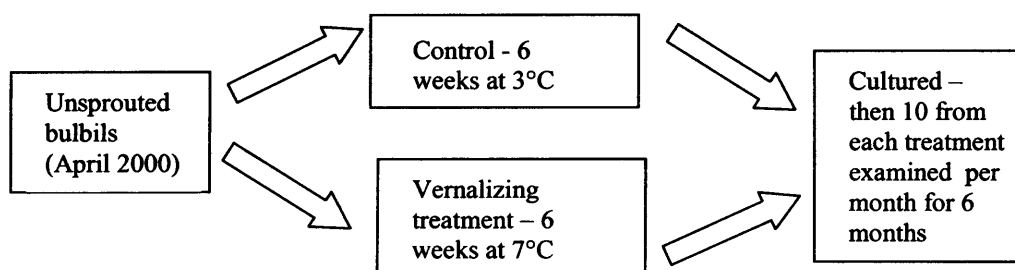


Figure 57: Treatment given to unsprouted bulbils to examine the effect of a vernalization on vegetative bulbils (control temperature of 3°C having been used for storage of unsprouted bulbils and demonstrated not to promote flowering; 7°C having been used successfully for vernalization in *A. ampeloprasum* var. *porrum* - leek).

Larger plants were also examined; eight sprouting, mature bulbs of unknown age (SH11/01) were selected at random from those of size 3 cm diameter and above. These had been sampled during November, 2001, so were unlikely to have been subjected to low temperatures sufficient to constitute a vernalization treatment. These were planted in pots with free draining compost, comprising 10 parts of proprietary brand multipurpose compost, with 1 part grit and 1 part sand. Half were

selected at random and were given a vernalizing treatment of 7°C for 6 weeks (as for the unsprouted bulbils), the other half grown at a constant temperature of 18° to maintain active growth. Both treatments had 8h photoperiods, with a light intensity of 19.5 $\mu\text{Mm}^{-2}\text{s}^{-1}$, (LI-COR LI 118B light meter), approximately 750 lux (mean of 3 readings), (TES-1334 Digital Illuminance Meter).

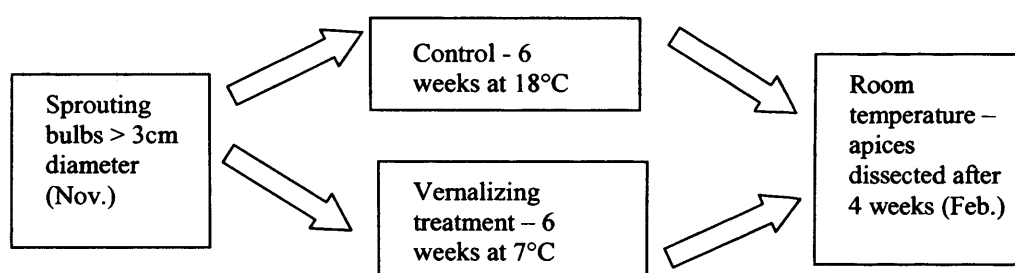


Figure 58: Treatment given to sprouting bulbils to examine the effects of vernalization on vegetative plants (control temperature maintained active growth, vernalizing treatment adopted from work with *A. ampeloprasum* var. *porrum* – leek).

After the 6-week treatments, the plants were all transferred to room temperature at ambient light levels. Temperature was monitored to ensure that no vernalizing conditions could develop during this period. After a further 4 weeks, they were dissected and examined for signs of floral development.

5.5.1.2 Gibberellic acid

The possibility of simulating the maturity/vernalization requirement with the use of Gibberellic acid (GA₃) was investigated. Since GA₃ molecules are known to be thermolabile at $\geq 60^\circ\text{C}$ (Sigma, pers. comm., 2000) particularly at concentrations of over 100 ppm, two alternative methods were used to add GA₃ to the growth medium/explant. For concentrations up to (and including) 100ppm, the GA₃ was added to the medium before autoclaving. For a larger range of concentrations, 0 –

750 ppm (Table 21), the GA₃ was dissolved in absolute ethanol, and then applied to the surface of the medium in aseptic conditions after autoclaving. The ethanol evaporated, leaving the GA₃ on the surface on which the explant was placed. This method was chosen as being the closest analogy to the more common method of applying GA₃ in absolute ethanol to the surface of the plant. As the explants are small, with large amounts of surface area, it was felt the ethanol could significantly damage the tissue if added direct to the explant. Source material was unsprouted bulbils collected in August 2000 (SH00), with 10 explants per treatment. The medium was 3% sucrose, 0.8% Agar Technical No. 3, with Gamborg's B5 salts. Explants were cultured for 6 months then dissected and examined by dissection microscope (x 40).

Table 21: Concentrations of GA₃ tested to induce flowering *in vitro*

GA ₃ concentration
50ppm 0.000145M
100ppm 0.000289M
350ppm, 0.00101M
500ppm, 0.00145M
750ppm, 0.00217M

5.5.1.3 Maturity

In season two and season three, only small numbers of plants produced inflorescences, although all the plants were of the same age in each season. This showed that maturity to flower is not a direct function of age, though it may be an indirect function for example, level of assimilates accumulated, size of apex. This has been measured in *Alliums* as bulb size and leaf number (1.7.1.1). Therefore, plants from two sources were monitored to provide data on bulb weight and size, and

leaf number with respect to floral determination. Whole plants collected from the wild in March 2001 (SH0301, Appendix F) comprised plants of known size, whilst JS00 plants, sourced from a supplier comprised plants of known age. Using these two sources could establish suitable characteristics that could be usefully used to characterise floral competence in this species. Additionally, when whole plants were originally sampled from Holywell Bay (SH00, Chapter 2) and grown on, it was noted that none flowered the following year. These had not been in any way selected for size, but were located for sampling by the scape, as there was no foliage at this time (August 2000). Therefore, all of these plants comprised daughter bulbs of varying sizes. This may indicate that daughter bulbs will not flower the following season, irrespective of size. Known daughter bulbs were also examined, for correlation between bulb size and floral competence.

SH0301 (2.0) was collected as growing plants in Spring, therefore, there was no information on bulb age or weight at the beginning of the season's growth as was done with other samples. Bulb height and width were recorded, in those plants that were still intact at the time of sampling. However, as the bulb decays during spring, in some cases the bulb was shrinking, necrotic or completely missing. Since growth is slow during the winter months, the size of the bulb at the beginning of the growth season (Autumn) and in early Spring before necrosis is likely to be comparable. Therefore, 21 plants from SH0301 of over 2 cm width were planted in an outdoor bed at UCW as previously, and randomly sampled at monthly intervals. These were dissected, photographed and wax embedded, sectioned and stained (see Table 13; 3.3.1) for assessment of the developmental state. The developmental state was examined for correlation with bulb size. Number of bulblets, number of leaves and

leaf length were also recorded to examine for other indicators that could be used to predict floral competence.

JS00 plants were weighed and measured (height and width of bulb) before planting, at the beginning of the growth season in October. This was compared with those found to be floral, and examined for a correlation between weight and floral determination, and age and floral determination. Two plants were sampled each month from November onwards; one was cultured (see Table 18; 4.3) for 6 months so that any inflorescence development would be visible, the other was wax embedded, stained and sectioned for examination under a light microscope (see Table 13; 3.3.1). Additionally in March and April, one plant was selected at random for dissection and the fresh apex observed by SEM

The response of daughter bulbs with respect to size and floral competence was also examined. Twenty-five daughter bulbs (SH0301, sampled in March) over 3cm width were also planted, and grown till the following summer. The number of plants that became floral was recorded.

5.5.2 Results

5.5.2.1 Vernalization

None of the vernalized or unvernallized explants (from bulbils) showed any development associated with floral determination. They remained vegetative, no scape was produced, but many developed new storage tissue. However, mature plants grown on after vernalizing treatments grew normally, the vernalized plants having slightly shorter leaves than the non-vernalized plants (Fig. 59), possibly as a

result of having been exposed to lower temperatures. Vernalized plants exhibited 100% flowering, whilst all non-vernalized plants remained vegetative.

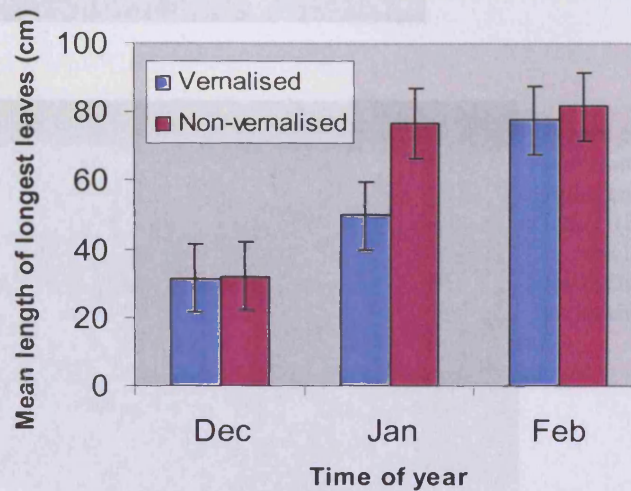


Figure 59: Mean length (cm) of the longest leaf of each plant of vernalized and non-vernalized plants (SH11/01)

The vernalized (floral plants) all had 13 leaves, 12 produced outside the first daughter bulb, then another leaf, then the second daughter bulb and the scape (Fig. 60-62).

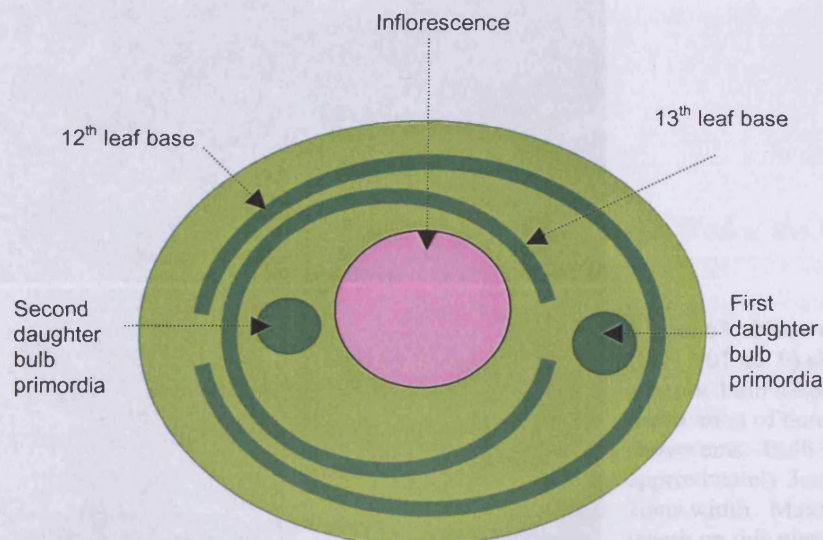


Figure 60: Schematic of scape and daughter bulb formation relative to leaf bases.



Figure 61: 7 bulblets developing in vernalized mature plant (floral) SH11/01 no. 66. (Scale in mms). Outermost side shoot – innermost side shoot is shown left – right.

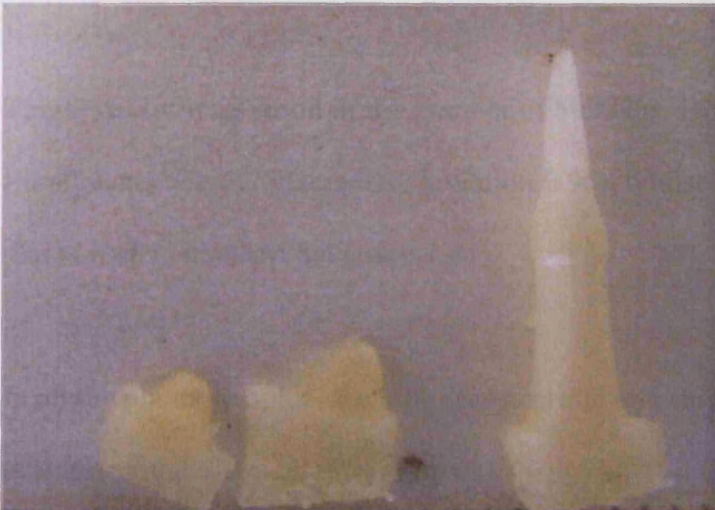


Figure 62a: Daughter bulbs, outermost to innermost left to right, and scape with spathe intact (SH11/01 no. 66). (Scale in mms). Scape is approximately 6mm, spathe is approximately 7mm tall.



Figure 62b: Developing inflorescence (SH11/01 no 66) shown intact above. Spathe has been removed, inflorescence is approximately 1.5mm tall, 1mm width.



Figure 63: Non-vernalized plant (SH11/01 no. 9) showing necrotic bulb tissue and desiccation of outer leaf. Scale shows cms. Bulb is approximately 3cms height x 2cms width. Maximum leaf length on this plant is 94cms.

The non-vernalized (non-floral) plants had more leaves, 2 developing 16 and 2 developing 17 leaves including primordia.

There was little variation in the number of bulblets visible. The mean number for the floral plants was 5.75 (standard deviation 0.96), whilst the mean for the non-floral plants was 5 (standard deviation 1.41).

In all the plants sampled, the bulb was necrotic and shrivelled. The outer leaf was desiccated and disintegrating (Fig. 63).

5.5.2.2 Gibberellic Acid

None of the explants showed any development associated with floral determination. They remained vegetative at all concentrations, and under both application methods.

5.5.2.3 Maturity

Plants sampled in April were all floral (Table 22). Those sampled at the beginning of May were 50% floral ($n = 6$), whilst 60% ($n = 5$) of those sampled at the end of May were floral. In June, 50% were floral. In all these plants, the inflorescence was clearly visible to the naked eye, and development was well advanced. Bulblets were present in the axils of the leaves. However, in samples examined during the later part of the season, i.e. mid-May onwards, most of the bulblets were external to the

parent plant, with only small numbers beneath the outer leaves. This suggests that bulblet production ceases during the latter part of the growth season.

Table 22: Summary of floral status of bulbs of known size, sampled in March and grown in an outdoor bed and sampled at approximately 2 weekly intervals until June (SH0301)

Date of sampling & Plant number	Width at planting (cm)	Developmental state	No. of Bulblets	Leaf length (cm)	Leaf number
23 4 01					
51	4.4	Floral		84	11
28	3.1	Floral	2	81	11
46	3.1	Floral	4	94	11
20	3.2	Floral	5	81	11
7 5 01					
34		Floral	7	92	10
49		Vegetative	4		7 + st + 2
15	3.4	Floral	4	92	9
48	1.9	Vegetative		69	6 + st
2	4	Floral	6	85	10
6	2.8	Vegetative	2		7+st + 2
22 5 01					
38	2.8	Vegetative	3e + 1	56	6 + st + 4
37		Vegetative	2e + 1	57	5 + st + 4
13		Floral	2e + 2	75	11
19	3.4	Floral	3e	72	11
27	4.1	Floral	1e	62d	11
4 6 01					
22	4	Floral	2e	75d	11
59	3.6	Floral	5e	71d	8
16		Vegetative	0	57d	6 + st + 5
55		Vegetative	0	74d	8 + st
41		Vegetative	0	51d	5 + st + 4
59	3.6	Floral			

e = bulblets are external to the parent bulb, rather than internal in leaf axils

d = dieback of the leaf is visible

st = storage tissue; figures indicate number of leaves external to the storage tissue, then the number of primordia developing inside the storage tissue

Leaf length diminishes from mid-May onwards, with chlorosis and dieback

becoming visible from the end of May. In all plants where floral development is not observed, a storage layer develops within the leaves, enclosing the sprout leaves for next season's growth.

All bulbs of which the size was known at the time of planting in March, showed a clear divide between the size of the non-flowering and the size of those flowering, i.e. those 3cms and above were floral, those below this size were vegetative (Table 23).

Table 23: Comparison of bulb width with the developmental state (SH0301)

Bulb width (cm)	Developmental state
1.9	Vegetative
2.8	Vegetative
2.8	Vegetative
3.1	Floral
3.1	Floral
3.2	Floral
3.4	Floral
3.4	Floral
3.6	Floral
3.6	Floral
4.0	Floral
4.0	Floral
4.0	Floral
4.1	Floral
4.4	Floral

It was also noted that none of the small bulbs under 2cm width (SH0301) planted (n = 25) to provide stocks for future work flowered the following season, confirming that smaller bulbs are not florally competent. However, these data indicate that bulb size can be a useful diagnostic criterion for floral competence.

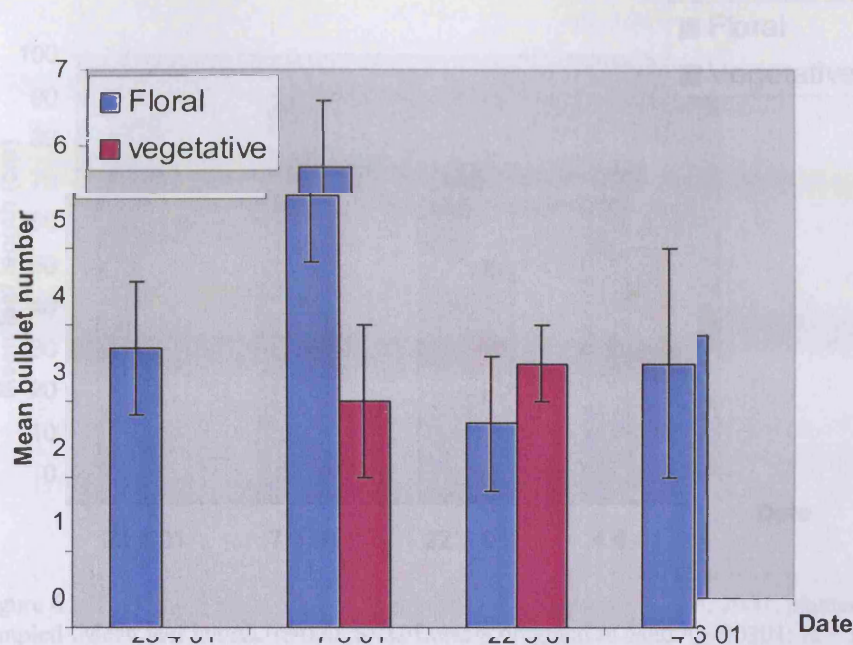


Figure 64: Mean bulblet numbers (\pm SE) in floral and vegetative material, obtained in March, 2001, and planted at UCW, then sampled from April to June (SH0301; $N = 18$)

Over the period of sampling, the mean bulblet number for floral plants ($n = 11$) was 3.09, standard deviation 1.81; mean bulblet number for vegetative plants ($n = 7$) was 1.85, standard deviation 1.85. However, there is no trend observable that would make bulblet numbers a useful indicator of floral or vegetative states. Vegetative plants were not selected in the April sampling, so data were lacking for this month (Fig. 64). In early May there were more bulblets in floral than vegetative material, by late May there were more bulblets in vegetative material. In June, the floral material showed a mean of 3.5 bulblets per plant, but there were none in the vegetative samples

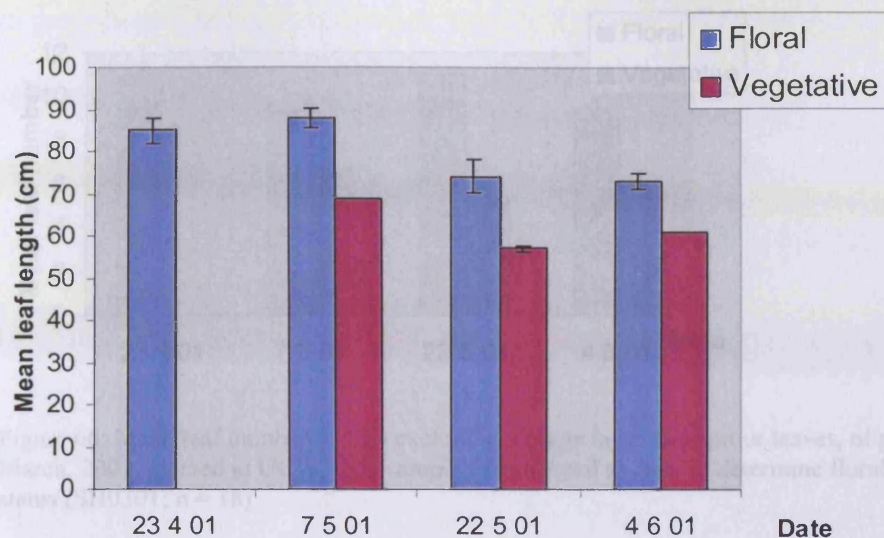


Figure 65: Mean leaf length (cm) (\pm SE) of plants obtained in March, 2001, planted at UCW, then sampled from April to June to determine floral or vegetative status (SH0301; N = 18)

The leaves in floral material are consistently longer than in vegetative material (Fig. 65). However, in the sample considered as a whole, the mean of 73.77 ± 3.12 cm suggested that this characteristic could not be used to identify floral material as the variation is too high and the difference in length between vegetative and floral plants too small. It would be expected that floral plants would be larger than vegetative plants, if it is considered that bulb size (over 3 cm) can be used to indicate floral competence (see 5.5.2.3). Peak leaf length appears to be reached in early May, the leaves becoming shorter before necrosis is visible at the end of May.

If the number of leaves is counted as only those external to the storage layer, then the number of leaves is consistently smaller in vegetative plants than in floral plants (Fig. 64) with all the floral plants having 11 leaves, whilst vegetative plants had fewer (5 – 8) (Table 23).

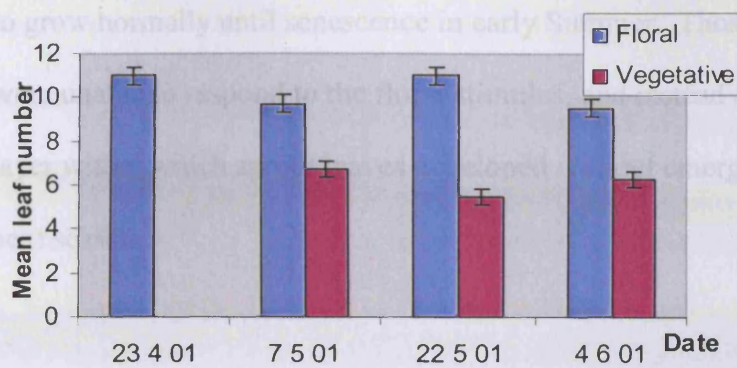


Figure 66: Mean leaf number (\pm SE) excluding storage layer and sprout leaves, of plants obtained in March, 2001, planted at UCW, then sampled from April to June to determine floral or vegetative status (SH0301; $n = 18$)

However, if the storage layer is included as a modified leaf, and also the sprout leaves, then there is little difference between the floral and vegetative plants (Fig. 67).

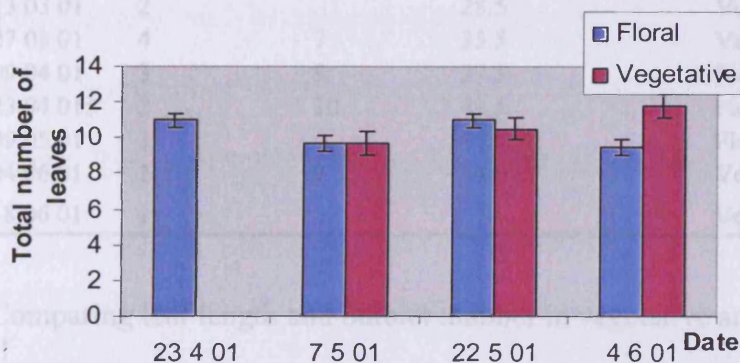


Figure 67: Mean leaf number (\pm SE) including the storage layer and sprout leaves, of plants obtained in March, 2001, planted at UCW, then sampled from April to June to determine floral or vegetative status (SH0301; $n = 18$)

These data from plants of known size at time of sampling (March) indicated that plants large enough to be florally competent (over 3 cm) and having produced 11 leaves (including primordia), were then able to respond to the floral stimulus, producing an inflorescence, with the main bulb dividing into two daughter bulbs. The daughter bulbs developed one in each of the leaf axils of the 10th and 11th leaves. No more leaves developed at this time, and those leaves (including primordia) continued

to grow normally until senescence in early Summer. Those plants smaller than this, were unable to respond to the floral stimulus, and instead developed a new storage layer within which sprout leaves developed without emerging, presumably until the next season.

The bulbs of known age (JS00) provided little floral material (Table 24) just three of the ten plants sampled having inflorescences.

Table 24: Summary of development of 3rd season plants (JS00), obtained in dormant state, planted in October and grown in outdoor beds, then sampled at intervals

Date	Bulblet No.	Leaf No.	Leaf length (cms)	Floral status
11 12 00		4		Veg.
16 01 01		6	1.3	Veg.
12 02 01	2	7		Veg.
13 03 01	2		28.5	Veg.
27 03 01	4	7	25.5	Veg.
09 04 01	3	9	37.5	Floral
23 04 01	2	10	49.5	Floral
09 05 01	3		45.0	Floral
04 06 01	2	9	54.0	Veg.
18 06 01	1			Veg.

Comparing leaf length and bulblet number in vegetative and floral samples showed no correlation between any of these data and floral competence (Appendix 13). Leaf number indicated that there were more leaves on floral plants than on vegetative plants, but these data were too few for meaningful analysis. It was noted that the leaf number of floral plants was less than that recorded for the assessment of plants of known size (SH0301) above. However, this was thought to be due to losses from the seasonal senescence.

The large daughter bulbs (SH0301) of unknown age but larger than 3cm width (n = 25) failed to produce any floral material. This apparent contradiction to the work

above where this size was indicated as a marker of floral competence is unexpected. However, as discussed earlier, indications of size in relationship to floral competence are not straightforward, and may reflect other factors of greater importance, such as assimilate levels, or apex size. It is therefore apparent, that first generation daughter bulbs require further investigation before using to produce predictable floral material, or else should be discarded or grown on.

5.5.3 Summary

The work described in this chapter was carried out in search of identifiable features of the floral response for *Allium ampeloprasum* var. *babingtonii*.

Chronological and developmental age were considered separately and the mean length and number of leaves that formed were compared in vegetative and floral plants. Bulb size and bulblet number were also compared.

The key findings of the work reported here were: -

- The vegetative phase of this species has been documented to provide the foundation for a developmental timetable;
- Competence to flower can be usefully assessed by the bulb width – over 3 cm (the critical mass for this species) indicated that the plant has completed juvenile growth, passing into the mature phase, and is therefore competent to flower;
- Vernalization treatments of 6 weeks at 7° C led to flowering, provided that bulbs had passed through the juvenile phase and reached critical mass;
- Application of GA₃ did not stimulate flowering in this species

- Floral material usually has 11 leaves (including primordia), but these cannot be accurately counted until floral development is well advanced, or by dissection;
- Floral plants generally had longer leaves but this did not give an accurate indicator of the floral/vegetative state. Neither could bulblet number be accurately linked with flowering.

The physiology of *A. ampeloprasum* var. *babingtonii* was similar to those described by Bothmer (1974) in the Aegean members of the *Ampeloprasum* complex. It had one fleshy, colourless modified leaf for storage (storage cataphyll), forming the bulk of the bulb. This probably similarly nourishes the sprout in the early stages through Winter, before declining in Spring. It was surrounded by a bladeless sclerified protective leaf. This structure was apparent in the bulbil, and this pattern was maintained throughout the vegetative life of the plant. This was dissimilar to the pattern described by Jones and Mann (1963) for wild *A. ampeloprasum* species, which they described as having two storage leaves, and often with multiple cloves. The basal bulb plate was a modified stem, which regenerated every vegetative period, unlike the cultivated *A. Ampeloprasum* var. *porrum* or *A. kurrat*, both of which are biennial. The old bulb plate (basal plate) remained attached to the new bulb plate (basal plate) eventually disintegrating during the following season, as described by Bothmer, (1974) for Aegean members of the *Ampeloprasum* complex.

During the first season, up to four leaves were likely to be produced with a small proportion of plants achieving critical mass by the end of the season, and therefore flowering following vernalization during the winter growth of the second season.

During the second season, the inflorescence (if the plant is florally determined) developed in the Spring, reaching maturity during the Summer. If the plant is vegetative, approximately eight leaves were produced, and critical mass was reached in many plants. Although many of the bulbs obtained commercially, were sold as being two years old, subsequent experience growing this plant, suggests that developing daughter bulbs may cause some difficulty in ascertaining age in mixed groups, and that age can only be precisely ascertained if plants are lifted, separated and recorded each year. Most plants will reach critical mass in the second season, flowering in the third season, following vernalization.

Failure to induce flowering by application of GA³ meant that future work was dependent on obtaining populations of plants of critical mass (3 cm) or above, these also being identified later in the season by having 11 leaves. Identification of a vernalizing treatment meant that mature plants could be treated to reliably produce 100% flowering when required.

6.0 Floral Development in *Allium ampeloprasum* var. *babingtonii*

Vegetative characteristics were identified in Chapter 5, together with the requirement for a minimum size of bulb (>3 cm width) combined with a vernalizing treatment (6 weeks at 7°C to induce floral development in *Allium ampeloprasum* var. *babingtonii*).

In Chapter 6, this was used to identify plants of known floral status. These were used to examine the timing of floral determination, and the development of the inflorescence. The floral developmental timetable was identified using tissue culture (see 4.4, Table 17) to establish the timing of floral determination, allied with light and electron microscopy to record the developmental progress of the inflorescence (Section 3.2.1, Table 13).

6.1 Floral score

A developmental scoring system was developed for describing the progress of inflorescence development. This was based on the sequence of floral organ initiation in *Allium* spp. noted by Brewster (1994), and the observed sequence in this study as inflorescence development proceeded. The florets of a single inflorescence were in many stages of development at any one time, with those on the summit developing ahead of those on the flanks (Cottrell, 1999; Treu, 1999). A single floret on the centre of the inflorescence summit appears before the others and continues to develop ahead of the others (Section 5.4.2, Fig. 56; Section 5.5.2, Fig. 62b).

Therefore, the development of this apical floret was used as a marker for developmental progress (Table 25). Anthesis (flower opening) was not included, as the florets do not fully open and are not shed (Treu 1999), but remain attached to the inflorescence until significant tissue degeneration causes loss of the floral tissues in Autumn/Winter, depending on weather conditions.

Table 25: Developmental Scoring system for *Allium ampeloprasum* var. *babingtonii* and var. *ampeloprasum*

Floral Score	Physiological development
1	1 st daughter bulb
2	2 nd daughter bulb – adjacent to scape
3	Spathe primordia – arises as a ring around a central dome
4	Cyme apparent as irregular region and the bracts are also developing
5	Outer tepals visible on apical floret
6	Inner tepals visible on apical floret
7	Outer stamens visible on apical floret
8	Inner stamens with tricuspidate filaments visible on apical floret
9	Carpels arise as ‘U’ shaped primordia
10	Carpels are fused with style developing
11	Tepals develop pigment

6.2 Methods and materials

Plant material from a number of sources (Table 26) was used to identify the timing of floral determination and the development of the inflorescence. Initial source material was purchased comprising twenty *A. ampeloprasum* var *babingtonii* and twenty *A. ampeloprasum* var. *ampeloprasum* (2 years old at time of planting in October 2000; JS00p bab and JS00p amp; see 2.0). This was supplemented by whole plants (var. *babingtonii*) collected during March 2001 (SH0301) (located by the presence of the leaves, but without any size criteria) and whole plants (var. *babingtonii*) collected during November 2001 (large bulbs greater than 3 cm diameter, located by the leaves, not having flowered the previous season (see 2.0), all these being from the Cornish Population 1 (Treu, 1999). Further data were obtained from plants collected from the same population during February 2002 (SH0202).

Plants sampled during August 2000 (SH00) failed to produce any floral plants and are not included here. These had flowered the previous season, and it was surmised that newly formed daughter bulbs were not subject to the same maturity requirement

for floral competence, otherwise measured by a bulb diameter greater than 3 cm (see 5.5.2.3).

Table 26: Summary of plants used to establish timing of floral determination and development of the inflorescence of *Allium ampeloprasum* var. *babingtonii*, with *A. ampeloprasum* var. *ampeloprasum* for comparison

Plants	Acquisition	Details
SH00 var. <i>babingtonii</i>	August 1999	Bulbils from Cornish population, grown on at UCW to provide bulbs of known age
JS00p var. <i>babingtonii</i>	September 2000	Known 2 year old bulbs
JS00p var. <i>ampeloprasum</i>	September 2000	Known 2 year old bulbs
SH0301 var. <i>babingtonii</i>	March 2001	Plants located by leaf from Cornish population, unknown history or age
SH1101 var. <i>babingtonii</i>	November 2001	Plants located by leaf and having a minimum diameter of 3cm at the widest point
SH0302 var. <i>babingtonii</i>	March 2002	Plants located by leaf and having a minimum diameter of 3cm at the widest point but excluding daughter bulbs

6.2.1 Floral determination

Plants of known age (3rd season plants JS00p var. *babingtonii*) were planted in October, 2000. At monthly intervals from November, 2000, one plant was selected at random. This was cultured for approximately one month (see 4.4; Table 17) then assessed for floral development by dissection under stereo microscope (up to x 40); sampling was continued until May 2001 (see also 5.5.2.3; Table 24). Var. *ampeloprasum* was cultured similarly and also assessed for floral development in the same way.

In addition, plants from SH99 (bulbils collected from Cornwall in August, 1999, then grown on at UCW) were sampled from July 2001 (the end of the second season's growth) until March, 2002 (the latter part of the third season's growth). Ten were

selected at random each month, and cultured for one month (see 4.4; Table 17) then assessed for floral development by dissection. These were of unknown size at the time of selection; therefore the samples would be likely to contain both vegetative and floral plants.

6 2.2 Inflorescence development

Data were gathered from a number of samples as available, comprising plants of known age (JS00 var. *babingtonii* and var. *ampeloprasum*, SH99) (see also 6.2.1), and plants of unknown age sampled from the Cornish population during March 2001 (SH0301 – no selection for size), November 2001 (SH1101 – over 3 cm) and March 2002 (SH0302 – over 3 cm, no daughter bulbs). These were transplanted to outdoor beds at UCW and grown on then sampled as required (Table 26).

JS00 var. *babingtonii* and var. *ampeloprasum* were sampled at random at monthly intervals, wax embedded, sectioned and stained (see 3.3; Table 13), then assessed for floral development. Additionally, two plants were recorded using a low vacuum scanning electron microscope (SEM), (JEOL 5200LV at 3% vacuum) one during March and one during April.

SH0301 were sampled at random, 16 being dissected in March to give a firm indication of floral status, of which two were photographed through SEM., then further plants were examined during April, May and early June 2001 (see also 5.5.2.3, Table 22). SH1101 were sampled at monthly intervals, wax embedded, sectioned and stained from December 2001 until April 2002, in order to provide data on the earlier stages of development.

SH0302 were also sampled monthly, 6 being dissected during March to give a mean floral score for the population at that time (these were recorded using SEM), then five in April and May and three in June, to record developmental progress. These were all wax embedded, sectioned and stained.

6.3 Results

6.3.1 Floral determination

Of the 17 JS00 plants sampled during this period, only three were floral (5.4.2; Table 20a). These were sampled from 9 4 01 to 9 5 01, and all were in the early stages of floral development, scoring 2 (second daughter bulb developing), 2, and 4 (cyme and bract developing) respectively (see also Appendix 13). It was difficult to draw any firm conclusions about the timing of floral determination from these limited data. However, it may be suggested that floral determination is likely to occur in the early part of the year, (Figure 68) though any firm conclusions must await further data.

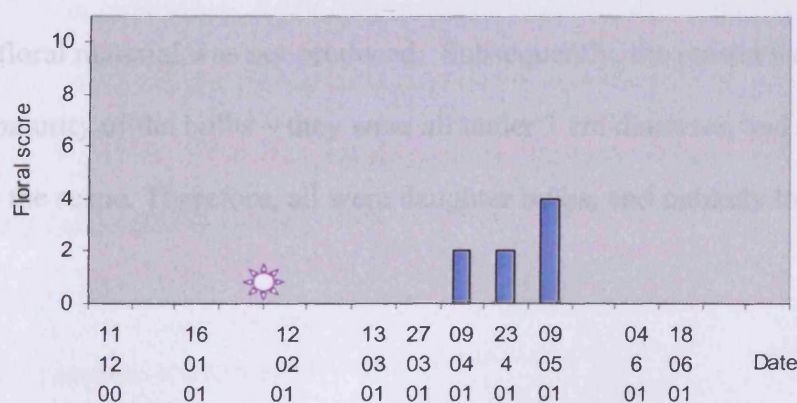


Figure 68: Floral scores of 3rd season plants (JS00) with suggested full development shown at 25 7 01. 🌸 indicates approximate suggested period of floral determination

This was compatible with the need for vernalization (6 weeks at 7°C being shown to produce 100% flowering in bulbs over 3cm in diameter; see 5.5.2.1). This was likely to occur during the months of November – March when mean temperatures are 4 – 5 °C with a maximum of 6 – 8 °C and a minimum of 0.6 – 1.5 °C (www.metoffice.com, accessed June 2004), following which temperatures began to rise (Figure 69).

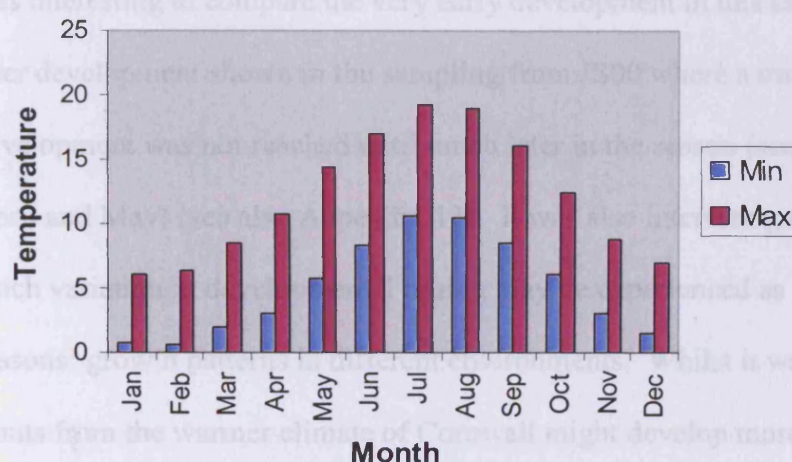


Figure 69: Mean monthly maximum and minimum temperatures (°C) for the UK, 1971 – 2000 (www.metoffice.com accessed June, 2004).

Plants sampled from the Cornish population (SH00) were placed in culture as for JS00, but floral material was not produced. Subsequently, the reason was identified as the immaturity of the bulbs – they were all under 3 cm diameter, and had been located by the scape. Therefore, all were daughter bulbs, and unlikely to flower (see 5.5).

The SH99 plants provided only one plant that was clearly identifiable as floral (Appendix 14). This was sampled on 10 1 02, and assayed on 7 02 02. It was well developed, (floral score 7), spathe 0.9 cm height, the apical floret clearly developed

and raised above the other primordia. All samples in the following months showed clearly developing storage tissue as a single bulb and showing no division into daughter bulbs, therefore were considered to be vegetative. This, combined with the data from JS00, suggested that determination may occur early in the year, during January or early February. However, there were no data to identify the timing between determination and floral development, so conclusions must be tentative. It was interesting to compare the very early development in this sample, with the much later development shown in the sampling from JS00 where a much earlier stage of development was not reached until much later in the season (scoring 2, 2 and 4 in April and May) (see also Appendix 13). It was also interesting to speculate how much variation in developmental timing may be experienced as a result of previous seasons' growth patterns in different environments. Whilst it was to be expected that plants from the warmer climate of Cornwall might develop more rapidly than at UCW, plants from Wales (JS00) might be expected to have experienced similar temperatures to UCW, resulting in a similar phenology. These data are small in number, and no firm conclusions may be drawn, but it was apparent that there was wide variation in the timing of developmental patterns. None of the var.

ampeloprasum sampled during the early part of the year showed any signs of floral development, therefore it was impossible to draw firm conclusions about timing of floral determination in this species.

6.3.2 Inflorescence development

SH99 provided little data, only one plant being floral (floral score 7, mid-January, see 6.3.1). JS00 produced only 3 floral plants (see Figure 68 and Appendix 13),

these being at a much earlier stage of development in April and May than the one from SH99.

SH0301 had seven plants (of 16 sampled) that showed floral development, with a mean floral score of 6.8 at the time of sampling in March, 2001 (Appendix 15). The floral score was highly variable throughout the monthly samples (Appendix 15) following this, for example, ranging from 5 to 9 in April (total number of floral plants = 14). By June, all floral plants had reached a floral score of 11, the inflorescence being fully developed with all organs present, anthers exerted, and the tepals fully coloured.

SH1101 provided few plants that were flowering. Four plants were floral in March, but none before that. This was surprising as all plants were selected for critical mass and should have been florally competent. It can only be speculated that plants were set back by transplanting during a vulnerable stage of growth, perhaps failing to reach the minimum number of leaves at the time that temperatures were appropriate for vernalization. This would imply that the apex was not mature enough to respond to the vernalization signal at this time.

SH0302, also selected for minimum size and excluding daughter bulbs, failed to produce floral material. Many of these were dissected subsequently, and it was apparent that abortion of the inflorescence had occurred, at least in some of the plants. A withered inflorescence was apparent in some, whilst others did not show any trace of inflorescence, but had produced two daughter bulbs, presumably aborting at an earlier stage of floral development. Others did not show any

development associated with the floral state, and it was not possible to ascertain whether the floral state had not developed, or whether abortion had occurred at such an early stage of development as to leave no physically apparent trace. Floral plants sampled in March 2002 (SH0302) produced 6 plants with a mean floral score of 4.2. However, if all the floral scores from these samples are shown together, a picture of the variation in floral development can be constructed (Figure 70).

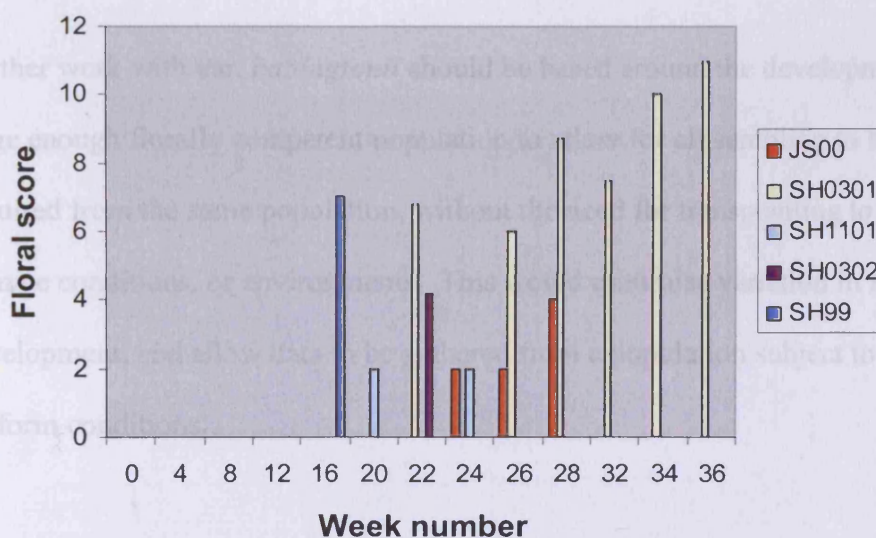


Figure 70: Summary of mean floral scores from plants sampled at different times from the Cornish population. Week 0 is the first week in October.

This illustrates clearly that there is considerable variation in the timing of floral development in this species. Moreover, the plants are clearly affected by climate differences and by transplant trauma, even though all due care was taken to avoid damage. Generally, those plants sampled from Cornwall in March, are more advanced than those plants grown on and sampled at UCW, although SH99 presents an anomaly. However, this is one plant, and may represent atypical results. If this is discounted, it could be surmised that determination probably occurs in Cornish populations during January, and in February for populations grown at UCW. Development of floral organs is rapid, with most floral organs being formed fairly

quickly (tepals forming during February/March) followed by maturation of the floral organs and elongation of the scape. Full inflorescence development (floral score 11) then occurs during week 36 (end of June) for Cornish populations, and a little later for populations at UCW. Var. *ampeloprasmus* produced only one floral plant from this sample, (JS00 D amp.), therefore no attempt is made to draw conclusions about the timing of inflorescence development.

Further work with var. *babingtonii* should be based around the development of a large enough florally competent population to allow for all sampling to be done as required from the same population, without the need for transplanting to other sites, climate conditions, or environments. This would minimise variation in floral development, and allow data to be gathered from a population subject to relatively uniform conditions.

The floral var. *babingtonii* plant produces the first daughter bulb from an axillary bud, followed by another leaf. The second daughter bulb is then produced in the next axil, followed by the spathe. In contrast, although *A. ampeloprasmus* var. *ampeloprasmus* generally follows a similar growth pattern, the daughter bulbs produced in the axils are multiple, two or three being produced in each axil. However, a sample of one plant cannot be assumed to be typical, therefore, conclusions must be extremely tenuous. However, as all the vegetative plants of this species sampled also produced multiple bulblets on rhizomes rather than the single bulblet produced by var. *babingtonii*, this plant may invest more heavily in clonal offspring through bulblet production, than var. *babingtonii* does, perhaps as compensation for not producing bulbils in the inflorescence.

Var. *babingtonii* develops as might be predicted from comparison with other members of the *Alliaceae*, such as *A. sativum* (garlic) and *A. cepa* (1.7). The height of the spathe increases rapidly initially as the florets develop. The spathe is approximately 3.5 cm height by April, when the tepals are being formed, with the scape just 9.4 cm rising rapidly to approximately 65 cm in just two weeks (Figure 71).

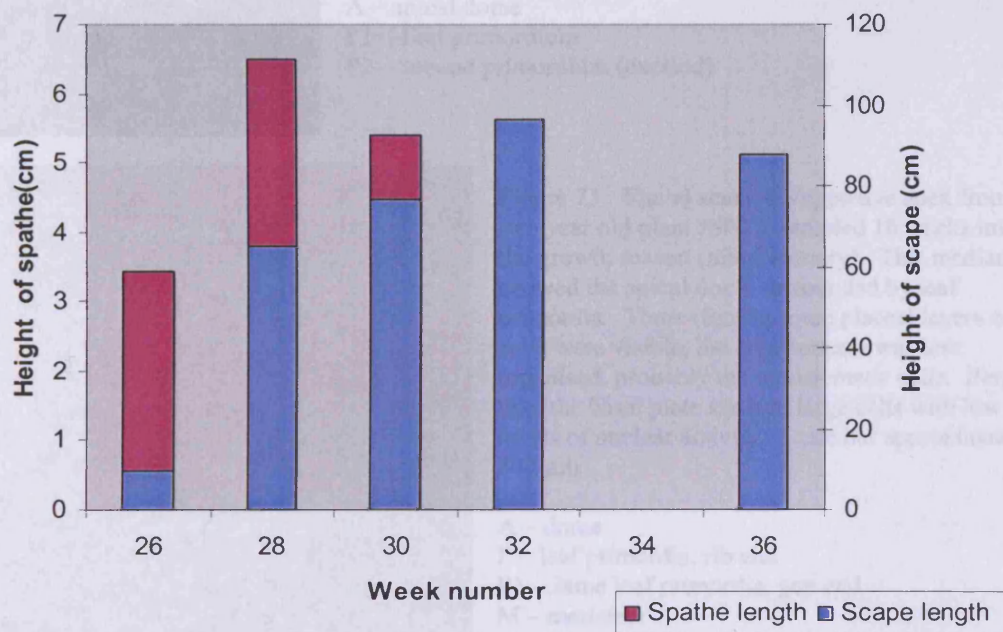


Figure 71: Inflorescence size in SH0301 plants sampled from Cornish population, March 2001, week zero is early October, 2000, for comparison with samples taken at other times from this population. From week 28, the spathe size diminishes as it splits, desiccates and dehisces.

6.3.3 Photographic summary of development of inflorescence from the vegetative state (floral score 0) to full floral development (floral score 11) in *A. ampeloprasum* var. *babingtonii*



Figure 72: **Floral score 0:** SEM of bulb of unknown age, 2 months into the growth season (December), vegetative apex (SH00 4). The apical dome was clearly visible measuring approximately 250 x 350 μm. This was a mature daughter bulb, selected from the Cornish population by location of the scape, the parent bulb dividing into two daughter bulbs following the development of the scape and inflorescence. The emerging shoot was narrow and wide, the angle of divergence of the leaves was 180°, each leaf enfolding the next younger leaf; six leaves have been removed (see also Figure 52, Chapter 5).

A – apical dome
P1 – first primordium
P2 – second primordium (excised)

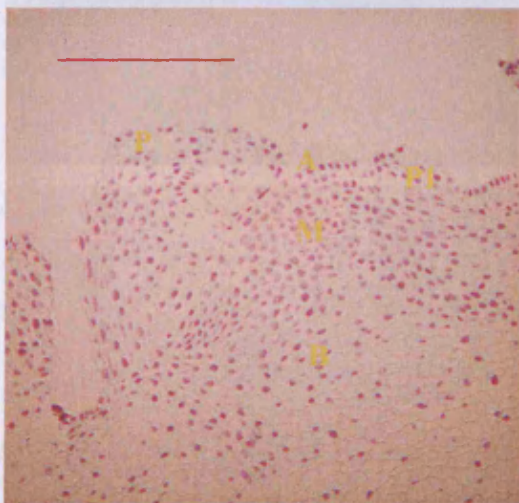


Figure 73: **Floral score 0:** Vegetative apex from two year old plant JS00 E sampled 16 weeks into the growth season (mid-February). This median LS showed the apical dome surrounded by leaf primordia. Three (four in some places) layers of cells were visible, the area beneath was less organised, probably the meristematic cells. Beneath this, the basal plate showed large cells with low levels of nuclear activity. Scale bar approximately 300 μm

A – dome
P – leaf primordia, rib end
P1 – same leaf primordia, gap end
M – meristem
B – basal plate

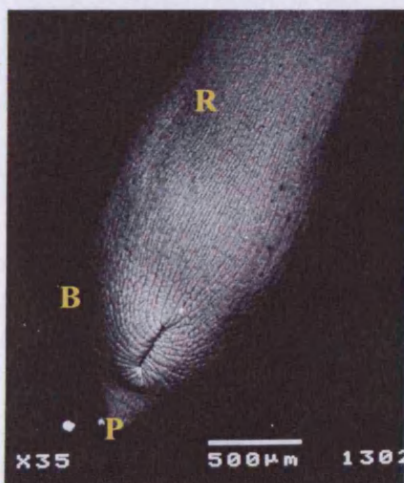


Figure 74: **Floral score 0:** Vegetative plant of unknown age, 16 weeks into the growth season (February). SH0015A SEM of bulblet developing on side shoot beneath 2nd leaf. The apical dome was surrounded by a protective layer (the first 'leaf') which becomes the storage layer. Most side shoots were produced during the winter and spring. They developed on rhizomes from the basal plate, the tip thickening as the bulblet develops. Ultimately, the rhizome withered separating the bulblet from the parent plant. However, no bulblets were observed to sprout during the course of these experiments, except when the parent plant was developing an inflorescence.

R – rhizome
B – bulblet
P – protective layer fully enclosed the apical dome

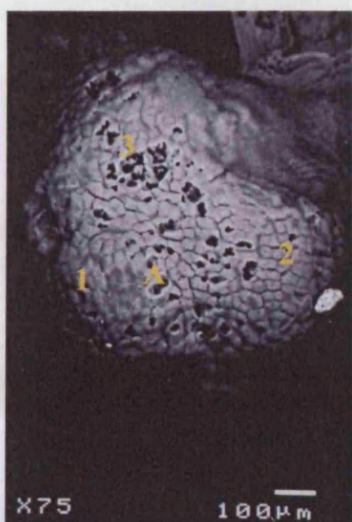


Figure 75: **Floral Score 2**; JS00 F, floral plant two years old at time of planting, sampled in week 26 (April) of growth season following. The apical dome was swelling and irregular in outline measuring approximately 900 x 800 μm. Daughter bulbs were developing (Figure 74). The spathe was not apparent, neither were any cymes or recognisable primordia. However, the cell divisions on the apex suggested at least two centres of activity, and possibly a third.

- A – apical dome
- 1 – focus of cell division
- 2 – smaller focus of cell division
- 3 – possibly another focus of cell division

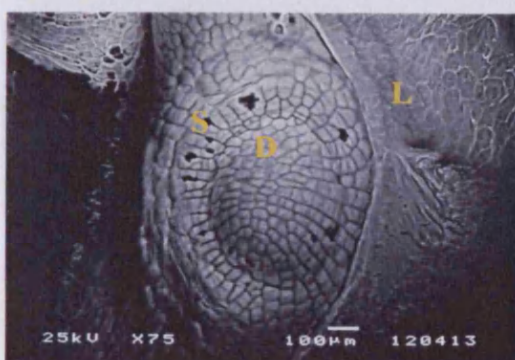


Figure 76: **Floral score 2**, (JS00 F as above); the daughter bulb forming in the apex of the 11th leaf adjacent to the developing scape. The central dome was arising surrounded by a ring of storage tissue similar to that found in bulbil formation.

- L – 11th leaf base
- D – apical dome
- S – storage tissue

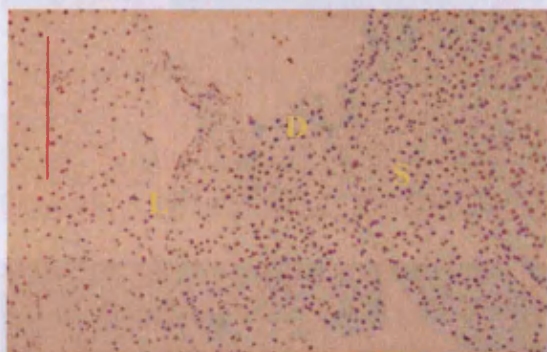


Figure 77: **Floral score 2**; TS of two year old plant, sampled in the 24th week (early April) of the growth season (JS00 A) showing daughter bulb (approximately 600 x 300 μm) developing adjacent to the eleventh leaf.

- L – 11th leaf base
- D – daughter bulb
- S – scape

Scale bar approx 500 μm

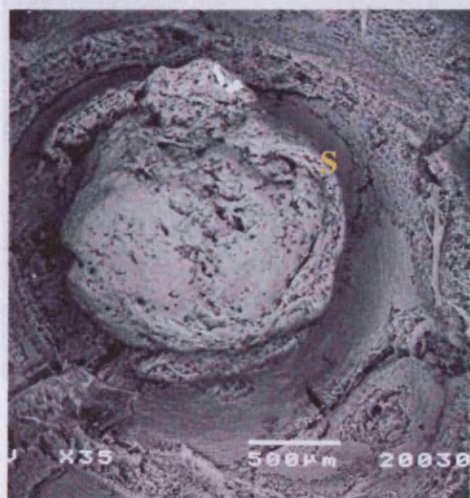


Figure 78: **Floral score 2**; plant of unknown age, selected from Cornish population in March 2002, and then dissected SH0302 7. The incomplete spathe was removed, revealing the apex as an uneven dome, without defined primordia, measuring approximately 1000 x 1000 μm. This was larger than Figure 73 and had an identifiable spathe, not present in that photograph, suggesting that scape elongation and apical dome enlargement may precede development of the spathe.

- S – spathe base

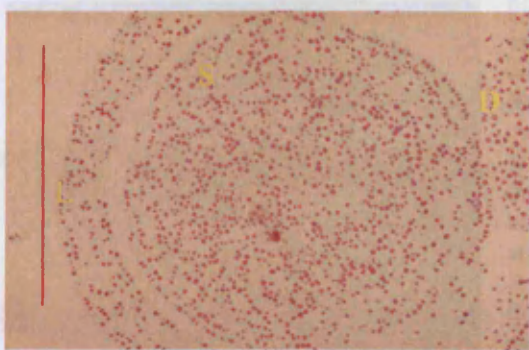


Figure 79: **Floral score 2**; TS of two-year-old plant sampled in 24th week of the growth season (early April) JS00 A through the developing scape; the final leaf was encircling the scape, with the developing daughter bulb on the opposite side. The scape is approximately 300 μ m to base of inflorescence. Scale bar approximately 1000 μ m
S – scape
L – leaf
D – daughter bulb

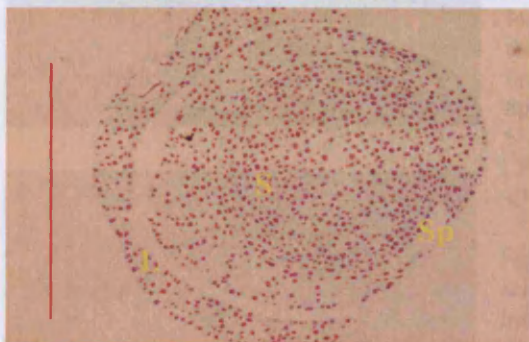


Figure 80: **Floral score 2**; TS of JS00 A (as above) spathe separating from inflorescence, approximately 140 μ m from inflorescence base; spathe edges meet at right hand side, not quite opposite the point where the leaf edges meet (bottom left). Inflorescence width is approximately 1000 μ m at this point. Scale bar approximately 1000 μ m.
Sp – spathe where edges meet
S – scape
L – leaf where edges meet

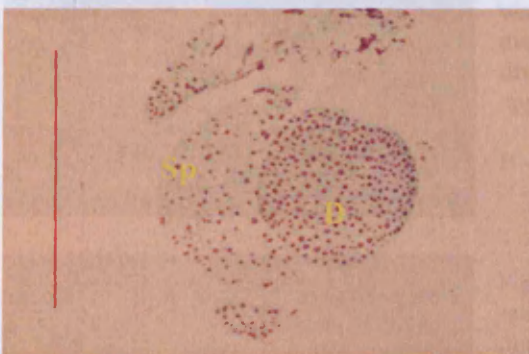


Figure 81: **Floral score 2**; TS of JS00A (as above) showing the apex of the developing inflorescence; the spathe is not complete, only partially enclosing the dome at this point, measuring approximately 265 μ m from the point of separation from the inflorescence. Inflorescence measures approximately 375 μ m in height. Scale bar approximately 1000 μ m.
Sp – spathe
D - dome

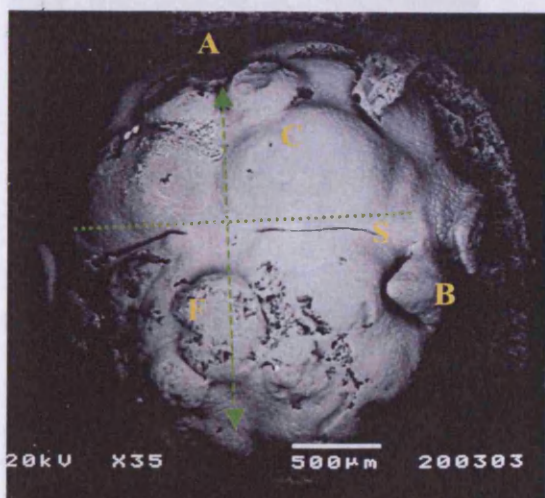


Figure 82: **Floral score 3**; Plant of unknown age sampled from Cornwall March 2002, and dissected immediately (SH0302 8). The incomplete spathe was removed and the symmetrical nature of the inflorescence could be seen (dashed line shows developmental axis and indicates the location of the daughter bulbs). It also seemed that the inflorescence developed in two parts; the upper half - slightly larger - following a slightly different pattern to the lower half (above and below dotted line) with a seam where the two areas meet. The inflorescence was approximately 1800 μ m width. The developing domes may represent cymes – particularly the larger ones which have smaller domes arising, whilst the smaller ones, for example the centre dome in the bottom half, may represent a floret

primordium. The flat pointed primordium in the right hand side was a bract.

C – cyme
F – floret

S – seam
A – developmental axis

B - bract

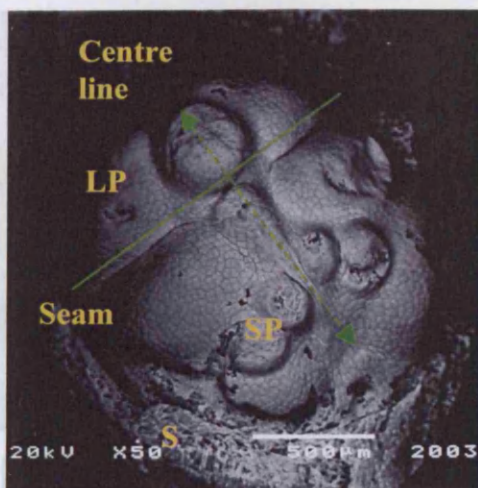


Figure 83: **Floral score 3**; Plant of unknown age sampled March 2002 from Cornish population and dissected immediately (SH0302 19). The spathe (removed) fully enclosed the developing inflorescence, but the cymes were not readily recognisable. There were several centres of development, and it was apparent that the head developed in two parts, a smaller part and a larger part, with a 'seam' between the two parts. The arising centres were more or less symmetrical about the centre line and these were probably a combination of floret primordia – more likely on the apex – and cymes – more likely on the flanks (the arrows indicate the location of the daughter bulbs). It was also possible that these centres of development further subdivide before becoming cymes. The inflorescence diameter was approximately 1300 µm.

S – spathe

LP – large primordium

SP – small primordium

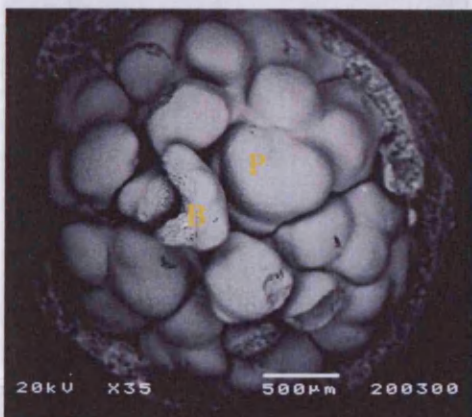


Figure 84: **Floral score 4**; Plant of unknown age selected from Cornwall, March 2002, and dissected immediately (SH0302 11). The central outswelling was developing as a bract, the seam between the two sections was still visible, whilst the cyme primordia now have many smaller primordia developing which are still dividing into smaller centres. The inflorescence was approximately 2000 µm diameter.

B – bract

P – primordium dividing into two centres

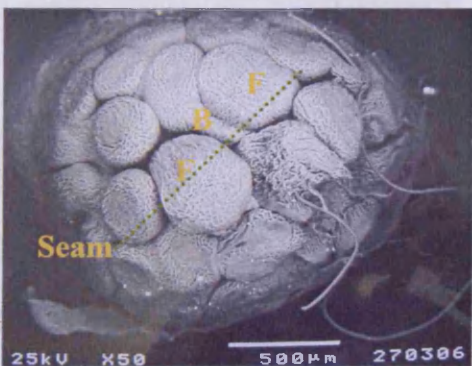


Figure 85: **Floral score 4**; Plant of unknown age, sampled from Cornwall March, 2001 and dissected immediately (SH0302 D2). The primordia were slightly more defined than in Figure 82. The two largest primordia were developing either side of the central bract noted in Figure 82. The seam was still partially visible. The inflorescence diameter was approximately 1100 µm.

B – bract

F – floret primordia

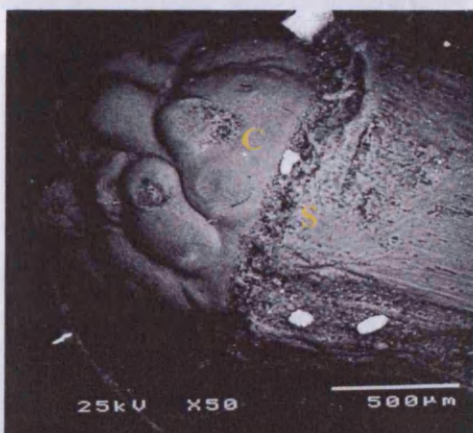


Figure 86: **Floral score 4**; Two year old plant sampled during week 28 of the growth season, early May (JS00 K) spathe removed. Although apical primordia were well defined, the flanks showed irregular developmental areas that probably corresponded to cymes. The inflorescence diameter was approximately 1000 µm.

C – cyme

S – spathe (removed)



Figure 87: **Floral score 5**; Plant of unknown age sampled from Cornwall, March 2002 (SH0302 16) and dissected immediately. The seam was still apparent dividing the two halves of the developing inflorescence, and the two apical florets, one on each half, were showing tepal primordia for both whorls. The outer stamens were also present as small outswellings. Inflorescence diameter approximately 2300 µm

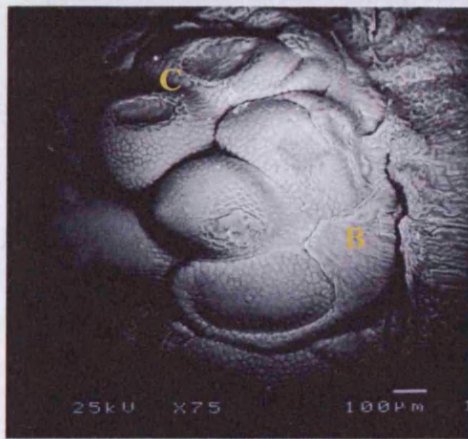


Figure 88: **Floral score 5**; SH0301 28, an inflorescence from a plant of unknown age, sampled during week 26 (late April) with the spathe removed. Individual florets were not yet apparent on the lower sides of this inflorescence. Cymes were developing and they were subtended by bracts around the base of the inflorescence, though the cymes themselves showed little recognisable features.

C – cyme
B - bract

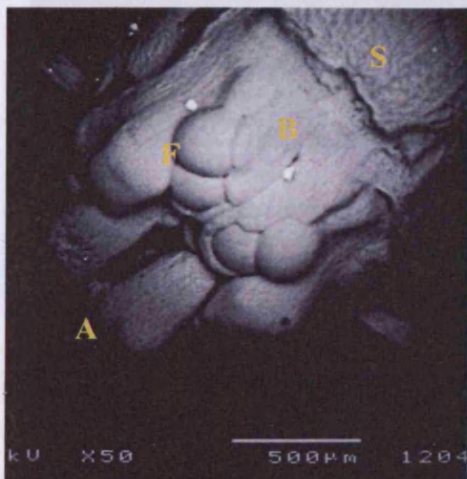


Figure 89: **Floral score 5**; SH0301 28 as above, SEM showing the other side of the same inflorescence. Here the bracts (or bracteoles) are well-defined, protecting groups of floret primordia. It was unclear whether these were the bracts subtending the inflorescence, or bracteoles subtending cymes. Inflorescence width was approximately 1300 µm

S – spathe (removed)
B – bract/bracteole
F – floret
A – floret near apex with tepal primordia developing

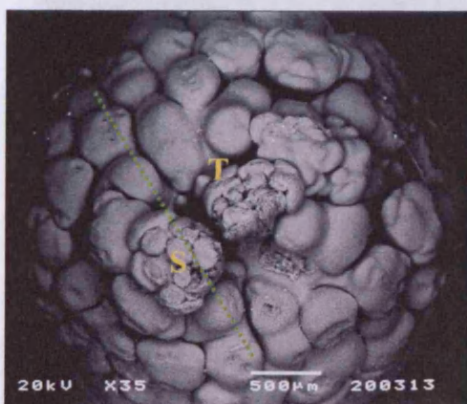
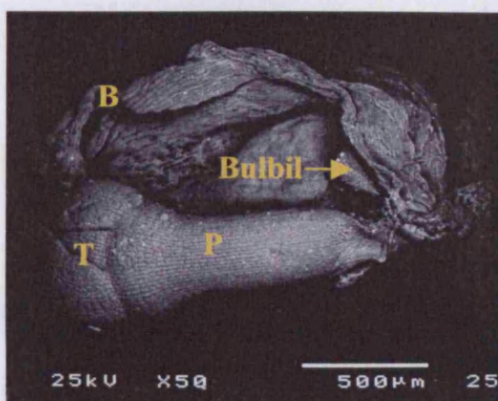


Figure 90: **Floral score 7/8**; Plant of unknown age sampled from Cornwall in March 2002, and dissected immediately (SH0302 23). Both whorls of tepals were visible as were both whorls of stamens. However, the tricuspidate filaments were not developed, neither were the carpels. The inflorescence measured approximately 2500µm in diameter. The seam separating the larger and small parts of the inflorescence was still visible. Development continued to be more or less symmetrical about the developmental axis

T – tepals
S - stamen



vegetative reproduction and forming bulbils.

B – bracteole

P – pedicel

T – tepals

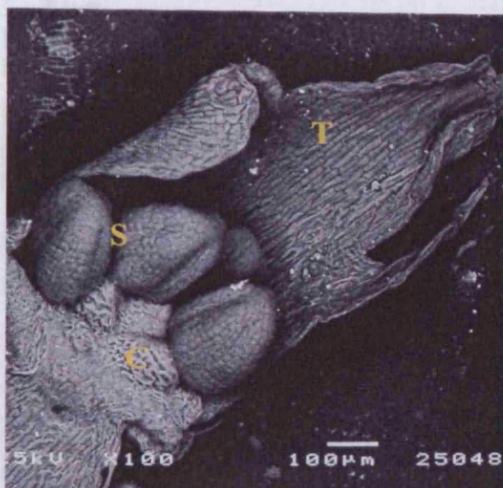


Figure 92: **Floral score 7/8**; SEM of half of the apical floret SH0301 34, plant of unknown age examined in week 28 (early May) of the growth season. The two whorls of stamens had developed with the anthers swelling and the locules visible, but the filaments had not yet developed in either whorl of stamens. There was some swelling of tissue where the carpels will arise, but no recognisable organs.

T – tepal

S – stamen

C – carpel primordia

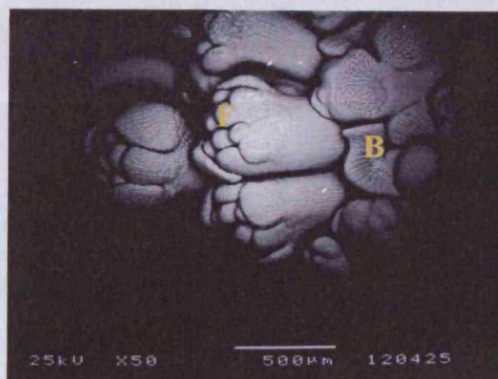


Figure 93: **Floral score 9**; plant of unknown age sampled from Cornwall in March 2001, dissected in week 26 of growth season (late April) SH0301 46. Although apical florets were well developed showing tepals and stamens, with carpels arising, the flanking primordia were still relatively unformed, with floret primordia showing little definition. Bracteole primordia were apparent around some of the floral primordia.

F – floret

B – bracteole

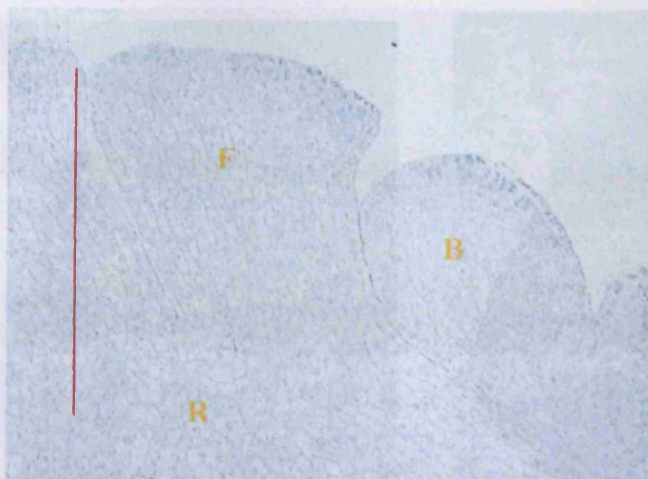


Figure 94: **Floral score 9**; Plant of unknown age sampled from Cornwall in March 2001, and dissected immediately (SH0301 D5). This LS showed the development of bulbil and floret primordia. The shape was clearly different, with the floret primordia broadening at the top for the development of the tepals, whilst the stem narrows and elongates. The bulbil was rounded, wider in the middle than the top, with layers beginning to define indicating the formation of layers. The apical shoot of the bulbil was enclosed within this storage tissue. The bulbil appeared to be developing in the axil of

the floret. To the left was another floret developing, whilst to the right is the subtending bracteole. Scale bar approximately 1000 μ m.
F – floret
B – bulbil
R – receptacle of inflorescence

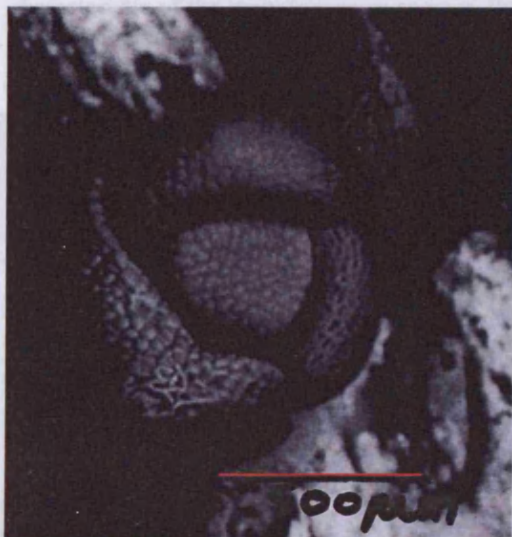


Figure 95 : **Floral score 10**, plant sampled from Cornwall in March 2001, and dissected in week 30 (late May). This SEM (SH0301 13 flanking floret) shows a floret with outer tepals, but no further floral organs developed. However, it was part of an inflorescence where the apical floret showed all the floral organs including carpels (score 10). This illustrated the range of development in the inflorescence, with the apical floret being consistently considerably more advanced than the flanking florets. Scale bar approximately 500 μ m

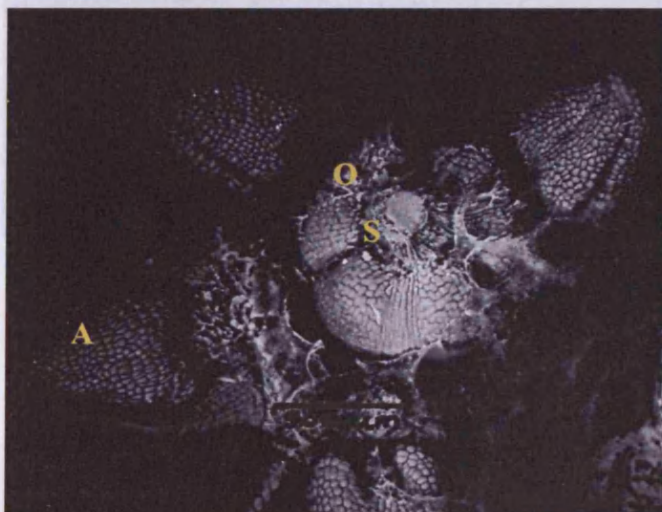


Figure 96: **Floral score 10**; mature apical floret SH0301 13 sampled week 30 (end May); anthers were well developed, but the filaments had not yet elongated. The carpels had developed into bilocular ovaries and the style was beginning to elongate.

O – ovaries
S – style
A – anthers

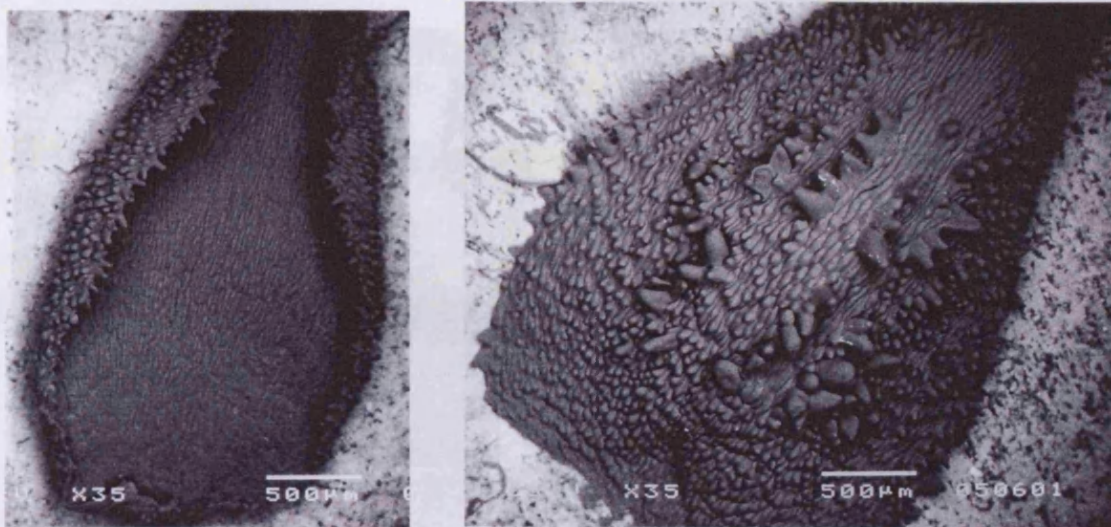


Figure 97: **Floral score 10**; SH0301 59, adaxial and abaxial surfaces of tepal. Note that the adaxial surface is smooth, featureless, whilst the abaxial surface is papillate, this being a defining characteristic of the *ampeloprasum* species, the larger papillae forming 'ribs' running the lengthwise along the tepal. It was later noted (floral score 11) that the colour of the tepals initially developed in these papillae.

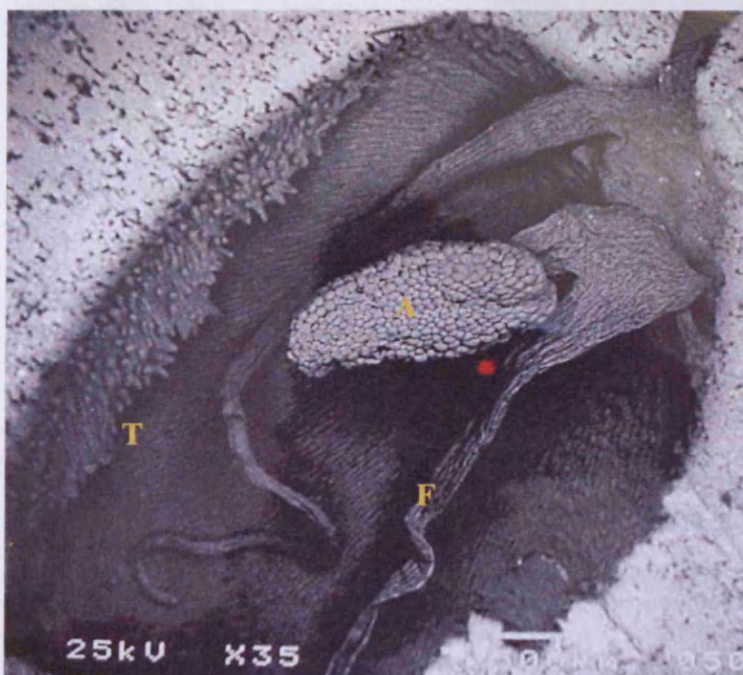


Figure 98: **Floral score 10**; Plant of unknown age sampled in Cornwall in March 2001, and dissected in week 32 (early June) (SH0301 59). The tricuspidate filaments (inner whorl) were fully developed, shown against the subtending tepal. The dorsifixed anthers were borne on the middle shorter filament, whilst the outer filaments were extremely elongated but did not develop anthers. Stamens in the outer whorl were borne singly without the extra filaments, but were otherwise similar in size and morphology.

A – anther
F – extended filament
T – tepal

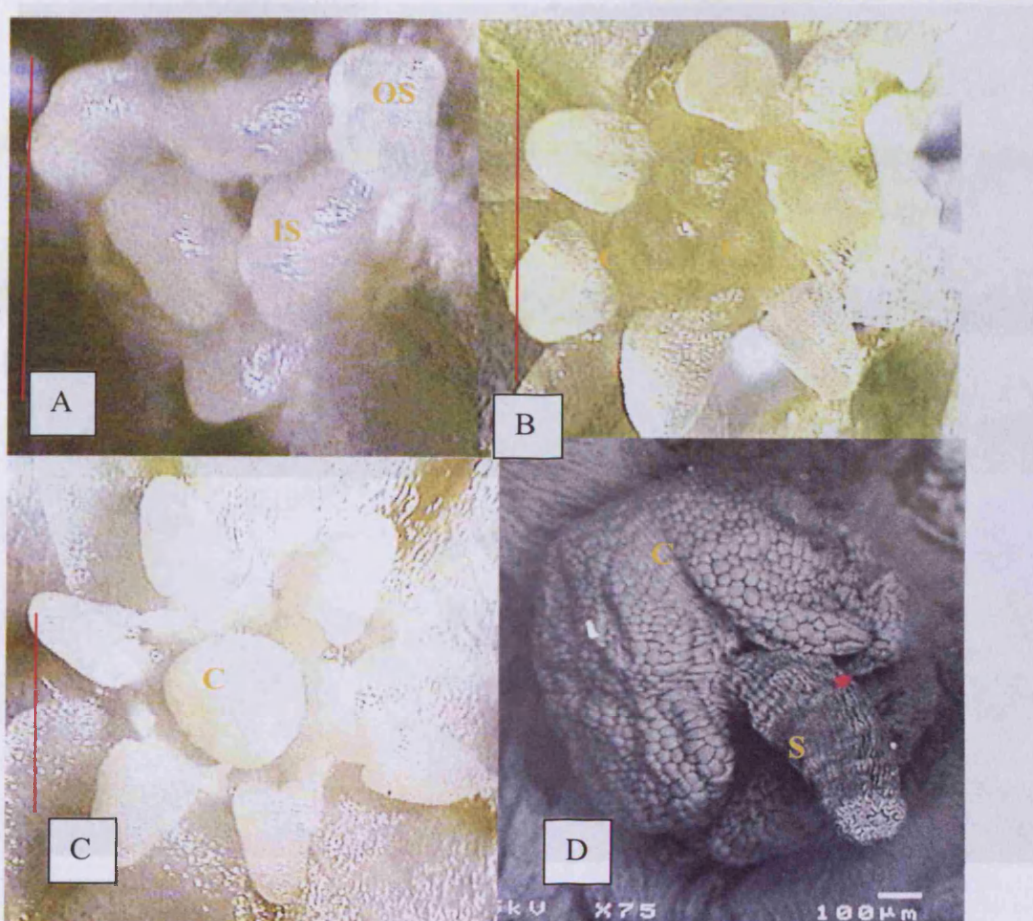


Figure 99: Views of carpel development (floral score 10/11).

A: Plant of unknown age sampled from Cornwall in March 2001 and dissected in week 28 of growth season (early May) (SH0301 2). Both whorls of anthers were formed and distinct from each other, but the filaments had not yet elongated in either whorl. There was no gynoecium apparent. Scale bar approx. 2 mm

B: Plant of unknown age sampled from Cornwall in March 2001 and dissected in week 30 of growth season (late May) (SH0301 19). The carpels arose as three distinct primordia curved in towards a central fourth primordia – the developing style. This morphology was reminiscent of the formation of bulbils and bulblets as well as the vegetative apex. Scale bar approx. 2 mm

C: Plant of unknown age sampled from Cornwall in March 2001 and dissected in week 30 of the growth season (late May) (SH0301 13). It was no longer possible to differentiate between the outer and inner stamens, the anthers being similar in size and development, and the filaments not yet elongated. The carpels had become con-joined into one almost spherical gynoecium with the style primordium (as observed in 'B') fully enclosed within the gynoecium. Scale bar approx. 2 mm

D: Plant of unknown age sampled from Cornwall in March 2001 and dissected in week 32 (early June) (SH0301 59). The bi-locular nature of the carpels was reflected in the outward morphology, with the three carpels each contributing to the gynoecium. The terete style was protruding. By this stage the carpel was approximately 1000 μm in diameter. The tepals were not yet coloured in this floret, therefore the floral score was still 10, despite the apparent maturity of the floral organs.

(A – C digital camera through dissecting microscope, D SEM)

OS – outer stamens

IS – inner stamens

C – carpels

S - style

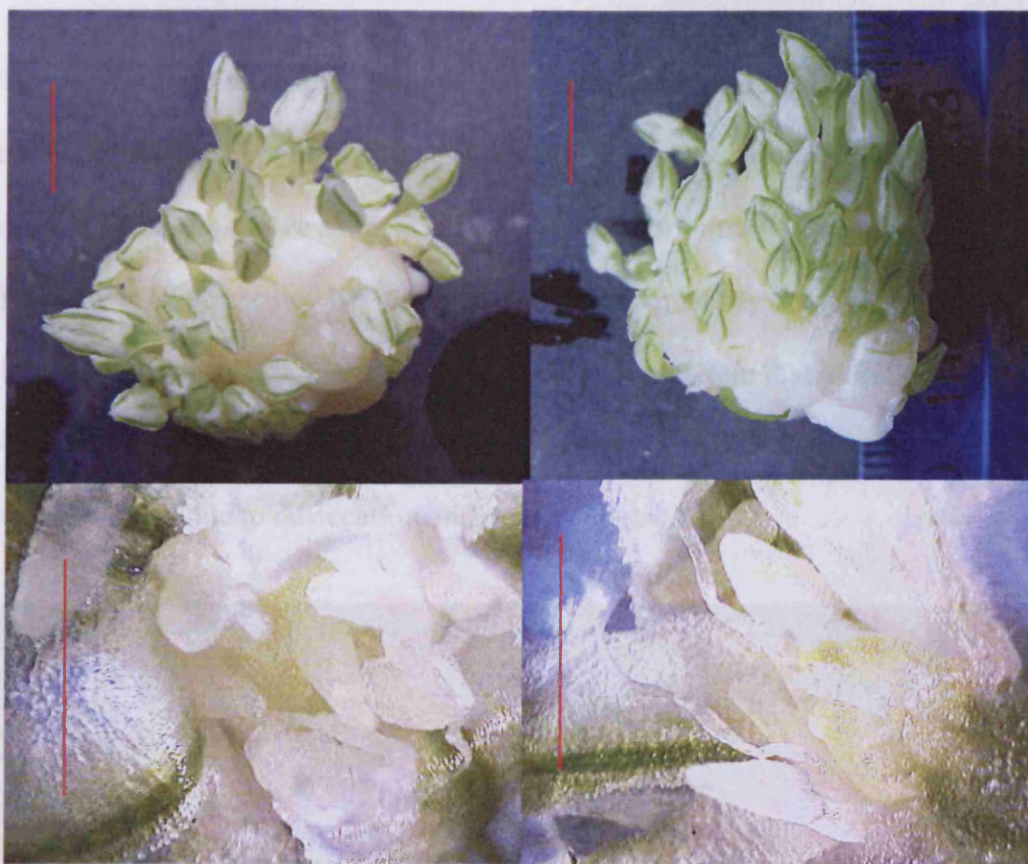


Figure 100: **Floral score 10**; Plant of unknown age sampled from Cornwall in March 2001, and dissected in week 30 (late May) (SH0301 27).

View of inflorescence from the apex and the lateral (scale bar 10 mm) view plus view into the apical floret (approximately length 4 mm) showing the development of the terete style, and the floret bisected laterally showing the extended filaments of the inner stamens (scale bar 2 mm).

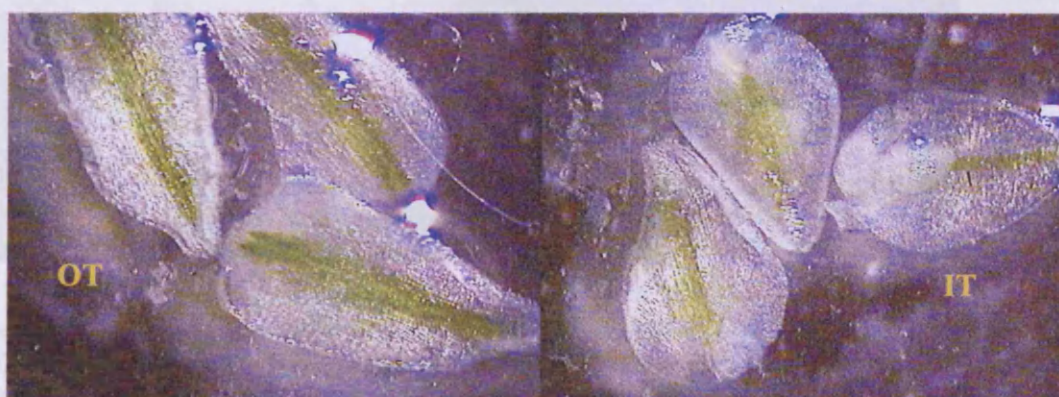


Figure 101: Plant as above, showing outer tepals and inner tepals of apical floret. The green central vein is noticeable, but the pigment has not yet developed in the tepals. Outer tepals were obovate/truncate whilst the inner tepals were oblanceolate.

OT – outer tepals

IT – inner tepals

Florets were observed to develop without bulbils, but bulbils did not develop without florets, supporting the hypothesis that bulbils develop as the lower part of the cyme which has reverted to the vegetative state. There may be one or more florets, and one or more bulbils within each bracteole. No attempt was made to quantify the numbers of florets and bulbils within each bracteole, as it was not possible to ascertain that bulbil development had ceased until the inflorescence was fully developed, at which time the bracteoles were difficult to identify due to desiccation and decay (Figure 102).

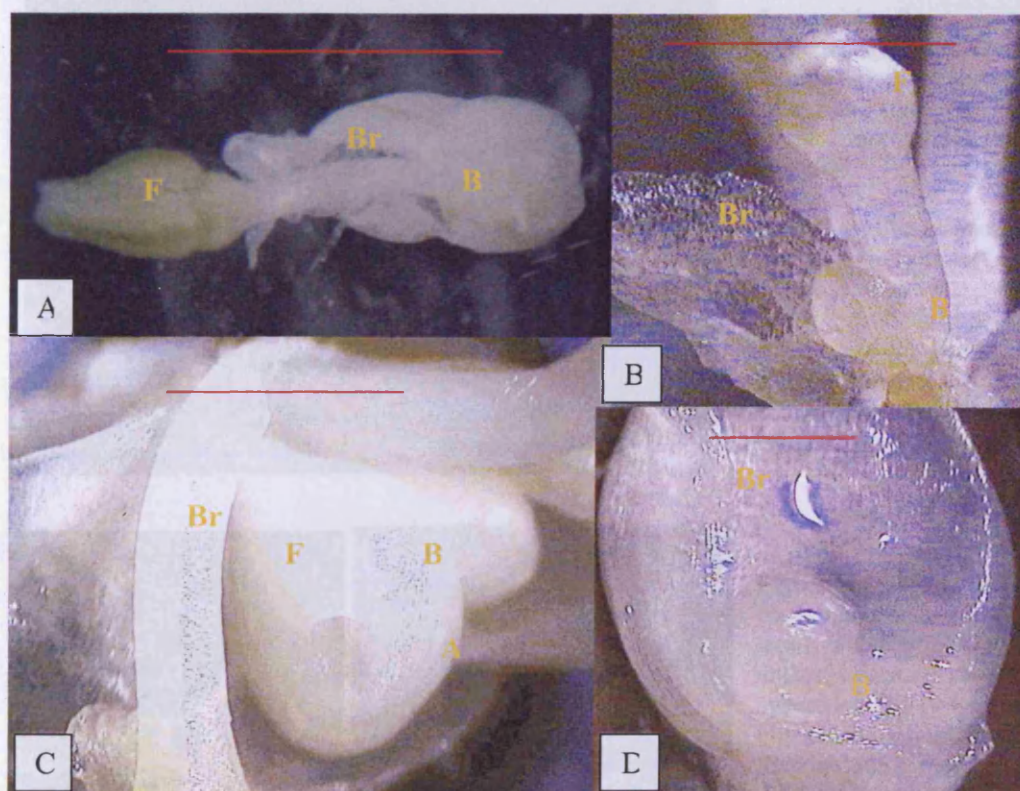


Figure 102: Floral score 10; A: Bract with one floret and one bulbil Scale bar approx. 2mm. B: floral score 9 (SH0301 19) Bract with two florets and an immature bulbil. This shows the storage tissue arising around the inner shoot before closure. Scale bar approx. 1 mm. C floral score 10 (SH0301 13) 2 bulbils and 2 florets within one bract, the storage tissue is still not fully closed around the shoot on the largest bulbil. Scale bar approx. 0.5mm. D: (SH0301 34) Bulbil developing within bract. Scale bar approx. 0.5 mm
F = floret B = bulbil,
Br = bract S = shoot.

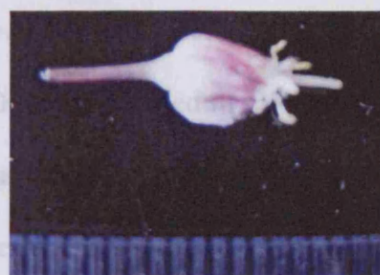


Figure 103: Floral score 11; all floral organs were developed and tepals were coloured SH0301 53. The inflorescence was approximately 14.4 cm height x 7.5 cm wide including secondary inflorescences. Secondary inflorescences were well developed on sturdy scapes, and the spathe was desiccated but still attached. Bulbils were green and well developed, the apical dome being fully enclosed (compare with floral score 10 where neither florets nor bulbils are fully coloured). Traces of bracts were still apparent as papery layers around bulbils. Anthers and style were exerted in all mature florets, though the tepals remain unopened.

Secondary inflorescences had small numbers of bulbils and/or florets, smaller than those on the main inflorescence, and developing a little later. In this figure, the anthers were not exerted in the secondary inflorescences, while those of the main inflorescence were exerted.

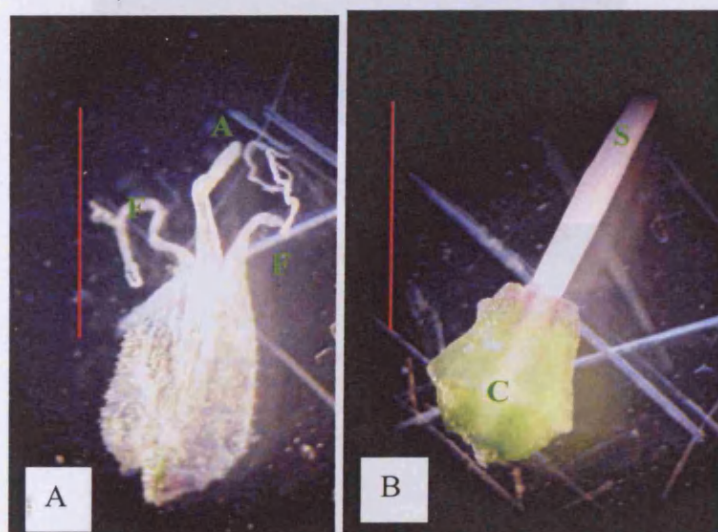


Figure 104: Floral score 11; A – inner tricuspidate stamen attached to inner tepal. The central filament carried the anther, whilst the outer two grow approximately two-three times longer, but remain crumpled. Their function may include protection of the developing organs, either mechanically or by contributing to the humidity. B – the fully developed carpels and extended plain style, all tepals and stamens removed.

Scale bar approximately 4mm.

6.3.4 Inflorescence development in *A. ampeloprasum* var. *ampeloprasum*

Only one of the plants sampled was floral, JS00 D amp., sampled in week 30 (21 5 01) floral score 9. This had ten leaves prior to spathe development, with three daughter bulbs forming from the previous two lateral buds (Figure 105).



Figure 105; **Floral score 9** var. *ampeloprasum*. This species produces three daughter bulbs from each previous lateral axillary bud. Apart from this and the multiple bulblets produced on rhizomes from axillary buds, the bulb is indistinguishable from var. *babingtonii*. The largest daughter bulb in this figure is approximately 10 mm from base to apex. (Scale shows cm and mm)



Figure 106; the spathe was indistinguishable from those of var. *babingtonii*. (Scale in mm)



Figure 107; the inflorescence was similar to that of var. *babingtonii*, with florets developing all over the inflorescence, the apical florets developing ahead of the lateral. There were no bulbils and few bracteoles could be seen. (Scale in mm)



Figure 108; the apical floret resembles that of var. *babingtonii*. The stamens were in two whorls aligned with the two whorls of tepals. The carpels were developing at this point, though the filaments had not yet extended. Scale bar approx. 2 mm

considered in future work. These could relate to environmental influences such as temperature, light exposure, nutritional availability etc.

6.3.5 Bulbil/floret numbers

The number of bulbils on each inflorescence was highly variable (Figure 109).

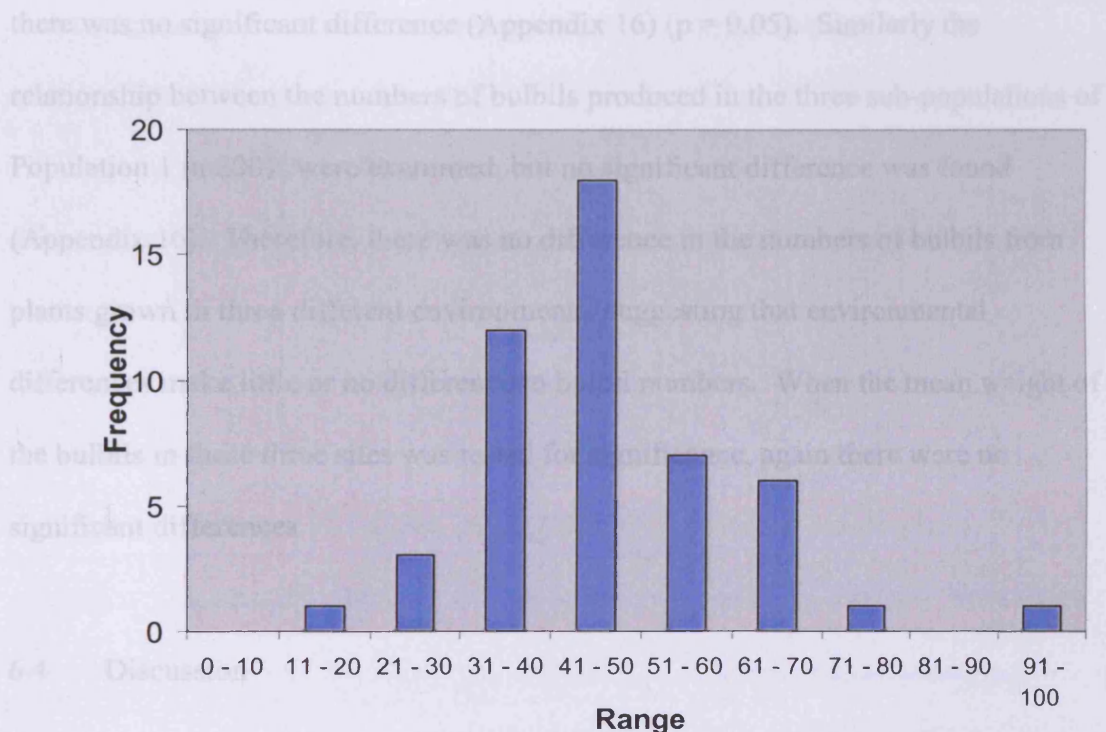


Figure 109: Numbers of bulbils on inflorescences collected from Cornwall (SH99). Mean 45.5 ± 1.95 ; median 44, $n = 49$

Treu (1999) recorded means of 49.5 ± 2.0 (population 1, $n = 70$), 45.1 ± 4.8 (population 38, $n = 10$) and 60.6 ± 3.3 (population 39, $n = 10$). When these means were compared with those calculated for SH1101 (three sub-populations from Population 1), and the regression co-efficient calculated, there was a weak positive correlation ($R = 0.57$) (Appendix 16). This probably reflected the larger inflorescence size, but factors such as the timing of the signals to produce bulbils as an alternative to florets must be

considered in future work. These could relate to environmental influences such as temperature, light exposure, nutritional availability etc.

The relationship between the number of bulbils and florets produced in 1999 and 2001 was also examined, using non-parametric ANOVA (Barnard *et al.*, 2001) but there was no significant difference (Appendix 16) ($p = 0.05$). Similarly the relationship between the numbers of bulbils produced in the three sub-populations of Population 1 in 2001, were examined, but no significant difference was found (Appendix 16). Therefore, there was no difference in the numbers of bulbils from plants grown in three different environments, suggesting that environmental differences make little or no difference to bulbil numbers. When the mean weight of the bulbils in these three sites was tested for significance, again there were no significant differences.

6.4 Discussion

Plants must be exposed to sufficient low temperatures for sufficient time to have been vernalized, and this is unlikely to have happened before January. Because Inflorescence development is well advanced by March, February, (as predicted in Figure 68), is likely to be when floral determination occurs.

Generally, the first daughter bulb was enfolded in the leaf axil and was morphologically visible after the tenth true leaf that, in turn, was produced after the initial bladeless sprout leaf. This was followed by the eleventh leaf, which arose at 180° angle of divergence from the previous leaf, with the second daughter bulb

arising in the axil of this leaf. The spathe was produced adjacent to the second daughter bulb, arising in a horseshoe shaped primordium similar to that formed by developing leaves, which rose up and enclosed the developing inflorescence.

Floret primordia emerged first on the summit of the inflorescence, simultaneously with the cymes that arose on the flanks as irregular shaped outswellings. Bracts arose just above the spathe around the circumference. Bracteoles developed on the inflorescence, enclosing both florets and bulbils. However, bulbils were not recorded outside bracteoles, although florets frequently arose independently of bracteoles. The number of bulbils and florets within a bracteole varied with up to four florets and up to two bulbils. No attempt was made to quantify this relationship, as it was not apparent whether production of bulbil/floret primordia had ceased in early inflorescences. Conversely, waiting for full maturity to develop led to unreliable data, as bracteoles had desiccated and in some cases had been shed. This made identification of individual cymes difficult to ascertain with any accuracy.

Floral organs developed outer whorls first. The inner stamens are tricuspidate, the anthers attached to the central shorter filaments. The outer cusps were crumpled within the floret, possibly being unable to emerge as the florets never fully opened. These cusps frequently reached several millimetres in length. Carpels began to develop before the cusps were fully developed. They had three 'U' shaped primordia, which bent around to form two ovules in each carpel. When they were joined, the plain, terete stigma developed, protruding with the anthers beyond the tepals.

Var. *ampeloprasum* developed similarly, but without the bulbils. Leaf number was similar, as was the physiology of the inflorescence as far as could be ascertained from one inflorescence.

Floret and bulbil number in var. *babingtonii* was highly variable, probably depending at least partly on the size of the inflorescence, which may be related to the size of the bulb and the accumulation of assimilates. No factors were identified as contributing to bulbil numbers per inflorescence, or mean bulbil weight, or the bulbil/floret ratio.

7.0 Expression of a putative homologue to the *Arabidopsis* meristem gene, *LEAFY*

The *LEAFY* gene and its homologues are central to the flowering processes in a number of plants, although its precise role varies with genus and species (see 1.8.3).

Examination of expression of this gene in *A. ampeloprasum* var. *babingtonii* could improve understanding of flowering processes in general in this species, and in particular, strengthen the suggestion that bulbil production in *Allium* species may be indicative of a reversion to the vegetative state (Aura, 1963; Brewster, 1994; Kamenetsky and Rabinowitch, 2002).

DNA was extracted from *A. ampeloprasum* var. *babingtonii*, purified and stored at -80°C. No *LEAFY* primers for *Alliums* were available; therefore already available primers designed from a highly conserved area of *RFL* (Rice *LFY/FLO* homologue) (Kyoizuka *et al.*, 1998) were used to isolate a partial putative *LFY* homologue, using the Polymerase Chain Reaction (PCR). These were chosen, as rice (*Oryza sativa*) is closer to *Allium* spp. being also a monocot, than other species for which *LFY* homologues have been isolated. Examination of expression of *RFL* in rice using these primers (Kyoizuka *et al.*, 1998) showed that it was expressed predominantly in very young panicles, but not in mature florets, mature leaves or roots. It was also expressed in epidermal cells in young leaves at vegetative growth stages and was thought to be involved in panicle branching. Transgenic *Arabidopsis* plants constitutively expressing *RFL* were used to test function. In most 35S-*RFL Arabidopsis* plants, the inflorescence meristem was not transformed to floral meristem. Instead, cotyledon, rosette leaf, petal and stamen

development were severely affected, showing that whilst its function was distinctly different from that of *LFY* in *Arabidopsis*, where it was first characterised, its role in floral development was still significant (Kyozyuka *et al.*, 1998).

PCR products from amplification of *A. ampeloprasum* var. *babingtonii* DNA using these primers were checked by gel electrophoresis, then eluted, and ligated into the pGEM® Vector and transformed into *E. coli*. Resulting colonies containing the ligated plasmids were screened by PCR amplification with M13 F and R primers, and the products checked by gel electrophoresis. Those with appropriate sized fragments were sequenced, and checked for homology with known *LEAFY* sequences.

A specific primer pair was then designed, and used to examine expression of the putative *LEAFY* homologue in vegetative and floral apices, and leaf material of *A. ampeloprasum* var. *babingtonii* by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction).

7.1 Methods and materials

7.1.1 Degenerate primers used to locate a putative *LEAFY* homologue

Kyozyuka *et al.* (1998) designed primers (*RFL*), to amplify rice *FLO/LFY*-like genes in rice (*Oryza sativa*) basing them on highly conserved sequences found in *FLO/LFY* (Figure 110A and B).

RFL	MDPND-AFSA	AHPFRWDLGP	PAPAPVPPPP	PPPPPPPPAN	V-PREL---E	ELVAGYQVRM	55
LFY	***EGFTSGL	FRWNPTRALV	Q**P*****L	QQQ*VT*QTA	AFGMR*GGL*	G*FGP**I*F	60
FLO	***DAFLFKW	D*RTALPQPN	RL LDA*A***	***QA*SYS	MR***GGL*	**FQA**I*Y	60
RFL	STVARISELG	FTASTLLAMT	ERELDDMMAA	LAGLFRWDL	LGERFGLRAA	LRAERGLMS	115
LFY	Y*A*K*A***	*****VG*K	DE**EE**NS	*SHI***E**	V***Y*IK**	V****R**QE	120
FLO	Y*A*K*A***	**VN***DMR	DE***E**NS	*CQIF*****	V***Y*IK**	V****R*IDE	120
RFL	LGG-----RH	HGHQSGSTVD	GASQ--EVLS	DEHDMAGSGG	MGDDDNRRM	VTGKKQAKKG	168
LFY	EEEEESSR*R	*LLL*AAGDS	*THHALDA**	Q*D*WT*LSE	EPVQQDQTD	AA*NNGGGGS	180
FLO	EE---VR*R	*LLL---GD-	-TTHALDA**	QE---*LSE	EPVQQE--KE	AM*SGGGGV*	165
RFL	S----AARKG	FKARRKKVDD	LRLDMQEDM	DCCDEDGGGG	SESTESSAGG	GGGERQREHP	224
LFY	GYWDAGQGKM	**QQQRRRK	KPMLTSVETD	EDVNEGEDDD	GMDNGNGGS*	L*T*****	240
FLO	GVWEMMGAG*	R**PQRRRKN	YKGRSRMASM	EE-DDDDDD	ETEGAEDDEN	IVS*****	225
RFL	FVVTEPGEVA	RAKKNGLDYL	FHLYEQCRLF	LLQVQSMAL	HGHKSPTKVT	NQVFRYAKKV	284
LFY	*I*****	*G*****	*****D*	*****TI**D	R*E*C*****	*****S	300
FLO	*I*****	*G*****	*****D*	*I**TI**E	R*E*C*****	*****A	285
RFL	GASYINKPKM	RHYVHCYALH	CLDEEASDAL	RRAYKARGEN	VGAWRQACYA	PLVDISARHG	344
LFY	*****	*****	*****N**	***F*E****	**S*****K	***N*AC***	360
FLO	**N*****	*****	***A**N**	***F*E****	*****K	***A*A**Q*	345
RFL	FDIDAVFAAH	PRLAIWYVPT	RLRQLCHQAR	SSHAAAAAAL	PPPLF-----	-----	389
LFY	W*****N**	***S*****	K*****LE*	NNAV*****	VGGISCTGSS	TSGRGGCGGD	DLRF 424
FLO	W***TI*N**	***S*****	K*****AE*	**A*V**TSS	ITG-----	---GG-PAD	HLPF 396

Figure 110A: Comparison of amino acid sequences encoded by *RFL*, *FLO*, and *LFY*. Asterisks show conserved amino acids. Blue areas show regions that *RFL* primer sequences were derived from. Adapted from Kyojuka *et al.*, 1998).

RFL F	5'-TAC/TATA/CAAC/TAAA/GCCA/G/C/TAAA/GATG-3'
RFL R	5'-AGCC/TTG/TGTG/TGGG/C/AACA/GTACCA-3'

Figure 110B: *RFL* primers used to amplify rice gene *RFL* and for amplification of the putative *LFY* homologue from *A. ampeloprasum* var. *babingtonii*. (*ABL**LFY* *Allium ampeloprasum* var. *babingtonii* *LFY*)

The primers were obtained from Sigma-Genosys.

7.1.2 Extraction of *A. ampeloprasum* var. *babingtonii* genomic DNA

The CTAB method of extraction used was initially based on Saghai-Marooof *et al.* (1984) and used by Treu (1999) with var. *babingtonii* (Appendix 17). However, initial work to locate a *LFY* homologue produced inconsistent results. Additionally, the DNA degraded

during storage at 4°C, -20°C and -70°C. The specificity of the primers was varied, by altering both annealing temperature and concentration of MgCl₂ (data not presented). However, results were still inconsistent. Inhibitory products may have been present in the extraction; therefore, the DNA was cleaned with Sepharose CL6B (Appendix 17). Test reactions were carried out using tubulin primers (Tub pos 63 F: 5' ATG AGY GGY GTS ACS TGC T and Tub neg 2 R: 5' GTA GGA NGA GTT CTT GTT CTG, data not presented), and including tobacco DNA (*Nicotiana*) in combination with *Allium* DNA as well as each sample of DNA alone, to assess the quality of the extracted DNA. The products were examined by gel electrophoresis and viewed under UV (ultraviolet light). Tobacco DNA alone produced clear banding, but in combination with *Allium* showed much fainter PCR products, confirming the presence of contaminants in the var. *babingtonii* DNA that inhibited the reactions. Therefore, a new extraction of DNA from var. *babingtonii* was further purified using equilibrium centrifugation in Caesium chloride/Ethidium bromide (Appendix 17). A vegetative plant was selected at random from stock grown at UCW (SH00 9B), and 3 g of young leaf was excised over ice to prevent degradation. Young leaf was chosen, as it was less likely to have suffered damage or contamination by other microorganisms, being protected within enfolding older leaves. This yielded approximately 150 ng/μl genomic DNA as judged by gel electrophoresis. When checked using gel electrophoresis, the band was clear with no visible degradation. Therefore, this DNA was used for further PCR amplifications.

Tubulin primers Pos 63 F and Neg 2 R were used as positive control with known *Arabidopsis* DNA and SDW as a negative control. The products were checked using gel electrophoresis, and viewed under UV.

7.1.3.2 Elution

The products were cut out of the gel under aseptic conditions, and the DNA extracted using the QIAquick Gel Extraction Kit following the manufacturer's protocol (Appendix 17). The same size products from both DNA concentrations were combined.

7.1.3.3 Ligation

Fragments were ligated into the pGEM®-T Vector System 1 (Promega, A3600), using the supplied protocol (Appendix 17). These vectors had been prepared by cutting with *EcoRV* and adding a 3' terminal thymidine to both ends. The single 3'-T overhangs at the insertion site improving the efficiency of ligation of the PCR product, by preventing re-circularization of the vector and providing a compatible overhang for the single 3'-A overhangs of the PCR product.

7.1.3.4 Transformation

Competent XL-1 Blue sub-cloning grade cells (Stratagene) were defrosted on ice and 50 µl was placed in each of two 14 ml polypropylene Falcon tubes. Competent cells have been soaked in ice-cold salt solution (e.g. 50 mM CaCl₂), which improves binding of plasmids to cell walls. Two micro litres of ligated plasmid was added to each tube, one containing the large fragment, one containing the small fragment. These were incubated

for 20 min on ice, and then heat shocked for 45 s at 42 °C. This stimulates the transport of the plasmids into the cell. After incubating for a further 2 min on ice, 0.9 ml SOC (Appendix 17) was added and the mixture placed in a shaking incubator at 225-250 rpm, 37°C, for 30 min. The resulting cultures were plated (200 µl) onto LB plates (+ Ampicillin + Xgal + IPTG) prepared as Appendix 17. Two plates were prepared for each size fragment. These were incubated for 17 h at 37°C. The plasmids possess a gene conferring antibiotic (ampicillin) resistance; therefore, any cells not containing the plasmid are destroyed by the ampicillin in the medium. The *E. coli* cells used have a mutation resulting in the inability to synthesise the α subunit of β -galactosidase. The plasmids also possess the gene for this subunit, but it is cleaved when the plasmid is opened for ligation. Therefore, cells that have not ligated the supplied insert, can synthesise β -galactosidase, whilst those that have been transformed without the insert cannot. X-gal (5-bromo-4-chloro-3-indoyl- β -Dgalactopyranoside) is an analogue of galactose, which is also metabolised by β -galactosidase. It is incorporated into the substrate, where it is metabolised by able colonies to give a blue colour. Therefore, white colonies possess the plasmid with the desired insert, and blue colonies do not.

7.1.3.5 PCR to amplify the fragments

For each white colony, plus one positive and one negative (blue) control, the reaction mixtures containing M13F and R primers (Genosys 10µM) were prepared (Appendix 17). Five colonies with the large insert and five with the small insert were selected. Each colony was touched with a sterile pipette tip, which was then stirred into the reaction mixture, then the remainder used for liquid cell culture to maintain the cells.

The products were amplified by PCR using M13 F: 5' GTAAAACGACGGCCAGT and M13 R: 5' AACAGCTATGACCATG which bind to the vector sequences, then checked on a gel.

7.1.3.6 Sequencing

The QIAprep Spin Plasmid Kit (Quiagen 27106) was used to extract plasmids from the transformed cells, in preparation for sequencing following the manufacturers' protocol (Appendix 17). The cultures were centrifuged, then the pellets resuspended in buffer P2. Buffer P2 (NaOH/SDS) solubilizes and denatures unwanted cell components, then is neutralized with a high salt buffer (N3) to precipitate further unwanted components. The product is centrifuged, and then applied to a silica-gel membrane that selectively adsorbs the plasmid DNA in high salt buffer. The membrane is washed twice to remove salts etc, by centrifugation, then the product eluted in a low salt buffer: Buffer EB (Appendix 17). The product was checked on a gel to confirm the presence of the plasmid (3-4 kbp), and the remainder sequenced using 3100 Genetic Analyser (Applied Biosystems).

Initial sequences were confused, suggesting mixed colonies. Therefore, S1 was re-plated, grown on as before for 17 h at 37°C, and single colonies re-selected, amplified by PCR (samples 1 – 10) as 7.1.3.5, plasmids extracted and sequenced as 7.1.3.6. The resultant sequences were aligned using the Seqman module of Lasergene (DNASar). Similar sequences were compared with those of known *LEAFY* (and *LEAFY* homologue) sequences using BLAST (Basic Local Alignment Search Tool, National Centre for

Biotechnology Information [http; www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast). This gave information about the homology of the putative *LFY* homologue and its relationship with known sequences.

7.2 Results

7.2.1 Extraction of PCR-clean DNA from *A. ampeloprasum* var. *babingtonii*

High molecular weight DNA was successfully extracted from the *A. ampeloprasum* var. *babingtonii* material. The size of the fragment was greater than 12 kb and was estimated to be at a concentration of 150 ng/μl. The band was precise, with no smearing, demonstrating no degradation of the DNA (Figure 111).

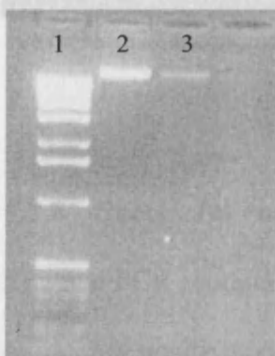


Figure 111: Extraction of DNA from *A. ampeloprasum* var. *babingtonii* using Caesium chloride purification (Appendix 17). Lane 1, 1 kb ladder; lane 2 1 μl genomic DNA; lane 3 1 μl made up to 1/10 dilution with SDW genomic DNA

7.2.2 PCR amplification of putative *LFY* homologue from *A. ampeloprasum* var. *babingtonii*

Two PCR products were obtained from amplification of the *A. ampeloprasum* var. *babingtonii* genomic DNA (Figure 112) of approximately 250 bp and 300 bp. Both products were larger than the fragment obtained by Kyojuka *et al.*, (1998) in rice (235 bp), which may reflect sequence divergence. Since var. *babingtonii* is hexaploid, (Treu, 1999) it was possible that the *LFY* homologue may be present as more than one allele, which could be reflected in the two different sizes of PCR products. Increased product

yield was obtained with higher levels of template (3 μ l compared with 2 μ l, lanes 3 and 2 respectively), indicating no inhibition of PCR at this concentration. There was no contamination as indicated by the SDW control, and good product yield with the tubulin primers. These produced very bright bands, probably as a result of multiple genes of the β -tubulin family in the genome.

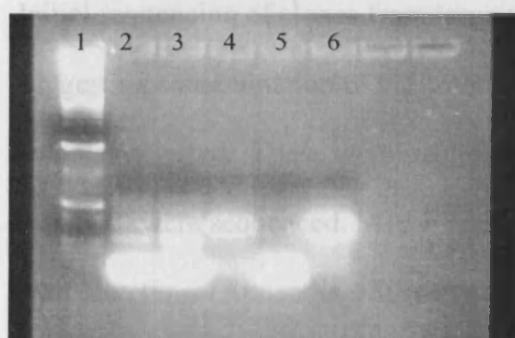


Figure 112; DNA extracted from *A. ampeloprasum* var. *babingtonii*, and amplified with ABLFY F and R.

Bands viewed under UV. Lanes numbered left to right; Lane 1 - 1 kb ladder

Lane 2 - genomic DNA 2 μ l + ABLFY F & R

Lane 3 - genomic DNA 3 μ l + ABLFY F & R

Lane 4 - genomic DNA 2 μ l + Tub pos 63 and neg 2 (+ve control)

Lane 5 - no DNA = ABLFY F & R (-ve control)

Lane 6 - *Arabidopsis* DNA + Tub pos 63 and neg 2 (+ve control)

Var. *babingtonii* DNA with ABLFY F and R shows

two bands at both concentrations, at approximately 245 kbp and 298 kbp.

7.2.3 Successful amplification of putative *LFY* homologue clones by colony PCR

The two PCR product sizes above (7.2.2) were cloned separately and colony PCR used to identify positive colonies. Products would necessarily be larger than the inserts, because these primers hybridise to M13 sites outside the ends of the insert (insert + 251 bp). This PCR successfully amplified ten inserts, 5 each of the large and small fragments (Figure 113).

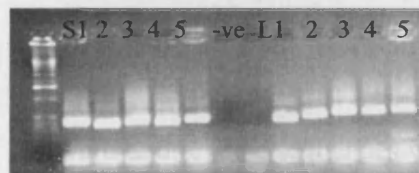


Figure 113: Gel electrophoresis of PCR products following amplification of inserted sequence with M13F and R primers. Reading left to right, small fragment S1 - 5, -ve controls with blue colony and water, large fragment L1-5. Lower bands were primer dimers.

There was clearly a size difference in some inserts, both in the small and the large fragment clones. The SDW –ve control showed that there was no contamination, but the blue colony should have produced a band of approximately 250 bp.

7.2.4 Sequencing of putative LFY homologue clones

Initial sequencing of clones from large and small fragments gave mixed results suggesting contamination of the colonies. Therefore, S1 was re-cultured, and 10 colonies selected. These were amplified as previously, and six of them (clones 1, 4, 5, 8, 9 and 10) were sequenced. M13 F and R primers were used to sequence the clones in both directions (Appendix 18) and the clones were found to align closely with each other (Figure 114).

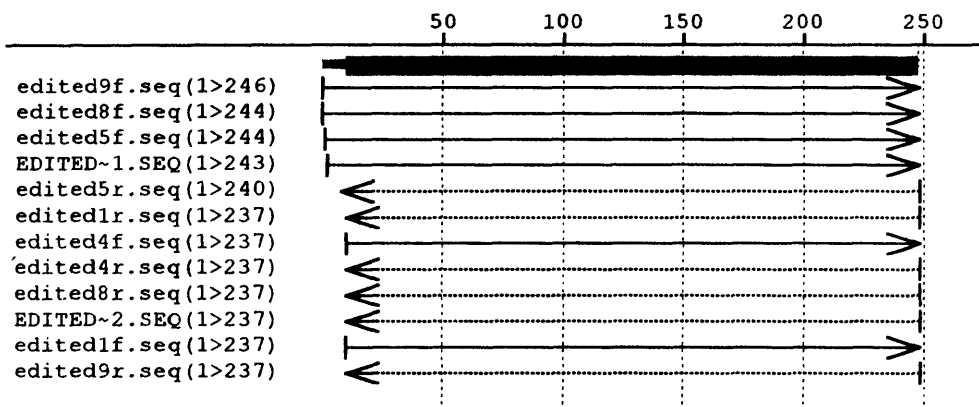


Figure 114; Seqman programme aligning sequences from clones 1, 4 5 8 9 and 10 (F and R) (small fragments). Note that 10 F and R is shown as EDITED 1 and 2. For full sequences see Appendix 18. All forward and reverse sequences for each clone were checked and found to be identical. All sequences produced unambiguous traces, leaving little room for doubt as to sequences. Each fragment was checked for completeness by identifying the plasmid sequence at each end of the insert.

Forward and reverse sequences were checked and found to be identical. All the DNA sequences produced strong, unambiguous traces, however the sequences were not identical to each other, and homology ranged between 69% and 94 % (Figure 116).

Since var. *babingtonii* is hexaploid, it was possible that these clones may represent different alleles. The sample comprised DNA from one plant only, (SH00 9B) (Figure 115), so this suggested that either the plant contained six different alleles of the putative *LFY* gene or that the genetic variation noted by Treu (1999) was the product of high levels of genetic instability. It is also possible that the situation in this species is more complex, and that there is more than one *LFY* homologue. A further alternative would be PCR error. This is possible since high fidelity Taq polymerase was not used for the amplification step.

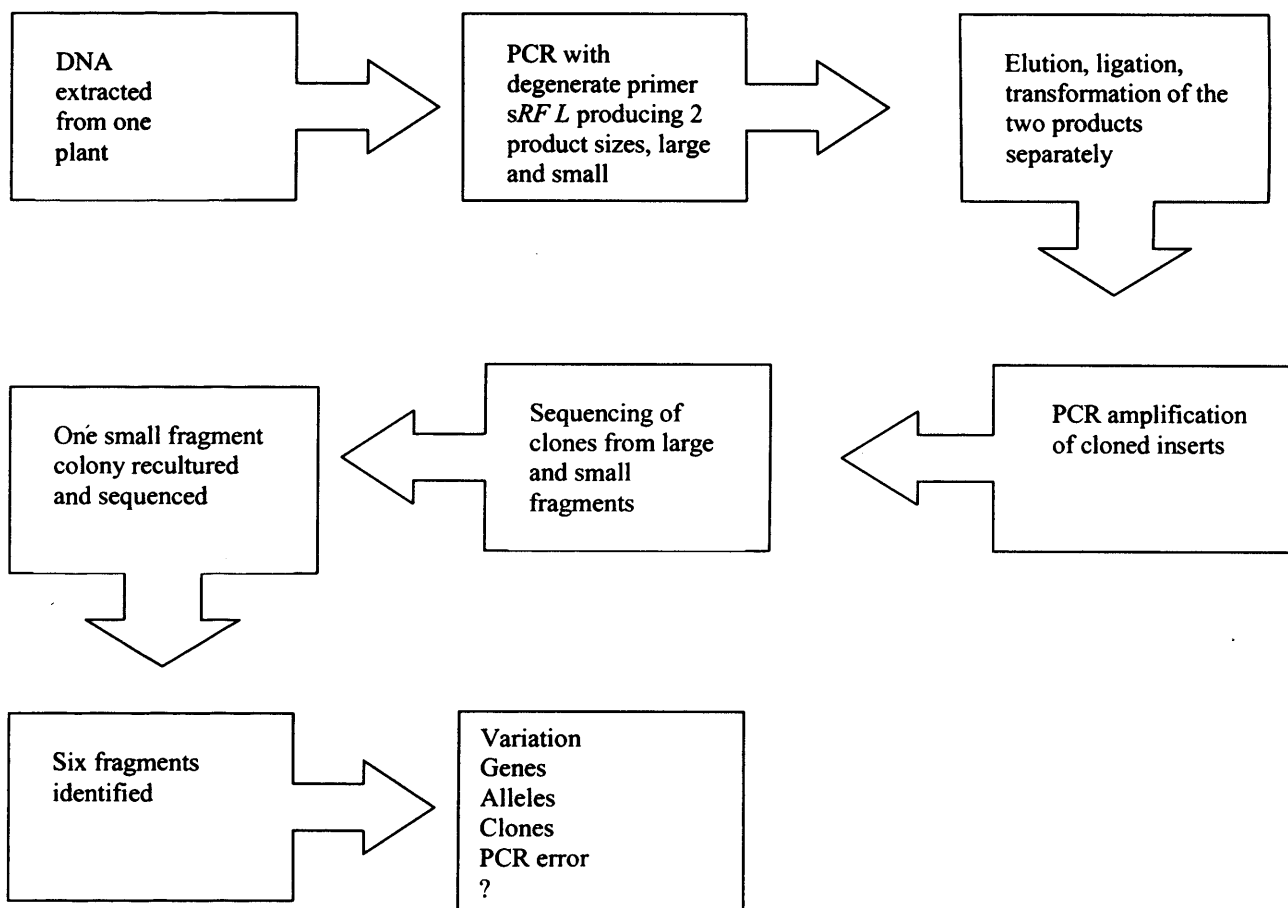


Figure 115: Summary of experimental methods for the extraction of DNA and sequencing of the putative *LFY* homologue

All six fragments showed high levels of consensus (Figure 116).

Figure 116: Sequences of six putative ABLFY fragments obtained from bacterial clones, aligned using CLUSTAL with consensus.

Clone 10	TACATCAATAAGCCCAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCCTAGAAGAGGACACATCAAACGCCCT	8
Clone 1	TATATCAATAAGCCCAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCCTAGAAGAGGACACATCAAACGCCCT	8
Clone 4	TATATCAA CAAGCCTAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCCTAGAAGAGGACACATCAAACGCCCT	8
Clone 5	TATATCAATAAGCCGAAGATGAGGCATTATGTCCATTGCTAAGCTTTGCAATTGCCTCGATGAAGAAACATCATATGCACT	8
Clone 8	TATATCAATAAGCCGAAGATGAGGCATTATGTCCATTGCTACGCTTTGCAATTGCTGGATGAAGAAACATCATAGCACT	8
Clone 9	TACATCAATAAGCCGAAGATGAGGCATTATGTCCATTGCTACGCTTTGCAATTGCCTGGATGAAGAAACATCATAGCACT	8
Consensus	TAYATCAAY AAGCC AAGATGAGRCAYTAYGTLCAYTGCTAMGCYYTSCAYTGMCTVGAWGARGA ACPTCAWAHGCCT	8
	90 100 110 120 130 140 150 160	
Clone 10	CCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCCATTGCTG	1
Clone 1	CCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCCATTGCTG	1
Clone 4	CCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCCATTGCTG	1
Clone 5	CCGTGATACTAAAAGCATCGAGGAGAAAAATGTGGGAGCATGGAGACTAACGTGCTATCCGCATATAGTGGTGATTTCCG	1
Clone 8	CCGTTGATACTACGAGCAACGAGGAGAAAAATGTGGGAGCATGGAGACAAGCGTGCTATCAGCATATAGTAGTGATTTCCG	1
Clone 9	CTATTGATACTACGAGCAACGAGGAGAAAAATGTGGGAGCATGGAGACAAGCGTGCTATCAGCATATAGTAGTGATTTCCG	1
Consensus	CYRWHGAKMCTWMRAGSADCGWGGAGARAATGTGGGAGCATGGAGRCWARCKTGCTAYTMRGMYTWCDCYSATTTKRC	1
	170 180 190 200 210 220 230	
Clone 10	GTCGTCAAGGTTGGGATATTGACTCCATTTTTAACTCTCATCCCTCGTCTTTCTATTTGGTACGTCCCCACAAAGCT	236
Clone 1	GTCGTCAAGGTTGGGATATTGACTCCATTTTTAACTCTCATCCCTCGTCTTTCTATTTGGTACGTCCCCACCAAGCT	236
Clone 4	GTCGTCAAGGTTGGGATATTGACTCCATTTTTAACTCTCATCCCTCGTCTTTCTATTTGGTACGTCCCCACCAAGCT	236
Clone 5	CGCAACACAATTGGGATGTAAACGTGCTTTACAATGCACATCTGAGGCTGTCGATTTGGTACGTCCCCACCAAGCT	236
Clone 8	AGCAGCACAATTGGGATGTGGATGCCGTTTTCAATTCACATCTGAGGATGTCGATTTGGTACGTCCCCACCAAGCT	236
Clone 9	CGCAGCACAATAGGGATGTGGATGCCGTTTTCAATTCACATCCGAGGTTTTTCGATTTGGTACGTCCCCACCAAGCT	236
Consensus	VKCRDCAMRRTWGGGATRTDRAYKYCRTTTTWYAAAYKWCATCYBMGKHKTCKATTTGGTACGTCCCCACMAAGCT	236

The fragments showed high levels of similarity with known sequences for *LFY* and *LFY* homologues/orthologues. For example, clone 1 was 96% identical to a *LFY* gene fragment in *Silene coeli-rosa*, 83% similar to *Arabidopsis thaliana LFY* gene fragment, and scored over 108 hits, all of which were *LFY* (homologue/orthologue) fragments or proteins (BLAST database). It therefore seemed likely that the fragments located with the *ABLFY* degenerate primers were part of larger *LFY* homologues, with more than one *LFY* gene, or up to six alleles of a single gene represented.

The 236 bp fragments were translated for comparison with other *LFY* homologues. However the ORF was interrupted in three of the clones (clones 5, 8 and 9) suggesting that some of the divergence in these clones was due to PCR error. The amino acid sequence of the remaining clones was identical, again indicating multiple genes or multiple alleles of a single gene.

The 78 amino acid translated sequence was aligned with the rice *RFL* sequence (Figure 117). As expected, the *A. ampeloprasum* var. *babingtonii* sequence aligns with amino acids 288 to 365 of the *RFL* sequence.



Figure 117: Alignment of the partial *A. ampeloprasum* var. *babingtonii* putative *LFY* translated sequence with the full rice RFL sequence, using Clustal W.

The translated sequence was further compared to six other *LFY* homologues over the same region of the protein. The genes showed high levels of homology ranging from 77% to 96 % (Fig. 118) with the consensus showing 57 amino acids homologous to all (Fig. 119).

	Percent similarity						
	<i>Allium</i> var. <i>babingtonii</i>	<i>Arabidopsis</i> <i>thaliana</i>	<i>Brassica</i> <i>oleraceae</i>	<i>Eucalypt</i> <i>usgrandis</i>	<i>Antirrhinum</i> <i>majus</i>	<i>Petunia</i> <i>inflata</i>	<i>Oryza</i> <i>sativa</i>
<i>Allium</i> <i>ampeloprasu</i> <i>m</i> var. <i>babingtonii</i>		87.1	84.6	84.8	92.3	93.5	76.9
<i>Arabidopsis</i>			96.1	86.0	91.0	91.0	84.6
<i>Brassica</i> <i>oleraceae</i>				84.8	88.4	88.4	84.6
<i>Eucalyptus</i> <i>grandis</i>					87.3	87.3	78.4
<i>Antirrhinum</i> <i>majus</i>						96.1	82.0
<i>Petunia</i> <i>inflata</i>							82.0
<i>Oryza sativa</i>							

Figure 118: Percent similarity of amino acid sequence for *ABLFY* fragment, compared with the homologous fragment from other known sequences. (*Petunia inflata* AF030171, Souer *et al.*, 1998; *Arabidopsis thaliana* NM 124479 Weigel *et al.*, 1992; *Eucalyptus grandis* AY640314 Southerton *et al.*, 1998; *Brassica oleracea* Z18362, Anthony *et al.*, 1993, *Oryza sativa* Kyozuka *et al.*, 1998, *Antirrhinum majus* M55525, Coen *et al.*, 1990.)

	10	20	30	40	50
rice	YINKPKMRHYVHCYALHCLDEEASD	NALRRRAFKERGENVGAWRQACYP	PLV		
arabidopsis	YINKPKMRHYVHCYALHCLDEEAS	NALRRRAFKERGENVGAWRQACYP	PLV		
brassica	YINKPKMRHYVHCYALHCLDEEAS	NALRRRAFKERGENVGAWRQACYP	PLV		
eucalyptus	YINKPKMRHYVHCYALHCLDEEAS	NALRRRAFKERGENVGAWRQACYP	PLV		
FLO	YINKPKMRHYVHCYALHCLDEEAS	NALRRRAFKERGENVGAWRQACYP	PLV		
petunia	YINKPKMRHYVHCYALHCLDEEAS	NALRRRAFKERGENVGAWRQACYP	PLV		
allium	YINKPKMRHYVHCYALHCLDEEAS	NALRRRAFKERGENVGAWRQACYP	PLV		
Consensus	YINKPKMRHYVHCYALHCLDEEAS	NALRRRAFKERGENVGAWRQACYP	PLV		
	60	70			
rice	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
arabidopsis	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
brassica	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
eucalyptus	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
FLO	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
petunia	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
allium	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
Consensus	DI SAR-HGWDIDAVFNAHPRLSIWYVPTK				
Consensus	I R G DID F HPRL IWYVPT				

Figure 119: Amino acid sequence for *ABLFY* fragment aligned with six other sequences of LFY homologues over the same region of the protein, using ClustalW (see legend to Fig. 118 for sequence details).

A phylogenetic tree was used to compare the translated sequence from the *ABLFY* fragment with amino acid sequences from the same region of a selection of known LFY

homologues (Figure 120). This showed the fragment to be most similar to a sequence from *Petunia inflata* (AF030171, Souer *et al.*, 1998) and much more distant from the corresponding sequence in *Oryza sativa* (Kyojuka *et al.*, 1998). This was surprising, as monocots might be expected to be more similar to each other than to dicots. However, as the fragments were all derived from the highly conserved region of the gene, firm conclusions about the relationships must await sequencing of the whole gene.

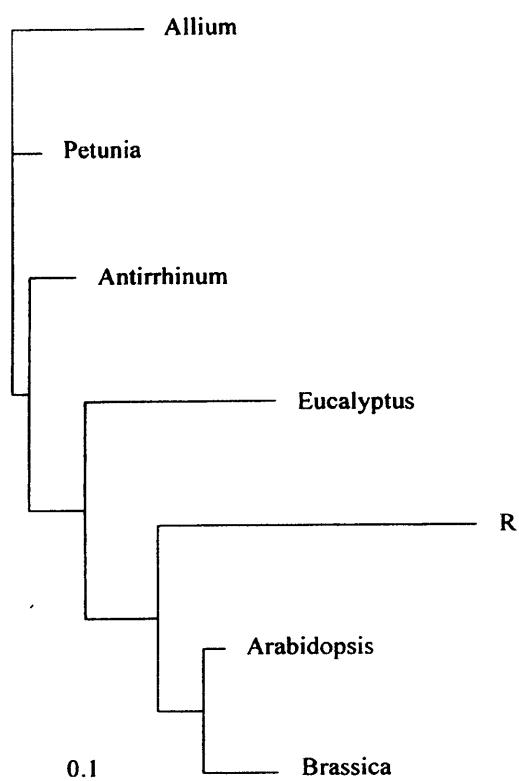


Figure 120: Phylogenetic tree using the Clustal method with Weighted residue weight table. This compared the cloned sequence from *Allium ampeloprasum* var. *babingtonii* with other LFY homologues over the same region (see legend to Fig. 118 for sequence details).

7.3 Expression of the putative *LEAFY* homologue in var. *babingtonii*

Specific primers were designed from these sequences (above) for *ABL* *LFY* *Allium ampeloprasum* var. *babingtonii* *LFY*, using the sequences from the six clones

(Appendix 18). It was not possible to find sequences that were completely homologous, but comparison of the sequences resulted in design of a primer with only one wobble in the forward primer. Expression of *ABLFY* was compared in vegetative and floral apices, and leaf material. Leaf material was included as some species (e.g. *Oryza sativa*, rice - Kyozuka *et al.*, 1998), have shown *LFY* homologue expression in young leaves, as well as the more commonly investigated expression in floral processes. RNA was extracted from fresh var. *babingtonii* plants, grown at UCW. Apices were checked during dissection for inflorescence development, or the development of storage tissue (indicating vegetative state). RNA was extracted, cleaned, then cDNA was synthesised with reverse transcriptase. This first strand was amplified using PCR with specific *ABLFY* primers derived from the identified *LFY* homologue, and the product checked by gel electrophoresis. The bands obtained in vegetative, floral and leaf material were compared.

7.3.1 Methods and materials

7.3.1.1 RNA extraction

RNA was extracted from three sources; known floral material (SH0302, 8 plants selected at random) was dissected over ice as rapidly as possible. Known vegetative plants (smaller than 3 cm diameter) were also dissected (16 plants selected at random), and fresh young leaf material from the same plants was also excised over ice. All samples were then flash frozen in liquid nitrogen and stored at -70°C. TRI-Reagent (Sigma T-9424) was used to extract RNA following the manufacturers' protocol (Appendix 17). The product was checked by gel electrophoresis, showing intense bands

at approximately 1000Kb. Concentrations were checked on the Genequant (Pharmacia RNA/DNA Calculator). Leaf RNA = 574 µg/ml; Floral RNA = 1408 µg/ml; Vegetative RNA = 1124 µg/ml. The remainder was stored at -70°C. Traces of DNA were removed by treating the samples with a DNase inhibitor; RQ1DNase was incubated with the RNA for 30 min, and then halted with RQ1 DNase Stop Solution to terminate the reaction. The DNase was inactivated by incubation according to the manufacturers' protocol (Appendix 17). The products were again checked by gel electrophoresis to confirm that the RNA was present and not degraded.

7.3.2 Complementary DNA synthesis

First strand cDNA was synthesised from the isolated RNA using M-MLV Reverse Transcriptase (Promega M530A) (Appendix 17) and stored at -70°C.

7.3.3 PCR

The cDNA was amplified using the specific primers (Sigma-Genosys) designed from the six sequences described in section 7.2. These primers were largely based on the original degenerate primers, with all but one of the wobbles removed:

ABLFY F 5' ATCAATAAGCCBAAGATGAG 3'
MW 6161, Tm 52.6°C

ABLFY R 5' TAGCTTGGTGGGGACGTA 3'
MW 5611 Tm 61.7°C

The first strand cDNA was amplified as double stranded DNA using HotStar Taq DNA Polymerase (QUIAGEN), which was provided in an inactive state with no polymerase activity at ambient temperatures. This reduced the formation of misprimed products and

primer-dimers at low temperatures. It was activated by a 15 min. incubation step at the beginning of the PCR programme. The programme was the same as that previously used to amplify *LFY* fragments from genomic DNA with degenerate primers (see 7.1.3.1). SDW was used as a –ve control, +ve control was tubulin (Pos63 Neg 2) primers (Appendix 17), and the results were checked by gel electrophoresis.

7.4 Results

Complementary DNA extracted from floral apices clearly showed a band when amplified with *ABLFY* F and R, indicating that the *LFY* homologue was expressing in these tissues (Lane 5 Figure 121). *ABLFY* was not expressed in the vegetative apices or the young leaf material (Lanes 3 and 4 Figure 121).

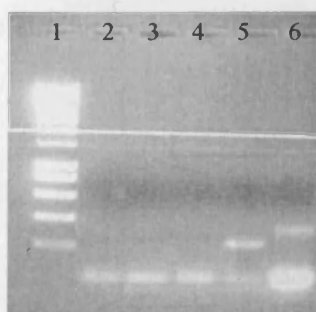


Figure 121: Expression of *ABLFY* in young leaves, vegetative apices, and floral apices; Lanes from left to right; 1 - 1 kb ladder; 2 - SDW –ve control; 3 - leaf cDNA; 4 - vegetative cDNA; 5 - floral cDNA; 6 - leaf cDNA with tubulin +ve control.

This result was reproducible, therefore, it can be considered that *ABLFY* was expressed in floral apices, but not vegetative apices or in young leaves.

This result was reproducible; therefore, it suggests that *ABLFY* may be involved with the development of floral apices in this species. The tubulin primers produced a band of the expected size in Lane 6 (Figure 122), confirming that the leaf cDNA was present and of PCR quality. The negative control confirmed that contamination had not occurred.

7.5 Summary

Work presented in this chapter has demonstrated the presence of a *LFY* homologue in *A. ampeloprasum* var. *babingtonii*. It seemed to be present in more than one form, six being amplified; though three of these definitely contained PCR errors. The other three translated to identical protein sequences, suggesting that any errors in PCR had not affected the encoding of the amino acid sequences. Early results showed the probable presence of at least two sizes of *LFY* homologue, however, the larger size was not investigated. As *A. ampeloprasum* var. *babingtonii* is hexaploid, there may be more than one allele of the gene present. However, beyond the fact that there is genetic variation in this clonally reproducing population (Treu, 1999), there is little information, about the genome of this species. The fragment size was much as expected, being similar to that located in *Oryza sativa* by the same degenerate primers.

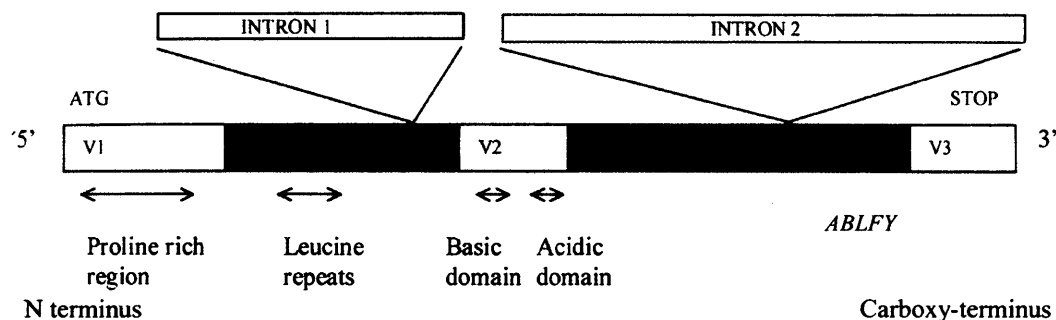


Figure 122: Generalised structure of the *LFY* gene, showing the two conserved regions (black), variable regions (V1, V2 and V3), intron positions, and regions of putative transcriptional activation function (double headed arrows), with approximate position of *ABL'FY*. Adapted from Allnutt, 2000.

LFY genes encode proteins of between 359 and 415 amino acids (*ELF1*, Southerton *et al.*, 1998 to *BOFH*, Anthony *et al.*, 1993). There are two highly conserved regions with three smaller variable regions (Mouradov *et al.*, 1998) (Figure 122), this conservation being apparent across a wide range of species, including a gymnosperm (*Pinus*), and a

monocot (*Oryza*) (Mouradov *et al.*, 1998). The *ABLFY* fragment was located in the second conserved region, very close to the third variable region (Figure 123). *LFY* and its homologues show no significant similarities to other sequenced genes in the databases, although they do have domains recognised as transcription factors (Mermod *et al.*, 1989; Struhl, 1989; Latchman, 1990; Hahn, 1993).

Further, it has been demonstrated that this gene may be involved in floral processes, as for so many of the published *LFY* homologues. Many of these have shown expression in vegetative material, (e.g. Hempel *et al.*, 1997; Blásquez *et al.*, 1997; Bradley *et al.*, 1996; Anthony *et al.*, 1996; Franco-Zorilla *et al.*, 1998; Bradley *et al.*, 1997) but only in pea (*Pisum*) (Hofer *et al.*, 1997) has it been shown to have a role in vegetative development. Moreover, the role of *LFY* homologues in the floral transition has been demonstrated in mutant phenotypes. Blásquez *et al.*, (1997) suggested that differences in the expression of *LFY* in SD and LD grown *Arabidopsis*, indicated that a threshold level may have to be reached in this species to confer floral fate on meristems. However, it is not possible to draw any firm conclusions, regarding the *ABLFY* function in possible floral reversion and the development of bulbils. This must await further work, such as *in situ* hybridization, and comparison with *A. ampeloprasum* var. *ampeloprasum*. Additionally, this fragment of *ABLFY* probably represents only a small part of the whole gene; further sequencing to obtain the whole gene would also provide further information regarding the function and role of this important gene.

8.0 General Discussion

The aims of the work presented in this thesis were:

- 1 To establish a population of *Allium ampeloprasum* var. *babingtonii* as a source of material of known age
- 2 To develop a protocol for the maintenance of *Allium ampeloprasum* var. *babingtonii* in culture
- 3 To establish a histological protocol for use with *Allium ampeloprasum* var. *babingtonii*
- 4 To construct a developmental timetable for *Allium ampeloprasum* var. *babingtonii* for vegetative and floral growth *in vivo*
- 5 To identify the nature and timing of floral determination in *Allium ampeloprasum* var. *babingtonii*
- 6 To identify and clone a homologue to the meristem identity gene *LEAFY* in *Allium ampeloprasum* var. *babingtonii* and examine its expression in the floral transition.

8.1 Establishment of a population of *A. ampeloprasum* var. *babingtonii*

Much of the work in this thesis has been restricted by the numbers of mature plants available for examination of the floral state. Although large numbers of plants, (approximately 800 as newly harvested bulbils, SH99) were planted in the first year, this was reduced by poor sprouting rates, probably influenced by poor drainage. Moreover, as it became apparent that plants were unlikely to reach floral maturity until their third year, their use was limited to the examination of vegetative development, the remainder being used to examine flowering in 2002 and reported on in Chapter 7 of this thesis.

Further supplies of plants were available in small numbers as whole plants from the Cornish site (Population 1), and those purchased from John Shipton Nurseries. The Cornish plants were always of unknown age, the first being obtained in August 2000. Since these were located by their scape, they had all flowered in 2000, and none of them flowered the following year. Populations obtained in 2001 yielded only small numbers of flowering plants, many of them appearing to abort developing inflorescences. Nevertheless, it has been possible to gain much data from these samples.

8.2. Tissue culture

A protocol for growth *in vitro* has been established. Many factors were examined, including surface sterilization, nutrition, fluence levels and photoperiod. Initial numbers of contaminations were high, and this may always be a factor in field-grown sources. Surprisingly, the use of hypochlorite and other chemical sterilants seemed to make little difference. This may relate to the structure of the bulbils and bulbs. Both have hard necrotic tissue that is continuous with the living tissue, in the case of the bulbils, this was the point of attachment to the inflorescence, in the case of older bulbs, this was the remains of previous basal plate tissue from earlier seasons' growth. The best method found to produce clean cultures was to remove all external tissues from the bulbil or bulb, rinsing only in sterile distilled water, which provided approximately 90 % clean samples. Hypochlorite supplied as domestic bleach appeared to be detrimental to sprouting *in vitro*, and this may be linked to salt content. Whole bulbils *in vitro* rooted but did not at any point sprout leaves. This curious phenomenon may be related to the seasonality of the plant, where root growth precedes leaf

growth, although some cultures were continued for up to three months, without showing sprouting. It was also possible that some environmental cue was necessary to induce leaf sprouting, and that this mechanism is compromised during excision of explants, most of which (but not all) sprouted in culture. It was unlikely to be linked with nutrition, since explants sprouted on the same media. However, growth in culture was always much less than *in vivo*, and this must call into question the validity of any deductions regarding development *in vivo*, when extrapolated from growth *in vitro*. Shoot elongation was used as an estimate of growth, as it was easy to monitor without compromising the culture, and non-destructive.

Comparisons of different media and carbon sources often made little difference to shoot elongation. Hence these findings are dissimilar from other work, notably, by Parfitt *et al.*, 2004 in press) on *Pharbitis nil*, that show differential effects of carbon supply on development. Decisions regarding culture conditions were often made based on data that was not statistically significant, but simply better when examined by eye. Since many of the samples were small, there being losses due to contamination, failure to sprout, or production of abnormal growth, it was possible that the tests failed to identify genuine differences in shoot elongation under different conditions. Nevertheless, the protocol developed allowed for the maintenance of cultures, apparently healthy and normal, with developing shoots.

A further difficulty encountered in culture, was the development of a new bulb, followed by dieback of the leaves, and the establishment of dormancy. This appeared to be stimulated by longer daylengths, although shortening the daylength did not reverse the process in plants that had become dormant. This was likely to be related to the phenology of the plant, where

dormancy naturally occurs in summer, unlike model short and long day plants that depend on critical night or day length (see Vince-Prue *et al.*, 1984).

8.3 Establishment of a histological protocol

Satisfactory protocols have been established for wax embedding, and staining. This has been used to establish in some detail the physiology of the plant, both in vegetative and floral states. It was initially difficult to achieve adequate penetration of the tissues, probably related to the physiology of the plant, where the modified stem structure (basal plate) means that many different tissues are in close proximity to each other, and that any sample of the shoot apex includes not only leaf tissue, but stem, root and storage tissue as well. A further complication could be the enfolding nature of the leaves, which restricts penetration of the fluids into the centre of the sample. This was largely solved by increasing the times of immersion in the fixing and wax-penetration processes, though this did sometimes lead to destruction of more fragile tissues, such as developing bulblets, and florets. Once this was achieved, the sectioning process was straightforward, but considerable losses were experienced during the staining processes. This was solved, by pre-subbing the slides, to provide greater adhesion during processing. This protocol was tested on different tissues of different ages, reliably providing high quality slides for examination.

8.4 Developmental timetable *in vivo*

The bulb structure comprised one cataphyll, as for the Aegean members of the *Ampeloprasmus* complex (Bothmer, 1974), which senesced in early spring. This was protected by a bladeless

sclerified protective leaf. Within the cataphyll, the first leaf was a bladeless sprout leaf which formed the basal sheath of the plant, the subsequent bladed leaves arising within this. The basal plate was a modified stem, which regenerated in each season, the old basal plate remaining attached to the base of the plant, again similar to those of the Aegean members of the *Ampeloprasum* complex, and possible reflecting the origins of the species. The short, heart-shaped (LS) stem was similar to that of *A. cepa*. The bulbil structure was similar to that of the bulb, with a single modified leaf scale forming the storage tissue, and enclosing the emerging shoot that developed through a fissure in the storage tissue. Formation of this layer was sometimes incomplete, leaving the shoot partially exposed. Bulbils were generally sessile or with a very short stem, but also arising occasionally on longer stems and secondary inflorescences with florets. The shoot may have several leaf layers within the bulbil prior to planting. Formation of the shoot within the bulbil was not visible until about October, when bulbils tend to dehisce.

Interestingly, the root primordia were asymmetrical, being present largely to one side of the point of attachment to the inflorescence. Sprouting commenced in Autumn, following initial root growth.

During the first year of development the plant produced up to 3 leaves and 2 bulblets arising from axillary buds beneath the leaves, which reached approximately up to 20cms in length by May, beginning dieback in June/July, becoming dormant in August. During subsequent years, the bulb continues to increase in girth and leaf number following the same pattern of

winter/spring growth and late summer dormancy. The single storage layer (present in the bulbil, and developed each year in bulbs) was probably used to support winter survival and growth. The remainder of the bulb is then discarded in spring, becoming necrotic, and the bulb (if not florally determined) then produces new storage tissue. This phenology was typical of poor competitors, utilising resources such as light and nutrition at times of the year when most competitors are dormant or slow growing. Up to eight leaves are likely to be produced and four bulblets in the second year, but these are subject to loss during sampling, and a more accurate figure could well be higher. Leaves die back from spring onwards, and may be lost due to pests, diseases or mechanical damage. Maximum leaf length is reached in May, when the leaves begin to dieback more noticeably.

A very small number of bulbs reached critical mass ($<1\%$), under these growing conditions, and produced an inflorescence during the second season. The majority developed an inflorescence during the third season. In floral plants, no storage tissue was produced in the parent bulb, rather two daughter bulbs develop from axillary buds and these develop storage tissue forming daughter bulbs that sprout the following season.

Bulblet production apparently slows around the time of inflorescence development, most bulblets being external, with none visible in the axils of inner leaves. It was likely that resources were channelled into inflorescence or daughter bulb production at this time. Within the storage tissue, leaf primordia were formed for the following season's growth, and up to 5 primordia were observed.

None of the bulblets produced sprouted during the time of this work, except in a small number of cases, when the plant was floral. This may relate to suppression of apical dominance mechanisms at this time, avoiding competition from close plants, but allowing for temporal distribution.

Floral plants develop 11 leaves, first daughter bulb, one more leaf, second daughter bulb and then the scape. Plants that are not florally determined develop a new storage layer, visible in March/April, and this can be used as an indicator of the vegetative state. Daughter bulb formation can also be used as evidence of early floral determination. Other plants growing in nearby beds as ornamentals in clay soil (likely to have a high nutrient content) were observed to all flower after 14 leaves (data not presented). This is consistent with Brewer's suggestion (1994) that the leaf number reflecting critical mass may increase in situations of high nitrogen availability.

The bulbils appeared to develop at the base of the cymes, enfolded by a bracteole. This would support the hypothesis that they reflect some kind of floral reversion, towards the end of inflorescence development, but conclusions must await further investigations.

8.5 The timing of floral determination

Only small amounts of data were obtained for this, so conclusions must be tentative.

However, it seems likely that determination occurs in late winter/spring following

vernalization. This could be established more precisely if a large population of known size was established, and cultured *in vitro* in sufficient numbers, say from December to March. It was apparent that floral development proceeds at different rates in different plants in the same plot, so determination timing may be similarly variable.

8.6 Location and cloning of a *LFY* homologue and examination of its expression in floral and vegetative tissues

The use of primers based on highly conserved sequences in the rice *LFY* homologue, *RFL* (Kyoizuka, 1998) yielded two PCR fragments as possible *LFY* homologues. The presence of two products might indicate that *Allium* has two divergent *LFY* genes as has been found in some other species. Homologues to *LFY* have been identified in a range of higher plants including: *Petunia* (Souer *et al.*, 1998), *Cucumis sativa* (Liu *et al.*, 1998), *Oryza sativa* (Kyoizuka *et al.*, 1998), *Populus balsamifera* (Rottmann *et al.*, 2000), *Welwitschia mirabilis* (Frohlich. and Parker, 2000), *Nicotiana tabacum* (Kelly *et al.*, 1995), *Pisum sativum* (Hofer *et al.*, 1997), *Brassica oleracea* (Anthony *et al.*, 1993) and, *Lycopersicon esculentum* (Molinero-Rosales *et al.*, 1999). Species as diverse as *Pinus radiata* (Mellerowicz *et al.*, 1998; Mouradov *et al.*, 1998;). *Eucalyptus globulus* (Southerton *et al.*, 1998) and *Malus* (Wada *et al.*, 2002) have been shown to have two *LFY* homologues, (see also section 1.8.3, Table 7), with Albert, *et al.*, (2002) suggesting that in most gymnosperms investigated to date, two divergent *LFY* paralogs are found, with sex-specific expression in conifers and that the predominantly male-expressed gymnosperm copy identifies with the single *LFY* gene in flowering plants. Frohlich and Parker (2000) theorize that the evolutionary lineage leading to

flowering plants originally had two copies of this gene, but that one copy was lost, developmental control of flower organization deriving more from systems active in the male reproductive structures of the gymnosperm ancestor rather than from the female, with ovules being ectopic in the original flower. It would be interesting to pursue these evolutionary relationships in var. *babingtonii* as further data become available. However as only one of the products was analysed further it is also possible that the larger fragment was a non-specific PCR product. When the smaller fragment was sequenced, it yielded six versions of the fragment, with varying degrees of homology to each other. Three of these are suspected to be at least in part due to PCR error. However, it seems likely that the others represent genuine variants. As discussed above, these could represent different *LFY* genes. Alternatively as *A. ampeloprasum* var. *babingtonii* is hexaploid, they could represent alleles of the same gene. Comparison of the *Allium LFY* to the databases revealed high level of homology to other *LFY* genes from other species and it is proposed that *ABLFY* is also a *LFY* homologue. Although the partial cDNA was most closely homologous to the *Petunia LFY* gene (Souer *et al.*, 1998), this small fragment represents only part of one highly conserved region, therefore, conclusions regarding genuine phylogenetic relationships must await extension of the sequence. This needs to be confirmed by sequencing the whole gene. To establish further whether it is indeed a functional homologue, it could be transformed into *Arabidopsis* to investigate the effects of over-expression. This type of functional analysis has been used to establish a functional homology for several of the *LFY* homologues in other species, including *FLO* from *Antirrhinum* (Carpenter and Coen, 1990; Coen *et al.*, 1990), *ELF* in *Eucalyptus* (Southerton *et al.*, 1998) and *RFL* in *Oryza sativa* (Kyoizuka, *et al.*, 1998). Interestingly, when 35S-RFL

expression was compared with 35S-*LFY Arabidopsis* plants, development of cotyledons, rosette leaves, petals and stamens was severely affected, rather than the expected conversion of inflorescence meristems to floral meristems, demonstrating that *RFL* function is distinct from that of *LFY*. Further work (Chujo *et al.*, 2003) showed that *RFL*, when introduced into *Arabidopsis lfy* mutants, partially rescued the mutation, suggesting that the functions overlap. However, again there were abnormal patterns of development such as leaf curling, bushy appearance and the transformation of ovules into carpels.

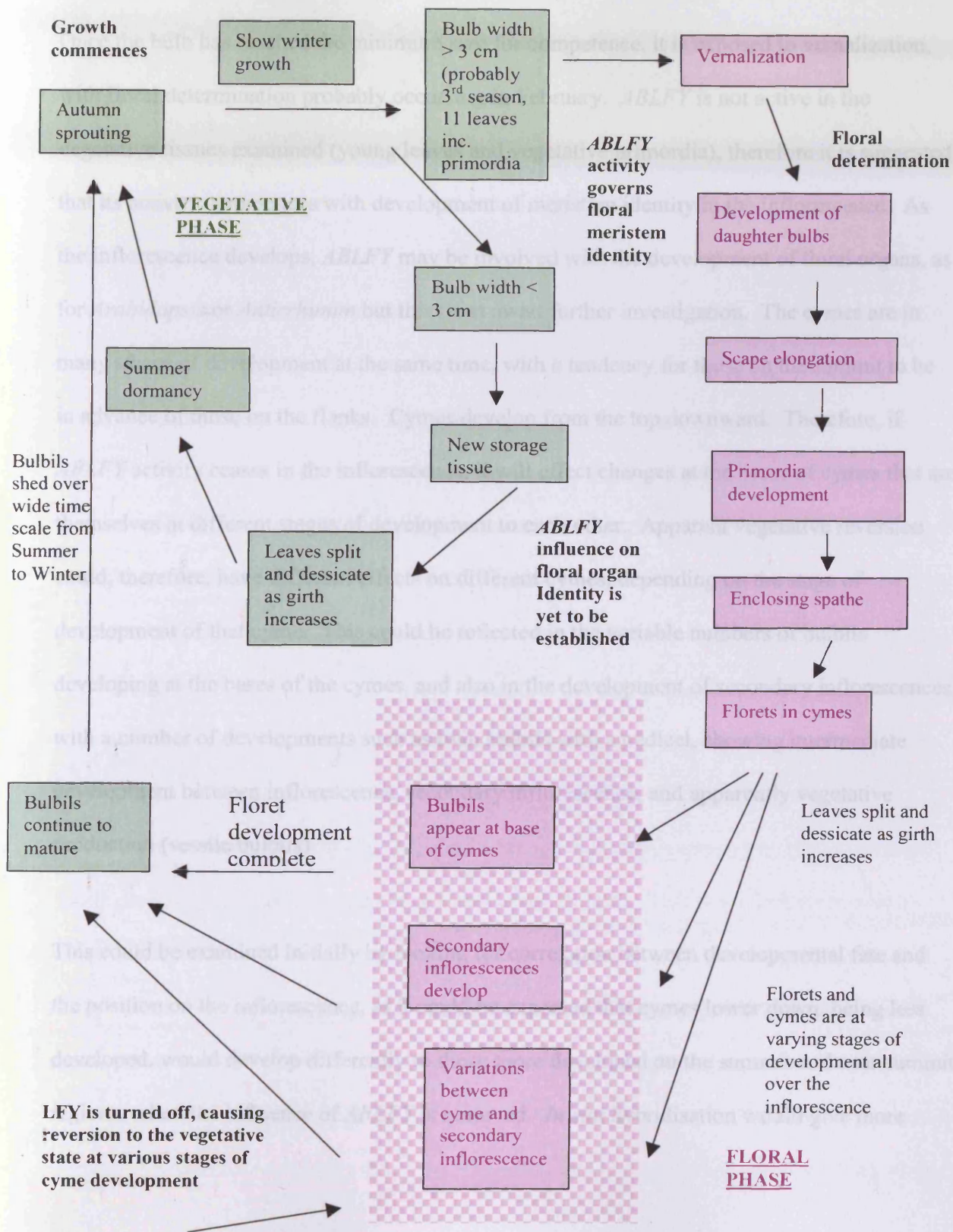
The expression of *ABLFY* was examined, proving positive in floral apices, but not in vegetative apices or in leaf material (Figure 124). It might be suggested that the lack of expression in leaf material reflects the fact that it is not expressed in response to photoperiod, which is largely detected in the leaves by the phytochrome group. However, *FLO* in *Antirrhinum* is also expressed in a similar way to *ABLFY* in var. *babingtonii*, i.e. the gene product is usually only detected in floral apices apart from occasionally in older vegetative meristems. This is in contrast to most plants examined to date where there are low levels of expression in vegetative tissues, followed by rapid increases in expression as part of the conversion to the floral meristem. In spite of this apparent similarity in pattern, *Antirrhinum* flowers in response to photoperiod rather than to maturity followed by vernalization. *FLO* is expressed in young bracts subtending the floral meristem, in the early floral meristem itself, and continues in all cell layers and meristematic regions during the early stages of floral organ initiation, apart from the stamens (Coen *et al.*, 1990; Huijser *et al.*, 1992). This is in contrast to *LFY* expression in *Arabidopsis*, where it is expressed in the floral meristem, and the floral

organ primordia, apart from carpels, as well as in leaf primordia. The *LFY* homologue *BOFH* in *Brassica oleracea* var. *botrytis* (cauliflower) also shows strong links with floral growth, with no expression detected in vegetative material, and expression throughout all stages of curd development (Anthony *et al.*, 1993, Anthony *et al.*, 1996). Interestingly, *BOFH* is switched off at high temperatures, causing a reversion to the vegetative state, reminiscent of the suggestion that floral reversion can be triggered in *Alliums* by exposure to high temperatures (e.g. Brewster, 1994). This species also flowers in response to vernalization following a period of juvenility (Atherton *et al.*, 1987; Aditya and Fordham, 1995), representing another similarity with var. *babingtonii*. Aditya and Fordham (1995) also attempted to bypass these requirements by the application of gibberellic acid, finding that although flowering was advanced by 3-5 days in one cultivar, another cultivar failed to respond at all. Since vernalization is largely detected in the apex of plants (but also may be detected in any actively dividing region of cells) (see 1.7.1.2), it might be expected that some of the responses might also be detected most easily in these tissues. *Malus* (apple) has two *LFY* homologues, of which one *ALF1*, is only expressed in floral buds, whilst the other, *ALF2* is expressed in both vegetative and floral meristems, as well as in floral organs (Wada *et al.*, 2002). Over-expression of *ALF1* and *ALF2* in transgenic *Arabidopsis* showed accelerated flowering, though the effect of *ALF1* was much weaker than *ALF2*, and it was concluded that both had a role to play in floral differentiation in *Malus* (Wada *et al.*, 2002). Interestingly, this is another species that requires vernalization in order to flower, and only flowers on wood that is at least two years old (Brickell, 1992). Should the larger fragment prove to be another *LFY* homologue, comparison of expression of the two genes, could elucidate the potentially

differing roles, as has been examined in *Malus*. *In situ* hybridization would be a useful method to further examine expression of this important gene and its possible alleles/homologues, and allow examination of its role, not only in the floral meristem, but also in floral organ development.

Thus, the work to date on var. *babingtonii* presented in this thesis allows the development of a tentative model for the regulation of flowering in this species (Fig. 123).

Figure 123: Putative function of *ABLFY* in the developmental timetable of *Allium ampeloprasum* var. *babingtonii*



Once the bulb has reached the minimum size for competence, it is exposed to vernalization, with floral determination probably occurring in February. *ABLFY* is not active in the vegetative tissues examined (young leaves and vegetative primordia), therefore it is suggested that its activity commences with development of meristem identity in the inflorescence. As the inflorescence develops, *ABLFY* may be involved with the development of floral organs, as for *Arabidopsis* or *Antirrhinum* but this must await further investigation. The cymes are in many stages of development at the same time, with a tendency for those on the summit to be in advance of those on the flanks. Cymes develop from the top downward. Therefore, if *ABLFY* activity ceases in the inflorescence, it will effect changes at the bases of cymes that are themselves at different stages of development to each other. Apparent vegetative reversion could, therefore, have different effects on different cymes, depending on the stage of development of that cyme. This could be reflected in the variable numbers of bulbils developing at the bases of the cymes, and also in the development of secondary inflorescences, with a number of developments such as twin bulbils with a pedicel, showing intermediate development between inflorescence, secondary inflorescence, and apparently vegetative production (sessile bulbils).

This could be examined initially by looking for correlation between developmental fate and the position on the inflorescence, as it could be expected that cymes lower down, being less developed, would develop differently to those more developed on the summit and near summit regions, when the influence of *ABLFY* is removed. *In situ* hybridisation would give more

detailed information that could be linked with the apparently vegetative state expressed in the production of bulbils.

8.7 Future directions

8.7.1 Stock population

Further work should be based on the development of a large population, where the size of the bulb is monitored, and so can be selected as being florally competent and given uniform vernalizing treatments. Selection for uniformity of size would also probably lead to more uniform development, making manipulation and study of growth patterns easier. The plants can be grown in John Innes No. 1 compost in pots, but will cease growth quite quickly, unless transplanted to larger containers, and preferably into Outdoor beds. They are poor competitors, requiring efficient weed suppression, prone to rotting off in badly drained conditions, but also requiring liberal supplies of water in well-drained conditions.

8.7.2 Floral competence

It was apparent that bulbs began laying down storage in spring, close to the time when floral development is initiated, requiring high levels of assimilates. This was likely to be why a critical mass of 3 cm diameter was needed before competence to flower was achieved.

Daughter bulbs over this size failed to flower the following season, apparently requiring further development before competence. It was observed that some daughter bulbs grown as ornamentals (data not presented) did flower in the following season, and these were generally very large bulbs (approximately 4 or 5 cm diameter), but no records were kept on these plants.

If the critical size of 3 cm width was reached then vernalization was necessary to induce flowering. Vernalization at 7° C for 6 weeks induced 100% flowering in bulbs (not daughter bulbs) over 3 cm, but this was the only treatment tested, and this could be further examined. Development of a simpler and preferably shorter treatment would make manipulation of this species easier.

8.7.3 The expression and role of ABLFY

This thesis has gone some way to identifying a putative *LFY* homologue and examining its role in floral development in this species. However, many conclusions are tentative and must await further work to clarify and confirm these suggestions. The larger fragment (approximately 298 kb) has yet to be investigated, and if found to be a second divergent *LFY* gene would greatly increase the complexity of *LFY* regulation of flowering. As a hexaploid, var. *babingtonii* might be expected to have up to six alleles of the *LFY* gene, depending on the origins of this sterile clonal species, and the out-crossing or otherwise of the ancestral species. The majority of *Allium* species are sexual, out-breeding, non-hybridising species (Stace, 1989), with outcrossing encouraged by protandry (Currah and Ockenden, 1978) and natural cytoplasmic sterility (Jones and Clarke, 1943). Self-pollination is estimated at only 5-25% in cultivated *Alliums* (Berninger and Buret, 1967). The small fragment produced six sequences, of which three could be alleles, the other three being less likely as they did not code for amino acids without gaps, whilst questions as to the identity of the large fragment remain unanswered. *Alliums* are known to have evolved as a result of genome duplications and

rearrangements; therefore, it is possible that some of the fragments could be the result of duplications.

While most *Alliums* are diploid, polyploidy occurs among botanical varieties of the cultivated forms of *A. ampeloprasum*, *A. schoenoprasum*, *A. chinense* and *A. tuberosum* as well as in wild species such *A. ampeloprasum* var. *babingtonii* and *A. oreoprasum*. Counts for *A. ampeloprasum* vary from $2n = 16$ to $2n = 56$ (Mathew, 1996). Whilst domestication itself would not account for changes in ploidy, it is likely that *A. ampeloprasum* spp. arose from ancestors of different ploidy levels (Fritsch & Friesen, 2002). All commercial leek cultivars are probably tetraploid, though opinions differ as to whether it is allo- or autotetraploid (De Clercq & Van Bockstaele, 2002). Burke *et al.* (2000) comment that natural hybridization is most prevalent in plant *genera* which comprise outcrossing perennials with some mechanism for clonal reproduction; this description would certainly apply to many *A. ampeloprasum* spp. As a hexaploid, *A. ampeloprasum* var. *babingtonii* could be allopolyploid, autopolyploid, or a combination of the two events. Treu (1999) was unable to identify six clear groupings of homologous chromosomes, therefore it was not possible to comment on the origins of the ploidy level. Identification and sequencing of all alleles present in this species would allow inferences to be made regarding the auto- or allo- ploid nature of this plant, and therefore inferences can be made as to its ancestry, provided the genetic variation noted by Treu (1999) is not so great as to interfere with identification of recognisable sequences for comparison. Comparison of the sequences with those of similar *A. ampeloprasum* varieties, may also indicate the parent plant or plants. Location of a *LFY* homologue in *A. ampeloprasum* var.

ampeloprasum, sequencing and data on expression, a variety that is very similar in many ways to var. *babingtonii* being most different in the sexual method of reproduction, could also provide data that might clarify the progress of the inflorescence development in var. *babingtonii*.

Klass and Friesen (2002) studied molecular markers concluding that *Allium* spp. are of ancient origin. However, *A. ameloprasum* var. *babingtonii* does not appear to have been recorded before early 19th century (1.1), suggesting that it might be of relatively recent origin. The history of *A. ameloprasum*, both as a wild species and as various domesticates has been well documented, e.g. De Clercq and Van Bockstaele, (2002) (1.2). Schön & Martens, (2000), suggest that ancient asexuals should either have genetically silenced transposable elements or be free of them. Comparison of the neutral sequence divergence between alleles of *A. ameloprasum* var. *babingtonii* and similar close relatives such as *A. ameloprasum* var. *ampeloprasum*, and analysis of transposon content, whilst beyond the scope of this work, could provide information about the history of var. *babingtonii* and the length of time since it diverged from the ancestral lines.

In situ hybridisation could be used to confirm and clarify the role of *ABLFY* in flowering and the development of the floral organs in this species. Real time PCR would allow the levels of expression of *ABLFY* to be quantified. Both these techniques may also elucidate its role in the production of bulbils. It would be interesting, and potentially useful in understanding the production of bulbils in this species, to attempt to manipulate bulbil production by variation in

environmental parameters such as temperature, and examine *ABLFY* expression in these manipulated inflorescences.

Further work could involve the use of RACE (random amplification of cDNA ends) to extend the fragment, and therefore sequence the whole gene for more comprehensive comparisons. The genetic variation noted by Treu (1999) might lead to location of *ABLFY* mutants, providing a further tool for investigation into the function of this important gene.

Transposons may significantly affect genome structure as their copy numbers increase through self-replication and provide a source of mutations, by their insertion in or near genes, (Kumar and Bennetzen, 1999; Charlesworth & Wright, 2001). Furthermore, Soltis and Soltis (1999) suggest that the process of polyploidization can facilitate the production of new gene complexes, leading to rapid evolution, these changes being mediated by the presence of transposons. These are considered to be junk DNA or genomic parasites, needing meiotic recombination for invasion and multiplication in the host (Schön & Martens, 2000). In their review (1999), Kumar and Bennetzen suggest that as retrotransposons play a major role in determining the size of plant genomes, those plants with large genomes might be the result of successful colonization and amplification of retrotransposons. The adaptation to a wide range of habitats could therefore be explained in terms of the recent evolution of var. *babingtonii*, with the process of polyploidization mediating the production of new gene complexes facilitated by large numbers of active transposons that also contribute to the genome size. Certainly, Treu (1999) noted that there was variation in the karyotypes, especially between

nucleolar organising regions (NOR's) in both number and morphology, many of which did not conform to the types normally found within section *Allium*. He further contrasts this apparent instability with the remarkable degree of NOR uniformity and stability with other studies within section *Allium*, but notes that in this respect var. *babingtonii* is consistent with other species known to be sterile. Investigations into the genetic variation thus outlined, may lead to insights into the genome size and identification of transposon activity all of which may contribute to the individual nature of this species.

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Appendix 1

Nurseries supplying *Allium ampeloprasum* var. *babingtonii*

Edulis

Contact: Paul Bamey
Address: 1 Flowers Piece
Ashampstead
RG8 8SG
United Kingdom
Telephone No: (01635) 578113
Fax: (01635) 578113
Email: edulis.2000@virgin.net
www.edulis.co.uk
Website:

Lodge Lane Nursery & Gardens

Contact: Rod or Diane Casey
Address: Lodge Lane, Dutton
Nr Warrington
WA4 4HP
United Kingdom
Telephone No: (01928) 713718
Fax: (01928) 713718
Email: rod@lodge-lane-nursery.co.uk
Website: www.lodge-lane-nursery.co.uk

Lisdoonan Herbs

Contact: Barbara Pilcher
Address: 98 Belfast Road
Saintfield
BT24 7HF
United Kingdom
Telephone No: (028) 9081 3624
b.pilcher@pop.dial.pipex.com
Email:

Poyntzfield Herb Nursery

Contact: Duncan Ross
Address: Nr Balblair, Black Isle, Dingwall
Ross & Cromarty
IV7 8LX
United Kingdom
Telephone No: (01381) 610352*
Fax: (01381) 610352
Email: info@poyntzfieldherbs.co.uk
Website: www.poyntzfieldherbs.co.uk

Arne Herbs

Contact: A Lyman-Dixon & Jenny Thomas
Address: Limeburn Nurseries, Limeburn Hill, Chew Magna
Bristol
BS40 8QW
United Kingdom
Telephone No: (01275) 333399
Fax: (01275) 333399
Email: lyman@lyman-dixon.freemove.co.uk
Website: www.arneherbs.co.uk

Natural Selection

Contact: Martin Cragg-Barber
Address: 1 Station Cottages, Hullavington
Chippenham
SN14 6ET
United Kingdom
Telephone No: (01666) 837369
Email: martin@worldmutation.demon.co.uk
Website: www.worldmutation.demon.co.uk

The Herb Garden & Historical Plant Nursery

Contact: Corinne & David Tremaine-Stevenson
Address: Pentre Berw, Gaerwen
Anglesey
LL60 6LF
United Kingdom
Telephone No: (01248) 422208 ymobile 07751 583958

Email: The_Herb-Garden@hotmail.com
Website: www.HistoricalPlants.co.uk

John Shipton (Bulbs)

Contact: John Shipton & Alison Foot

Address: Y Felin, Henllan Amgoed

Whitland

SA34 0SL

United Kingdom

Telephone No: (01994) 240125

Fax: (01994) 241180

Email: bluebell@zoo.co.uk

Website: www.bluebellbulbs.co.uk

(The Royal Horticultural Society, 2003)

Appendix 2

Cultivated (edible) *Allium* species and their areas of cultivation (Fritsch & Friesen, 2002).

Botanical names of the crop groups	Other names used in the literature	Area of cultivation	English names
<i>A. altaicum</i> Pall.	<i>A. microbulbum</i> Prokh.	South Siberia	Altai onion
<i>A. ampeloprasum</i> L.			
Leek group	<i>A. porrum</i> L. <i>A. ampeloprasum</i> L. var. <i>porrum</i> (L.) J. Gay	Mainly Europe, North America	Leek
Kurrat group	<i>A. kurrat</i> Schweinf. Ex Krause	Egypt and adjacent areas	Kurrat, salad leek
Great-headed-garlic group	<i>A. ampeloprasum</i> L. var. <i>holmense</i> (Mill.) Aschers. Et Graebn	Eastern Mediterranean, California	Great-headed garlic
Pearl-onion group	<i>A. ampeloprasum</i> var. <i>sectivum</i> Lued.	Atlantic and temperate Europe	Pearl onion
Tarée group		Iran	Tarée irani
<i>A. canadense</i> L.		Cuba	Canada onion
<i>A. cepa</i> L.			
Common onion group	<i>A. cepa</i> ssp. <i>cepa</i> / var. <i>cepa</i> , <i>A. cepa</i> ssp. <i>australe</i> Kazakova	Worldwide	Onion. Common Onion
Ever ready onions	<i>A. cepa</i> var. <i>perutile</i> Stearn	Great Britain	Ever-ready onion
<i>Aggregatum</i> group	<i>A. ascalonicum</i> auct. hort., <i>A. cepa</i> var. <i>aggregatum</i> G. Don, var. <i>ascalonicum</i> Backer, ssp. <i>orientalis</i> Kazakova	Nearly worldwide	Shallot, potato onion, multiplier onion
<i>A. consanguineum</i> Kunth		North East India	

<i>A. x cornutum</i> Clem. Ex vis.	<i>A. cepa</i> var. <i>viviparum</i> auct.	Locally in South Asia, Europe, Canada, Antilles	
<i>A. chinense</i> G. Don	<i>A. bakeri</i> Rgl.	China, Korea, Japan, South-East Asia	Rakkyo, Japanese scallions
<i>A. fistulosum</i> L.		East Asia, temperate Europe and America	Japanese Bunching onion, Welsh onion
<i>A. hookeri</i> Thw.		Bhutan, Yunnan, North-West Thailand	
<i>A. kunthii</i> G. Don	<i>A. longifolium</i> (Kunth) Humb.	Mexico	
<i>A. macrostemon</i> Bunge	<i>A. uratense</i> Franch., <i>A. grayi</i> Regel	China, Korea, Japan	Chinese garlic, Japanese garlic
<i>A. neapolitanum</i> Cyr.	<i>A. cowanii</i> Lindl.	Central Mexico	Naples garlic
<i>A. nutans</i> L.		West and South Siberia, Russia, Ukraine	
<i>A. obliquum</i>		West Siberia, East Europe	Oblique onion
<i>A. oschaninii</i> O. Fedtsch.		France, Italy	French shallot
<i>A. x proliferum</i> (Moench) Schrader			
East Asian group	<i>A. aobanum</i> Araki, <i>A. wakegi</i> Araki	China, Japan, South-East Asia	Wakegi onion
Eurasian group	<i>A. cepa</i> var. <i>viviparum</i> (Metzg.) Alef. <i>A. cepa</i> var. <i>proliferum</i> (Moench) Alef.	North America, Europe, North-East Asia	Top onion, tree onion, Egyptian onion, Catawissa onion
<i>A. pskemense</i> B. Fedtsch.		Uzbekistan, Kyrgystan, Kazakhstan	
<i>A. ramosum</i> L.	<i>A. odorum</i> L., <i>A. tuberosum</i> Rottle. Ex Sprengel	China and Japan, world-wide now	Chinese chive, Chinese leek

<i>A. rotundum</i>	<i>A. scorodoprasum</i> ssp. <i>rotundum</i> (L.) Stearn	Turkey	
<i>A. sativum</i> L. Common garlic group	<i>A. sativum</i> . var. <i>sativum</i> , <i>A. sativum</i> var. <i>typicum</i> Rgl.	Mediterranean area, also world-wide	Garlic
<i>Longicuspis</i> group	<i>A. longicuspis</i> Regel	Central to South and East Asia	
<i>Ophioscorodon</i> group	<i>A. sativum</i> var. <i>ophioscorodon</i> (Link) Döll	Europe, also world-wide	
<i>A. schoenoprasum</i> L.	<i>A. sibiricum</i> L.	World-wide in temperate areas	Chive
<i>A. ursinum</i> L.		Central and North Europe	Ransoms
<i>A. victorialis</i> L.	<i>A. microdictyon</i> Prokh., <i>A. ochotense</i> Prokh.	Caucasus, Japan, Korea, Europe	Long-rooted onion, long-rooted garlic
<i>A. wallichii</i> Kunth	<i>A. platyphyllum</i> Diels, <i>A. lancifolium</i> Stearn	East Tibet	

Appendix 3

The location and habitat characteristics for the populations of *Allium ampeloprasum* var. *babingtonii* sampled by Treu (1999).

Population number	Location	Habitat and estimated population numbers
1	SW 784567-771584 Perranporth – Mount Cubert Road. Footpath from Mount to Holywell	Mainly lightly wooded, often damp, streamsides, also field margins and grassy verges. Sandy soil especially further north. Est. 5000 +
4	SW 784486. B3284 Road, 2 km NW of Shortlandsend from A30	Grassy roadside verge. No soil record. Est. 50 +
5	SW 72249 on B3277, 1 km SE of St. Agnes	Grassy roadside verge. No soil record. Est. 40 +
6	SW 836580 Trewerry Mill	Grassy with bramble roadside/streambank. Light stony soil. Est. 100 +
11	SW 936796 New Polzeath	Dense bramble undergrowth. Light stony soil. Est. 200 +
14	SW 877736 opposite village hall on B3276, St. Merryn	Grassy roadside and damp ditch. High organic matter soil. Est. 60
16	SW 644240 Loe Bar	Limited grassy vegetation on beach c. 80m from shore. Soil almost completely sand. Est. 70 +
17	SW 658210 – 656216 Church Cove	Grassy/scrubby roadside. Light stony soil. Est. 200 +
20	SX 408514 Tregonhawke Nr. Millbrook	Scrubby vegetation including Gorse. Very peaty soil. Est. 150 +
21	SW 731249 entrance to Tremyne Woods	Mature woodland margin. Rich stony soil. Est. 100 +
24	SX 144966 Crackington Haven	Grassy roadsides/streamsides. One area (estimated 20m ² , is exclusively this species) Light stony soil. Est. 1000 +
25	SW 440253 Trewoofe	Open woodland/roadside. Light stony soil. Est. 50
29	SX 152545 Trevelyan	Grassy roadside/hedge. Light stony soil. Est. 200
30	SW 756229 Tregidden	Grassy roadside/hedge. No soil or number record.
32	SW 923435 S. of Tregony nr. Hay Barton Farm	Grassy roadside/hedge. No soil record. Est. 55
33	SW 867369 turn 2 km NW of St. Just in Roselands (A3078) toward Lanhay	Grassy roadside/hedge. No soil or number record
38	SW 878358 Porthcurnick beach, Porthscathon	Sea cliff and sea wall. Clay soil. Est. 250 +
39	SW 837638 Newquay – Padstow road	Grassy roadside. Light stony soil. Est. 200 +
43	SW 987712 Treneague	Grassy roadside. No soil record. 100 +

Adapted from Treu (1999).

Appendix 4

Examples of tissue culture in *Allium* species

<i>Allium</i> species	Media	PGRs	Environmental conditions	Tissue	Reference
<i>A. carinatum</i>	BDS	None, 10 μ M Kinetin, 10mM IAA 5 μ M 2,4-D, 40 μ M BAP, 5 μ M NAA	25°C dark	Bulbils, roots, shoots,	Havel and Novak, 1988
<i>A. cepa</i>	B5; 0.06% CaCl ₂ .H ₂ O; 0.8% glutamine; 8% sucrose BDS	None 0.1mg/l 2,4-D & NAA, 1mg/l 2,4-D & 0.1mg/l NAA, 0.1mg/l 2,4-D & NAA & 1mg/l BAP, 1mg/l NAA & BAP, 1mg/l NAA & 0.1mg/l BAP, 0.1mg/l NAA & 1mg/l BAP	25°C; 16h photoperiod	Ovules/whole flower buds	Godwin <i>et al.</i> , (undated)
<i>A. fistulosum</i> x <i>cepa</i>	B5, BDS, 0.2% Gelrite; 0.75g/l MgCl ₂	0.75 mg/l picloram, 2.0 mg/l BA, 2.5g/l proline, 0.7 mg/l 2,4-D, 2.0 mg/l BA	12h photoperiod; CD; 25°C	Inflorescence; basal plate	Lu <i>et al.</i> , 1989
<i>A. ampeloprasum</i> var. <i>porrum</i>	MS, 0.6% agar, 2% sucrose, B5	1 ppm NAA, 8ppm 2-iP, 2ppm IAA	16 h photoperiod, 25°C	Inflorescence, basal disk	Baumunke-Wende, 1989
<i>A. sativum</i>	MS	0.5 mg/l benzyladenine	8 h photoperiod	Segments of basal plants, leaves, meristem, flower buds, opened flowers, anther	Bhojwani <i>et al.</i> , 1983
<i>A. wakegi</i>	15% coconut milk, 0.7% agar;	2 mg/l 2,4-D, 2 mg/l NAA, 2 mg/l kinetin	CD, 26°C	Internal bulb tissues	Seo & Kim, 1988
<i>A. ampeloprasum</i> <i>A. sativum</i> , <i>A. schoenoprasu</i>	LS-basic, 2mmol/l NH ₄ NO ₃ , 3% sucrose,	0.011 mmol/l NAA, 0.008 mmol/l BAP	12h photoperiod, 26°C	Leaf discs, flower buds	Rauber & Grunewaldt, 1988

<i>m</i> <i>A. sativum</i>	0.8% agar MS, B5, 3% sucrose, 0.95% agar	1 mg/l NAA, 2 mg.l IBA		Stem tips	Nagasawa & Finer, 1988
<i>A. sativum</i>	BDS, 8g/l agar, 30g./ sucrose	2,4-D, KIN, IAA	CD 25°C	Leaf	Dolozel and Novak, 1984
<i>A.</i> <i>ampeloprasum</i> <i>var. porrum</i>	MS, 5% sucrose	Benzyladenine, NAA	24h photoperiod, 25°C	Shoot tip, basal plate, bulblet, inflorescence	Ziv <i>et al</i> , 1983
<i>A. sativum</i> <i>A. fistulosum</i> , <i>A. fistulosum x</i> <i>cepa</i>	MS B5	2 mg/l kinetin, 2 mg/l IAA, 1 mg/l 2,4-D 4.5 µM 2,4-D, 4.6µM kinetin	CD, 28°C	Shoot tips Seeds	Novak, 1981 Shahin & Kaneko, 1986
<i>A. cepa</i> , <i>A.</i> <i>sativum A.</i> <i>ampeloprasum</i> <i>var porrum</i>	BDS, B5	1 µM NAA, 0.1µM NAA, 5µM NAA, 1 µM BA, 5µMBA, 40µM BA, 0.1µM KIN, 10µM KIN, 50µM KIN, 10µM IAA, 5µM 2,4-D,	CD for 7 days, then 16h photoperiod, 25°C	Meristem, embryos, flower heads, basal plates, cloves, bulbils,	Novak <i>et al.</i> , 1986
<i>A. cepa</i>	MS , half strength salts, 0.7% agar, pH 5.9, 100mg.; myo-inositol, 0.5 mg/l thiamine-HCl, 1.0 mg.l pyridoxine-HCl, 5.0 mg/l nicotinic acid, 30g/l sucrose	BAP, NAA, IBA	8 h photoperiod, 16h photoperiod. 20°C	Scales, shoots produced <i>in</i> <i>vitro</i>	Hussey and Falavigna, 1980

Appendix 5

Location of *Allium ampeloprasum* var. *babingtonii* Population 1 (Treu, 1999)



Ordnance Survey Explorer™ 104 reference SW784567 – 771584

Scale bar = 1kilometre

Small numbers of plants were located along the SE part of the path, but the vast majority of plants were located at the NW end of the path, where it emerges from the Holywell Bay Holiday Park, and this is where sampling was done.

Location	Sample numbers	Total
A	1 – 10	10
B	11 – 12	2
C	13 – 15	3
D	16 – 18	3
E	19	1
F	20 – 26	7
G	27 – 36	10
H	37 – 43	7
I	44 – 48	5
J	49 - 82	34

Beginning at the most southerly end of this part of the population, each clump was labelled A, B, C., etc, and plants were sampled at every 10th inflorescence.

A1	49 bulbils	G27	36 bulbils	J60	32 bulbils
A4	48 bulbils	G30	51 bulbils	J61	48 bulbils
A5	90 bulbils	G32	41 bulbils	J62	59 bulbils
A6	53 bulbils	G34	41 bulbils	J63	44 bulbils
A8	43 bulbils	G35	41 bulbils	J64	39 bulbils
A9	25 bulbils	H40	42 bulbils	J65	36 bulbils
A10	70 bulbils	H41	52 bulbils	J67	28 bulbils
B12	66 bulbils	H43	38 bulbils	J69	36 bulbils
C15	52 bulbils	I44	47 bulbils	J70	30 bulbils
D16	38 bulbils	I46	33 bulbils	J72	65 bulbils
D17	40 bulbils	I48	25 bulbils	J73	45 bulbils
F20	46 bulbils	J47	36 bulbils	J75	45 bulbils
F21	40 bulbils	J51	62 bulbils	J77	44 bulbils
F22	59 bulbils	J53	61 bulbils	J79	48 bulbil
F23	49 bulbils	J54	66 bulbils	J82	65 bulbils
F24	31 bulbils	J56	50 bulbils		
F26	19 bulbils	J59	34 bulbils		

Appendix 6

Sampling of *A. ampeloprasum* var. *babingtonii* August 2000

Plant number	Number of bulbs
1	2
2	2
3	1
4	3
5	3
6	2
7	2
8	1
9	2
10	2
11	1
12	2
13	2
14	2
15	1
16	2
17	2
18	2
19	2

Those plants with only 1 bulb may have lost the second daughter bulb as a result of disease, damage or predation. Those apparently having three daughter bulbs may be so close to adjacent plants that deformation of bulb shape occurred, making identification of the true daughter bulbs uncertain. The plants were located by the scapes, and therefore all had flowered during the previous season. As roots were in active growth even though there was no active above-ground growth, care was taken to retain as much soil as possible during collection. Plants roots were packed in damp John Innes No. 1 compost, for transport, and then planted into outdoor beds at University College Worcester. The inflorescences remained attached to the plants, and were later removed (October 2000) and stored at 3°C.

Appendix 7

Whole plants selected from Holywell Bay Holiday Park, March 2001

Plant No.	Maximum leaf length (cms)	No of leaves visible	Maximum bulb width (cms)	Maximum bulb height (cms)
1	103	7	3.0	3.8
2	100	9	4.0	3.5
3	91	8	3.1	3.3
4	116	8	3.1	3.5
5	83	7	2.7	3.2
6	101	7	2.8	3.6
7	82	6	2.9	3.8
8	75	6	2.4	2.3
9	76	6	-	-
10	70	6	-	-
11	59	5	-	-
12	77	5	2.5	2.4
13	92	9	-	-
14	99	7	-	-
15	101	8	3.4	3.0
16	96	6	-	-
17	102	9	-	-
18	75	4	2.6	3.0
19	95	8	3.4	4.0
20	100	8	3.4	3.8
21	97	8	3.2	3.1
22	99	9	4.0	4.0
23	76	4	2.1	2.7
24	77	7	2.8	3.0
25	89	6	2.6	3.6
26	77	6	2.2	3.5
27	99	9	4.1	4.2
28	94	7	3.1	4.4
29	92	6	2.4	2.5
30	117	8	3.7	3.9
31	91	4	2.2	2.0
32	96	5	2.6	3.1
33	74	5	1.8	2.8
34	93	8	-	-
35	85	6	-	-

36	112	9	3.5	3.4
37	63	5	-	-
38	77	5	2.8	3.2
39	81	7	-	-
40	77	6	1.8	3.4
41	72	5	-	-
42	71	7	2.3	1.5
43	85	8	-	-
44	85	8	-	-
45	87	6	2.6	1.6
46	103	8	3.1	4.1
47	91	6	2.2	3.5
48	90	6	1.9	3.1
49	73	5	-	-
50	76	6	2.7	3.4
51	73	10	4.4	2.6
52	99	7	-	-
53	88	8	3.2	2.6
54	95	7	3.1	3.6
55	101	7	-	-
56	93	7	2.8	3.7
57	68	2	2.4	2.0
58	91	5	-	-
59	99	7	3.6	3.9
60	91	5	-	-
61	91	6	2.6	3.2
62	83	4	-	-
63	75	4	-	-
64	76	4	-	-

- Leaf length is measured from the base of the bulb (as bulb height is variable around the plant, the upper edge being quite ragged). It is only measured to the nearest cm, as leaf tips themselves were often quite ragged at the tips, as a result of mechanical or predator damage.
- Number of leaves is recorded as those that can be observed without causing damage to the apex.
- Bulb height is at the highest point from the point where the roots emerge, to the upper edge, which may be uneven
- Bulb width is at the widest point, the bulbs being noticeably asymmetrical.
- The storage tissue of many bulbs was necrotic and/or shrivelled; therefore, measurements of bulb dimensions may not reflect other aspects of plant size such as leaf number and length.

Appendix 8

Sampling of *A. ampeloprasum* var. *babingtonii* November 2001

Whole plants

Plant No.	Bulb width (cms)	Plant No.	Bulb width (cms)	Plant No.	Bulb width (cms)	Plant No.
1	4.5	31	3.6	61	3.0	91
2	3.1	32	3.2	62	3.1	92
3	5.3	33	3.6	63	3.2	93
4	4.0	34	3.7	64	3.7	94
5	4.2	35	3.9	65	3.7	95
6	4.0	36	4.0	66	4.1	96
7	3.4	37	3.7	67	3.5	97
8	4.5	38	3.3	68	3.0	98
9	4.0	39	3.9	69	3.3	99
10	3.3	40	4.0	70	3.2	100
11	3.4	41	4.2	71	4.5	101
12	3.6	42	4.2	72	4.1	102
13	4.6	43	3.4	73	3.2	103
14	4.3	44	3.7	74	3.2	104
15	3.2	45	4.4	75	4.1	105
16	3.6	46	3.1	76	3.2	106
17	3.9	47	4.1	77	3.3	107
18	3.3	48	3.8	78	3.0	108
19	5.0	49	5.0	79	4.7	109
20	4.8	50	3.3	80	5.6	110
21	4.2	51	4.0	81	4.2	111
22	4.6	52	3.4	82	3.1	112
23	4.4	53	3.1	83	3.4	113
24	5.2	54	3.1	84	4.9	114
25	4.6	55	4.0	85	4.4	
26	4.3	56	3.6	86	3.2	
27	3.4	57	4.4	87	3.6	
28	3.1	58	4.3	88	3.5	
29	3.0	59	3.3	89	3.8	
30	4.2	60	4.4	90	3.1	

- Selection was dictated by accessibility and the need to protect the amenity landscape
- Bulb width is measured at the widest point as previously

Inflorescences

Inflorescence No.	No. of bulbils	Total weight of bulbils (g)	Mean weight of bulbil (g)	No. of florets	Bulbil/floret ratio
1i	39	14.33	0.37	105	0.37
1ii	48	16.76	0.35	149	0.32
1iii	49	15.26	0.31	176	0.28
1iv	30	10.31	0.34	92	0.33
1v	58	17.76	0.31	154	0.38
Mean	45			135	0.33
Samples 1 i-v taken from streamside					
2i	35	12.37	0.35	125	0.28
2ii	84	15.48	0.18	185	0.45
2iii	49	10.72	0.22	155	0.32
2iv	58	9.98	0.17	145	0.40
Mean	57			153	0.37
Samples 2 i-v taken from field side					
3i	47			122	0.39
3ii	37	7.33	0.20	127	0.29
3iii	45	9.92	0.22	110	0.41
3iv	44	10.18	0.23	120	0.37
3v	44	7.66	0.17	27	1.63
Mean	43			101	0.43
Samples 3 i-v taken from far end of field					
Means for total samples					
Bulbils	Florets	Bulb/floret ratio			
48	128	0.38			

Bulbil/ floret ratio for samples taken by Treu (1999)	
Population 1	0.45
Population 38	0.36
Population 39	0.41

Appendix 9

Sampling of *A. ampeloprasum* var. *babingtonii*, March, 2002

Plant Number	Maximum leaf length (cms)
64	65
7	76
24	58
47	72
65	53
59	62
14	63
44	77
1	51
41	52

- Plants were selected by accessibility and the need to preserve the amenity landscape, selecting over as wide an area as possible
- Bulbs were desiccating and necrotic, therefore no record was taken of bulb size
- Without bulb size data, assessment of plant size in relation to maturity was entirely subjective
- Outer leaves were showing signs of necrosis or other physical damage, therefore maximum leaf length was recorded in only 10 sample plants

Appendix 10

Two year old bulbs of *Allium ampeloprasum* var. *babingtonii* obtained from John Shipton Nurseries in September 2000 (JS00bab)

Bulb	Width (cms)	Height (cms)	Weight (g)
A	3.9	2.4	19.27
B	3.6	2.9	16.08
C	3.0	2.2	14.13
D	3.6	2.6	14.09
E	3.0	2.2	12.78
F	3.0	2.2	13.46
G	2.5	2.0	10.47
H	2.7	2.1	9.72
I	2.4	2.0	7.08
J	2.2	1.8	6.75
K	2.2	2.2	4.26
L	2.4	2.0	5.53
M	2.2	1.8	5.95
N	2.2	1.8	4.93
O	2.2	1.7	4.58
P	1.8	1.6	3.85
Q	1.8	1.7	3.35
R	2.0	1.8	4.61
S	2.1	1.6	4.73

Mean bulb width: 2.57 ± 0.51 cms

Mean bulb height: 2.03 ± 0.27 cms

Mean bulb weight: 8.72 ± 4.24 g

Two year old bulbs of *Allium ampeloprasum* var. *ampeloprasum* obtained from John Shipton Nurseries in September 2000 (JS00amp)

Bulb	Width (cms)	Height (cms)	Weight (g)
A	4.0	3.2	29.7
B	3.7	3.2	19.84
C	3.5	2.7	18.14
D	3.8	3.0	18.09
E	3.6	2.7	16.47
F	3.2	3.0	16.22
G	3.5	2.8	14.68
H	3.2	2.7	14.42
I	3.4	2.9	13.54
J	3.0	2.5	11.87
K	2.3	2.2	5.94
L	3.0	2.6	11.01
M	2.7	2.5	9.93
N	2.6	2.6	9.05
O	2.7	2.5	9.11
P	2.6	2.8	9.07
Q	2.6	2.5	7.98
R	3.0	2.0	7.74
S	2.5	2.0	6.85
T	3.5	2.8	14.97

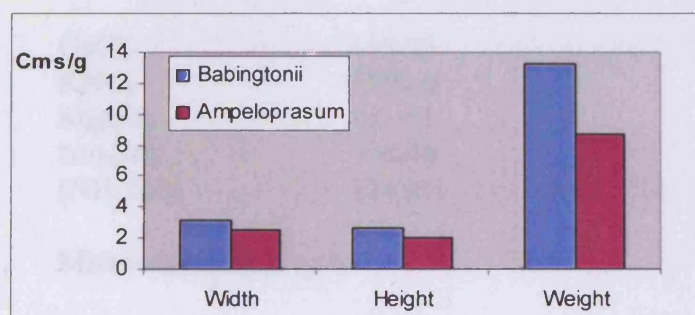
Mean bulb width: 3.12 ± 0.42 cms

Mean bulb height: 2.66 ± 0.25 cms

Mean bulb weight: 13.23 ± 4.38 g

Note: some of the bulbs clearly had flat sides, indicating recent division into daughter bulbs. Whilst both species were sold as 2 year old plants, this could not be independently verified.

It was noted that bulbs of *A. ampeloprasum* var. *babingtonii* were slightly smaller than bulbs of *A. ampeloprasum* var. *ampeloprasum*.



Comparison of *A. ampeloprasum* var. *babingtonii* and *A. ampeloprasum* var. *ampeloprasum*.

Appendix 11

Laboratory recipes

Gamborg's B5 Basal Salts;

Macro elements (mg/l):

CaCl ₂	113.23
KNO ₃	2500.0
MgSO ₄	121.56
NaH ₂ PO ₄	130.44
(NH ₄)SO ₄	134.00

Micro elements (mg/l):

CoCl ₂ .6H ₂ O	0.025
CuSO ₄ .5H ₂ O	0.025
H ₃ BO ₃	3.00
KI	0.75
MnSO ₄ .H ₂ O	10.00
Na ₂ MoO ₄ .2H ₂ O	0.25
ZnSO ₄ .7H ₂ O	2.00

FeNaEDTA	36.70
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Gamborg's vitamin Solution (1000x)

Myo-inositol	100.0 mg/ml
Nicotinic acid	1.0 mg/ml
Pyridoxine hydrochloride	1.0 mg/ml
Thiamine hydrochloride	10.0 mg/ml

Feulgen Stain

4g of Basic fuchsin (pararosaniline) was dissolved in 800ml of boiling distilled water. This solution was allowed to cool to 50°C and then filtered. 120ml of 1M HCl and 12g of potassium metabisulphite were added to the filtrate and left overnight in the dark. 2g of de-colourising charcoal was added, and the mixture filtered. The stain was stored in the dark at 4°C

Sulphur dioxide water

50ml of 1M HCl was added to 1l of distilled water. 5g of potassium metabisulphite was dissolved in this, the solution being made up fresh each time.

Subbing solution

A 1:1 mixture of 1% potassium dichromate and 1% gelatine (w/v) was made up. The solution was used without dilution for pre-subbing slides. 4 ml were added per litre to the water bath subbing solution.

Light green stain

A 0.2% w/v solution was made of Light green stain, in 98% ethanol.

Clarke's Fixative

3:1 (v/v) mixture of absolute ethanol and glacial ethanoic acid.

LB Broth (Luria-Bertani medium, Sambrook, J., Fritsch, E. F., Maniatis, T. 10989)

Add

- 950ml deionised water
- 10g bacto-tryptone
- 5g bacto-yeast extract
- 10g NaCl

Shake till dissolved. Adjust pH to 7.0 with 5N NaOH, adjust volume to 1 litre. Sterilise for 20minutes at 15lb/sq. in (1.034 bar).

M13 Forward and Reverse Primers (Genosys)

F: GTAAAACGAGGGCCAGT

R: GGAAACAGCTATGACCATG

SOC

Add

- 950ml deionised water
- 20g bacto-tryptone
- 5g bacto-yeast extract
- 0.5g NaCl

Shake till dissolved

Add 10ml 250mM KCl
Adjust pH to 7.0 with 5N NaOH
Adjust volume to 1 litre
Autoclave to sterilise, then cool to 60°C
Add 20ml sterile 1M solution glucose

Appendix 12

Tissue culture of *Allium ampeloprasum* var. *babingtonii*

1. Contamination in culture
2. Sterilising times and concentrations of hypochlorite
3. Techniques to improve surface sterilization
4. Chloramine B and sodium dichloroisocyanurate as surface sterilization agents
5. Minimal sterilisation
6. Media and fluence level
7. Sucrose concentrations
8. Carbon source and photoperiod
9. Nutritional restriction
10. Photoperiod and dormancy
11. Light effects on sprouting numbers of bulbils
12. Effect of storage period on dormancy
13. Culturing effects on dormancy
14. Photoperiod and dormancy

1. Contamination in culture

The bulbils (SH99, Chapter 2.0) were harvested during warm, humid weather, with light rain and no vegetative growth; therefore contamination was likely to be high (Lu *et al.*, 1989). Preliminary investigations with both whole bulbils and explants from bulbils in culture, showed a high degree of contamination. This was largely fungal with *Penicillium* and *Fusarium* predominant, and with some yeasts, frequently *Rhodotorula*, a widespread plant-borne yeast (James, *pers. com.* 1999). Both *Fusarium* and *Penicillium* are among the most common soil moulds, along with *Aspergillus* and *Mucor* (Brady, 1990).

Vigorous sterilisation techniques have been described in a number of reports on tissue culture of *Alliums*, e.g. Bhojwani *et al.*, 1982/3; Novák *et al.*, 1986; Novák, 1990;

Rodrigues *et al.*, 1997, though few record contamination rates nor the nature of the contamination. Lu *et al.*, (1989) noted that contamination was associated with field conditions, season and plant development, with less contamination occurring when harvested dry and least when vegetative growth was vigorous.

2. Sterilising times and concentrations of hypochlorite (as household bleach)

- Detergent ('Fairy®') was added to the hypochlorite as a wetting agent, (three drops per litre).
- After surface sterilisation, the plant material was placed into aseptic conditions in a laminar flow cabinet.
- Both explants and bulbils were then dipped in 70 % ethanol for 1 minute, and rinsed three times in SDW (1 minute each rinse).
- Culture vessels were 60ml powder jars with screw top lids, containing 20ml media, autoclaved at 121°C for 12 min. at 1.5 bar.
- The medium comprised Gamborg's B5 salts (Sigma, G5768) (Appendix 11), with 0.8% Agar Technical No. 3 (Oxoid L13), and 4 % sucrose w/v (Sigma S9031). Sucrose was used, as it usually gives optimum growth rates (Dodds and Roberts, 1995).

The temperatures at which *Alliums* have been cultured do not vary widely. High temperatures may cause abortion of the inflorescence (Brewster, 1994; Smith, 2000), whilst lower temperatures may encourage bulbing (Dragland, 1972; Brewster, 1994). Optimum temperatures for vegetative growth in *Allium ampeloprasum* var. *porrum* are between 20°C and 25°C (Rubatzky and Yamaguchi, 1997).

- Temperature was 25°C (Gallenkamp cooled incubator)
- Fluence was $19.5 \mu\text{M m}^{-2} \text{s}^{-1}$, (LI-COR LI 118B light meter), continuous light (CL).
- For comparison with earlier work, light levels were also measured in lux (TES-1334 Digital Illuminance Meter) mean 750 lux (3 readings).
- Culture vessels were rotated daily at random within the cabinet, to reduce any effects of environmental gradients within the cabinet.

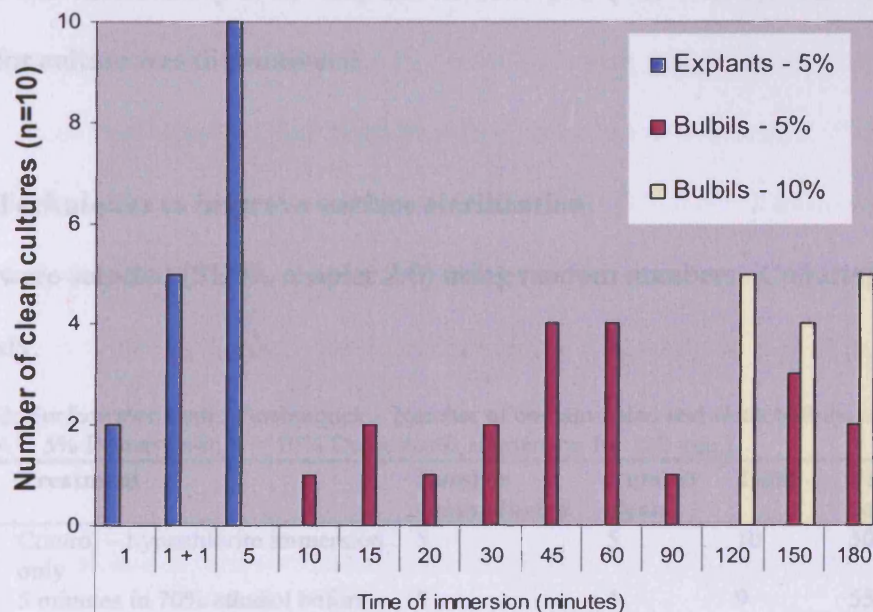


Figure 12-1: Percentage of uncontaminated cultures following surface sterilising treatments with immersion times of 1 – 180 min and 5-10% bleach, assessed after 21 days (n = 10 for each treatment).

There was a trend towards improved surface sterilisation with increasing times of immersion in the sterilising fluid, and at the higher concentration of 10%, but these also incurred 10% higher losses through necrosis offsetting any gain in viable cultures (Table 12-1).

Table 12-1: Summary of cultures of bulbils and explants developing roots or shoots

	Bulbils			Explants		
	Minimum	Maximum	Mean	Minimum	Maximum	Mean
Percentage contaminations	50	100	80	0	80	56
Percentage developing shoots (clean cultures)	0	0	0	40	50	47
Percentage developing roots (clean cultures)	0	100	57	0	20	9

None of the bulbils cultured developed shoots though some developed roots, whilst explants were more likely to develop shoots than roots (Table 12-2). Use of whole bulbils for culture was discontinued.

3. Techniques to improve surface sterilization

Bulbils were selected (SH99, chapter 2.0) using random numbers. Culturing was as previously.

Table 12-2: Surface sterilisation techniques - Number of contaminated and clean bulbils, assessed after 21 days (A = 5% Domestos®; B = 10% Domestos®, immersion for 120 min.).

Experiment	Treatment	Number contaminated	Number clean	Total	Percentage contaminated
A1	Control – hypochlorite immersion only	5	5	10	50
A2	5 minutes in 70% ethanol before sterilisation	5	4	9	55
A3	10 minutes in 70% ethanol before sterilisation	5	5	10	50
A4	Magnetic stirrer used during sterilisation	3	7	10	30
A5	Wash under running tap water for 5 minutes before sterilisation	7	3	10	70
B1	Control – hypochlorite immersion only	7	2	9	77
B2	5 minutes in 70% ethanol before sterilisation	5	4	9	55
B3	10 minutes in 70% ethanol before sterilisation	5	4	9	55
B4	Magnetic stirrer used during sterilisation	6	3	9	66
B5	Wash under running tap water for 5 minutes before sterilisation	5	5	10	50
Totals		53	42	95	

Significance was tested using χ^2 , a non-parametric test comparing the proportions of explants contaminated. The null hypothesis (H_0) stated that there was no difference in the proportion of contaminated and clean cultures in each treatment. At a probability of $p = 0.05$, d.f. = 9, $\chi^2_{\text{crit}} = 16.9$. The calculated value of $\chi^2 = 8.38$. Therefore, the null hypothesis was accepted. The use of 70% ethanol for 5 or 10 minutes, mechanical agitation, or pre-washing, did not improve surface sterilisation. Adding together figures for A and B for each treatment to give larger sample sizes: $\chi^2 = 1.994$. The critical value = 9.95 for 4 d.f., therefore there was no difference in proportions of contaminated and clean samples between the treatments. Similarly, adding together all treatments for 5% and 10% bleach, $\chi^2 = 0.90$. The critical value is 3.84 (1 d.f.), therefore there was no difference in the proportions of contaminated and clean samples between the concentrations of bleach. Use of these methods was discontinued.

4. Chloramine B and sodium dichloroisocyanurate as surface sterilization agents

- One g sodium dichloroisocyanurate was dissolved in SDW to give a final volume of 58 ml. Two ml of Igepal solution (1:49 Igepal:SDW v/v) were added, giving 1.7 % w/v sodium dichloroisocyanurate.
- Chloramine B was dissolved in SDW to give 5 % (w/v).
- Fifty bulbils were selected at random. Twenty bulbils were incubated in sterile distilled water, in a warm water bath at 40° C for 1 h (Puddephat, pers. com. 2000). The remainder were sterilised without this incubation step.

- Ten un-incubated bulbils selected at random were added to the sodium dichloroisocyanurate solution for 5 min, and then rinsed in SDW.
- Ten incubated bulbils were treated similarly (Table 12-3).
- Ten un-incubated bulbils selected at random were added to Chloramine B for 20 min then rinsed in SDW in a Schott bottle, which was then sealed, cleaned with 70% ethanol, and transferred to the laminar flow cabinet. Ten incubated bulbils selected at random were added to Chloramine B similarly.
- Ten un-incubated bulbils were rinsed only in SDW, before being placed in culture.
- The bulbils were rinsed twice more in SDW, and cultured on 20 ml sterile medium comprising Gamborg's B5 salts, with 0.8% (w/v) Agar Technical No. 3, and 4% (w/v) sucrose. The bulbils were cultured as above.

Table 12-3: Bulbils surface sterilised with sodium dichloroisocyanurate and Chloramine B, assessed after 21 days (n = 10 for each treatment)

Treatment	Percentage contaminated	Percentage clean
Sodium dichloroisocyanurate 1.7% w/v	80	20
Chloramine 5% w/v	90	10
Incubation (60mins.) + Sodium dichloroisocyanurate w/v	-	-
Incubation (60 mins.) +Chloramine 5% w/v	50	50
Control	-	-

These data were tested for significance using χ^2 as above. H_0 states that there is no difference in the proportion of contaminated and clean explants, using these different sterilants. At 2 d.f, $p = 0.5$, the critical value of $\chi^2 = 5.99$. The observed value of $\chi^2 = 4.553$. Therefore, the difference in these results was not significant, and the H_0 is accepted. Although there was no control data for comparison, the numbers of clean explants following these treatments was so low that use of these sterilants was discontinued.

5. Minimal sterilisation

- Bulbils were selected at random and prepared by removal of all surface tissue
- They were cultured as above

Table 12-4: Explants with all surface tissue removed and given minimal surface sterilisation by rinse in SDW or immersion in hypochlorite, assessed after 34 days (n = 10 for each treatment)

Treatment	Percentage clean	Percentage contaminated	Percentage sprouting	Mean shoot length
A. Rinse in sterile distilled water only	90	10	90	13.0mm
B. 1 minute in sterilant, then 3 rinses in sterile distilled water	80	20	50	11.8mm
C. 3 minutes in sterilant, then 3 rinses in sterile distilled water	90	10	10	14.0mm
D. 5 minutes in sterilant, then 3 rinses in sterile distilled water	100	0	60	4.2 mm

Testing for significance in the proportions of clean explants compared with contaminated explants, using χ^2 gave an observed value of $\chi^2 = 2.222$. The critical value was 7.81, $p = 0.05$, 3 d.f.; therefore there was not a significant difference in the number of clean explants using these sterilisation methods (Table 12-4).

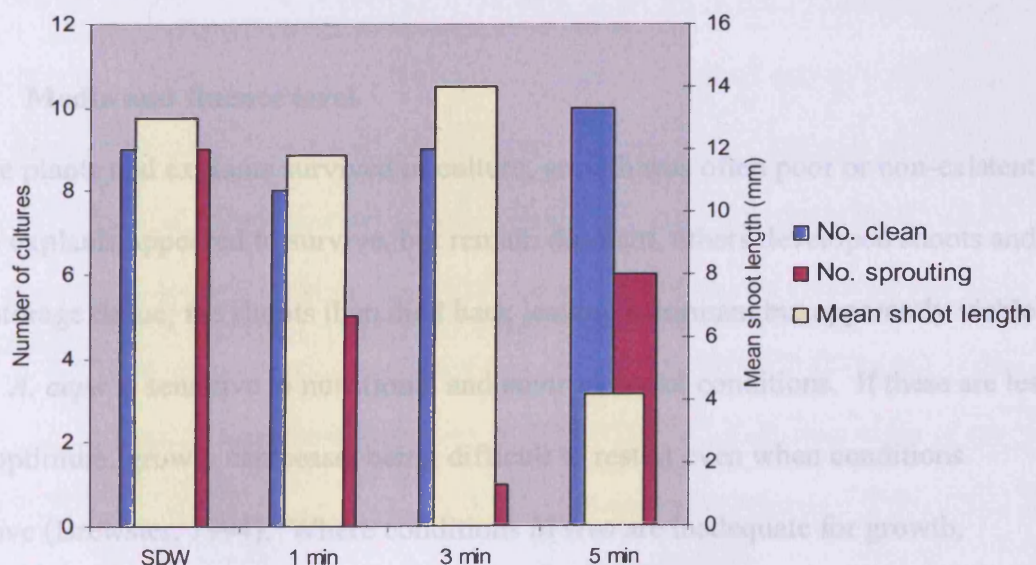


Figure 12-2: Comparison between numbers of clean and sprouting explants, following different immersion times in hypochlorite

Testing for significance in the number of explants sprouting compared with the number of explants not sprouting, using χ^2 , gave an observed value of 13.132 compared with a critical value of 11.34 for 3 d.f. at $p = 0.01$. There was a very significant difference in the number of explants sprouting, using these different sterilising methods. The highest proportion of sprouting explants (90%) was in the treatment involving no hypochlorite immersion, just a rinse in SDW (Figure 12-2).

Analysis of the length of the shoots (Figure 12-2) using the Kruskal-Wallis Test (non-parametric test comparing the median of each sample group) gave $K = 6.37$. The critical value was 7.81 at $p = 0.05$, therefore there was no difference in the medians of each sample. Although the use of hypochlorite did not significantly affect the numbers of clean explants, it did significantly reduce the numbers of explants sprouting. Therefore, rinsing in SDW was adopted for all future work.

6. Media and fluence level

Where plants and explants survived in culture, growth was often poor or non-existent. Some explants appeared to survive, but remain dormant, others developed shoots and new storage tissue; the shoots then died back leaving a dormant but apparently viable bulb. *A. cepa* is sensitive to nutritional and environmental conditions. If these are less than optimum, growth can cease, being difficult to restart even when conditions improve (Brewster, 1994). Where conditions *in vivo* are inadequate for growth, abortion of the inflorescence can occur (Brewster 1994).

- The higher light levels (Sanyo Growth Cabinet) were $95.2 \mu\text{M m}^{-2} \text{s}^{-1}$, mean 4500 lux (minimum 3400 lux, maximum 5500 lux).

- The lower light levels (Gallenkamp Cooled Incubator) were $19.5 \mu\text{M m}^{-2} \text{ s}^{-1}$, mean 750 lux (minimum 550 lux, and maximum 1390 lux).
- All samples were cultured under continuous light (CL) at 25°C.

Cultures were assessed after 30 days, when both contamination and chlorosis were recorded. Analysis of contamination in each of the six different conditions, using χ^2 , gave a value of 19.435. The critical value was 15.09 ($p = 0.01$, d.f. = 5). Therefore, the result was very significant. If data relating to sample 1 (White's medium, high light levels) was removed from the test, then the observed value of $\chi^2 = 6.208$, the critical value is 9.49 at $p = 0.05$, and there was no significance in the remaining samples. Therefore, this sample contributed most to the significance level. The numbers of explants sprouting under these conditions was analysed similarly. The observed value of $\chi^2 = 8.49$. The critical value = 11.07 when d.f. = 5 and $p = 0.05$. Therefore, there was no significant difference in the numbers of explants sprouting under these conditions. The medians of the shoot length were compared using the Kruskal-Wallis test. The observed value of $K = 7.47$. At 4 d.f., the critical value = 9.49, therefore there was no significant difference in the shoot lengths. Examination of the ranks of the shoot lengths using the non-parametric ANOVA (Barnard, *et al.*, 2001) also showed no significance in the variation of shoot length as a result of the light level ($K_{\text{obs}} = 0.089$, $K_{\text{crit}} = 3.86$, $p = 0.05$). However examination of the effect of the medium on shoot length was significant ($K_{\text{obs}} = 4.2235$, $K_{\text{crit}} = 3.86$, $p = 0.05$), whilst the interaction between these factors was also not significant ($p = 0.05$, $K_{\text{crit}} =$

5.99, $K_{obs} = 2.70$). Therefore, the media and fluence levels examined did not significantly affect shoot length.

7. Sucrose concentrations

- Explants were prepared as previously (Figure 29) ($n = 10$ for each treatment)
- Medium was Gamborg's B5 basal salts, with 0.8 % agar
- All samples were cultured under continuous light (CL) at a fluence of $19.5 \mu\text{M m}^{-2} \text{ s}^{-1}$ at 25°C .

Table 12-5: Contamination and survival numbers for explants cultivated in media supplemented with different sucrose concentrations. Assessed at 30 days; mean length is per surviving and developing explant. Viable is assessed as surviving, clean, and developing ($n = 10$ per treatment)

Sucrose	1%	2%	3%	4%	6%	8%
Number clean	10	10	10	10	4	8
Number viable	7	5	5	4	2	4
Mean shoot length	4.7mm	5.8mm	12.8mm	11.75mm	11.5mm	8mm
Chlorosis	None	None	None	None	None	None

The overall percentage of contaminations was 13.3%. Analysis of the number of contaminated and clean explants under different sucrose concentrations, using χ^2 gave an observed value of $\chi^2 = 9.34$. At 5 d.f., $p = 0.05$, the critical value of $\chi^2 = 11.07$. Therefore, there was no significant difference between the number of contaminated and clean explants under the different treatments. Analysis of the number of viable explants using χ^2 , gave a value of 5.466. At $p = 0.05$ and 5 d.f., the critical value was 11.07, therefore the observed differences were not significant. The Kruskal-Wallis test comparing differences in medians for non-parametric data was used to evaluate differences in shoot length, giving $K = 6.116$. The critical value at 5 % was 10.75. Therefore, there was no significant difference between the medians of the shoot length of the populations cultured at different sucrose levels. Spearman's Rank correlation

(non-parametric test for association) was used to examine the relationship between sucrose concentrations and contaminations. $R = 0.8$. The 2 tailed test at $p = 0.05$, gives a critical value of $R = 0.886$. Therefore, there was no correlation between sucrose concentrations and number of contaminations. Similarly, there was no correlation between sucrose concentrations and the number of viable explants ($R_{\text{observed}} = 0.828$, $p = 0.05$, $R_{\text{critical 2-tailed}} = 0.886$). However, these values are close to the critical values, so more rigorous testing may identify a correlation.

8. Carbon source and photoperiod

- Explants were all prepared as previously (Figure 29)
- They were sterilised by rinsing with SDW and cultured in Magenta vessels with 50 ml media (Gamborg's B5 basal salts, 0.8 % w/v agar)
- fluence was $19.5 \mu\text{M m}^{-2} \text{s}^{-1}$, 750 lux at 25°C)
- All contaminated samples were discarded.
- Those that exhibited bulbing and dormancy were not included in shoot length measurements, as it was not possible to obtain accurate measurements. Shoots that failed to elongate, or became abnormal were discarded.

Table 12-6; Explants cultured on a medium supplemented with glucose at 3% or 6%, assessed after 3 months (12 explants per treatment)

	3%		6%	
	14 h	24 h	14 h	24 h
Treatment number	1	2	3	4
Number contaminated	3	4	2	3
Number bulbing	1	1	1	1
Number with distorted growth	1	1	2	2
Number viable	7	6	7	6
Mean shoot length (mm)	24.2	20.2	11.67	11.80

Table 12-7; Explants cultured on medium supplemented with sucrose at 3% or 6%, assessed after 3 months (12 explants per treatment)

	3 %	6 %
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	14 h	24 h	14 h	24 h
Treatment number	5	6	7	8
Number contaminated	1	2	1	1
Number bulbing	4	3	4	4
Number with distorted growth	3	5	2	2
Number viable	4	2	5	5
Mean shoot length (mm)	21.5	18.0	7.4	12.2

Table 12-8: Explants cultured in medium supplemented with fructose, after 3 months (12 explants per treatment)

	3%		6%	
	14h	24h	14h	24h
Treatment number	9	10	11	12
Number contaminated	2	2	3	2
Number bulbing	5	2	1	3
Number with distorted growth	2	2	2	2
Number viable	3	6	6	5
Mean shoot length	9.6	9.33	9.2	7.0

These data suggested that contamination was lowest on sucrose (Table 12-6) and highest on glucose (Table 12-5), whilst bulbing is lowest on glucose and highest on sucrose. However, analysis using χ^2 to examine the difference in the ratios of contaminated and clean explants, between the 12 treatments showed no significant difference. The observed value of $\chi^2 = 5.225$, the critical value for 11 d.f. at $p = 0.05$, was 19.68. Therefore, there was no significant difference in the proportions of contaminated and clean explants in these treatments. When the summed data was analysed using Kruskal-Wallis test to rank the number of contaminations per carbon source, then the observed value of $K = 8.355$. The critical value is 5.99 (d.f. = 2, $p = 0.05$). Therefore, the difference in the medians of the ranks between the different sugars is significant, and the number of contaminations is significantly different between these sugars.

Analysing the numbers bulbing under each of the 12 treatments, the observed value of $\chi^2 = 16.726$. The critical value (d.f. = 11 $p = 0.05$) of $\chi^2 = 19.68$. Therefore, the differences in proportions of bulbing and non-bulbing explants, was not significant.

However, this was quite close to the critical value. When the results for each sugar were summed, then the observed value of $\chi^2 = 7.165$. The critical value was 5.99 (2.d.f. $p = 0.05$). Therefore, the difference in proportions of explants bulbing under different sugars was significant. Again, when the numbers were analysed using the Kruskal-Wallis test, using summed data for each sugar, then the observed value for $K = 7.038$. The critical value of $K = 5.99$ (d.f. =2, $p = 0.5$). This result was significant. These apparently contradictory results may suggest that there is some significant difference in the numbers of explants bulbing between the treatments, but that more stringent testing would be necessary to confirm this.

The highest number of viable explants was observed in the medium supplemented with glucose (26 compared with 16 on sucrose and 20 on fructose, out of a 48 explants grown on each sugar). The low viable numbers indicated not only losses due to contamination and bulbing, but also the production of distorted growth, this being particularly reflected in sucrose, where 25% of the explants are affected. When the number of viable explants in each of the 12 treatments was examined, the observed value of $\chi^2 = 6.519$. The observed value of $\chi^2 = 19.68$ (d.f. = 11, $p = 0.05$). Therefore, the difference in the proportions of viable and non-viable explants was not significant in these conditions. As the observed value was not close to the critical value, no further tests were done.

Mean shoot length was also higher on glucose. The lengths of the shoots of viable explants were examined, using the Kruskal-Wallis test to compare medians under each treatment. The observed value of $K = 21.82$. The critical value of $K = 19.68$ (d.f.11, p

= 0.05). Therefore, the difference in medians between the treatments was significant, with treatment 1 and 2 (3% glucose at 14 and 24 h photoperiods) contributing most to the significance.

9. Nutritional restriction

- Explants were prepared as previously
- These were grown on with 3% sucrose, Gamborg's B5 basal salts, 0.8% agar
- Fluence was $19.5\mu\text{M m}^{-2} \text{ s}^{-1}$ at 25°C
- Gamborg's Vitamin Solution was included in the medium at a concentration of 1ml l^{-1} in accordance with the manufacturer's recommendations
- Contamination, shoot growth and viability were compared after 3 months

Table 12-9: Shoot lengths assessed after approximately 3 months (0 growth indicates that the explant did not at any time produce a sprout; there was no bulbing at this time).

Treatment	50ml exc. Vitamins	100ml exc. vitamins	50ml inc. vitamins	100ml inc. vitamins
	0	23mm	Cont	4mm
	88mm	50mm	82mm	48mm
	0	0	10mm	0
	0	118mm	Cont	0
	56mm	49mm	9mm	Cont
	0	64mm	cont	43mm
	Cont.	74mm	135mm	193mm
	43mm	152mm	4mm	67mm
	0	0	190mm	Cont
	7mm	34mm	Cont	70mm
	0	0	4mm	Cont
	0	Cont	0	0
Number contaminated	1	1	4	3
Number viable	4	8	7	6

χ^2 was used to evaluate the number of contaminated explants (Table 12-8). The observed value of $\chi^2 = 3.65$. The critical value (d.f. = 3, $p = 0.05$) of $\chi^2 = 7.81$.

Therefore, there was no significant difference in the proportions of clean and

contaminated explants between these treatments. However, the least contamination was in treatments 1 and 2 which did not have added vitamins.

χ^2 was used to evaluate the number of viable explants in each of the 4 treatments (Table 12-8). The observed value of $\chi^2 = 2.92$. The critical value (d.f. = 3, $p = 0.05$) of $\chi^2 = 7.81$. Therefore, there was no significant difference in the proportions of viable and non-viable explants in these treatments. However, the largest number of viable explants was in treatment 2 (100ml ex. vitamins).

Mean shoot length was longer in explants cultured on 100 ml of medium compared with 50 ml medium (Table 12-8). The Kruskal-Wallis test was used to evaluate the lengths of the shoots in each of the treatments (Table 32). The observed value of $K = 3.82$. The critical value (d.f. = 3, $p = 0.05$) of $K = 7.81$. Therefore, the difference in length was not significant. This was also analysed using non-parametric two-way ANOVA, but again the results were not significant.

Although there was no bulbing apparent at this time, after a further 4 weeks, bulbing became well developed (Table 12-10).

Table 12-10: Bulbing numbers after 4 months in culture

	50ml exc. Vitamins	100ml exc. vitamins	50ml inc. vitamins	100ml inc. vitamins
Number bulbing (total) percentage	2 (4) 50%	6 (8) 75%	2 (7) 28%	2 (6) 33%

This was tested using χ^2 . The observed value of $\chi^2 = 3.912$. The critical value = 7.81 (d.f. = 3, $p = 0.05$). Therefore, there was no significant difference in the proportions

of explants bulbing under these treatments. However, 67 % of those explants grown without vitamins showed bulbing after 4 months, whilst only 31 % of those cultured with vitamins showed bulbing after 4 months.

10. Photoperiod and dormancy

Table 12-11: Effects of photoperiod on dormancy

	24 h photoperiod	14 h photoperiod
No. of dormant plants	9	8
No. of sprouting plants	7	9

11. Light effects on sprouting numbers of bulbils

Twenty bulbils were chosen at random; half were placed on damp filter paper in a Petri dish in total darkness; the other half was placed on damp filter paper in a Petri dish in continuous light.

Both were grown at 20°C (Sanyo Growth Cabinet 95.2 $\mu\text{M m}^{-2} \text{s}^{-1}$, mean 4500 lux) and sprouting numbers were compared after two and four weeks (Figure 12-3).

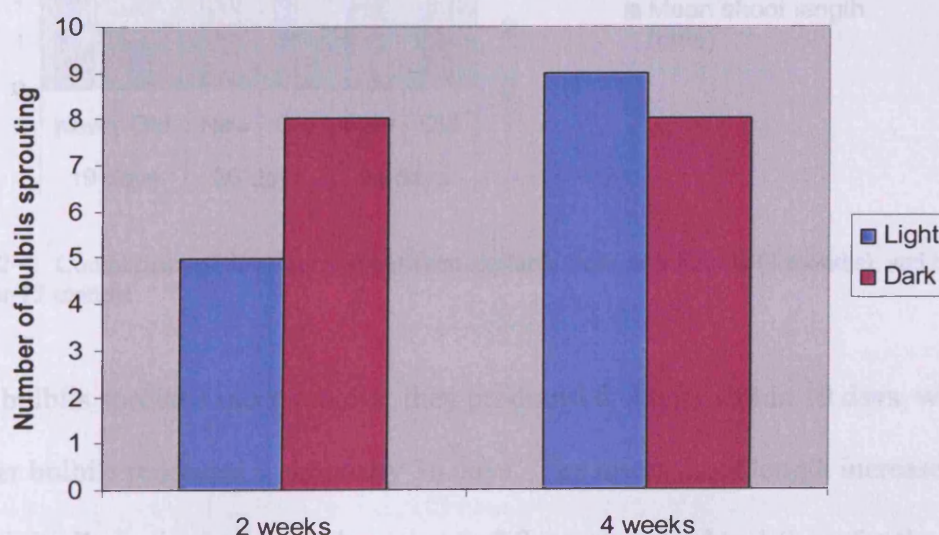


Figure 12-3: Effects of light on sprouting numbers of bulbils

After two weeks, five bulbils had sprouted in CL compared with eight in continuous dark. However, by four weeks, there was little difference with nine sprouting in CL and 8 in continuous dark.

12. Effect of storage period on dormancy

- Bulbils were selected at random, excised as previously (Figure 29), and rinsed in SDW
- They were cultured at 25°C, 14 h photoperiod.
- Medium was B5, with 3 % w/v glucose, 0.8 % w/v agar
- Fluence was $95.2 \mu\text{M m}^{-2} \text{s}^{-1}$, mean 4500 lux (Sanyo Growth Cabinet)

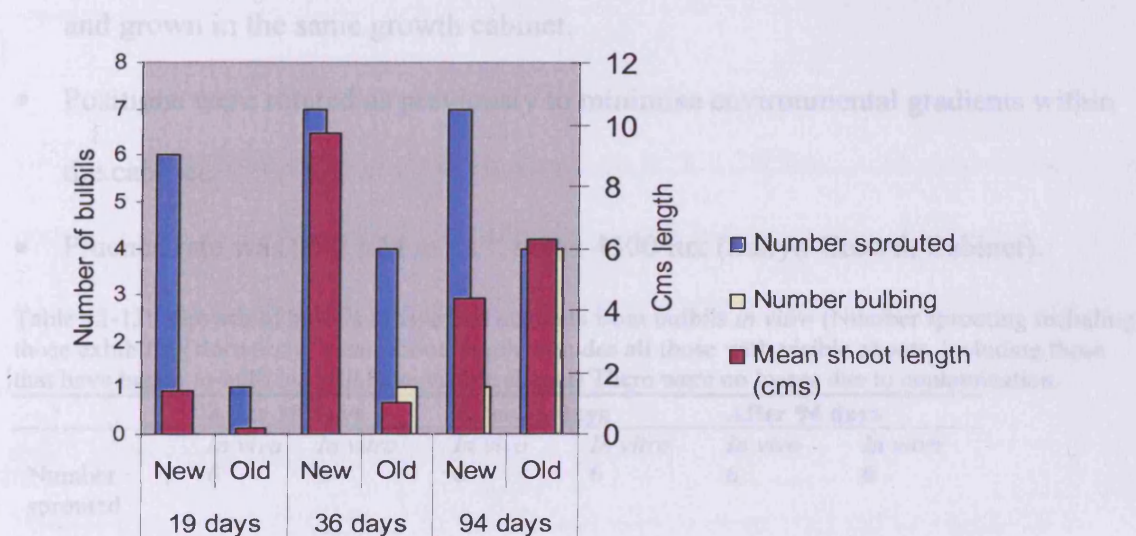


Figure 12-4: Comparison of development between explants from new bulbils (3 months), and bulbils stored for 15 months

Newer bulbils sprouted more rapidly; they produced 6 shoots within 19 days, whereas the older bulbils produced 4 shoots by 36 days. The mean shoot length increased rapidly initially in the fresh bulbils, rising to 9.7 cm compared to 1.0 cm for the older bulbils. However, after this point, dormancy and shoot dieback reduce the mean shoot length, to 4.4 cm for fresh bulbils. The figure of 6.3 cm for the older bulbils reflects

growth of one shoot only, the rest having become dormant by this time. Bulbing also proceeded faster on older bulbils than fresh. Fresh bulbils produced only one explant bulbing by 94 days, whilst older bulbils produced one bulbing explant by 36 days, rising to 4 by 94 days.

13. Culturing effects on dormancy

- Bulbils and explants were grown at 25°C, 14h photoperiod
- The medium for explants was B5, with 3 % (w/v) glucose, 0.8 % (w/v) agar.
- The whole bulbils were placed approximately 2 cm deep in compost ('Homebase' multipurpose, with approximately 10 % v/v added horticultural grit) in 9 cm pots and grown in the same growth cabinet.
- Positions were rotated as previously to minimise environmental gradients within the cabinet.
- Fluence rate was $95.2 \mu\text{M m}^{-2} \text{s}^{-1}$, mean 4500 lux (Sanyo Growth Cabinet).

Table 12-12: Growth of bulbils *in vivo* and explants from bulbils *in vitro* (Number sprouting including those exhibiting dormancy; mean shoot length includes all those with visible shoots, including those that have begun to bulb but still have visible shoots) There were no losses due to contamination.

	After 19 days		After 36 days		After 94 days	
	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>
Number sprouted	6	5	6	6	6	6
Mean shoot length (cm)	10.2 (n = 6)	2.4 (n = 5)	19.7 (n = 6)	2.2 (n = 2)	21.7 (n = 6)	0 (n = 0)
Number exhibiting dormancy	0	0	0	4 (67%)	0 (0%)	6 (100%)

These data suggested that there is no difference in numbers sprouting, between those grown *in vivo* and those grown *in vitro* (Table 12-9). However, the difference in the shoot lengths was readily apparent, the mean of those grown *in vivo* being larger by a

factor of 4.25 at 19 days rising to 8.9 after 36 days, reflecting both the growth in the whole plants, and the dieback of the shoot as bulbing develops. Additionally the explant shoot was chlorotic compared with whole plant growth. The Mann-Whitney test for differences in medians (1-tailed) was used to examine the significance of the difference in shoot lengths, between 19 days and 36 days. After 94 days, mean shoot length shows little increase from 36 days for those grown *in vivo*, whilst those *in vitro* have all bulbed and no longer have shoots. The observed value of $T = 36$; the critical value for $T = 33$ ($p = 0.005$); therefore, this difference is very highly significant, and the median of the sprouting lengths is very highly significantly larger *in vivo* than *in vitro*.

Bulbing occurred in 4 out of 6 explants *in vitro* within 36 days, with none of the whole plants *in vivo* (out of 6 sprouting) showing signs of bulbing within this period. After 94 days, all of those *in vivo* were still sprouting, whilst none of those *in vitro* were in active growth, but had bulbed and become dormant.

14. Photoperiod and dormancy

Bulbils from SH99 (Chapter 2.0) were selected at random, excised as Figure 29 to remove all external tissue, and grown in culture on 20 ml B5 medium with 3% sucrose, 0.8% agar at 25°C, CL at a fluence of $19.5 \mu\text{M m}^{-2} \text{s}^{-1}$.

After 6 months in culture, approximately half were dormant (Table 12-10). These were allocated at random to CL and 14 h photoperiods and maintained in culture on 20 ml B5 medium with 3% sucrose, 0.8% agar at 25°C, at a fluence of $19.5 \mu\text{M m}^{-2} \text{s}^{-1}$

Sub-culturing was continued at monthly intervals. Effects of the change in photoperiod on dormancy were assessed after 4 months

Table 12-13: The effects of change of photoperiod on sprouting and dormancy of explants after four months in vitro

	Explants initially dormant		Explants initially sprouting	
	Plant no.	Shoot length (mm)	Plant no.	Shoot length (mm)
14 h photoperiod	63	Dormant	13	3
	37	Dormant	73	22
	31	Dormant	25	8
	36	Dormant	57	23
	68	Dormant	38	13
	50	Dormant	48	6
	27	Dormant	8	8
	29	Dormant	17	5
			65	Dormant
24 h photoperiod	53	Dormant	39	Dormant
	44	Dormant	24	Dormant
	32	Dormant	12	13
	33	Dormant	43	Dormant
	52	Dormant	64	Dormant
	71	Dormant	19	Dormant
	51	Dormant	56	Dormant
	69	Dormant		
	16	-		
	42	-		
	47	-		
Total of dormant plants sprouting after 4 months	0/19	Total of initially sprouting plants dormant after 4 months	7/16	

When tested for significance using χ^2 adjusted by Yates correction factor (d.f. = 1) then the observed value of $\chi^2 = 6.2115$ at $p = 0.05$. Therefore, this result was significant and there was a difference in the numbers of explants sprouting or becoming dormant under the 14h photoperiod and CL treatments.

Appendix 13

Development of third season plants (JS00)

Table of raw data here

All 3 floral plants were sampled in the period from 9 4 01 to 9 5 01 (Fig. 66)

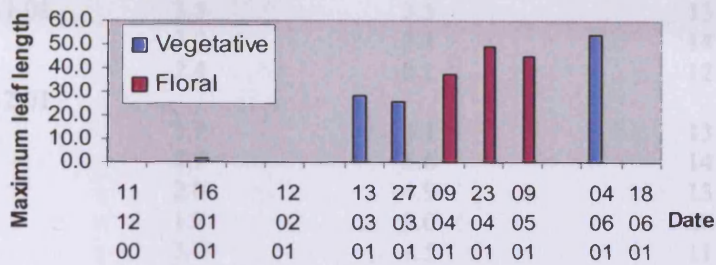


Figure 66: Maximum leaf length in 3rd season plants (JS00), planted in October following dormancy, grown in outdoor beds, and sampled at intervals and examined for floral development.

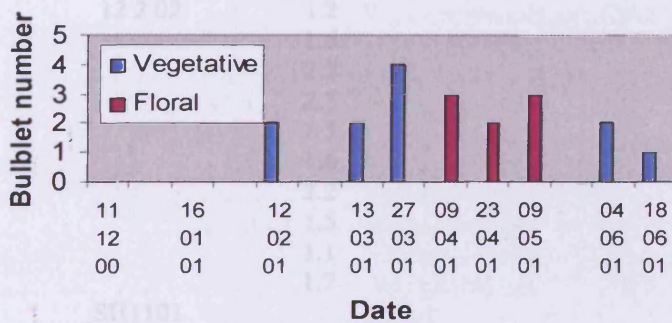


Figure 40: Bulblet number in 3rd season plants (JS00)

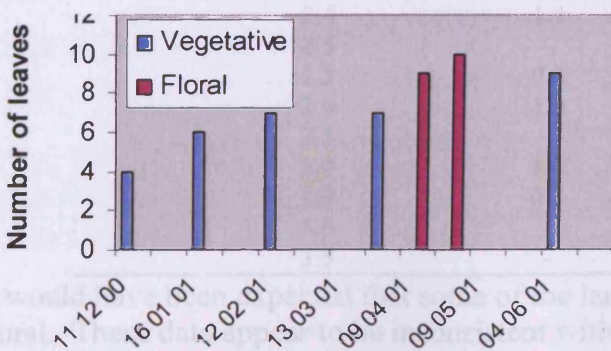


Figure 41: Number of leaves in 3rd season development (JS00)

Appendix 14

Third season plants (SH99) sampled monthly, cultured for approximately 4 weeks, then dissected to assay for floral development. Contaminated cultures were not included.

Date	Plant	Stage	Method	Score	Floral	Inflorescence				
Date	Bulb Width at sampling (cm)	Length of shoot at assay (cm)	Total no. of leaves (removed for culture plus removed at assay)	(cm)	Floral Score	(cm)				
Immediate dissection										
6 11 01	3.5	3.5	13	0						
24 3 01	1	0	3.0	Wax embedding	3.1	14	0			
3	4	2.4	SEM	6.1	1	0.3	12	0.2	0	0.25
3 12 01	0		SEM							
4	6	3.2	Wax embedding	4.1	4.8	13	0.4	0	0.35	
5	9	4.2	Wax embedding	5.6		14	0.3	0	0.3	
6	3	2.0	Wax embedding	4.9	2.0	13	0.2	0	0.2	
7	4	1.7	Wax embedding	2.0		9		0		
8	3	3.0	Wax embedding	4.5		11		0		
9	0	1.7	Wax embedding	2.7		10		0		
10	0		Wax embedding							
9 1 02	0	2.1	Wax embedding	1.9	1.5	10	0.3	0	0.3	
11	0	1.4	Wax embedding	6.1		8		0		
1 15	0	1.5	Wax embedding	-		-		-		
16	0	3.0	Wax embedding	0.1		10		0		
Two weekly sampling										
10 4 01	38	0	2.8	SEM	4.6	11		0		
11	54	0	2.9	SEM	4.5	12		7		
23 4 01	28	3	2.4	SEM	2.2	9	0.3	0	0.4	
46	5		SEM		5.7	2.5				
12 2 02	0	1.2	Wax embedding	25.2	7.1	-	0.7	-	0.4	
30	3	1.6	Wax embedding	4.3	2.5	12	0.3	0	0.3	
9 5 01	34	4	2.7	SEM	72.0	3.8	1.1	0	1.9	
15	9	2.3	Wax embedding	43.0	6.0	12	1.1	0	0.7	
40	0	1.5	Wax embedding			12		0		
2	10	1.6	Wax embedding	76.0	5.7	12	1.3	0	1.3	
46	0	2.2	Wax embedding			-		-		
6	0	1.5	Wax embedding			11		0		
21 5 01	19	9	1.1	Wax embedding	86.5	5.7	1.4	-	1.0	
	10	1.7	Wax embedding	76.5	5.1	12	1.4	0	1.1	
SH1101	0		SEM	-						
14 3 02	0	2.2	SEM			-				
27	10	2.4	Wax embedding	1.4	87.0	4.3	3.3	0	2.8	
4 6 01	39	20	2.5	Wax embedding	-	96.5	4.7	-	1.8	
16	0	2.2	Wax embedding	0.4		11		0		
33	0	2.6	Wax embedding	1.0		12		0		
41	0	2.5	Wax embedding	-		-				
18 6 01	50	11	3.4	Dissection	1.0	10	1.4	0		
4 7 01	53	11	2.9	Dissection	0.7	87.5	1.4	0		
All floral plants examined during the two weekly sampling intervals in bulbs to be the same										

It would have been expected that some of the larger bulbs (highlighted) would have been floral. These data appear to be inconsistent with the suggestion that critical mass is approximately 3 cm width. However, floral development appears to proceed at different rates in different plants, so this is inconclusive (see results for JS00, 6.3.1, Figure 66)

Appendix 15

Floral development of *Allium ampeloprasum* var. *babingtonii* (SH0301) sampled from the Cornish population in March, 2001 as growing plants of unknown history

Date	Plant no.	Floral Score	Method	Scape length (cm)	Spathe height (cm)	Inflorescence height (cm)	Inflorescence width (cm)
Immediate dissection:							
24 3 01	1	0	Wax embedding				
	2	4	SEM	}	0.5	0.2	0.25
	3	0	SEM				
	4	6	Wax embedding		4.8	0.4	0.35
	5	9	Wax embedding			0.3	0.3
	6	5	Wax embedding		2.0	0.2	0.2
	7	0	Wax embedding				
	8	8	Wax embedding				
	9	0	Wax embedding				
	10	0	Wax embedding				
	11	9	Wax embedding	1.5	1.8	0.3	0.3
	13	0	Wax embedding				
	15	0	Wax embedding				
	16	0	Wax embedding				
Two weekly sampling							
10 4 01	30	0	SEM				
	54	0	SEM				
23 4 01	28	5	SEM	2.5	1.6	0.8	0.4
	46	5	SEM	5.7	2.5		
	51	9	Wax embedding	25.2	7.1	0.7	0.6
	20	5	Wax embedding	4.2	2.5	0.3	0.3
9 5 01	34	8	SEM	72.0	7.8	1.1	1.0
	15	9	Wax embedding	48.0	6.0	1.1	0.7
	49	0	Wax embedding				
	2	10	Wax embedding	76.0	5.7	1.4	1.3
	48	0	Wax embedding				
	6	0	Wax embedding				
21 5 01	19	9	Wax embedding	66.5	5.7	1.8	1.0
	13	10	Wax embedding	76.5	5.1	1.8	1.3
	38	0	SEM				
	37	0	SEM				
4 6 01	27	10	Wax embedding	87.0	4.2	3.3	2.9
	59	10	Wax embedding	96.5	4.7	1.7	1.8
	16	0	Wax embedding				
	55	0	Wax embedding				
	41	0	Wax embedding				
18 6 01	50	11	Dissection		Desiccated	2.4	
4 7 01	53	11	Dissection	87.5	Desiccated	4.4	

All floral plants examined during the two weekly sampling had bulbils in the inflorescence

Appendix 16

Bulbil/floret numbers

1. Number of bulbils on each inflorescence (SH99) collected from population 1 in August 1999:

A1	49 bulbils	A4	48 bulbils	A5	90 bulbils
A6	53 bulbils	A8	43 bulbils	A9	25 bulbil
A10	70 bulbils	B12	66 bulbils	C15	52 bulbils
D16	38 bulbils	D17	40 bulbils	F20	46 bulbils
F21	40 bulbils	F22	59 bulbils	F23	49 bulbils
F24	31 bulbils	F26	19 bulbils	G27	36 bulbils
G30	51 bulbils	G32	41 bulbils	G34	41 bulbils
G35	41 bulbils	H40	42 bulbils	H43	38 bulbils
I44	47 bulbils	I46	33 bulbils	I48	25 bulbils
J47	36 bulbils	J51	62 bulbils	J53	61 bulbils
J54	66 bulbils	J56	50 bulbils	J59	34 bulbils
J60	32 bulbils	J61	48 bulbils	J62	59 bulbils
J63	44 bulbils	J64	39 bulbils	J65	36 bulbils
J67	28 bulbils	J69	36 bulbils	J70	30 bulbils
J72	65 bulbils	J73	45 bulbils	J75	45 bulbils
J77	44 bulbils	J79	48 bulbils	J82	65 bulbils

Mean bulbil number, 45.5 ± 1.95 ; standard deviation 35.5 ; median 44.

2. Bulbils and florets on each inflorescence (SH1101) collected from population 1, November, 2001

Inflorescence No.	No. of bulbils	Total weight of bulbils (g)	Mean weight of bulbil (g)	No. of florets	Bulbil/floret ratio
1i	39	14.33	0.37	105	0.37
1ii	48	16.76	0.35	149	0.32
1iii	49	15.26	0.31	176	0.28
1iv	30	10.31	0.34	92	0.33
1v	58	17.76	0.31	154	0.38
Mean	45		0.336	135	0.33
2i	35	12.37	0.35	125	0.28
2ii	84	15.48	0.18	185	0.45
2iii	49	10.72	0.22	155	0.32
2iv	58	9.98	0.17	145	0.40
Mean	57		0.23	153	0.37
3i	47			122	0.39
3ii	37	7.33	0.2	127	0.29
3iii	45	9.92	0.22	110	0.41
3iv	44	10.18	0.23	120	0.37
3v	44	7.66	0.17	27	1.63
Mean	43		0.205	101	0.43
Standard deviation				42	0.09
Mean for whole population	48			128	0.38

Samples 1 i – v were taken from damp, shady ground on a streamside

Samples 2 i – iv were taken from sunny dry ground with little competition on a field edge

Samples 3 i – v were taken from sunny dry ground with much competition on a path edge

3. Samples taken by Treu (1999)

Population number	Mean bulbil no.	Mean bulbil weight (g)	Mean floret number	Bulbil/floret ratio
1 (n = 70)	49.5±2.0	0.24±0.01	110.1±6.6	0.45
38 (n = 10)	45.1±4.8	0.22±0.03	124.2±16	0.36
39 (n = 10)	60.6±3.3	0.28±0.03	147±10.4	0.41

4. Analysis

There was apparently much variation in both bulbil and floret numbers. If varying bulbil numbers were a reflection of varying inflorescence size, there should be a relationship between the bulbil number and the floret number, with a correlation between bulbil number and the bulbil/floret ratio. However, when the data from SH1101 was combined with the data from Treu (1999), the coefficient of linear regression was only 0.5721, showing only a weak positive correlation between bulbil and floret numbers.

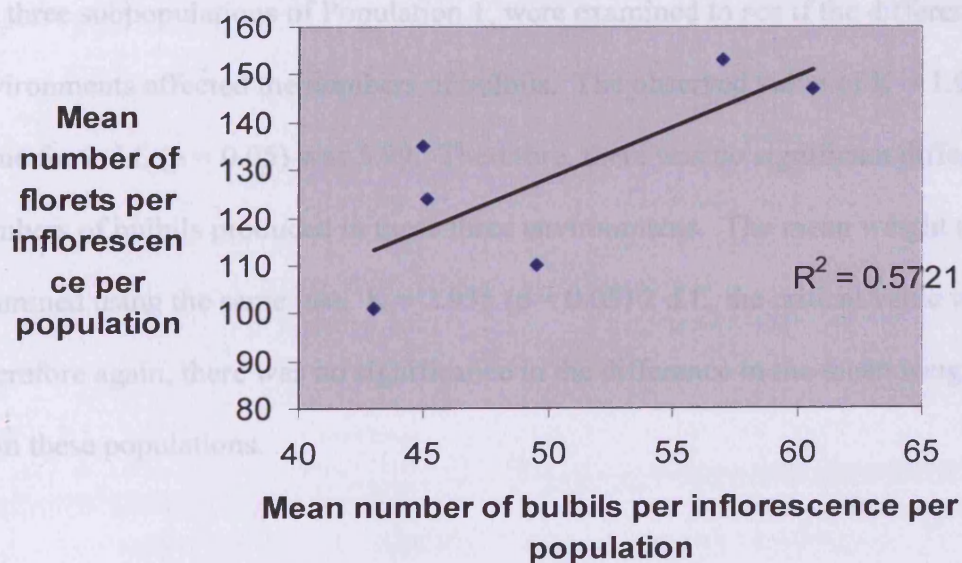


Figure: Data for bulbil number/floret number from Treu 1999, (Table above) and mean bulbil/floret numbers from SH1101 (above).

Some correlation between the bulbil numbers and floret numbers was to be expected since a larger inflorescence could be expected to have more of each. This result suggested that the relationship was more complex, and could perhaps be linked with the development of the

cyme, and the timing of the response to the signal that produced the bulbils as an alternative to florets.

The variation in bulbil numbers between SH99 and the three sub-populations in SH1101 was tested using non-parametric Anova (Barnard, 2001). At 3 d.f. ($p = 0.05$) the critical value was 7.81. The observed value was $K = 1.384$. Therefore, there was no significant difference in the mean numbers of bulbils in each of these samples. This was further tested by comparing SH99 with SH1101 (treated as one population) to examine the relationship bulbil numbers from one year to another, also using non-parametric Anova. At 1 d.f. ($p = 0.05$) the observed value of K was 0.22, compared with a critical value of 3.84. Therefore, there was no significant difference in the variation in bulbil numbers between the samples taken from Population 1 in 1999 and 2001. Similarly, the relationship between the bulbil numbers from the three subpopulations of Population 1, were examined to see if the different growing environments affected the numbers of bulbils. The observed value of $K = 1.986$. The critical value for 2 d.f. ($p = 0.05$) was 5.99. Therefore, there was no significant difference in the numbers of bulbils produced in these three environments. The mean weight of the bulbils was examined using the same test. $K = 2.935$ ($p = 0.05$) 2 d.f., the critical value was 5.99. Therefore again, there was no significance in the difference in the mean weight of the bulbils from these populations.

Similarly, the variation in floret numbers in SH1101 (3 sub-populations from Population 1) was examined using non-parametric Anova for 2 d.f., ($p = 0.05$). The critical value was 5.99, the observed value was $K = 4.1657$. Therefore, there was no significant difference in the mean numbers of florets in each of these samples, and the variations in the environments were not shown to have any effect on the floret numbers.

Appendix 17

Protocols for extraction, amplification and sequencing of DNA and RNA from *Allium ampeloprasum* var. *babingtonii*

1. Extraction and cleaning of genomic DNA
2. Sepharose CL6B cleaning of genomic DNA
3. Purification of genomic DNA by equilibrium centrifugation in Caesium chloride/ethidium bromide (CsCl/EtBr) gradient
4. Summary of QIAQUICK Gel Extraction Kit Protocol
5. The Polymerase Chain Reaction (PCR)
6. Summary of protocol for ligation using pGEM®-T Vector System 1
7. Preparation of LB plates
8. SOC growth medium
9. PCR to extract insert from *E. coli* colonies
10. Mixture for liquid culture of transformed cells
11. Summary of extraction of high-copy plasmid DNA from overnight cultures
12. Summary of extraction of RNA using TRI-Reagent
13. Summary of method for cleaning RNA
14. cDNA synthesis
15. PCR of cDNA

1. Extraction and cleaning of genomic DNA from *Allium ampeloprasum* var. *babingtonii* (adapted from Saghai-Marooof *et al.* 1984).

- 50 ml of 2 x CTAB isolation buffer was made up comprising 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% w/v CTAB (hexadecyltrimethylammonium bromide) with SDW
- 2 x 25 ml was preheated in sterile 50 ml Corning tubes to 65°C
- Young leaves were removed from *Allium ampeloprasum* var. *babingtonii* plants, cooled in liquid nitrogen and stored at – 70°C.
- Two pieces approximately 3 g were removed and ground in a pre-chilled pestle and mortar (-20°C) with liquid nitrogen
- Half was added to each tube of 2 x CTAB isolation buffer and then incubated at 65°C for 20 minutes.
- The mixture was placed into four round bottom centrifuge tubes and 12.5 ml SEVAG (24:1 dichloromethane:isoamyl alcohol) was added to each in a fume cupboard and mixed gently, releasing pressure as necessary
- Lids were sealed with Nesco film, and mixture was rocked to mix for 30 min. (Denley Suprarocker)
- The tubes were centrifuged for 20 min. at 8000 rpm, using a Sorvall SS34 Rotor in a RC5C Sorvall Centrifuge (DuPont)
- The top (aqueous) layer was transferred to sterile 50 ml centrifuge tubes, yielding approximately 10 ml in each tube
- Approximately 2.5 x volume of 100% ethanol was added to each tube at -20°C and stored overnight at this temperature to precipitate the DNA
- Tubes were centrifuged at 3000 rpm for 10 min. at 4°C (Beckman GS-GR), and the supernatant removed

- The pelleted DNA was washed with 70% ethanol, then centrifuged at 3000 rpm for 15 minutes as above
- The supernatant was discarded and the samples were dried at room temperature for 5 min
- Pellets were resuspended in 400 μ l Tris_{10mM} EDTA_{0.1mM} buffer and stored at 4°C.

2. Sepharose CL6B cleaning of genomic DNA

- Sepharose CL6B was washed 2 x in TE and autoclaved in 0.5 vols of TE prior to use
- A hole was made in the base of a 0.5ml Eppendorf, which was then placed inside a 2ml Eppendorf.
- Glass beads, washed, autoclaved and in TE were pipetted into the inner tube.
- Sepharose CL6B was shaken, then 500 μ l pipetted over the top of the glass beads. This was microfuged at 3000 rpm for 3 minutes.
- The outer tube was replaced with a clean tube. DNA was added (50 μ l), then microfuged for 3 min. at 3000 rpm. The resultant liquid was stored at 4°C.

3. Purification of genomic DNA by equilibrium centrifugation in Caesium chloride/ethidium bromide (CsCl/EtBr) gradient

- 10 ml CsCl/EtBr solution was made comprising
 - 0.5 ml 1 M Tris-HCl pH 8.0
 - 0.2 ml 0.5 M EDTA pH 8.0
 - 0.1 ml 10 mg/ml ethidium bromide
 - 7.53 g CsCl
 - and SDW to 10 ml
- Density was adjusted to 1.55 g/ml
- 0.75ml CsCl/EtBr solution was added to each (2) DNA pellet prepared as described in section 17.2, and warmed for 20 minutes at 65°C and gently rocked till resuspended
- The two samples were combined, and a further 2 ml CsCl/EtBr was added
- Samples were transferred to polycarbonate centrifuge tubes and filled with CsCl/EtBr solution. Caps were sealed onto the tubes with a crimper (DuPont) and placed into a Sorvall TV-865 rotor
- Samples were centrifuged in an OTD65B Sorvall Ultracentrifuge (DuPont) at 40,000 rpm 25°C, for 21 h
- The DNA band was removed under UV illumination and placed in a sterile tube
- One volume butan-1-ol (Analar BDH) saturated with Tris/EDTA (added as equal volume Tris 10 mM EDTA 1 mM pH 8.0, shaken, allowed to stand and then pipetted off as top layer) was shaken with the DNA then allowed to settle
- The EtBr was removed as an upper pink layer. Butan-1-ol saturated with Tris/EDTA was added three more times and removed as before, removing all EtBr
- The DNA solution was transferred to sterile dialysis tubing (Sigma D0405, boiled for 10 min in 200 ml 50 mM EDTA), placed in 1 l dialysis buffer (10 mM Tris/1 mM EDTA pH 8.0) and placed on a magnetic stirrer for 12 h. The dialysis buffer was changed and then dialysis resumed for another 12 h.

- The remaining solution was removed from the tubing and stored at 4°C in sterile tubes

The presence of DNA was checked, using Gel Electrophoresis. 1 µl DNA was added to 20 µl SDW and 2 µl loading buffer. The gel comprised 50 ml TAE buffer with 1.5 % agarose, stained with 5µl ethidium bromide (10 µg/ml).

50 x TAE -	242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8, made up to 1 litre with SDW
10 x DNA loading buffer -	20 mM EDTA, 50% (v/v) Glycerol, 0.05% (w/v) bromophenol blue, stored at minus 20°C or 4°C when diluted

The gel was run for 40 min. at 60V, with 10 µl 1 kilobase DNA ladder (50ng/µl Gibco BRL), then photographed under UV light.

The DNA amounts of the samples were quantified using a Genequant (Pharmacia RNA/DNA Calculator) and then standardised to 100 pg/µl with SDW.

4. Summary of QIAQUICK Gel Extraction Kit Protocol as used for *A. ampeloprasum* var. *babingtonii*

- Add 3 volumes Buffer QG to 1 volume of gel
- Incubate at 50 °C for 10 min vortexing every 2-3 min
- Confirm that colour is yellow
- Add 1 volume of isopropanol
- Apply the sample to the QIAquick column place in collection tube and centrifuge for 1 min at 10,000 g (13,000 rpm)
- Discard flow through
- Wash with 0.75 ml of buffer PE to QIAquick column, stand for 5 min. then centrifuge for 1 min. at 10,000 g. Repeat, and stand for 5 min
- Centrifuge the column again for 1 min, then place column in clean microfuge tube
- Add 30 µl Buffer EB warmed to approximately 50 °C (10mM Tris-Cl, pH 8.5) to the centre of the membrane, stand for 5 min, and then centrifuge for 1 min.

5. The Polymerase Chain Reaction (PCR)

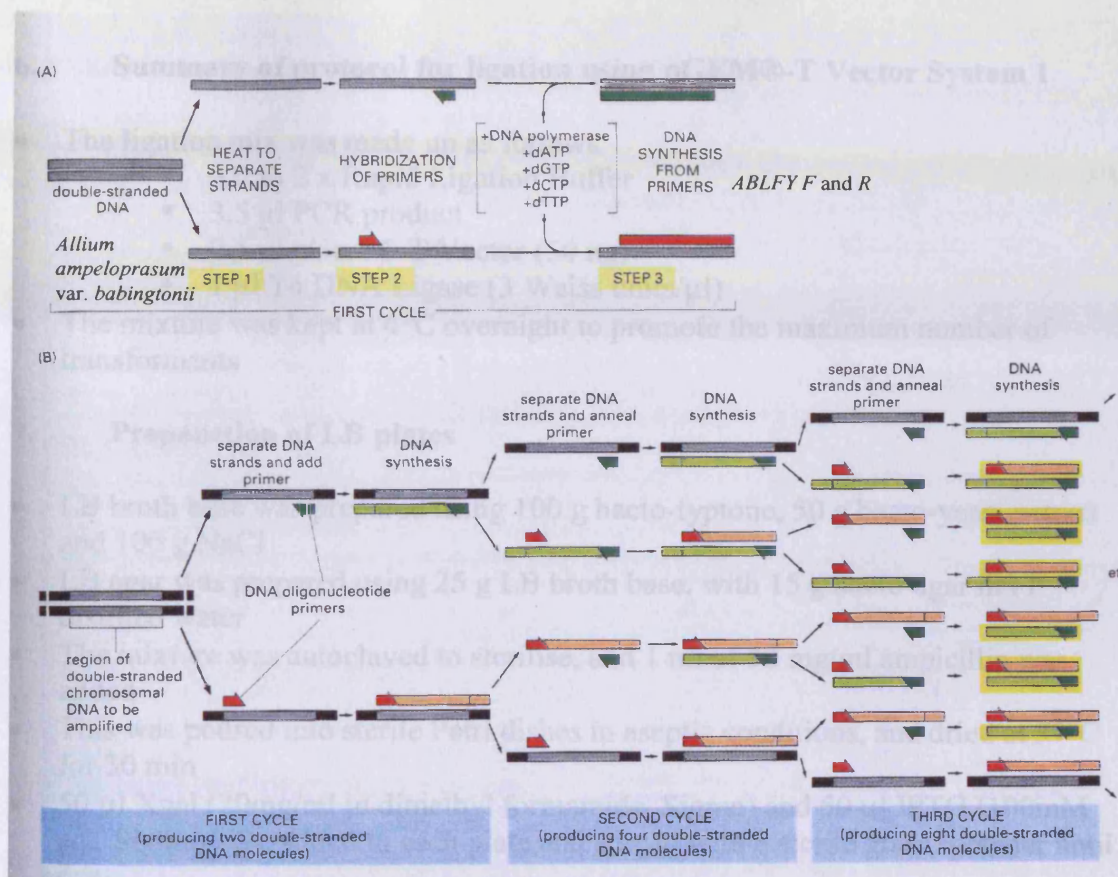


Figure 17-1; Amplification of DNA using the PCR technique: knowledge of the DNA sequence to be amplified is used to design two synthetic DNA oligonucleotides, each complementary to the sequence on one strand of the DNA double helix at opposite ends of the region to be amplified. These oligonucleotides serve as primers for *in vitro* DNA synthesis, which is carried out by a DNA polymerase, and they determine the segment of the DNA that is amplified.

(A) PCR starts with double-stranded DNA, and each cycle of the reaction begins with a brief heat treatment to separate the two strands (step 1). After strand separation, cooling of the DNA in the presence of a large excess of the two primer DNA oligonucleotides allows these primers to hybridize to complementary sequences in the two DNA strands (step 2). This mixture is then incubated with DNA polymerase and the four deoxyribonucleoside triphosphates so that DNA is synthesised, starting from the two primers (step 3). The cycle is then begun again by a heat treatment to separate the newly synthesized DNA strands. The technique depends on the use of DNA polymerase isolated from a thermophilic bacterium (*Thermus aquaticus*); this polymerase is stable at much higher temperatures than eukaryotic DNA polymerases, so it is not denatured by the heat treatment shown in step 1. It therefore does not have to be added again after each cycle of reaction.

(B) As the procedure is carried out over and over again, the newly synthesized fragments serve as templates in their turn, and within a few cycles, the predominant DNA is identical to the sequence bracketed by and including the two primers in the original template. In practice, 20-30 cycles are required for useful DNA amplification. Each cycle doubles the amount of DNA synthesized in the previous cycle. A single cycle takes only about 5 minutes and automation of the whole procedure now enables cell-free cloning of a DNA fragment in a few hours, compared with several days required for standard cloning procedures. Of the DNA put into the original reaction, only the sequence bracketed by the two primers is amplified because there are no primers attached anywhere else. In the example illustrated, three cycles of reaction produce 16 DNA chains, 8 of which (boxed in yellow) are the same length as and correspond exactly to one or the other strand of the original bracketed sequence shown at the far left; the other strands contain extra DNA downstream of the original sequence, which is replicated in the first few cycles. After three more cycles, 240 of the 256 chains will correspond exactly to the original sequence, and after several more cycles, essentially all of the DNA strands will have this unique length (Adapted from Alberts *et al.*, 1998).

6. Summary of protocol for ligation using pGEM®-T Vector System 1

- The ligation mix was made up as follows
 - 5.0 µl 2 x Rapid Ligation Buffer
 - 3.5 µl PCR product
 - 0.5 µl pGem®-T Vector (50 ng)
 - 1 µl T4 DNA Ligase (3 Weiss units/µl)
- The mixture was kept at 4°C overnight to promote the maximum number of transformants

7. Preparation of LB plates

- LB broth base was prepared using 100 g bacto-typtone, 50 g bacto-yeast extract and 100 g NaCl
- LB agar was prepared using 25 g LB broth base, with 15 g bacto-agar in 1 l distilled water
- The mixture was autoclaved to sterilise, and 1 ml of 50 mg/ml ampicillin was added
- This was poured into sterile Petri dishes in aseptic conditions, and dried at 37°C for 30 min
- 50 µl Xgal (20mg/ml in dimethyl formamide, Sigma) and 50 µl IPTG (100mM aq., Sigma) were added to each plate and spread with a sterile glass spreader until dry
- plates were incubated at 37°C for at least an hour to allow evaporation of the demethyl formamide

8. SOC growth medium

- 20 g bacto-tryptone
- 5 g bacto-yeast extract
- 0.5 g NaCl
- 20mM glucose
- made up to 1 l with SDW , autoclaved and stored at -20°C

9. PCR to amplify insert from *E. coli* colonies

- 18 µl Reddymix™ PCR Master Mix (Abgene® AB0575/LD).
- 1 µl each M13 F and R (Genosys 10µM)

PCR was carried out with the following conditions:

- 95 °C for 15 min
- 50 °C for 1 min
- 72 °C for 1 min
- 95 °C for 1 min }

- 50 °C for 1 min }
• 72 °C for 1 min } 33 cycles
- 95 °C for 1 min
- 50 °C for 1 min
- 72 °C for 1 min
- products stored at 4 °C

10. Mixture for liquid culture of transformed cells

- 2 µl ampicillin 1µl/ml of 50mg/ml stock (Sigma A9518)
- 2 ml LB broth
- prepared in aseptic conditions
- 100µl for each culture
- incubate at 37°C, 250 rpm, 5-6 h (Gallenkamp Orbital Incubator)

Then

- Add 100 µl of liquid culture
- 3 ml LB broth
- 3 µl ampicillin
- incubate at 37°C, 250 rpm, overnight

11. Summary of extraction of high-copy plasmid DNA from overnight cultures of *E. coli* in LB (Luria-Bertani) medium with 50 µg/ml ampicillin, using QIAprep Spin Plasmid Kit

- 1.25 ml of each culture was placed into each of two 1.5 ml Eppendorf tubes
- cultures were microcentrifuged at 8000 rpm for 3 min
- supernatant was removed and one sample of pelleted bacteria resuspended by pipetting in 250 µl Buffer P1, then added to second sample and used to resuspend second pellet
- 250 µl of Buffer P2 was added and the tube gently inverted 4-6 times
- Within 5 min, 350 µl Buffer N3 was added to neutralize P2 and provide high salt content to precipitate unwanted components, tube was gently inverted to mix
- Tube was microcentrifuged for 20 min. at 13000 rpm
- QIAprep columns were placed into 2 ml collection tubes and supernatants pipetted onto columns
- Columns were centrifuge at 13000 for 1 min, then flow-through discarded
- 0.75 ml Buffer PE was added to the column, allowed to stand for 5 min, and then centrifuged at 13000 rpm for 1 min
- flow through was discarded and a further 0.75 ml Buffer PE was added to the column, allowed to stand for 5 min then centrifuged at 13000 rpm for 1 min
- Flow-through was discarded and the tube centrifuged again at 13000 rpm for 1 min
- The columns were placed into clean 1.5 ml Eppendorfs and 50 µl Buffer EB (at 60°C) was added to the centre of the column
- After 5 min, the column was centrifuged at 13000 for 1 min

- Concentrations were estimated using the GeneQuant (Pharmacia RNA/DNA Calculator)
- Product was stored at -80°C until sequencing on 3100 Genetic Analyser (Applied Biosystems)
- The product was run on a gel to check for the presence of the plasmid , using 2 µl product, with 8 µl SDW and 2 µl loading buffer.
- Bands should be present at approximately 3 kb

12. Summary of extraction of RNA using TRI-Reagent

- All equipment was RNA dedicated and sterilised, work areas cleaned with RNaseZap (Ambion 9780)
 - 200 mg of plant material was excised, placed in foil and flash frozen in liquid nitrogen
 - Pestles and mortars were sterilized by autoclaving, then stored at - 20°C till required
 - Plant material was ground to fine powder then 2ml TRI-Reagent added to each sample and reground
 - The paste was divided equally into two Eppendorf tubes, vortexed and allowed to stand at room temperature for 5 min
 - The tubes were microcentrifuged at 12000 rpm at 4°C for 10 min
 - The supernatants were transferred to clean Eppendorf tubes, 0.2 ml of chloroform was added to each tube, and they were vortexed for 15 s
 - After standing at room temperature for 5 min, the mixture was microcentrifuged at 12000 rpm for 15 min. at 4°C
 - The aqueous (top) layer was transferred to clean Eppendorf tubes
 - 0.5 ml isopropanol was added to each tube and allowed to stand at room temperature for 10 min
 - The tubes were microcentrifuged at 12000 rpm for 10 min. at 4°C, then the supernatant removed
 - The pellets were washed in 75% ethanol, vortexing for 15 s
 - The tubes were microcentrifuged at 12000 rpm for 10 min. at 4°C
 - The supernatant was removed and the pellet allowed to air dry
 - The pellets were resuspended in 50 µl SDW and the tubes combined
- 10 µl was checked by gel electrophoresis, the remainder stored at - 70°C. (Note, the electrophoresis tank and comb were cleaned with 0.1 M NaOH (4 g/l) to protect against RNase contamination). The gel comprised 1% agarose, in 50ml TAE buffer with 2µl ethidium bromide.

13. Summary of method for cleaning RNA

The DNase digestion reaction was set up as follows:

- 2 µg RNA
- 2 µl RQ1 DNase 10 x buffer (Promega M198A)
- 2 µl RQ1 DNase (Promega, M610A) 1000 U/ml
- made up to 20 µl with SDW
- the mix was incubated at 37°C for 30 min

- 2 µl RQ1 DNase Stop was added (Promega, M199!)
- mix was incubated at 65°C for 10 min
- 10 µl product was checked on an electrophoresis gel, as for RNA extraction above
- 10 µl were used for first strand cDNA synthesis

14. cDNA synthesis

- 10 µl of DNase treated RNA (as above) were placed in a 0.5 ml Eppendorf tube
- 1 µl Oligo (dt) 15 (500 µg/ml) (Deoxy poly T primer which anneals to poly A tail of RNA was added
- the mix was incubated at 70°C for 10 min, then cooled on ice for 10 min
- 6 µl 5 x 1st strand buffer (GibcoBrL Y00146) was added
- 2 µl of 0.1M DTT (dithiothreitol) was added as stabilizer (Gibco BrL Y00147)
- 1 µl 10mM dNTPs was added
- the mix was incubated at 42°C for 2 min
- 1 µl reverse transcriptase was added (M-MLV Reverse Transcriptase, Promega M530A)
- the mix was incubated at 42° for 50 min
- the mix was then inactivated by incubating at 70°C for 15 min
- this produced single strand cDNA which was stored at -70°C

15. PCR of cDNA

The reaction mix was as follows:

- 47 µl Reddymix
- 1 µl ABLFY F (1µ/µl in SDW)
- 1 µl ABLFY R (1µ/µl in SDW)
- 1 µl cDNA
- -ve control used SDW instead of cDNA
- +ve control used Tub Pos 63 and Neg 2 instead of ABLFY F and R

16. Tubulin primers

POS63 = TGA GYG GYG TSA CST GCT

TUB2 = GTA GGA NGA GTT CTT GTT CTG
Allnutt (2000)

Appendix 18

Forward and reverse sequences for six putative *LFY* alleles extracted from *Allium* var. *ampeloprasum* var. *babingtonii*, identified by comparison with BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information <http://www.ncbi.nlm.nih.gov/blast.database>)

Clone 1f

ATCAATAAGCCCAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCTAGAAGAGGACACATCAAACGCC
CTCCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCC
ATTGCTGGTTCGTCAAGGTTGGGATATTGACTCCATTTTAACTCTCATCCTCGTCTTTCTATTTGGTACGTCCCC
ACCAAGCTA

Clone 1r

TAGCTTGGTGGGGACGTACCAAATAGAAAGACGAGGATGAGAGTTAAAAATGGAGTCAATATCCCAACCTTGACG
ACCAGCAATGGCAACAAGGGGTTTGTAACAAGCTTGCTCCACGCGCCTACATTCTCGCCACGCTCCTTGAAGGC
TCTTCGGAGGGCGTTTGATGTGTCTCTTCTAGGCAGTGGAGGGCGTAGCAGTGTACGTAGTGTCTCATCTTGGG
CTTATTGATATA

Clone 4f

ATCAACAAGCCTAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCTAGAAGAGGACACATCAAACGCC
CTCCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCC
ATTGCTGGTTCGTCAAGGTTGGGATATTGACTCCATTTTAACTCTCATCCCCGTCTTTCTATTTGGTACGTCCCC
ACCAAGCTA

Clone 4r

TAGCTTGGTGGGGACGTACCAAATAGAAAGACGGGGATGAGAGTTAAAAATGGAGTCAATATCCCAACCTTGACG
ACCAGCAATGGCAACAAGGGGTTTGTAACAAGCTTGCTCCACGCGCCTACATTCTCGCCACGCTCCTTGAAGGC
TCTTCGGAGGGCGTTTGATGTGTCTCTTCTAGGCAGTGGAGGGCGTAGCAGTGTACGTAGTGTCTCATCTTAGG
CTTGTGATATA

Clone 5f

ATCAATAAGCCGAAGATGAGGCATTATGTCCATTGCTAAGCTTTGCATTGCCTCGATGAAGAAACATCATATGCA
CTCCGTCGATACTAAAAGCATCGAGGAGAAAAATGTGGGAGCATGGAGACTAACGTGCTATCCGCATATAGTGGTG
ATTTTCGGCGCAACACAATTGGGATGTAAACGTCGTTTACAATGCACATCTGAGGCTGTGATTGGTACGTCCCC
ACCAAGCTA

Clone 5r

TAGCTTGGTGGGGACGTACCAAATCGACAGCCTCAGATGTGCATTGTAAACGACGTTTACATCCCAATTGTGTTG
CGCCGAAATCACCCTATATGCGGATAGCACGTTAGTCTCCATGCTCCACATTTTCTCCTCGATGCTTTTAGTA
TCGACGGAGTGCATATGATGTTTCTTCATCGAGGCAATGCAAAGCTTAGCAATGGACATAATGCCCTCATCTTCGG
CTTATTGATATAAAT

Clone 8f

ATCAATAAGCCGAAGATGAGGCATTATGTCCATTGCTACGCTTTGCATTGACTGGATGAAGAAACGTCATAAGCA
CTCCGTTGATACTACGAGCAACGAGGAGAAAAATGTGGGAGCTTGAGACAAGCGTGCTATCAGCATATAGTAGTG
ATTTTCGGAGCAGCACAATTGGGATGTGGATGCCGTTTCAATTACATCTGAGGATGTGATTGGTACGTCCCC
ACCAAGCTA

Clone 8r

TAGCTTGGTGGGGACGTACCAAATCGACATCCTCAGATGTGAATTGAAAACGGCATCCACATCCCAATTGTGCTG
CTCCGAAATCACTACTATATGCTGATAGCACGTTGTCTCCAAGCTCCACATTTTCTCCTCGTTGCTCGTAGTA
TCAACGGAGTGCTTATGACGTTTCTTCATCCAGTCAATGCAAAGCGTAGCAATGGACATAATGCCCTCATCTTCGG
CTTATTGATATA

Clone 9f

ATCAATAAGCCGAAGATGAGGCATTATGTCCATTGCTACGCTTTGCATTGCCTGGATGAAGAAACATCATAAGCA
CTCTATTGATACTACGAGCAACGAGGAGAAAATGTGGGAGAATGGAGACAAGCGTGCTATCAGCATATAGTGGTG
ATTTCCGCGCAGCACAAATAGGGATGTGGATGCCGTTTTCAATTCACATCCGAGGTTTTCGATTGGTACGTCCCC
ACCAAGCTA

Clone 9r

TAGCTTGGTGGGGACGTACCAAATCGAAAACCTCGGATGTGAATTGAAAACGGCATCCACATCCCTATTGTGCTG
CGCCGAAATCACCCTATATGCTGATAGCAGCTTGTCTCCATTCTCCACATTTTCTCCTCGTTGCTCGTAGTA
TCAATAGAGTGCTTATGATGTTTTCTTCATCCAGGCAATGCAAAGCGTAGCAATGGACATAATGCTCATCTTCGG
CTTATTGATGTA

Clone 10f

ATCAATAAGCCCAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCTAGAAGAGGACACATCAAACGCC
CTCCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCC
ATTGCTGGTGGTCAAGGTTGGGATATTGACTCCATTTTAACTCTCATCCTCGTCTTTCTATTGGTACGTCCCC
ACAAAGCTA

Clone 10R

TAGCTTTGTGGGGACGTACCAAATAGAAAGACGAGGATGAGAGTTAAAAATGGAGTCAATATCCCAACCTTGACG
ACCAGCAATGGCAACAAGGGGTTTGTAAACAAGCTTGCTCCACGCGCTACATTCTCGCCACGCTCCTTGAAGGC
TCTTCGAGGGCGTTTGATGTGTCCTCTTCTAGGCAGTGGAGGGCGTAGCAGTGACGTAGTGTCTCATCTTGGG
CTTATTGATGTA

NB

- Blue type indicates the primer sequence
- Forward and reverse sequences were identical for all bacterial clones
- All sequences produced strong traces without ambiguity
- Red type indicates plasmid remnant

	10	20	30	40	50	60	70	80
Clone 10	TACATCAATAAGCCCAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCTAGAAGAGGACACATCAAACGCCCT	80						
Clone 1	TATATCAATAAGCCCAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCTAGAAGAGGACACATCAAACGCCCT	80						
Clone 4	TATATCAATAAGCCCAAGATGAGACACTACGTACACTGCTACGCCCTCCACTGCCTAGAAGAGGACACATCAAACGCCCT	80						
Clone 5	TATATCAATAAGCCCAAGATGAGGCATTATGTCCATTGCTAAGCTTTGCATTGCCTCGATGAAGAAACATCATATGCACT	80						
Clone 8	TATATCAATAAGCCCAAGATGAGGCATTATGTCCATTGCTACGCTTTGCATTGACTGGATGAAGAAACGTCATAAGCACT	80						
Clone 9	TACATCAATAAGCCCAAGATGAGGCATTATGTCCATTGCTACGCTTTGCATTGCCTCGATGAAGAAACATCATAAGCACT	80						
Consensus	TATATCAATAAGCCCAAGATGAGGCATTATGTCCATTGCTACGCTTTGCATTGACTGGATGAAGAAACGTCATAAGCACT	80						

	90	100	110	120	130	140	150	160
Clone 10	CCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCCATTGCTG	160						
Clone 1	CCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCCATTGCTG	160						
Clone 4	CCGAAGAGCCTTCAAGGAGCGTGGCGAGAATGTAGGCGCGTGGAGGCAAGCTTGTTACAAACCCCTTGTTGCCATTGCTG	160						
Clone 5	CCGTGATACTAAGCAACGAGGAGAAAATGTGGGAGCATGGAGACTAAGCTGCTATCCGCATATAGTGGTGATTTCGG	160						
Clone 8	CCGTGATACTAAGCAACGAGGAGAAAATGTGGGAGCATGGAGACAAGCGTGCTATCAGCATATAGTAGTGATTTCGG	160						
Clone 9	CTATTGATACTAAGCAACGAGGAGAAAATGTGGGAGCATGGAGACAAGCGTGCTATCAGCATATAGTGGTGATTTCGG	160						
Consensus	CYRWHGAKMCTWMRAGSADCGWGGMGARAATGTGGGCMMDTGGAGRCWARCKTCYTA YMMRCMYMTWGTDCYSATTCKG	160						

	170	180	190	200	210	220	230
Clone 10	GTCGTCAAGGTTGGGATATTGACTCCATTTTAACTCTCATCCTCGTCTTTCTATTGGTACGTCCCCACCAAGCT	236					
Clone 1	GTCGTCAAGGTTGGGATATTGACTCCATTTTAACTCTCATCCTCGTCTTTCTATTGGTACGTCCCCACCAAGCT	236					
Clone 4	GTCGTCAAGGTTGGGATATTGACTCCATTTTAACTCTCATCCTCGTCTTTCTATTGGTACGTCCCCACCAAGCT	236					
Clone 5	CGCAACACAATTGGGATGTAAACGTCGTTTACAATGCACATCTGAGGCTCTCGATTGGTACGTCCCCACCAAGCT	236					
Clone 8	AGCAGCACAAATTGGGATGTGGATGCCGTTTTCAATTCACATCTGAGGATCTCGATTGGTACGTCCCCACCAAGCT	236					
Clone 9	CGCAGCACAAATTGGGATGTGGATGCCGTTTTCAATTCACATCCGAGGTTTCGATTGGTACGTCCCCACCAAGCT	236					
Consensus	VKCRDCAMRRRTGGGATRTDRAYKYCRFTTWYAAKYKWCATCYBMCKHTKCTCKATTGGTACGTCCCCACCAAGCT	236					

Specific ABLFY primers

ABLFY F

edited9f.seq (1>246) -> TC--CCGGATTATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited8f.seq (1>244) -> GN--CC--ATTTATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited5f.seq (1>244) -> GTCC--ATTTATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 EDITED~1.SEQ (1>243) -> TCC--ATTTATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited5r.seq (1>240) -> ATTTATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited1r.seq (1>237) -> TATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited4f.seq (1>237) -> TATATCAACAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited4r.seq (1>237) -> TATATCAACAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited8r.seq (1>237) -> TATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 EDITED~2.SEQ (1>237) -> TATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited1f.seq (1>237) -> TATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA
 edited9r.seq (1>237) -> TATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA

Kcgtcc--ATTTATATCAATAAGCCAAAGATGAGCAATATGTCCATTGGCTACGCTTTCATTGGCTGGATGAAGAAACA

edited9f.seq (1>246) -> TCATAAGCACTCTATTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited8f.seq (1>244) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited5f.seq (1>244) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 EDITED~1.SEQ (1>243) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited5r.seq (1>240) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited1r.seq (1>237) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited4f.seq (1>237) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited4r.seq (1>237) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited8r.seq (1>237) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 EDITED~2.SEQ (1>237) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited1f.seq (1>237) -> TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT
 edited9r.seq (1>237) -> TCATAAGCACTCTATTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT

TCATAAGCACTCCGTTGATACTACAGCAACGAGGAGAAATGTGGAGATGGAGCAAGCTTGTTACCATATAGT

edited9f.seq (1>246) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited8f.seq (1>244) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited5f.seq (1>244) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 EDITED~1.SEQ (1>243) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited5r.seq (1>240) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited1r.seq (1>237) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited4f.seq (1>237) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited4r.seq (1>237) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 EDITED~2.SEQ (1>237) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited1f.seq (1>237) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA
 edited9r.seq (1>237) -> GGTGATTTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA

GGTATTCGGGCGAGCACAATAGGGATGTGATGCCCTTTTAAATCAGATCGAGGTTTCATTGGTACGTCCGCA

edited9f.seq (1>246) -> CCAAGCTA
 edited8f.seq (1>244) -> CCAAGCTA
 edited5f.seq (1>244) -> CCAAGCTA
 EDITED~1.SEQ (1>243) -> CCAAGCTA
 edited5r.seq (1>240) -> CCAAGCTA
 edited1r.seq (1>237) -> CCAAGCTA
 edited4f.seq (1>237) -> CCAAGCTA
 edited4r.seq (1>237) -> CCAAGCTA
 edited8r.seq (1>237) -> CCAAGCTA
 EDITED~2.SEQ (1>237) -> CCAAGCTA
 edited1f.seq (1>237) -> CCAAGCTA
 edited9r.seq (1>237) -> CCAAGCTA

ABLFY R

