# THE ROLE OF POLYAMINE CATABOLISM IN *ARABIDOPSIS THALIANA* DEVELOPMENT AND SENESCENCE

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- *In the Name of God the Most Merciful and the Most Compassionate* -

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*This thesis is dedicated to:*

*The soul of My Father*

*For his love, support and encouragement in the years of my life.*

*And to My Mother*

*For her endless love, sacrifice, encouragement, support and for being my first teacher in this life.*

## Abstract

Plant polyamines (PAs) are involved in several physiological processes and their application delays senescence. PA catabolism via amine oxidase (AO) activity is essential in regulating PA levels and generating compounds essential in the physiological interactions during development and stresses. AOs include polyamine oxidases (PAOs) and copper amine oxidases (CuAOs). In Arabidopsis, CuAOs are encoded by a gene family of ten members.

The expression pattern of each *AtCuAO* gene was investigated in individual Arabidopsis leaves at four developmental stages using real-time PCR (qRT-PCR). Seven members of the family showed distinct patterns of expression during leaf development and senescence. These can be divided into two groups: the first is highly expressed at early stages whereas transcripts of the other group, which includes *AtCuAO4*, reached maximum levels at senescence. Growth and senescence of three *AtCuAO4* over-expression lines and two *AtCuAO4* mutants were compared to wild-type (WT) under optimal conditions. Results demonstrated a clear phenotypic response to *AtCuAO4* mutation represented by late flowering and delayed senescence, although dark-induced senescence was unaffected. Since previous studies on gibberellic acid (GA)-deficient mutants showed a delay in flowering, mutant lines were treated with gibberellin, which rescued the mutant phenotype. Furthermore, the levels of some GA biosynthetic and flowering transcripts (by qRT-PCR) were lower in the mutants than in WT. Content of PAs both pre-bolting and post-bolting was altered in *AtCuAO4* mutation.

To avoid functional redundancy between *AtCuAOs*, two different artificial microRNA (amiRNA) clones were transformed into Arabidopsis. The amiRNAs were predicted to silence several *AtCuAOs* simultaneously. Artificial microRNA plants showed a wide range of phenotypic variations indicating the potential value of this approach for investigating the function of *AtCuAOs*.

Overall, this work suggests the possibility of partial functional redundancy between *AtCuAOs*, an indirect role of these genes in senescence retardation and a link between PAs and GA signalling.

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# List of Acronyms and Abbreviations

| *Abbreviation* | *Name or term in full* |
|---|---|
| **4-MUG** | 4-Mu-β-D-GLUCURONIDE |
| **ABA** | Abscisic acid |
| **ADC** | Arginine decarboxylase |
| **AOs** | Amine oxidases |
| **bp** | base pairs |
| ***AtCuAO3*-SP** | *AtCuAO3* with short protein |
| **BASTA** | Glufosinate-ammonium PESTANAL® |
| **BR** | Brassinosteroid |
| **Cad** | Cadaverine |
| **cDNA** | Complementary deoxyribonucleic acid |
| **chl** | Chlorophyll |
| **CuAOs** | Copper binding diamine oxidase |
| **DAP** | 1,3-diaminopropane |
| **DIA** | Diamino heptane |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxy-ribonucleotide triphosphate |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EtBr** | Ethidium bromide |
| **FW** | Fresh weight |
| **gDNA** | Genomic DNA |
| **GABA** | $\gamma$-aminobutyric acid |
| **GA** | Gibberellic acid |
| **Gent** | Gentimycin |
| **$H_2O_2$** | Hydrogen peroxide |
| **HPLC** | High performance liquid chromatography |
| **JA** | Jasmonic acid |
| **Kan** | Kanamycin |
| **Kb** | Kilo base |
| **LB** | Lysogeny broth medium |
| **LD** | Long-day conditions |
| **MeJA** | Methyl jasmonate |
| **MS** | Murashige and Skoog basal salt |
| **MU** | 4-Methylumbelliferone |
| **n** | Number of replicates |

| *Abbreviation* | *Name or term in full* |
|---|---|
| **NaCl** | Sodium chloride |
| **$Na_2CO_3$** | Sodium carbonate |
| **ODC** | Ornithine decarboxylase |
| **ORF** | Open reading frame |
| **PA** | Polyamine |
| **PAO** | Polyamine oxidase |
| **PCA** | Perchloric acid |
| **PCD** | Programmed cell death |
| **PCR** | Polymerase chain reaction |
| **Put** | Putrescine |
| **RNA** | Ribonucleic acid |
| **Rif** | Rifampicin |
| **RT** | Room temperature |
| **qRT-PCR** | The real-time quantitative reverse transcriptase PCR |
| **rpm** | Revolutions per minute |
| **SA** | Salicylic acid |
| **SAM** | Shoot apical meristem or *S*-adenosylmethionine (context specific) |
| **SAMDC** | *S*-adenosylmethionine decarboxylase |
| **SD** | Short-day conditions |
| **SDW** | Sterile distilled water |
| **SE** | Standard error |
| **Spd** | Spermidine |
| **SPDS** | Spermidine synthase |
| **Spec** | Spectinomycin |
| **Spm** | Spermine |
| **SPMS** | Spermine synthase |
| **TAE** | Tris-acetate-EDTA |
| **T-DNA** | Transferred deoxyribonucleic acid |
| **UTR** | Untranslated region |
| **WT** | Wild type |
| **X-GlcA** | 5-bromo-4chloro-3-indolyl-β-D-glucuronic acid (cyclohexyl ammonium salt) |

# Chapter One

## Introduction

# 1. General Introduction

Developmental senescence in old leaves is a genetically programmed process that aims to support actively growing parts of the plant through nutrient re-mobilization from the dying leaves (Hortensteiner and Feller 2002). During senescence, changes in metabolism are fundamental to enhance plant growth and reproduction and the ageing leaves must remain alive until nutrient transportation is complete (Buchanan-Wollaston *et al.* 2005). Leaf senescence can be initiated by several factors either external such as biotic and environmental stress conditions or internal such as phytohormones, metabolic compounds and developmental age (Guo and Gan 2012). In general, increasing understanding of the metabolism and regulation of this critical developmental process either pre- or post-harvest may contribute to reduction in deterioration rate of the harvested products which in turn leads to higher quality and lifespan extension of crop plants as well as consumer satisfaction (Hewett 2006). Exogenous treatment of plants with growth regulators and manipulation of endogenous growth regulator levels contribute to the reduction of the effects of stress on crop plants, and/or delay senescence through controlling the physiological and biochemical processes (Peleg and Blumwald 2011).

Polyamines (PAs) play a well-established role in the response of plants to different stresses and are involved in a wide range of crucial cellular processes during growth and development under stress or non-stress growth conditions (Alcazar *et al.* 2006). Moreover in many plant species PA application delays ageing and senescence by inducing DNA synthesis and mitotic activity and by inhibiting the rise in protease, RNase, and peroxidase activities (Dumbroff 1990). Hence, PAs can have a fundamental role in expanding the postharvest shelf life of vegetables and fruits due to their properties in maintaining membrane stability and homeostasis which are essential for different biochemical and physiological processes (Paliyath et al. 2009).

PA catabolism is essential in regulating PA concentrations, controlling physiological processes in cells and in response to abiotic and biotic stresses (Angelini *et al.* 2010; Cona *et al.* 2006; Wimalasekera *et al.* 2011b). However, the exact roles of PAs in both delaying senescence and plant response to stresses remain unclear (Alcazar et al. 2006; Moschou

*et al.* 2012). In plants, degradation of PAs via the action of CuAOs contributes to the production of alkaloids (Kuznetsov and Shevyakova 2007; Moschou et al. 2012), but regulation of their expression has not been fully investigated.

## 1.1. Polyamines

Polyamines (PAs) are nitrogenous molecules that are ubiquitous in a wide range of living organisms (Anjum *et al.* 2014; Guo and Gan 2005). The first discovered PA was spermine (Spm), as crystals in human semen, upon microscopic examination by Anton Van Leeuwenhoek in 1678, while putrescine (Put) was found two centuries later and eventually spermidine (Spd) was discovered in 1927 (Mattoo *et al.* 2015). In the early twentieth century, PA chemical structure and biosynthesis pathways were determined (Wallace 2009). PAs have a low-molecular weight and an organic polycationic nature which facilitates their interaction with large anionic molecules such as RNA, DNA, protein and phospholipids, and negatively charged groups in membranes (Kuznetsov and Shevyakova 2007). These interactions lead to the stabilisation and regulation of the function of DNA, tRNA, membranes, some proteins such as enzymes as well as the regulation of ion channels (Bachrach 2005).

The most common PAs in plants are the tri-amine Spd, the tetra-amine Spm and their di-amine precursor Put (Bouchereau *et al.* 1999), which represent the main sinks of assimilated nitrogen due to their high intracellular concentrations (Moschou et al. 2012). The less common PAs are Cadaverine (Cad, present in legumes), diaminopropane DAP, nor-Spd, nor-Spm and thermo-Spm (the common Spm isomer in higher plants) (Gill and Tuteja 2010; Mattoo and Handa 2008). The three common PAs, Put, Spd and Spm, differ in their backbone length and their number of positive charges at cellular physiological pH (Figure 1-1) (Del Duca *et al.* 2014).

Owing to their multiple roles in plant growth and development, polyamines are also regarded as growth regulators (Menéndez *et al.* 2013). Durner (2013) also considered PAs as hormones for a number of reasons: these include their presence in all cells, the clear regulatory control they perform through growth and development, and their efficiency at micro-molar titres.

In plants, PAs are involved in a wide range of crucial cellular processes, for example controlling cell division and elongation, somatic embryogenesis, plant growth, differentiation, development, reproduction and senescence (Galston *et al.* 1997; Kasukabe *et al.* 2004; Tisi *et al.* 2011b; Walden *et al.* 1997). They occur in all parts of the plant cell including the nucleus where they modulate the cell cycle, genome expression and signalling (Kuznetsov and Shevyakova 2007). PAs are also responsible for the agronomically important traits. For example in tomato they affect traits such as fruit quality, phytonutrient content and vine life (Mattoo *et al.* 2006). The influence of PAs as anti-senescence agents may occur through retaining chlorophyll, maintaining the stabilisation of membranes, inhibiting the synthesis of ethylene, preventing the peroxidation of lipid and scavenging free radicals (Wimalasekera *et al.* 2011a). PAs play an important role in the protection of chloroplasts and thereby enhance the ability of plants to repair the damage caused by the environmental stresses (Shu *et al.* 2012b). When the plant is placed under stressful conditions, PAs are produced to enhance its tolerance (Gill and Tuteja 2010), and they play a fundamental role in protecting the plant against these changes in many ways: for example, through osmotic adjustment, maintenance of membrane stability and ROS-scavenging (Bouchereau et al. 1999).

Due to their association with anionic cellular macromolecules, PAs may be present in the plant cells as organic cations associated with other macromolecules or as low molecular weight compounds, and in some cases conjugated to phenols (Galston and Sawhney 1990). PAs are able to bind to other molecules via links of various types and strength including hydrogen bonds (electrostatic linkages) which cause conformational stabilization/destabilization of RNA, DNA, chromatin, and proteins; covalent bonds with hydroxycinnamic acids and photosynthetic complexes; and ionic bonds with negatively charged molecules (Del Duca et al. 2014).

PA concentrations in plants are much higher than any other phytohormones, and vary depending on the plant species, tissue, organ and developmental stage (Kuznetsov and Shevyakova 2007). The titre of PAs is carefully controlled in the cell (Moschou et al. 2012). Because of the wide range of PAs functions in plants, their homeostasis is critical and controlled during cell cycle progression and the processes of the cell division and

expansion (Paschalidis and Roubelakis-Angelakis 2005b). This is done by the regulation of their biosynthesis (Figure 1-1), conjugation, interconversion and their transport (Angelini et al. 2010; Kusano *et al.* 2008), along with their catabolism (Figure 1-2). This occurs through oxidative de-amination interactions at their primary or secondary amino groups by the action of amine oxidase enzymes, which contribute to PA homeostasis maintenance (Angelini et al. 2010; Cona et al. 2006). PA contents may also be affected by surrounding factors and modified as a response to external conditions like temperature and light, in addition to physical and chemical stresses which are able to alter PA titres in the cell (Galston and Sawhney 1990).

### 1.1.1. Polyamine biosynthesis

In plants, the key enzymes that participate in PA biosynthesis include arginine decarboxylase (ADC), ornithine decarboxylase (ODC), spermidine synthase (SPDS), spermine synthase (SPMS), and *S*-adenosylmethionine decarboxylase (SAMDC) (Bagni and Tassoni 2001). At the transcriptional, translational and post-translational levels, the activities of PA biosynthetic enzymes are controlled developmentally and environmentally (Martin-Tanguy 2001).

In most plant species, the first PA produced from the biosynthetic pathway of PAs is Put, which is the important intermediate precursor in the synthesis of higher amines Spd and Spm. Put forms either indirectly from L-arginine by ADC via Agmatine, or directly by the decarboxylation of L-ornithine via the activity of ODC (Figure 1-1) (Michael 2015). However, in Arabidopsis, *ODC* activity is absent and two different genes encoding *ADC* have been described (Hanfrey *et al.* 2001; Soyka and Heyer 1999). Conversion of Put to Spd and Spm needs successive addition of aminopropyl moieties. By sequential addition of an aminopropyl group generates from *S*-adenosylmethionine (SAM) via a reaction catalysed by SAMDC, Put is converted to Spd and the latter to Spm in a reaction catalysed by SPDS and SPMS successively (Figure 1-1) (Marco *et al.* 2011; Tiburcio *et al.* 1990).

In plant cells, PAs occur in different forms: as free (soluble [S-PA]), and/ or soluble-conjugated with small molecules such as phenolic acids (soluble-hydrolysed [SH-PA]),

and/ or insoluble-conjugated with various large molecules such as proteins (pellet-hydrolysed [PH]-PA) forms (Paschalidis and Roubelakis-Angelakis 2005b).

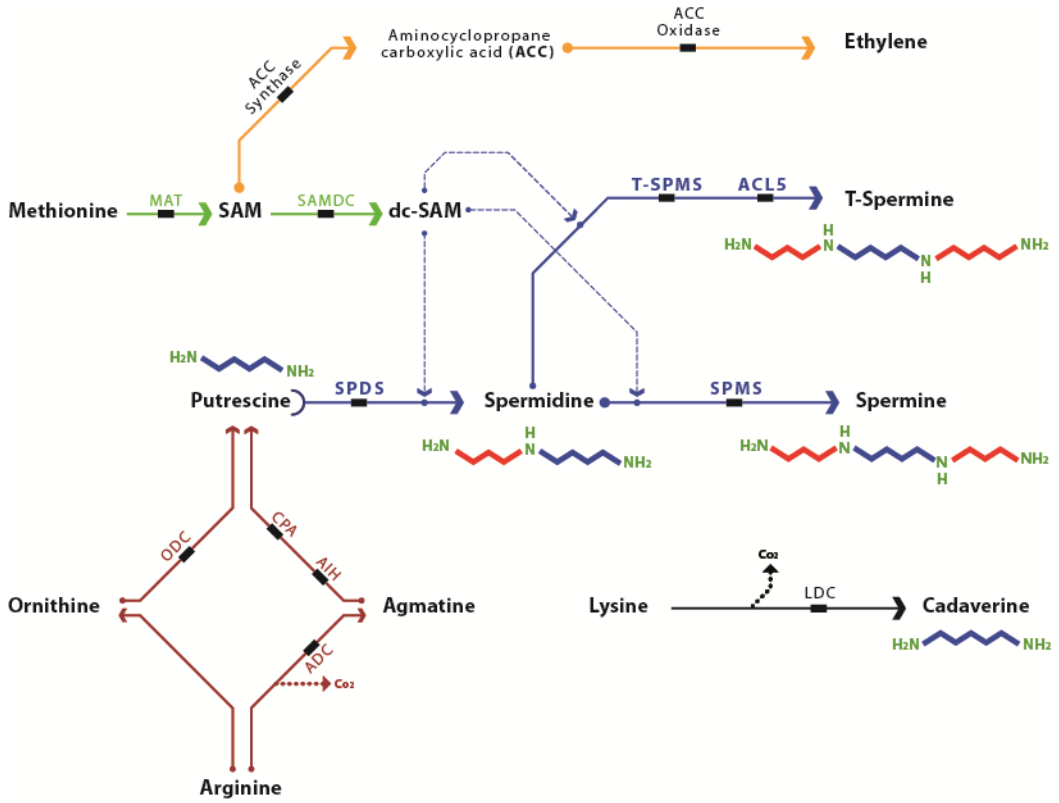



Figure 1-1 Biosynthetic pathways of polyamines in plants. The polyamines putrescine, spermidine, spermine and/or thermo spermine (T-Spermine) are consecutively derived from arginine through agmatine via arginine decarboxylase (ADC), agmatine iminohydrolase (AIH), and N-carbamoylputrescine amidohydrolase (CPA) activity or from ornithine via ornithine decarboxylase (ODC). *S*-adenosylmethionine (SAM) is derived from methionine via methionine adenosyltransferase (MAT) activity and acts as a precursor of decarboxylated *S*-adenosylmethionine (dc-SAM) via *S*-adenosylmethionine decarboxylase (SAMDC) activity or as a producer of ethylene. dc-SAM is utilized as a source of aminopropyl groups in Spd, Spm and T-Spermine biosynthesis in the presence of spermidine synthase (SPDS), spermine synthase (SPMS), and T-spermine synthase (T-SPMS) or acaulis5 (ACL5) respectively. Cadaverine is derived from the amino acid lysine via lysine decarboxylase (LDC) activity. Chemical structure of different PAs is shown in the figure (Kusano et al. 2008; Sobieszczuk-Nowicka *et al.* 2015).

Each PA molecule needs to be targeted to a particular intracellular site and/or a particular conjugated form (Imai *et al.* 2004b). In this regard, it has been found that loss-of-function mutant in Arabidopsis *ACL5*, one of the two spermine synthases, showed a severe defect in stem expansion and exogenous treatment with Spm was not sufficient to reverse stem phenotype of the mutated plants, while mutation of the other spermine synthase, *SPMS*, displayed normal growth (Hanzawa *et al.* 2000). Interestingly, the double mutants affected in both *SPMSs*, *acl5* and *spms*, showed the same stem phenotype observed in

*acl5* mutant. These results suggested that Spm produced through the activity of ACL5 is the only Spm implicated in stem elongation (Imai *et al.* 2004a).

### 1.1.2. Polyamine catabolism

*Amine oxidases* (*AOs*) are widely found in fungi, bacteria, higher plants and animals (Federico and Angelini 1991).

There are two classes of oxidative enzymes in plants which are responsible for the degradation of both exogenous and endogenous PAs (Kuznetsov and Shevyakova 2007). These enzymes can be classified, based on the cofactor involved, into two classes; copper-containing amine oxidases (CuAOs, EC 1.4.3.6), and flavin-containing polyamine oxidases (PAOs, E.C. 1.5.3.11) (Šebela *et al.* 2001). CuAOs are homodimeric enzymes, consisting of 70–90 kD subunits, in which each subunit contains a copper ion and the cofactor 2,4,5-trihydroxyphenylalanine quinone (TPQ) produced from an endogenous tyrosine residue (Angelini et al. 2010; Medda *et al.* 1995).

Plant development and stress responses are diversely affected by PAs depending on the expression levels of *AOs* which alter the PA/ hydrogen peroxide ($H_2O_2$) ratio and lead to different plant responses (Tisi *et al.* 2011a). *AOs* play several pivotal physiological roles in plant growth and development, as well as response to the environmental stresses, whether they be biotic or abiotic (Angelini et al. 2010; Cona et al. 2006; Moschou *et al.* 2008) via regulation of the cellular PA contents and primarily through production of some important compounds generated by AO activity, which are essential in the physiological interactions both during environmental stress and development (Martin-Tanguy 2001; Paschalidis and Roubelakis-Angelakis 2005a). These compounds are amino–aldehydes, 1, 3-diaminopropane, $\gamma$-aminobutyric acid (GABA) and $H_2O_2$ (Angelini et al. 2010).

PAOs oxidize the higher PAs Spd and Spm along with their acetylated derivatives at the secondary amino group (Cona et al. 2006). In contrast, CuAOs are more specific for the di-amines Put and Cad oxidizing them at the primary amino group. The action of CuAOs on Put yields $H_2O_2$, ammonium ($NH_4^+$) and 4-aminobutyraldehyde which then directly cyclizes to $\Delta^1$-pyrroline that is further converted to GABA by the influence of aldehyde

dehydrogenase (Kuznetsov and Shevyakova 2007; Petrivalsky *et al.* 2007) (Figure 1-2). By its transamination and oxidization, GABA can enter the Krebs cycle, which ensures the recycling of nitrogen and carbon from PAs (Wen and Moriguchi 2015).

GABA has a key role in signal transduction in many plants under stress conditions (Moschou et al. 2012). A critical role played by PA catabolism-derived GABA in protecting plants against salinity stress was demonstrated (Xing *et al.* 2007). Furthermore, GABA is an important metabolite that is implicated in various physiological processes such as carbon flow into the citric acid cycle, deterrence of insects, cytosolic pH regulation, and protection against oxidative stress (Bouche and Fromm 2004). On the other hand, $H_2O_2$ is a form of reactive oxygen species and has an established role in stomatal closure (Pei *et al.* 2000; She *et al.* 2004), root development (Dunand *et al.* 2007), and developmental programmed cell death (PCD) (Rogers 2005). PA oxidation-derived $H_2O_2$ has been involved in cell wall lignification and maturation during development, wound-healing, and reinforcement of cell walls during pathogen infection (Cona et al. 2006). As a signal molecule, $H_2O_2$ also mediates the expression of defence genes (Takahashi and Kakehi 2010). A rapid production of nitric oxide (NO) has also been described in Arabidopsis as a result of exogenous PA supplementation (Tun *et al.* 2006), and this reaction was attributed to the activity of the enzyme encoded by the *AtCuAO1* gene (Wimalasekera et al. 2011b).

The copper binding diamine oxidases also have the ability to oxidise Spd and Spm with a much lower affinity than for Put, producing $H_2O_2$ and $NH_4^+$, in addition to the production of 4-aza-8-amino-octan-1-al or 4,9-diaza-dodecan-1,12 dialdehyde respectively (Moschou and Roubelakis-Angelakis 2014; Moschou et al. 2012) (Figure 1-2). The oxidation of Cad by CuAO generates aminoaldehyde which converts into 1-piperideine, and ultimately into alkaloids (Kuznetsov and Shevyakova 2007) (Figure 1-2). There is also a class of CuAOs in plants which preferentially oxidise N–methyl–Put leading to the production of 4–methylaminobutanal which then converts into N-methylpyrrolinium cation, the precursor of alkaloids (Figure 1-2), but this class of enzymes can also oxidise Cad and Put (Moschou et al. 2012).

As enzymes, CuAOs occur at high levels in dicots, particularly in some Fabaceae (such as soybean, chickpea, lentil and pea); in these species CuAOs were detected as the most abundant soluble protein in the extra cellular fluid (Federico and Angelini 1991). As genes, *CuAOs* play an important developmental regulatory function which is species- and tissue-specific, and they have their own pattern of spatial-temporal expression (Cona et al. 2006). They also contribute to plant defence against plant pathogens and environmental factors (Angelini et al. 2010).

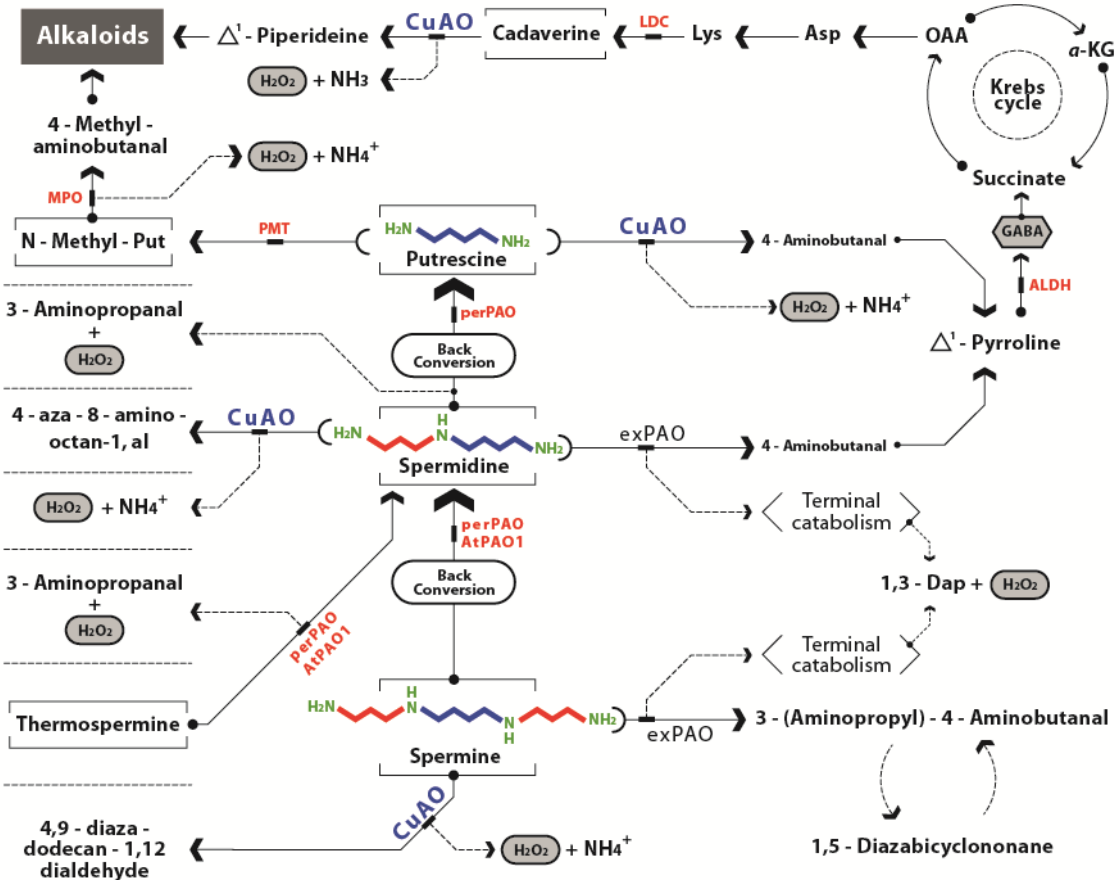



Figure 1-2 The polyamine catabolic pathways. AtPAO1, *Arabidopsis thaliana* polyamine oxidase; CuAO, copper amine oxidase; GABA, γ-aminobutyric acid; ALDH, aldehyde dehydrogenase; α-KG, α-ketoglutarate; LDC, lysine decarboxylase; MPO, N-methyl-putrescine oxidase; OAA, oxaloacetate; PAO, polyamine oxidase (per: peroxisomal, ex: extracellular); 1,3-Dap, 1,3-diaminopropane; PMT, putrescine-N-methyl transferase. Adapted from (Moschou et al. 2012).

In plants, *CuAO* genes have been characterised in only a few species to date including *Arabidopsis thaliana* (Moller and McPherson 1998; Planas-Portell *et al.* 2013) and chickpea (Rea *et al.* 1998). In Arabidopsis, copper-containing amine oxidase genes (*CuAOs*) form a gene family of ten members (Planas-Portell et al. 2013) (TAIR;

https://www.arabidopsis.org/) with different patterns of expression (Table 3-1). Six of the ten *CuAOs* are highly expressed in senescent leaves (eFP Browser; TAIR), although independent expression studies conducted by the PRESTA project (Breeze *et al.* 2011), and in the Cona lab (personal communication) are not always in full agreement (Table 3-1). Recently, it was indicated that only eight of the ten *Arabidopsis thaliana CuAOs* encode functionally active enzymes while the remaining genes (*AtCuAO3*-SP and *AtCuAO9*) are indeed consecutive fragments of the *AtCuAO4* gene, and new names of *AtCuAOs* (Table 1-1) were reported (Tavladoraki *et al.* 2016).

Table 1-1 Copper amine oxidase (CuAO) gene family in *Arabidopsis thaliana* (Planas-Portell et al. 2013; Tavladoraki et al. 2016).

| ***At gene code*** | ***Gene bank accession number*** | ***Gene common name*** | ***Gene new name*** |
|---|---|---|---|
| At4g14940 | NM_117580 | *AtAO1* or *ATAO1* | *AtCuAOβ* |
| At1g62810 | NM_104959 | *AtCuAO1* | *AtCuAOγ1* |
| At4g12270 | NM_117297 | *AtCuAO3*-SP | *AtCuAOε1* |
| At4g12290 | NM_117299 | *AtCuAO4* | *AtCuAOδ* |
| At1g31670 | NM_102902 | *AtCuAO5* | *AtCuAOα1* |
| At1g31710 | NM_102906 | *AtCuAO2* | *AtCuAOα3* |
| At3g43670 | NM_114235 | *AtCuAO7* | *AtCuAOγ2* |
| At1g31690 | NM_102904 | *AtCuAO8* | *AtCuAOα2* |
| At4g12280 | NM_117298 | *AtCuAO9* | *AtCuAOε2* |
| At2g42490 | AY120717 | *AtCuAO3* | *AtCuAOζ* |

Taking into account known important amino acid residues in the deduced amino acid sequences of the four functional AtCuAOs (AtAO1, AtCuAO1, AtCuAO2, AtCuAO3) (Planas-Portell et al. 2013), alignment of the enzymatic domains produced from the eight functionally active *AtCuAO* genes showed a high to full level of sequence conservation in the amino acid residues which are catalytically active in plant CuAOs (Figure 1-3). Furthermore, aspartic acid active site base, tyrosine modified to TPQ, and the copper binding histidine residues (Planas-Portell et al. 2013) are also fully conserved among Arabidopsis AtCuAO enzymes (Figure 1-3). Protein alignment shows that there are very few differences around the catalytic sites suggesting that the enzymes act on the same or very similar substrates. This suggests that the functional differences amongst the genes may be due to the timing of their expression or the localisation of the encoded protein within the cell.

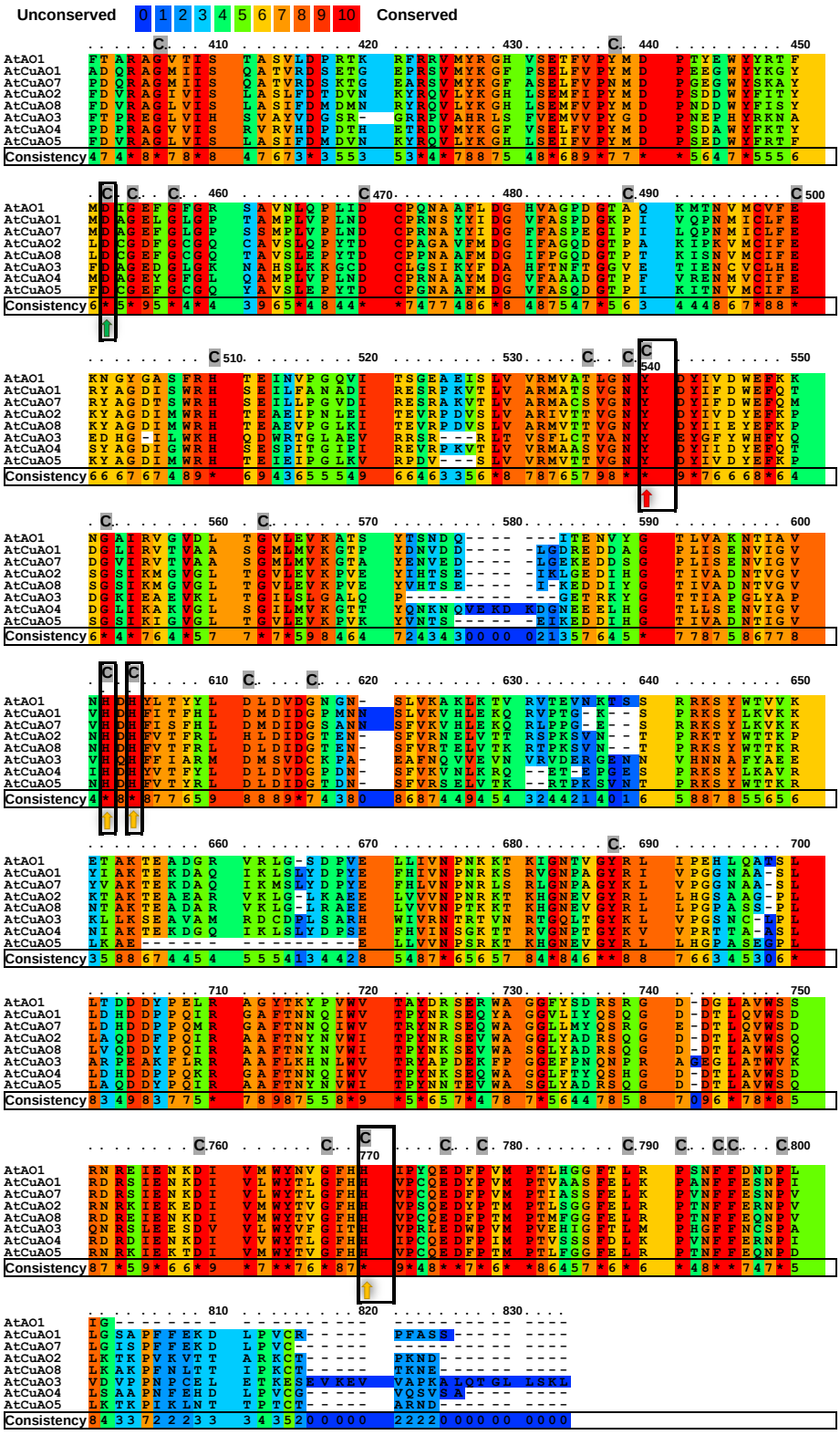



Figure 1-3 Alignment of amino acid sequences of the enzymatic domains of the functionally active AtCuAOs in *Arabidopsis thaliana*. Functional residues are marked with arrows: green for catalytically important residues (D), red for precursor of TPQ cofactor (Y), and yellow for residues involved in copper coordination (H). Totally conserved residues in most of the CuAOs are indicated by grey highlighted C. The conservation scoring was carried out using the multiple sequence alignment program PRALINE (http://www.ibi.vu.nl/ programs/pralinewww/).

During plant development and wound healing, the expression of some *CuAOs* is modulated (Rea et al. 1998; Rea *et al.* 2002). Moreover, *CuAO* expression is up-regulated in response to external stimuli such as hormones and pathogens. For example, methyl jasmonate (MeJA) is a potent promoter of *CuAO* gene expression in chickpea seedlings (Rea et al. 2002), and of soluble and particulate CuAO activities in barley leaves (Walters *et al.* 2002), while abscisic acid (ABA) is able to enhance the expression of *CuAO* in *Vicia faba* (An *et al.* 2008). Recently, a strong *AtAO1* gene expression was detected, by a promoter::*GFP-GUS* fusion, in the protoxylem of the transition, elongation, and maturation regions of *Arabidopsis thaliana* roots suggested the involvement of this gene in the final stages of protoxylem differentiation (Ghuge *et al.* 2015a). Jasmonic acid (JA) acts as a promoter of early vascular tissue differentiation (Cenzano *et al.* 2003), and xylogenesis (Fattorini *et al.* 2009). Treatment of *AtAO1*::*GFP-GUS* Arabidopsis transgenic plant roots with MeJA enhanced *AtAO1* expression in the protoxylem suggesting that AtAO1 plays a role in MeJA signalling leading to protoxylem differentiation (Ghuge et al. 2015a). Treatment of WT Arabidopsis seedlings with MeJA enhanced *AtAO1* expression in roots and led to early protoxylem differentiation, decrease in Put content and $H_2O_2$ accumulation, while *N,N1*-dimethylthiourea, the $H_2O_2$ scavenger, reversed this effect (Ghuge et al. 2015a). Likewise, Put application to WT Arabidopsis and *AtAO1* overexpression induced early protoxylem differentiation and showed higher $H_2O_2$ accumulation in the root zone, and *N,N1*-dimethylthiourea treatment reversed these effects, while *AtAO1* loss of function mutants (*Atao1*) did not response to either MeJA or Put treatments. (Ghuge et al. 2015a). These results suggested that PA-derived $H_2O_2$ via the action of AtAO1 plays a role in MeJA-induced early differentiation of protoxylem in Arabidopsis roots. In addition, Planas-Portell et al. (2013) reported that ABA, SA, MeJA and flagellin are able to up-regulate the expression of Arabidopsis *AtCuAO1* and *AtCuAO3*, whereas *AtCuAO2* transcript accumulation is unaffected by most of these stimuli except the clear induction observed in the expression level as a result of MeJA application and wounding. These results suggested the different role played by these genes in plant reaction to stresses where *AtCuAO1* and *AtCuAO3* are implicated in plant response to abiotic stress while *AtCuAO2* is more involved in plant defence responses against biotic stresses (Planas-Portell et al. 2013; Wimalasekera et al. 2011b). In agreement with this, it has been found previously that interactions between

pathogens and plants, such as interaction of powdery mildew fungus and barley, *Asochyta rabiei* and chickpea or Arabidopsis plants and nematodes, induce the activity of *CuAOs* (Angelini *et al.* 1993; Moller and McPherson 1998; Walters et al. 2002). An up-regulation in the mRNA of the two *AOs*, *CuAO* and *PAO*, has been reported during *Hordeum vulgare* leaf ageing indicating that the internal PA pool is also subjected to regulation in plant senescent cells (Ioannidis *et al.* 2014).

### 1.1.3. Interaction between polyamines and other phytohormones

Along with their interaction with other anionic macromolecules in the cell, the cross talk between PAs and almost all the major plant hormones plays an important role in performing the biological activities of PAs. In this regard, it has been reported that treatment with different phytohormones including ABA, cytokinins, or MeJA may affect PA metabolism and thus their homeostasis by altering the expression of genes responsible for polyamine biosynthesis and/or catabolism (Anwar *et al.* 2015). For example, ABA treatment enhanced the transcript of *SPDS3*, encoding spermidine synthase, in Arabidopsis (Hanzawa *et al.* 2002), and with stressors such as dehydration and high salt ABA stimulated *AtADC2* expression leading to Put accumulation and a decrease in Spd (Urano *et al.* 2003). Similar trend in PA homeostasis was found as a result of treatment of etiolated cucumber cotyledons with kinetin which stimulated PAO and down-regulated SAMDC activities (Sobieszczuk-Nowicka *et al.* 2007). Treatment of tobacco explants with MeJA up-regulated expression of *ODC*, *ADC*, and *SAMDC*, increased conjugation and oxidation of PAs and significantly inhibited organogenesis (Biondi *et al.* 2001).

Conversely, PAs are able to affect the biosynthesis of other hormones: the production of cytokinin and ABA is induced by PA treatments (Cuevas *et al.* 2009; Cui *et al.* 2010; Wang *et al.* 2009b), whereas the biosynthesis of ethylene and gibberellin (GA) is reduced in response to PAs (Alcazar *et al.* 2005; Hu *et al.* 2006). Put accumulation in the transgenic *Arabidopsis thaliana* plants 35S:*AtADC2* by constitutive overexpression of *ADC2*, one of the two genes encoding arginine decarboxylase in Arabidopsis, perturbed GA metabolism through down-regulating *AtGA20ox1* and *AtGA3ox1,3* expressions which led to plant dwarfism and late flowering (Alcazar et al. 2005). GA is involved in

several processes of plant growth and development including stem and hypocotyl elongation and leaf expansion (Matsuoka 2003). It is known in plants that GA enhances PA levels (Durner 2013). Stimulation of elongation of dwarf pea internode by GA is accompanied by an increase in ADC activity and thus PA concentrations, and primarily this elongation was due to increased cell division rather than cell elongation (Smith *et al.* 1985). Likewise, an increase in PA contents was associated with GA-induced α-Amylase activity in germinating barley seeds (Lin 1984). During early development of parthenocarpic fruit induced by auxins and gibberellins, expression of *SPDS* and *ODC* genes is induced and a transient increase in the activity of ADC and ODC and thus in the amount of free PAs has been recorded and was accompanied by a decrease in conjugated PAs (Alabadí and Carbonell 1998).

## 1.2. Leaf Senescence

Leaf senescence can be defined as a highly regulated process that eventually leads to cell death either in a particular organ or in the whole plant, and aims to replace the senescent organ with a younger and physiologically more active one (Krupinska and Humbeck 2008; Lim *et al.* 2007).

Environmental factors and various internal signals can regulate leaf senescence (Guo and Gan 2012) (Figure 1-4). Internally, plant hormones such as ethylene, Abscisic acid (ABA), brassinosteroid (BR), salicylic acid (SA) and JA are able to promote leaf senescence whereas this phenomenon is inhibited by cytokinins, PA, NO and gibberellins (Gan 2010; Khan *et al.* 2014). In addition, leaf senescence is markedly affected by exposing plants exogenously to some hormones such as ABA, SA, and JA which can promote leaf senescence (He *et al.* 2002; Morris *et al.* 2000; Zeevaart and Creelman 1988), whereas Spm retards this phenomenon (Serafini-Fracassini *et al.* 2002). Generally, exposing plants to environmental stresses that negatively affect plant growth and development, such as drought, radiation, extreme temperatures and light, pathogen infection, nutrient deficiency, presence of toxic chemicals in the surrounding environment and flooding, accelerates leaf senescence (Lers 2007). Globally, premature senescence which is induced by exposing plants to stresses is one of the major causes of crop losses as it can reduce plant growth and productivity (Mahajan and Tuteja 2005).

Transcriptome studies on Arabidopsis revealed that thousands of genes are down- or up-regulated during developmental or dark-induced leaf senescence (Buchanan-Wollaston et al. 2005; Lin and Wu 2004; van der Graaff *et al.* 2006).

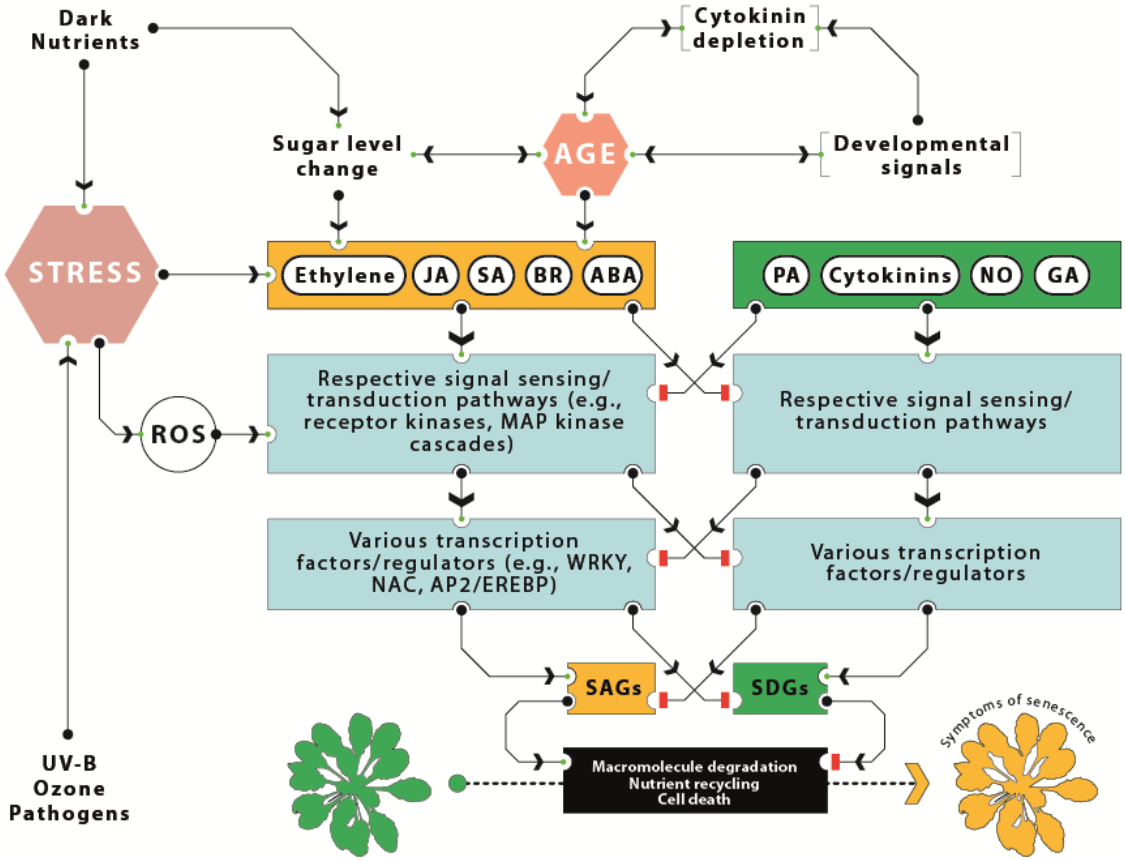



Figure 1-4 The molecular regulatory mechanisms by which external or internal signals induce or delay leaf senescence. Developmental signals like leaf position on the plant and the growth stage are able to initiate leaf senescence. Developmental senescence might be controlled by signals of photosynthetic status, sugar levels and/ or changes in cytokinin levels. Environmental factors that cause oxidative stress such as ozone or UVB irradiation or nutrient stress due to deficiency in nitrogen, water, etc are able to promote precocious senescence. Senescence inducing signals can achieve their influence via activating the expression of senescence associated genes (*SAGs*) and by inhibiting the expression of senescence down-regulated genes (*SDGs*). On the contrary, suppressing SAGs and activating SDGs lead to retardation in leaf senescence. Jasmonic acid (JA); salicylic acid (SA); brassinosteroid (BR); abscisic acid (ABA); polyamine (PA); nitric oxide (NO); gibberellin (GA). Pointed arrows indicate activation, blunt-end arrows indicate repression or inhibition. Adapted from Buchanan-Wollaston et al. (2003) and Guo and Gan (2005).

In old leaves, many changes in metabolism and gene expression occur due to the action of developmental senescence processes which are tightly regulated and genetically controlled (Lim et al. 2007). During developmental senescence, different leaves in an Arabidopsis rosette senesce at a different time and there are also differences in cell

senescence stages within an individual leaf. Studying developmental senescence is also inherently difficult due to the length of the experiments and requirement for tight environmental controls to avoid stress-induction. Various methods such as dark-induced senescence have been therefore used in order to induce senescence artificially and obtain a more synchronous process (Buchanan-Wollaston et al. 2005).

In a comparison between developmental senescence and senescence enhanced by darkness Buchanan-Wollaston et al. (2005) revealed that in both types of senescence, cellular constituents are dismantled and macromolecules such as nucleic acids, proteins, and lipids are degraded through up-regulation of many genes encoding enzymes implicated in these processes. However in developmental senescence photosynthesis persists at a reduced rate to provide energy for senescence progression and to deal with oxidative stress resulting from catabolism processes. In addition there is a high expression of flavonoid synthesis genes. In dark-promoted senescence, however, a rapid reduction in sugar levels could be the main signal for the process leading to degradation of lipids which represent an energy source in this case. Nevertheless, dark-induced senescence can be a useful model as it eliminates other confounding factors such as bolting and flowering.

Senescence associated genes (*SAGs*), chlorophyll loss, and yellowing in detached leaves are all induced by incubation in darkness (Weaver and Amasino 2001). Previous studies investigated the possibility of PA involvement in regulating dark induced senescence. In this regard, Legocka and Zajchert (1999) reported that incubation of barley leaf discs in the dark led to a massive accumulation of the polyamine Put, but this was associated with chlorophyll decline, increase in RNase activity and a rapid senescence. These reactions to darkness were reversible by exogenous Spd treatment which prevented the degradation of thylakoid membranes during senescence by enhancing their stabilization through the direct interaction with them (Legocka and Zajchert 1999). The concentration of exogenous PA applied also appears to influence the result. Spm at low concentrations (1-10 mM) was more active than other PAs in preventing chlorophyll degradation in detached leaves of oat in darkness (Kaur-Sawhney and Galston 1979).

The role of PAs in leaf senescence retardation might be through blocking the conversion of SAM to ACC (aminocyclopropane carboxylic acid) and of ACC to ethylene, a promoter of leaf senescence (Kaur-Sawhney et al. 2003), or by inducing the synthesis of the DNA and mitotic activity as well as inhibiting the rise in proteases, peroxidases, and RNases (Dumbroff 1990). However, quantitation of levels of various PAs during dark induced senescence of detached rice leaves suggested that the endogenous PAs may not play an important role in the control of dark-induced leaf senescence (Chen and Kao 1991). Recently, Sobieszczuk-Nowicka et al. (2015) investigated the involvement of PA metabolism in dark-induced senescence in barley *Hordeum vulgare* L leaves and they reported an enhancement in the expression of genes implicated in both pathways of PA metabolism, the anabolic and the catabolic, as well as an increase in the activity of enzymes implicated in the two pathways, indicating that the internal PA pool is subjected to regulation during senescence in barley. These results underline the contradictory effects of PAs in different plants.

Leaf senescence is of relevance to the storage of leafy vegetables. Factors which contribute to consumer attraction include leaf size uniformity, fresh appearance, colour and form, characteristic flavour and aroma, and lack of undesirable defects such as decay or yellowing (Cantwell and Reid 1993). Degradative processes taking place in leafy products exhibit many similarities to the events that take place during developmental leaf senescence where the most obvious visual change is chlorophyll degradation accompanied by protein and lipid loss and which eventually leads to cell death (Page *et al.* 2001). Exogenous signals such as nutrient availability, water deficit, and light intensity induce leaf senescence (Buchanan-Wollaston et al. 2003; Dangl *et al.* 2000; Lim *et al.* 2003; Lin and Wu 2004; Yoshida 2003) and typical post-harvest storage conditions for leafy vegetables include low light/dark, dehydration and lack of nutrients. Moreover, leafy products senesce rapidly at higher temperatures (Paull 1992), whereas visible senescence in broccoli is markedly retarded at low temperature (Page et al. 2001). Hence, a better understanding of the physiological processes underlying post-harvest leaf senescence is of direct relevance in controlling quality of leafy vegetables to increase shelf-life and reduce waste.

## 1.3. Regulation of flowering in Arabidopsis and PA involvement

Transition from vegetative growth to reproductive development is a result of responses to different endogenous and external signals that later integrated leading to flowering (Srikanth and Schmid 2011). Internally, this process is controlled by a complex network of genes (Quesada *et al.* 2004), as well as alteration in hormonal balance including gibberellins (Mouradov *et al.* 2002) and polyamines (Applewhite *et al.* 2000).

### 1.3.1. Gene regulation of flowering

In flowering plants, switching from vegetative to the flowering phase is the main developmental switch and is fundamental for successful sexual reproduction (Weingartner *et al.* 2011). This transition needs reprogramming of lateral primordia at the shoot apical meristem to transform the vegetative meristem into the inflorescence meristem which leads to the production of flowers instead of leaves (Hempel and Feldman 1995). The timing of floral induction is regulated by a complex network of genes that integrate the developmental and environmental cues (Amasino 2010). In *Arabidopsis thaliana*, the isolation of numerous loss-of-function mutants and the completion of the sequencing of the *A. thaliana* genome led to the identification of more than 180 genes involved in the control of flowering time (Fornara *et al.* 2010).

During the floral induction of *Arabidopsis thaliana*, five distinct genetic pathways regulate flowering (Figure 1-5): the photoperiod and vernalisation pathways control flowering in response to environmental signals, whereas the age, autonomous, and gibberellic acid (GA) pathways act more independently of environmental stimuli (Yeap *et al.* 2014). The core gene regulatory network of flowering time integration in *Arabidopsis thaliana* is composed of eight genes (Figure 1-5): *FLOWERING LOCUS C* (*FLC*), *SHORT VEGETATIVE PHASE* (*SVP*), *FLOWERING LOCUS T* (FT), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *AGAMOUS LIKE 24* (*AGL24*), *APETALA1* (*AP1*), *LEAFY* (*LFY*) and *FD* (Valentim *et al.* 2015).

The floral induction signals from photoperiod, vernalisation, autonomous, and GA-induced pathways are transmitted to two major regulators of flowering that

antagonistically regulate flowering, *FLC* and *CONSTANS* (*CO*) (Putterill *et al.* 1995; Samach *et al.* 2000). The *FLC* gene mediates the vernalisation and autonomous pathways and acts as a repressor of flowering (Kim and Sung 2014; Searle *et al.* 2006).

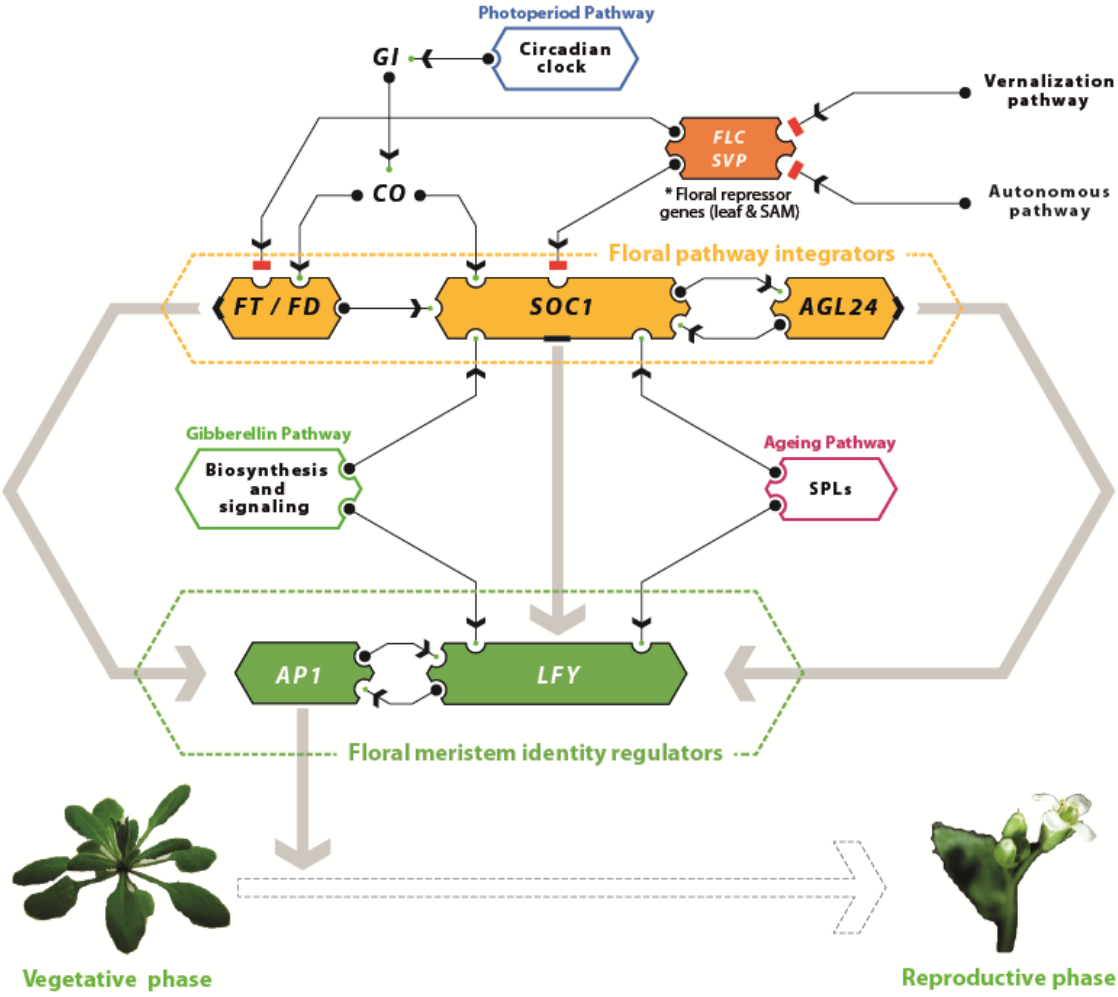



Figure 1-5 Simple model of the major genetic pathways regulating time of flowering in *Arabidopsis thaliana*. Five major signalling pathways control the switch from vegetative phase to flowering phase: autonomous, vernalisation, photoperiod, gibberellin and aging. The vernalisation and autonomous pathways repress the activity of *FLC*. The circadian clock regulates the transcription of the *GI* and *CO* genes, whereas light quality regulates CO protein abundance. In the photoperiod pathway, CO stimulates the expression of the floral integrators, *FT* and *SOC1* whereas the floral repressor *FLC* suppresses their expression. *LFY* is regulated mainly by the gibberellin pathway and acts as a regulator of the floral meristem identity genes. The aging pathway represses the flowering repressors (miR-156) and controls both the floral integrators and the regulators of the meristem identity. (*SPLs*) *SQUAMOSA PROMOTER BINDING LIKE*, (*FT*) *FLOWERING LOCUS T*, (*SOC1*) *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*, (*LFY*) *LEAFY*, (*FLC*) *FLOWERING LOCUS C*, (*SVP*) *SHORT VEGETATIVE PHASE*, (*AP1*) the MADS box factor *APETALA1*, (SAM) shoot apical meristem, (*AGL24*) *AGAMOUS LIKE 24*. Pointed arrows indicate activation, blunt-end arrows indicate repression or inhibition (Fornara et al. 2010; Kim and Sung 2014; Valentim et al. 2015).

*FLC* and *SVP* are expressed in leaves where they repress the expression of *FT* (Tao *et al.* 2012), and they are also expressed in the shoot apical meristem (SAM) where they repress the expression of *SOC1* (Deng *et al.* 2011). The *CO* gene, in contrast, mediates the photoperiodic pathway and acts as a floral activator (Suárez-López *et al.* 2001).

Both the *FLC* and the *CO* genes regulate the expression of floral pathway genes, *FT*, *SOC1* and *LFY*, that integrate signals from different flowering pathways and the level of their expression determine the exact time of flowering (Parcy 2005; Simpson and Dean 2002). *FLC* enhances the response of plant flowering to the environmental cues through suppressing flowering under non-inductive conditions, and the level of its mRNA is reduced in response to winter cold (vernalisation) which allows flowering to proceed (Michaels and Amasino 1999; Sheldon *et al.* 1999).Both floral integrators, *SOC1* and *FT*, are negatively controlled by *FLC*, but are the immediate targets of the transcription factor *CO* (Moon *et al.* 2005).

In Arabidopsis, the FT protein moves from the leaf phloem, where it is expressed, to the SAM where it forms a complex with the FD protein and directly promotes transcription of the floral integrator *SOC1* (Yoo *et al.* 2005) and of the downstream floral meristem identity gene *AP1* (Corbesier *et al.* 2007).

The expression of *AP1* is used as a marker to indicate the floral transition (Valentim et al. 2015). The initiation of flower development stage, through the direct positive feedback interaction between *AP1* and *LFY* (Valentim et al. 2015), terminates the process of floral induction, and by the time *AP1* is expressed, plants continue to flower autonomously of environmental signals, such as day length and light quality (Hempel *et al.* 1997). *LFY* is under a direct control of *SOC1* (Valentim et al. 2015), in addition to its regulation by the GA pathways and long days (Blázquez and Weigel 2000), and in contrast to *SOC1* and *FT*, it is not an immediate target of *CO* (Samach et al. 2000). *AGL24* is up-regulated via vernalisation and exists in leaf, SAM and flower bud tissue (Liu *et al.* 2008; Michaels *et al.* 2003). *AGL24* promotes flowering by direct induction of *SOC1* gene (Liu et al. 2008), and by activating the expression of *LFY* in response to inductive long day photoperiod (Lee *et al.* 2008).

On the other hand, the concentrations of the transcription factors *SQUAMOSA PROMOTER BINDING LIKE* (*SPLs*) increase with age (Wang *et al.* 2009a). The microRNA miR156 negatively regulates SPL proteins (Wang et al. 2009a). During the juvenile stage, the cellular level of miR156 is high and progressively decreases as the plant ages. Its overexpression, which reduces *SPL* expression, considerably delays flowering (Wu and Poethig 2006). SPLs promote flowering by initiating the expression of the floral integrator gene *SOC1* and the floral meristem identity genes *LFY* and *AP1* (Wang et al. 2009a; Yamaguchi *et al.* 2009).

Construction of the triple mutant *co-2 fca-1 ga1-3*, the genes that act in the long-day, autonomous and gibberellin pathways respectively, resulted in non-flowering plants under short or long day conditions, indicating that these three pathways are essential in promoting flowering under either short or long days, however, vernalisation rescued these triple mutant plants and promoted their flowering indicating the critical role of the vernalisation pathway in plant flowering even when these three pathways, long-day, autonomous and GA, are absent (Reeves and Coupland 2001).

In Arabidopsis, the floral development gene *SUPERMAN* (*SUP*) is involved in controlling cell proliferation in carpel and stamen primordia and in ovules (Ito *et al.* 2003), and in maintaining the stamen/carpel whorl boundary (Sakai *et al.* 2000). In the floral meristem, the expression of *SUP* is regulated by the floral meristem identity genes and by the floral organ identity genes (Sakai et al. 2000). Mutation of the Arabidopsis *SUP* resulted in flowers with increased number of stamens and reduced carpels as a consequence of the ectopic expression of the floral homeotic gene *APETALA3* (*AP3*) (Gaiser *et al.* 1995).

### 1.3.2. Hormonal regulation of flowering

Various hormones (GAs, ABA, BRs) play a key role in the regulation of the floral transition (Domagalska *et al.* 2010), and alteration in the balance of hormones affects flowering time (Blázquez *et al.* 2001).

In plants, GA promotes bolting, seed germination, leaf expansion, stem elongation, trichome development, pollen maturation, and development of fruit and seed (Achard and Genschik 2009; Sun 2008). Thus the phytohormone GA regulates the development and fertility of Arabidopsis flowers (Cheng *et al.* 2004), in addition to its prominent role in regulating the timing of the floral transition (Richards *et al.* 2001).

The mature flowers of GA-deficient mutants exhibited impaired floral development represented in reduced elongation growth of stamens and petals, and a block in anther development leading to male sterility due to the lack of mature pollen (Goto and Pharis 1999; Wilson *et al.* 1992).

In addition to their influence on Arabidopsis flowering time, mutations that disrupted GA pathways also affected other plant growth and development aspects including germination of seed, elongation of stem, and the floral development (Wilson et al. 1992). Furthermore, mutants with a defect in GA biosynthesis such as *ga1-ga5* showed clear alterations in plant phenotypes such as dark green leaves, retardation in the vegetative growth of shoots (dwarfism), and late flowering which are rescued by exogenous treatment with GA (Koornneef and Van der Veen 1980; Peng and Harberd 1997). GA deficient mutants also produced rosette leaves with a reduced size indicating the important role of GA in promoting leaf expansion which is attributed to its effect on cell proliferation and cell elongation.

The role of GA in promoting Arabidopsis flowering was demonstrated at first by the exogenous application of GA (Langridge 1957; Wilson et al. 1992). Furthermore, mutants that are defective either in GA biosynthesis (*ga1-5, gibberellin deficient*) or in GA signalling (*gai, gibberellin insensitive*) exhibited various defects in plant phenotype including dwarfism and late flowering (Koorneef *et al.* 1985; Koornneef and Van der Veen 1980). The GA pathway is essential in both short and long day conditions, however, the effect of mutating GA biosynthesis on flowering time is stronger under short days (Blázquez *et al.* 1998; Reeves and Coupland 2001; Wilson et al. 1992). In Arabidopsis, GA induces flowering through activation of the floral integrators *SOC1* and *LFY* (Blázquez et al. 1998), while the expression of *FT* is not regulated by the GA pathway

suggesting that both genes, *SOC1* and *FT*, act differently in integrating the flowering process (Moon *et al.* 2003).

Recently, Yamaguchi *et al.* (2014) reported that the switch from vegetative to flowering in Arabidopsis includes two stages: branching of the inflorescence and flowering. GA acts positively, then negatively, in controlling the onset of flower generation in Arabidopsis, indicating that GA enhances the termination of vegetative development as well as inhibiting flower formation. This was illustrated as follows: in the presence of bioactive GA, DELLA proteins (which restrain plant growth by repressing GA-dependent pathways) are degraded, however, activation of the transcription factor *LFY* causes reduction in GA levels by promoting the expression of gibberellin catabolism genes allowing accumulation of DELLA proteins which is required to up-regulate the *AP1* leading to, synergistically with *LFY*, flowering.

### 1.3.3. GA biosynthesis and metabolism

The major pathways for the formation and degradation of the bioactive GAs and the enzymes catalysing these reactions are shown in Figure 1-6. $GA_{12}$ and $GA_{53}$ are substrates for the final stages of GA biosynthesis and their oxidation by the action of GA20-oxidases (GA20ox) produces $GA_9$ and $GA_{20}$ respectively as immediate precursors of active gibberellins. Via GA3-oxidase (GA3ox) activity, the bioactive GAs, $GA_4$ and $GA_7$ are produced from $GA_9$, while the bioactive GAs, $GA_1$ and $GA_3$ are derived from $GA_{20}$ (Hedden and Phillips 2000; Paparelli *et al.* 2013).

GA20ox is the key enzyme in GA biosynthesis pathway and involved in stem elongation and shoot growth (Chiang *et al.* 2015). Plants overexpressing *GA20ox* showed acceleration in flowering time under long and short days (Croker *et al.* 1999). While *GA20ox* and *GA3ox* are involved in the biosynthesis of the bioactive GAs, *GA2-oxidase* (*GA2ox*) deactivates them via oxidation (Yamaguchi 2008). Overexpression of a bean *GA2ox* in Arabidopsis exhibited a range of alterations in plant phenotypes: extreme dwarf phenotype was exhibited by Arabidopsis plants with strong overexpression of the transgene *GA2-oxidase*, whereas plants with lower expression levels of the transgene

showed a semi-dwarf or WT phenotype due to the increased expression of both *GA20ox* and *GA3ox* genes (Hedden and Phillips 2000).

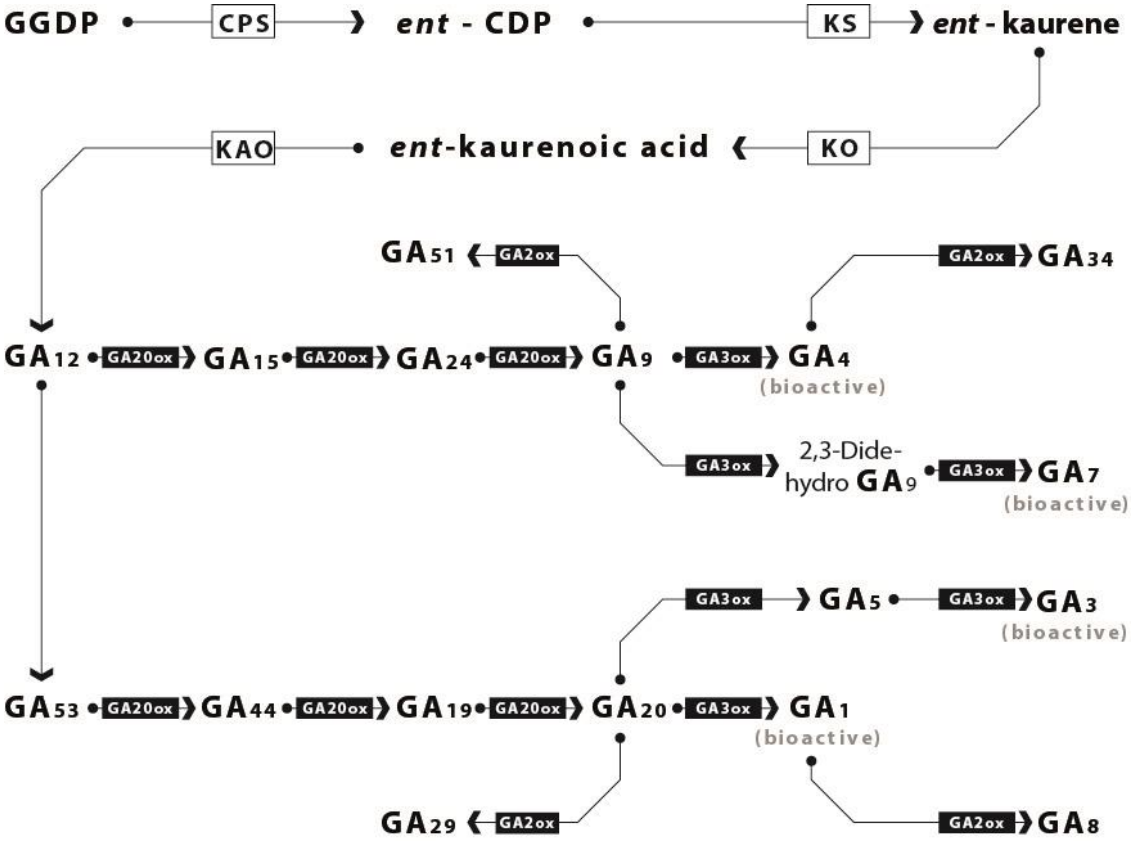



Figure 1-6 Gibberellin biosynthesis and degradation in plants. (GGDP) geranylgeranyl diphosphate, (CDP) *ent*-copalyl diphosphate, (*CPS*, *AtCPS1*) *ent*-copalyl diphosphate synthase, (*KS*, *AtKS1*) *ent*-kaurene synthase, (*KO*, *AtKO1*) *ent*-kaurene oxidase, (*KAO*, *AtKAO1*, *2*) *ent*-kaurenoic acid oxidase, (*GA20ox*) AtGA20-oxidase1-5, (*GA2ox*) AtGA2-oxidase1-5, (*GA3ox*) AtGA3-oxidase1-4. (Farrow and Facchini 2014; Hedden and Phillips 2000; Magome *et al.* 2004)

### 1.3.4. Role of PAs in flowering

PAs are implicated in flowering induction, differentiation of the flowers and in regulating fertility (Huang *et al.* 2004), in addition to their essential role in the germination of pollen and tube growth (Falasca *et al.* 2010; Song *et al.* 2001). In *Arabidopsis thaliana*, PAs are essential for flowering and altering their levels can affect the transition to flowering (Applewhite et al. 2000). Measurement of Put and Spd concentrations in different organs of *Arabidopsis thaliana* (Col-0) showed that these two PAs present in flowers at the highest concentration, with Spd prevailing, in contrast to other organs where Spd and Put

contents were lower (Applewhite et al. 2000). Application of Spd inhibitors that lower its endogenous levels completely inhibited bolting and flowering and plants were only able to overcome that developmental defect when transferred to inhibitor-free medium (Applewhite et al. 2000). Treatment of Arabidopsis plants with Spd under short day conditions where flowering is naturally late, increased its endogenous titres and enhanced flowering rate, treatment of the delayed-flowering mutant *CS3123* with Spd considerably hastened flowering (Applewhite et al. 2000). All of these results suggest a strong correlation between PAs and the physiological processes leading to reproductive development. In general, sexual organs of *Arabidopsis thaliana* wild-type (Col-0) such as buds, mature and immature siliques, and flowers accumulate all the major PAs, Put, Spd and Spm, at high titres under normal conditions, while in other organs such as leaves and roots, the major PAs present at less concentrations (Urano et al. 2003).

In both long- and the short-day plants, previous studies reported an increase in foliar PAs as a response to photoperiodic induction followed by their increase in the SAM where floral initiation eventually takes place (Hamasaki and Galston 1990; Havelange *et al.* 1996). The increase in PA levels enhances flowering events in different plants and alterations in PA titres were reported during the switch from vegetative to flowering phases. For example, while PAs were absent in young vegetative parts of tobacco plants, a progressive accumulation of the conjugated PAs, hydroxycinnamoyl amides, in apical leaves and ultimately in the floral organs was recorded as the plant started its reproductive stage (Martin-Tanguy 1985). Under short day conditions, the exogenous treatment of the short-day plant *Pharbitis nil* cv. Kidachi with Put increased the internal free PA levels and induced flowering (Wada *et al.* 1994). In contrast, application of inhibitors that may reduce PA levels inhibited flowering, for instance, a decline in endogenous Spd levels, as a result of treatment with dicyclohexylammonium sulfate (CHA), an inhibitor of Spd synthase, was associated with a delay in flowering (Batchelor *et al.* 1986). Likewise, inhibiting Put biosynthesis by $\alpha$-difluoromethylarginine (DFMA), an inhibitor of the ADC enzyme, or by $\alpha$-difluoromethylornithine (DFMO), an inhibitor of the ODC enzyme postponed flowering in tobacco (Burtin *et al.* 1991), chrysanthemum (Aribaud and Martin-Tanguy 1994), and in *Spirodela punctate* (de Cantú and Kandeler 1989). However, exceeding the optimal concentration of PAs may also negatively affect the

flowering process. In this regard, it has been reported that accumulation of Put as a result of overexpressing its biosynthetic gene, *ADC2*, delayed flowering time through disturbing gibberellin metabolism by down-regulating GA biosynthetic genes (Alcazar et al. 2005). The phenotype was rescued by exogenously applied $GA_3$ and was attributed to the decrease in the contents of bioactive GAs as a result of down-regulation of two GA-biosynthetic genes, *GA 20-oxidase* and *GA 3-oxidase*, suggesting a negative effect of elevated Put on GA metabolism.

PA titres can affect plant fertility across different species. In stem mustard (*Brassica juncea* var. tsatsai), male sterility has been correlated with PA levels, in both free and conjugated forms (Guo *et al.* 2003). Furthermore, sterile pollen resulted from silencing *SAMDC*, one of the key genes in polyamine biosynthesis, in tomato tapetal tissue (Sinha and Rajam 2013). The polyamine Spd appears to be essential in maintaining male fertility and pollen viability (Ma *et al.* 2012), and an increase in Spm and Spd levels along with up-regulation of *SAMDC* was found to be important for pollen germination and tube growth in tomato plants (Song et al. 2001).

## 1.4. Aims of the project

Prior work on PAs raised many interesting questions about the mechanism of their action in regulating plant growth, development, response to stresses and their involvement in plant senescence. Since some of the physiological actions of PAs were attributed to their catabolism, the aim of this study was to investigate the role of different *AtCuAO* genes in affecting development and senescence. Work was therefore carried out to test the following hypotheses:

- Each member of the *AtCuAO* family has a particular role in *Arabidopsis thaliana* growth, development, productivity and senescence and this is linked to a differential expression pattern.

- A reduction in *AtCuAO* expression will affect the endogenous PA balance resulting in an increase of putrescine content.

- An increase in putrescine content will affect the timing of flowering and this is related to an interaction with gibberellin signalling.

- Perturbation of the expression of *AtCuAO* genes that are highly expressed during leaf senescence will affect the timing or progression of leaf senescence.

- Silencing multiple members of the *AtCuAO* gene family will have a strong effect on Arabidopsis growth and development.

To accomplish the aim of this research and test the hypotheses set out above, different experimental approaches were used:

- The temporal expression of *AtCuAO* gene family members and mRNA distribution was studied in individual leaves of wild type Arabidopsis during vegetative and floral development under controlled conditions using two different expression-detection methods: quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and histochemical GUS assay **(Chapter 3)**.

- To investigate the involvement of *AtCuAO* in regulating plant growth and development, the phenotype of catalytically-repressed *AtCuAO4* mutants and transgenic plants overexpressing *AtCuAO4* was tracked under optimal growth conditions **(Chapter 4)**.

- The effects of *AtCuAO4* involvement in senescence was tested by comparing developmental and dark induced senescence by measuring changes in the visible leaf greenness and in the chlorophyll contents caused by mutation or overexpression of the *AtCuAO4* gene **(Chapter 4)**.

- *AtCuAO4* loss of function mutant lines were tested for changes in endogenous polyamines at two critical stages, pre- and post-bolting, by analysing the PA contents using High Performance Liquid Chromatography (HPLC) **(Chapter 4)**.

- In order to understand the possible interactions between PAs and gibberellins, mutant lines were treated with $GA_3$ at early stages and timing of flowering was scored along with analysing the transcription levels of selected genes involved in gibberellin biosynthesis **(Chapter 4)**.

- For a better understanding of the physiological role of *CuAOs* in plants, and to avoid issues related to the compensation effect of other gene family members, an artificial microRNA (amiRNA) approach was used to down-regulate multiple *AtCuAO* gene family members simultaneously and observable traits of *AtCuAO* silenced transformants were recorded **(Chapter 5)**.

The conclusions from these studies and the concept for the role of *AtCuAOs* is then discussed **(Chapter 6)**.

# Chapter Two

## Methodology

# 2. General Materials and Methods

This chapter explains the materials and methods used in two or more of the subsequent chapters of the present thesis. The specific methods and/or conditions related to the work specific to each chapter are described in the relevant chapter. Unless stated, all chemicals and reagents used in this work were obtained from Sigma, UK, (Sigma-Aldrich Co. LLC).

## 2.1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) were utilized in all experiments of this study as wild type. For different types of experiments, plants were grown differently but in general plants were grown either on a sterilized mix of sand and commercial multi-purpose compost (1: 3, v/ v respectively) or on autoclaved Murashige and Skoog (MS) (4.708 g/ L) basal salt (Duchefa Biochemie, Belgium), in 9 cm Petri dishes supplemented with 1 % DifcoTM agar and 1 % (w/ v) sucrose, pH 5.5- 5.7 with or without a selective agent that was added when the agar had cooled to just above the gelling point.

Where soil was used to grow plants, seeds were first immersed in water within an Eppendorf tube and then stratified in the dark at 4° C for 48 h. Seeds were then sown in 3.5×4×5 cm plastic tray pots filled with autoclaved wet sand and compost (1: 3) and kept in a controlled environment in a growth room or Sanyo-Fitotron growth chamber under the required conditions: long day (LD) conditions [16 h light (120-140 µmol $m^{-2}$ $s^{-1}$) and 8 h dark] at 21° C or short day (SD) conditions [8 h light (120-140 µmol $m^{-2}$ $s^{-1}$) and 16 h dark] at 21° C. Seeds were sown at a density of five per pot until the first two leaves emerged then each plant (seedling) was transferred to a bigger single plastic pot with a diameter of 9 cm. Plant pots were placed on trays with or without a capillary matting, and they were watered with tap water 3-4 times weekly to maintain 1 cm of water around base of pots and thus retain soil humidity.

For using MS media, seeds were first sterilized in microcentrifuge tubes as described in Section 2.2, then stratified as described above. After sprinkling seeds on the surface of MS media (by adhering seeds one by one to the tip of a sterile 1 ml pipette using suction

then releasing it onto the surface of the MS medium), they were allowed to dry then plates were sealed with 3M™ Micropore™ Medical Tape 1530-0 (BM, USA). The agar plates with seeds were then moved to the growth chamber or the growth room to grow under the required conditions as described above. Ten to fifteen days later, seedlings were transferred to 9 cm plastic pots (one plant/ pot) filled with sterilized wet sand and compost (1:3) and were left to develop in the same conditions.

In both cases, plates or pots were relocated to a new position in the growth chamber or growth room after each measurement in order to reduce position dependent variations.

## 2.2. Surface sterilization and sowing of Arabidopsis Seeds

In order to ensure that all seeds were free of any source of contamination, seeds were sterilized in a sterile Microflow Laminar flow workstation each time prior to propagation on MS media.

A small amount of seeds (~60-100 seeds) were surface sterilized in Eppendorf tubes using 1 ml of 10 % (v/ v) sodium hypochlorite. The tubes were inverted thoroughly for 2-3 min to ensure contact between all seeds and solution, and then they were centrifuged for a short spin (5 s) in an Eppendorf MiniSpin® microcentrifuge to accumulate them at the bottom of the tube. The supernatant was pipetted off to remove most of the sodium hypochlorite solution from the tube. Seeds were then washed in 1 ml of ethanol mix (Ethanol: sterile distilled water (SDW): hypochlorite; 7: 2: 1) for 2- 3 min and again inverted thoroughly. They were centrifuged for a short spin and the ethanol mix removed by pipetting. After that, seeds were washed with 1 ml SDW three to five times to remove all traces of hypochlorite. Seeds were then stratified for 48 h at 4° C, and after that sown on the surface of MS agar in Petri dishes and allowed to dry on the agar for several minutes. Dishes were then sealed with 3M™ Micropore™ Medical Tape 1530-0 (BM, USA).

## 2.3. Seed stocks of *Arabidopsis thaliana* used in this work

All the seed stocks used for the work described in this thesis are listed in Table 2-1.

Table 2-1 Seed stocks of *Arabidopsis thaliana*.

| Study type | Name | Selection/ Description | Chapter | Origin |
|---|---|---|---|---|
| Phenotyping | 35SCaMV::*AtCuAO4* (AT4G12290)-His tag | Kan*, (50 µg/ ml)<br>HZ*,OEX*,<br>P9 (T2)<br>P17 (T2)<br>P27 (T2) | 5 | Ghuge, S. (Cona, A group. Roma TRE Uni) |
| | SALK_072954.55.00.x | T-DNA insertional line for *AtCuAO4* (AT4G12290) [C#4] | 5 | NASC |
| | GK_011C04-013046 | T-DNA insertional line for *AtCuAO4* (AT4G12290) [BIS#4] | 5 | NASC |
| | amiRNA; CSHL_058443 | BASTA, HZ* | 6 | amiRNA constructs were cloned at ABRC (http://abrc.osu.edu) and transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana* plants in the present work |
| | amiRNA; CSHL_017399 | BASTA, HZ* | 6 | |
| Spatial and temporal expression | *AtCuAO7*::GUS (AT3G43670) | Kan*, (50 µg/ ml)<br>HZ*<br>Lines; T2-16 | 4 | Ghuge, S. (Cona, A group. Roma TRE Uni) |
| | *AtCuAO8*::GUS (AT1G31690) | Kan*, (50 µg/ ml)<br>HZ*<br>T1 | 4 | Ghuge, S. (Cona, A group. Roma TRE Uni) |
| | *SAG12*::GUS | HM* | 4 | Buchanan-Wollaston, V. Warwick Uni. |
| *AtCuAOs* temporal expression and as a control | WT* | *Arabidopsis thaliana* Columbia (Col-0) | 3, 4, 5, 6 | Cona, A; group. Roma, TRE Uni and the present work |

(* Kan: kanamycin; OEX: overexpression line; HZ: heterozygous or hemizygous; HM: homozygous; WT: wild type, Col-0).

## 2.4. Growth measurement

During the growth of the plants, selected growth parameters were recorded from the day of sowing as follows: bolting day was scored at the day of initiation of the inflorescence

stem distinguished by a visually discernible morphological change in the shoot apical meristem. Number of true rosette leaves was counted on the day of bolting excluding the two cotyledons. The first day a flower bud opened was recorded as the day of first flowering. In order to measure productivity rate, plants were scored for the formation of the first silique and the total number of siliques produced by the plant in the following week on the primary inflorescence, secondary inflorescence branches (at the axils of the cauline leaves), and inflorescence branches (grown out from the axillary buds subtended by rosette leaves).

## 2.5. DNA extraction

For genotyping purposes, DNA was extracted as described by Edwards et al. (1991) as follows: The frozen tissue (flash frozen in liquid nitrogen) of a cauline leaf from the floral stem was macerated using a sterile plastic pestle in a sterile Eppendorf tube at room temperature (RT) for about 10 s. Edwards extraction buffer (400 µl of 100 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS) was added and the sample was ground briefly in order to remove tissue from the pestle, then vortexed for 5 s. The extract was centrifuged at 13,000 rpm for 1 min in a microcentrifuge (Eppendorf MiniSpin®) and 300 µl of the supernatant transferred to a clean Eppendorf tube. The supernatant was then mixed with 300 µl of isopropanol and left at RT for 2 min. Following centrifugation at 13,000 rpm for 10 min using an Eppendorf MiniSpin® microcentrifuge, the pellet was allowed to dry for 30 min, and then the dried pellet was dissolved in 100 µl of SDW. DNA quality was then checked by PCR reaction (Section 2.9 and 2.11.1) using PUV primers (Table 2-2) that bind to the 18S ribosomal RNA (rRNA) as the rRNA is the most abundant type of RNA in the cell (Vandesompele et al. 2002).

## 2.6. RNA extraction

As ribonucleic acid (RNA) is susceptible to contamination by RNases, mortars and pestles used for RNA extraction were first soaked in 0.1 NaOH for 30 min, rinsed thoroughly with SDW, wrapped with aluminium foil, autoclaved at 120° C for 15 min and dried at 60° C. All plastic-ware was autoclaved, dried and containers opened shortly before use. Prior to using them, mortars and pestles were frozen at -20° C overnight.

RNA was isolated using an RNeasy Plant Mini kit (QIAGEN) as follows. Plant tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle and 100-150 mg of the fine powdered tissue was transferred into a 1.5 ml Eppendorf tube with 450 µl of buffer containing β-mercaptoethanol (1 β-mercaptoethanol: 100 RLT buffer supplied with the kit). The mixture was vortexed vigorously, transferred to a QIAshredder spin column (Lilac) placed in 2 ml collection tube, and centrifuged for 2 min at maximum speed 13,000 rpm in a microcentrifuge (Eppendorf MiniSpin®). Without disturbing the pellet, the supernatant of the flow-through was then carefully transferred to a new 2 ml microcentrifuge tube and 250 µl (0.5 volume) of absolute ethanol was added, mixed immediately by pipetting up and down and directly transferred to an RNeasy mini column (pink) placed in a 2 ml collection tube. The pink column was centrifuged for 30 sec at 10,000 rpm in a microcentrifuge (Eppendorf MiniSpin®) and the flow-through was discarded. To wash the column, 700 µl of RW1 buffer supplied with the kit was added, centrifuged for 30 sec at 10,000 rpm as above, and the flow-through was discarded. Next, 500 µl of RPE buffer was added onto the pink column and centrifuged for 30 s at 10,000 rpm as above and the flow-through was discarded. The last step was repeated but the centrifugation was for 2 min, then the collection tube containing flow-through liquid was discarded and the column was transferred into a new 2 ml collection tube. To dry the column membrane and remove any residual of RPE buffer which contains ethanol that may interfere with downstream reactions, the column was centrifuged for another 1 min as above. Finally, the column was transferred to a new 1.5 ml Eppendorf tube and 30-50 µl of warm RNase-free water was added directly on to the silica-gel membrane, left for 1 min at room temperature, and centrifuged for 1 min at 10,000 rpm as above to elute the RNA. Eluted RNA was stored at -80° C until further use. The concentration of RNA was measured as described in Section 2.11.2.

## 2.7. DNase treatment after RNA extraction

Prior to real time RT-PCR analysis, RNA extracts were treated with DNase to eliminate genomic DNA contamination and thus avoid amplification of genomic DNA. For each digestion, 1x RQ1 RNase-free DNase buffer (40 mM Tris-HCL pH 8, 10 mM $MgSO_4$, 1 mM $CaCl_2$; PROMEGA, Southampton, UK) and 3 µl RQ1 RNase-free DNase (1 unit/ µl; PROMEGA, Southampton, UK) were added to 2 µg RNA in PCR tubes, made up to

20 µl with SDW, mixed and incubated at 37° C for 30 min . To inactivate the DNase and terminate the reaction, 2 µl of RQ1 stop solution (20mM EGTA, pH 8, Promega) was added to the mix which was then incubated at 65° C for 10 min. Subsequently, treated RNA samples (1 µl) were checked via PCR (Section 2.9), using 18S rRNA primers (PUV primers, Table 2-2) to confirm that all genomic DNA was indeed digested.

## 2.8. cDNA Generation

The first strand cDNA was generated from DNase-treated RNA as follows: 2 µg of total RNA was mixed with 1 µl Oligo (dt) 15 Primer (500 µg/ ml, Promega), incubated at 70° C for 10 min, and the mix was then cooled on ice for 10 min. Afterwards, 6 µl of 5x M-MLV RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT, Promega), 1 µl of 10 mM dNTP mix, and 2 µl of 0.1 M DTT were added to each tube and the reaction mix was then incubated for 2 min at 42° C. To start the cDNA synthesis, 1 µl of M-MLV Reverse Transcriptase, RNase [H-] (Promega: 200 unit/ µl) was added and the reaction further incubated at 42° C for 50 min in a PCR machine. To terminate the reaction, the incubation temperature was increased to 70° C for 15 min. Samples were stored at -80° C for further expression analysis.

Quality of cDNA was tested by PCR (Section 2.9) using *Actin2* primers (Table 2-2) before proceeding to real-time RT-PCR.

## 2.9. Polymerase chain reaction (PCR)

PCR was performed in either a GeneAmp® PCR System 2700 (Applied Biosystems, USA), Techne Flexigene (Techne, UK), or Veriti® thermal cycler (Life Technologies, USA) PCR machine.

For each reaction the PCR volume was 25 µl and contained: 1 µl of each appropriate primer (10 µM; Table 2-2 and Table 2-3), 0.5 µl dNTP mix (10 mM, Promega), 5 µl of 5x Green GoTaq Flexi buffer (Promega), 1.5 µl $MgCl_2$ (25 mM), 0.125 µl GoTaq DNA Polymerase (5U/ µl, Promega), 14.9 µl SDW, and finally 1 µl of isolated DNA or cDNA. As a negative control to detect any contamination, 1 µl of SDW replaced the DNA template. All PCR reactions were started with a DNA denaturation step at 95° C for 2–3

min and ended with an extension for 7 min at 72° C. The number of cycles was between 35 and 40 of 1 min at 94° C, 1 min at 50-58° C (depending on the Tm ° C), and 1 min at 72° C. When using Hot Star Taq polymerase (5 U/ µl, Qiagen), the same protocol was used except that 2.5 µl of 10X PCR buffer (Qiagen) and 1 µl $MgCl_2$ (15 mM) were used, and the programme started with a denaturation step at 95° C for 15 min.

## 2.10. Quantitative real time RT-PCR

As assessed by NanoDrop (see Section 2.11.2), equal amounts of RNA of each sample were used for cDNA synthesis. To equalize transcript concentrations in cDNA samples, cDNAs were normalized prior to proceeding with the analysis of the samples by measuring the cDNA using the nanodrop and adjusting the concentration accordingly to 100 ng/ µl. Quantitative real-time PCR was performed in triplicate with SybrGreen using the first-strand cDNA as a template on an Agilent Mx3000P QPCR System (Agilent Technologies UK 28 Ltd., Stockport, UK).

The qRT-PCR mixture for each reaction was in a total volume of 20 µl containing: 400 nM of each primer, 60 ng of the single strand cDNA, and 10 µl of 2 x qPCRBIO SyGreen Mix LoROX (Applied Biosystems Ltd., London, UK), made up to 20 µl with SDW. The thermal profile was: 1 cycle at 95° C for 5 min, 40 cycles at 95° C for 15 s, 60° C for 30 s, 72° C for 30 s. After amplification, a dissociation curve analysis (from 60° C to 95° C where the temperature is increased by 0.5° C $s^{-1}$) was carried out to test primer specificity and check for the absence of primer dimers. Gene expression was calculated according to the 2-ΔΔCT ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001), using the equation $\Delta\Delta CT = 2^{-[\Delta Ct\ \text{treated-sample} - \Delta Ct\ \text{control-sample}]}$, with ΔCt = Ct target-gene – Ct reference-gene, where Ct refers to the cycle threshold.

The software used to control the thermocycler and to analyse data was the MxPro qPCR software (Agilent Technologies), and *Actin2* mRNA was used as a housekeeping gene to normalize target gene expression levels (see Table 2-3 for primer sequence). *Actin2* primers were used because the expression of this gene is high throughout leaf development and stable amongst leaf tissue (Winter et al. 2007).

## 2.11. Quantification of the nucleic acids

### 2.11.1. Agarose gel electrophoresis

PCR products were checked by electrophoresis of 12 µl of the reaction mixture on an agarose gel as follows. Depending on the size of nucleic acid to be separated, 1- 1.4 % (w/ v) agarose (Bioline, London, UK) was added to 1x Tris-acetate-EDTA (TAE) buffer (4.84 g/ L Tris, 1.142 ml/ L glacial acetic acid, 2 ml/ L 0.5M EDTA), heated using a microwave to dissolve the agarose, and cooled down to approximately 45° C. This was then mixed with ethidium bromide EtBr (1 µl of 10 mg/ ml EtBr per 10 ml of agarose gel solution) and poured into a tray with a comb to form wells and left at RT for 20 min to solidify the gel. The comb was removed, and the gel placed in a gel tank containing 1x TAE buffer covering the gel and each deoxyribonucleic acid (DNA) sample was loaded into a separate well.

Likewise, the integrity of the ribonucleic acid (RNA) was assessed by agarose gel electrophoresis after soaking all parts of the equipment, the gel tank, comb and tray, in 0.1 M NaOH for 20 min and then rinsing them with distilled water to prevent RNA degradation by RNase. Prior to pipetting RNA samples into wells, 2 µl 5x Green GoTaq Flexi buffer (Promega) was added to each sample.

The gel was electrophoresed in 1x TAE buffer, at 100 V for 30-40 min alongside 7-10 µl (400– 500 ng) $1Kb^{+}$ DNA ladder (1 µg/ µl, Invitrogen) as an indicator of DNA fragment size. The gel was then visualized under UV light and an image captured using a Gene Genius Bioimaging System (Syngene Ltd.).

### 2.11.2. Spectrophotometry

Concentration of both DNA and RNA expressed as ng/ µl was determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific), to analyze a sample of 1-1.2 µl at 260 nm. Ratios of 260/ 280 and 260/ 230 were also measured to assess nucleic acid purity.

## 2.12. Purification of PCR amplicons

PCR products were either purified directly from the PCR reaction mixture using a QIAquick PCR purification kit or gel purified using a QIAquick gel extraction kit as per the manufacturer's instructions provided in the QIAquick® Spin Handbook (QIAGEN). Before sequencing, DNA fragments were re-checked on a 1.7 % agarose gel (Section 2.11.1) and the concentration of the extracted PCR product was determined using a spectrophotometer (Section 2.11.2).

## 2.13. DNA sequencing

Sequencing reactions were performed using the Big Dye Terminator (v 3.1) (Applied Biosystems, Foster City, CA, USA), with an automated sequence analyzer ABI PRISM® 3730XL (Applied Biosystems), by Eurofins MWG Operon in the forward direction using appropriate primers (Section 2.14).

## 2.14. Primers

Primer pairs for each gene, analysed by either PCR or qRT-PCR, were designed with Primer3 (Rozen and Skaletsky 1999), on-line software (http://fokker.wi.mit.edu/primer3 /input.htm).

For high specificity, primers used for qRT-PCR were designed using 300 –500 nucleotides downstream of the open reading frame including the 3'UTR. The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/), the Munich Information Centre for Protein Sequences (MIPS; http://mips.helmholtz-muenchen.de/plant/), and the National Centre for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov/pubmed) databases were used as sources of information about coding sequences of genes.

Primers were all purchased from Sigma-Genosys Ltd, UK. They were all desalted-grade (scale 0.025), except *ssActin2* primers which were Reverse Phase-grade (scale 0.05). Primer sequences are listed in Table 2-2 and Table 2-3.

Table 2-2 Sequence of primers used for PCR

| Target | Primer | Primer sequence (5'-3') | Fragment size (bp) | Tm° C |
|---|---|---|---|---|
| *AtCuAO4* (At4g12290) (Flank the T-DNA) | *AtCuAO4* F<br>*AtCuAO4* R | CTGGTTACCCGATGATGACTA<br>TCGGTTATCTCGATCACTTGC | 283 | 50 |
| *AtCuAO4* (At4g12290) (Up-stream) | Up F<br>Up R | CTTTGCGCTTCGAGAAACTT<br>TGTGGTGTACCGGAAGTGAA | 200 | 58 |
| *AtCuAO4* (At4g12290) (Down-stream) | Down F<br>Down R | GAGTTACGCCGGAGATATTG<br>TAGCGTCACTTTTGGTCTCA | 199 gDNA<br>88 cDNA | 58 |
| SALK_072954.55.00.x Line C#4 | LBa1<br>*AtCuAO4* F | GATGGTTCACGTAGTGGGCCATCGC<br>CTGGTTACCCGATGATGACTA | 400 | 50 |
| GK-011C04-013046 Line BIS#4 | P6<br>*AtCuAO4* R | GAAGATAGTGGAAAAGGAAGGTGGCTC<br>TCGGTTATCTCGATCACTTGC | 200 | 50 |
| Gateway vector | ABB1<br>ABB2 | GGGGACAAGTTTGTACAAAAAAGCAGGCTAT<br>GGGGACCACTTTGTACAAGAAAGCTGGGTC | 500 | 55 |
| 18S rRNA | PUV2<br>PUV4 | ATGGTGGTAACGGGTGAC<br>TCCCATGCTAATGTATCCAGAG | 459 | 55 |
| *UBQ10* (At4g05320) | F<br>R | CACACTCCACTTGGTCTTGCGT<br>TGGTCTTTCCGGTGAGAGTCTTCA | ~400 | 58 |
| *AtCuAO4* Over-expression lines | 35SforW1<br>*AtCuAO4* R | AGGAGCATCGTGGAAAAAGA<br>TCGGTTATCTCGATCACTTGC | ~1075 | 52 |
| *Actin2* (At3g18780) | F<br>R | TGTGCCAATCTACGAGGG<br>TTTCCCGCTCTGCTGTTGT | 137 | 55 |
| *BASTA* | 229 Bar_R | TTTCGGTGACGGGCAGGACCG | 545 | 58 |
| MIR-443_F (amiRNA sequence) | F | AAAGGAGAACGTAAACGGTAT | | 58 |
| MIR-399_F (amiRNA sequence) | F | CGAGCAGCATTTAGCAACTAT | | 61 |
| 35Sp (Used with amiRNA) | | GATTGATGTGATATCTCCACTGACG | | 65.5 |
| amiRNA designed primers | F<br>R | AGGACGCATATTACACATGTTCA<br>TTGGCGACTCGGTATTTGGA | 158 | 60 |
| *AtCuAO3-SP* verification | F<br>R | TCGACTGCACAAAATCTTCG<br>CCCTTTTGGTGTTGTCGTCT | 579 | 58 |
| *AtCuAO9* verification | F<br>R | TGGGGTTATGAGAGCAAAG<br>GGAATGTGATGGAACCCAAG | 834 | 58 |

Table 2-3 Sequence of primers used for quantitative real time RT-PCR

| Gene / Locus | Primer | Primer sequence (5'-3') | Amplified fragment Size (bp) | | Tm° C |
|---|---|---|---|---|---|
| | | | cDNA | gDNA | |
| *AtAO1* (At4g14940) | F<br>R | CGTCAAACATATGATCATTGAAAA<br>GGCACGACCAACTTGAAAAT | 189 | 189 | 58 |
| *AtCuAO1* (At1g62810) | F<br>R | CCGAACAAGAAGCCTGAGTC<br>CGATGGCAGAACCATCTTTT | 109 | 109 | 58 |
| *AtCuAO3-SP* (At4g12270) | F<br>R | GGAACGCCATATGTGAGAGAG<br>GGTATCCGGAGCCAACTTTT | 152 | 545 | 58 |
| *AtCuAO4* (At4g12290) | F<br>R | CCGCTCCAAACTTTGAACAT<br>TCCGCATGAATATATGGATTCTC | 218 | 218 | 58 |
| *AtCuAO5* (At1g31670) | F<br>R | TGGTACACTGTCGGATTCCA<br>CGTTGGTGTGGTGTTGAGTT | 153 | 153 | 58 |
| *AtCuAO2* (At1g31710) | F<br>R | GACCGACCAACTTTTTCGAG<br>TCGAATCGTTACAGTCCATCA | 155 | 155 | 58 |
| *AtCuAO7* (At3g43670) | F<br>R | TTTTCCAGTGATGCCAACAA<br>TGAAAATGCACAGTGTCGAA | 156 | 156 | 58 |
| *AtCuAO8* (At1g31690) | F<br>R | TCAATCTCACCACCATTCCA<br>TTGGAATCTCTTTACAATTCCTCA | 114 | 114 | 58 |
| *AtCuAO9* (At4g12280) | F<br>R | TGGGGTTATGAGAGCAAAG<br>CATTTTCGGACAGAATCGTG | 137 | 234 | 58 |
| *AtCuAO3* (At2g42490) | F<br>R | GCTTGATCTTCCCCCTTCTC<br>CTCAAGATCCGGTGACGAAT | 172 | 172 | 58 |
| *ssActin2* (At3g18780) | F<br>R | ACATTGTGCTCAGTGGTGGA<br>CTGAGGGAAGCAAGAATGGA | 163 | 163 | 55 |
| *AtKS1* (At1g79460) | F<br>R | TTTACAGGAAGGACGATGGA<br>TACCTGCCAGATCAACTTGG | 122 | 122 | 55 |
| *AtCPS1* (At4g02780) | F<br>R | AACCGCTTCTGGATTTGTCT<br>TCCATTCCAAGTACAACCTTTC | 112 | 934 | 60 |
| *AtGA2ox1* (At1g78440) | F<br>R | GTACAACCTCTCGTCCTCATTGTCT<br>CTTCGCTGGACCTTCATTGAC | 76 | 76 | 58 |
| *AtGA3ox1* (At1g15550) | F<br>R | AGATCGTCTTTAGGGGTCCA<br>GAGCAAGATGCCTGCTATGT | 132 | 132 | 55 |
| *AtGA20ox1* (At4g25420) | F<br>R | CTGCTTGCGTAGCCAACACT<br>GGCATCAGCGAGGAGCTTATT | 122 | 122 | 65 |
| *SUP* (At3g23130) | F<br>R | CCATAAAGGATTCGAAGTTCA<br>AAACGGTAACAAGCGCATAC | 185 | 185 | 60 |
| *SOC1* (At2g45660) | F<br>R | CAGCATCACAAAGCACTGAG<br>TTTCTGTGTGCAAGGGAAAT | 161 | 161 | 55 |

## 2.15. Chlorophyll determination

Chlorophyll content was measured in leaves number 5 and 6 according to (Lichtenthaler 1987). Leaves were weighed and then powdered in liquid nitrogen using a sterile plastic pestle in a sterile Eppendorf tube. Methanol (99.8 %) was added, 10 % (w/ v), and then samples were incubated in complete darkness at 4° C for 24h. Samples were then centrifuged at 13,000 rpm in a microcentrifuge (Eppendorf MiniSpin®) for 5 min at RT. Magellan™ 701 software (TECAN) was used to measure the optical densities of 200 µl of the supernatant against a methanol blank at 665 and 652 nm using an Infinite M200, quad4 monochromator™ detection system (Tecan Austria, Austria) and 96-well flat bottom transparent microplates (Greiner Bio-One, Germany). Chlorophyll and carotenoid concentrations were then calculated according to Lichtenthaler and Buschmann (2001) as follows: Chl. a (µg/ ml) = 16.72 x $Abs._{665}$ - 9.16 x $Abs._{652}$; Chl. b (µg/ ml) = 34.09 x $Abs._{652}$ - 15.28 x $Abs._{665}$; Chl. a+b (µg/ ml) = 1.44 x $Abs._{665}$ + 24.93 x $Abs._{652}$; Carotenoid (µg/ ml) = (1000 x $Abs._{470}$ - 1.63 x Chl. a - 104.96 x Chl. b)/ 221. Then µg Chl. per mg fresh weight of plant tissue was calculated using the following equation: [Chl. a, b, total, or carotenoid (µg/ ml)] x volume methanol/ mg tissue.

## 2.16. Microscopy and Imaging

Visualization of different parts of the plant was performed at suitable magnifications either under a light microscope using an Olympus BH-2 microscope or under a dissecting microscope using a Nikon SMZ-2T. Images were captured using a Tucsen Camera (Tucsen imaging technology Co., Ltd. TCA-5.0 C).

## 2.17. Statistical analysis

All statistical work was carried out using SPSS 18.0 software. To identify the best test to use, the normality of data was tested among plant lines using a Shapiro-Wilk test and accordingly the parametric test, T-test, was used on normally distributed data whereas the non-parametric test, Mann-Whitney, was used on non-normal data. The statistical analysis of gene expression, chlorophyll contents, GUS activity among stages, and phenotype of GA treated plants was performed with one-way ANOVA. Significance of differences was determined if the *P* value was $\leq 0.05$ in all statistical tests used.

# Chapter Three

## Expression of *AtCuAOs*

# 3. Developmental regulation of *AtCuAO* gene family expression in *Arabidopsis thaliana*

## 3.1. Introduction

In all plant species studied to date, copper amine oxidase genes are found in multigene families (Moller and McPherson 1998; Tipping and McPherson 1995). In Arabidopsis, ten genes encoding putative copper-binding amine oxidases (*AtCuAOs*) have been predicted based on sequence homology (Table 3-1) (Arabidopsis genome database; TAIR; https://www.arabidopsis.org).

Four of the Arabidopsis *CuAOs*, *AtAO1* (At4g14940); *AtCuAO1* (At1g62810); *AtCuAO2* (At1g31710); *AtCuAO3* (At2g42490), have been characterized at the protein level. A peroxisomal localization has been reported for two of them, *AtCuAO2* and *AtCuAO3*, while *AtCuAO1* and *AtAO1* are predicted to be extracellular (apoplastic) proteins (Moller and McPherson 1998; Planas-Portell et al. 2013). CuAOs mediate polyamine (PA) catabolism in different cellular compartments of Arabidopsis (Planas-Portell et al. 2013). Most of the intracellular PA catabolism occurs in peroxisomes (Moschou et al. 2012). Recently Planas-Portell et al. (2013) studied three *CuAOs*, *AtCuAO1*; *AtCuAO2*; *AtCuAO3*, and found that these genes encode functional CuAOs that are able to oxidize putrescine (Put) and spermidine (Spd) but not spermine (Spm). Put and Spd are oxidized in the apoplast by AtAO1 and AtCuAO1 (Planas-Portell et al. 2013; Wimalasekera et al. 2011b), while this reaction is catalysed by AtCuAO2 and AtCuAO3 in the peroxisomes and co-localized with PA back conversion pathways catalysed by polyamine oxidases (PAOs). This suggests a tight organisation between the two catabolic enzyme machineries to preserve PA cellular content at the optimum level (Planas-Portell et al. 2013). Both spatial and temporal expression of *CuAOs* is regulated during plant development and it can be affected by environmental or endogenous stimuli (Angelini et al. 1990; Laurenzi et al. 2001; Moller and McPherson 1998; Paschalidis and Roubelakis-Angelakis 2005a; Rea et al. 1998). *AtCuAO1-3* are expressed in whole seedlings, rosette leaves, stems and flowers (Planas-Portell et al. 2013). *AtCuAO1* and *AtCuAO3* were similar in their expression pattern as shown by quantitative real time RT-PCR (qRT-PCR) using total RNA isolated from the whole plants but their expression increased during

plant development. *AtCuAO3* reached a peak in flowers whereas *AtCuAO1* peaked in stems and flowers suggesting the involvement of these genes in plant development. On the other hand, no change in *AtCuAO2* expression was observed during plant development although its transcript level was abundant in stems and low in other organs.

*AtAO1* expression also changes spatially with high expression in cells undergoing programmed cell death (PCD) such as developing xylem and lateral root cap cells (Moller and McPherson 1998). This is consistent with a need for high levels of amine oxidases (AOs) in tissues and cells undergoing wall rigidification or lignification as the AOs are thought to have a role in affecting the rigidity and stiffness of the cell wall and thus plant growth and development (Cona et al. 2003). Strong expression of *AtAO1* gene, detected by a promoter::*GFP-GUS* fusion, in guard cells of Arabidopsis leaves and xylem tissue of roots, suggested its involvement in balance between supply of water via root xylem and its loss via transpiration through stomata (Ghuge et al. 2015b). *AtAO1* expression is also high in developing leaves, hypocotyl, and style/ stigmatal tissue (Moller and McPherson 1998).

Expression analysis of *AtCuAO1, AtCuAO4* and *AtCuAO7* genes during plant development using qRT-PCR in Arabidopsis rosette leaves grown under normal conditions showed an increase in the transcript level of all the three genes at 6 and 8 weeks compared with 2 and 4 weeks and these changes were dramatic for *AtCuAO4* expression (Ghuge 2014).

Tissue and organ specific activity of the GUS reporter enzyme in six *AtCuAO*::GUS transgenic plants has been monitored in young seedlings (4-5 day old), and results revealed that promoters of *AtAO1, AtCuAO1,* and *AtCuAO7* are active in both roots and leaves, whereas *AtCuAO2* is root specific gene, and both *AtCuAO4* and *AtCuAO8* are leaf specific genes (Ghuge 2014). Overall the information on the expression of the *AtCuAO* gene family is fragmentary. Some of the *AtCuAO* genes have been studied by qRT-PCR analysis and some by GUS reporter gene assays either in the whole plant at specific stages or at different developmental stages, or in specific tissues at specific stages. Information on the expression of the whole family of *AtCuAO* genes is available by microarray

analysis on TAIR and PRESTA websites but the microarray data sources are not in full agreement regarding *AtCuAOs* expression during leaf senescence as shown in Table 3-1. Detailed information about stage specific changes in *AtCuAO* gene expression is needed for a full understanding of the gene regulatory network underlying development.

Table 3-1 Characteristics of the ten *AtCuAOs* in the *Arabidopsis thaliana* genome.

| ***CuAO* genes** | **Localization (P sort)** | **Expression in plant*** | **Expression during senescence*** | | | **Expression during stress*** |
|---|---|---|---|---|---|---|
| | | | **TAIR** | **PRESTA** | **Cona Lab** | |
| *AtAO1* (At4g14940) | Apoplastic | During early stages of vascular tissue development. | No | Yes | Yes | Highly expressed under different abiotic and biotic stresses |
| *AtCuAO1* (At1g62810) | Apoplastic | During different stages of plant development | Yes+ | Yes | ___ | Expressed in leaves under osmotic stress |
| *AtCuAO3*-SP (At4g12270) | Outside | During all the stages of plant development | Yes+ | maybe | ___ | Highly expressed in roots in response to abiotic stresses and in leaves in response to some biotic stresses |
| *AtCuAO4* (At4g12290) | Vacuole | During late seed germination and dry seeds | Yes+ | maybe | Yes | Highly expressed under different abiotic and biotic stresses |
| *AtCuAO5* (At1g31670) | ER (membrane) | During seed germination | No | No | ___ | Expressed in leaves during biotic stresses and in roots during abiotic stresses |
| *AtCuAO2* (At1g31710) | Peroxisome | Expressed in 13 plant structures during 8 growth stages | No | No | ___ | Expressed in roots and leaves in response to abiotic stresses and in leaves under biotic stresses |
| *AtCuAO7* (At3g43670) | Apoplastic | Expressed in 20 plant structures | Yes+ | maybe | Yes | Expressed in leaves under biotic and abiotic stresses |
| *AtCuAO8* (At1g31690) | Peroxisome | Expressed in 9 plant structures during 7 growth stages | No | maybe | ___ | Expressed in leaves in response to some abiotic and biotic stresses |
| *AtCuAO9* (At4g12280) | Cytoplasm | Highly expressed during late seed germination and dry seeds | Yes+ | maybe | ___ | Expressed under different biotic and abiotic stresses |
| *AtCuAO3* (At2g42490) | Peroxisome | Expressed in 22 plant structures during 13 growth stages | Yes+ | No | ___ | Expressed in roots and leaves as a response to abiotic stresses and in leaves under biotic stresses |

* Data from TAIR eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and PRESTA, the Arabidopsis project (http://www2.warwick.ac.uk). +, high level of expression; ___, not studied.

To date, no data are available on the expression pattern of all *AtCuAO* gene family members in leaves during development using qRT-PCR. The real time quantitative reverse transcriptase (qRT-PCR) technique is both a highly sensitive and specific method (Bustin 2000), that is generally accepted as a gold standard for relative gene expression measurement and is frequently used to confirm results of microarray data (Qin et al. 2006). Because it allows the specific detection of each member of the family, the qRT-PCR technique is indeed a good tool to carry out an analysis of the expression level of genes belonging to a very conserved gene family as was illustrated previously with members of four different gene families in *Arabidopsis thaliana* (Baud et al. 2004; Charrier et al. 2002; Orsel et al. 2002; Yokoyama and Nishitani 2001). This analysis method has therefore been used in the present chapter to validate available expression data on the *CuAO* gene family in wild type Arabidopsis. Single leaves, 5 and 6, were used to obtain an accurate representation of changes in the expression of different members of the family during four critical developmental stages in the life cycle of the plant (pre-bolting, at bolting, post bolting and at senescence) under controlled conditions. These two leaves were chosen because they are less shaded by other rosette leaves (Mullen et al. 2006), and because of their synchronized (concurrent) emergence and development under our growth conditions, and the synchronization of senescence and nutrient remobilization from these two leaves with reproductive growth (Breeze et al. 2011). For further confirmation and as the reporter gene *β*-glucuronidase (GUS) is helpful for better understanding of gene expression (Jefferson 1987), the expression of two members of *CuAO* gene family, *AtCuAO8* and *AtCuAO7*, was also examined in GUS reporter lines during Arabidopsis development. Promoter regions of around 1.8 and 2.5 kb upstream of the ATG were used in *AtCuAO8* and *AtCuAO7* GUS lines respectively (Ghuge 2014), for more details see Appendix B (c).

## 3.2. Materials and methods

### 3.2.1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) were used in this experiment. Seeds were stratified and sown on soil in plastic pots as described in Section 2.1 at a density of 1-2 plants per pot and grown under long day conditions until

the first two leaves emerged, then seedlings were thinned out to keep a single plant in each pot and leaves 5 and 6 were tagged with thread 18 days after sowing (DAS). During plant growth, leaves 5 and 6 were always harvested in the morning (to avoid any possible circadian effects), randomly selected to avoid any potential effects of position within the growth incubator, at specific time points starting at 20 DAS and continuing every week until almost full senescence was reached (41 DAS). Two biological replicates consisting of 20 leaves/ replicate, were collected at each stage. Samples were frozen in liquid nitrogen and then kept at -80° C until further use.

For promoter GUS (β-glucuronidase) analysis, *Arabidopsis thaliana* (Col-0) and transgenic lines expressing *SAG12*::GUS, *AtCuAO7*::GUS, *AtCuAO8*::GUS plants were grown *in vitro* (with kanamycin (Kan) at 50 μg/ ml for *AtCuAO7*::GUS, *AtCuAO8*::GUS or without for other plants) and then in soil in a controlled environment as described in Section 2.1. Samples were collected as described above except that sampling was from six plants at early stages while three plants were used at late stages for this purpose.

### 3.2.2. Primer design, sequence alignment and analysis

Primer sets of each *CuAO* gene were designed with Primer 3 software, as described in Section 2.14. To ensure the specificity and efficiency of designed primers during real-time PCR using cDNA as a template and under standard reaction conditions, primers were designed based on a specific set of criteria. These included primer length of 20-24 nucleotides, PCR amplicon lengths of 100- 200 bp and when possible, both primers of each pair (or at least one of them) were designed from the 3' UTR zone. BioEdit software was used for multiple sequence alignment of *AtCuAO* cDNAs and primer sequences. To determine the evolutionary relationship among the gene family members, using amino acid or nucleotide sequences, Arabidopsis *CuAO* members were used for Phylogenetic and molecular evolutionary analyses using Phylogeny.fr (http://www.phylogeny.fr) in "Advanced" mode using the MUSCLE 3.7 program for multiple alignments, PhyML 3.0 aLRT program for phylogenetic tree building based on an approximation of the standard likelihood ratio test, and TreeDyn 198.3 for tree rendering (Dereeper et al. 2008).

### 3.2.3. Testing primers and sequencing of PCR products

Primers were tested first on genomic DNA (gDNA) to confirm primer efficiency and specificity, and amplified fragments were then sequenced to confirm gene identity by aligning sequencing results with the original gene.

PCR reactions were performed, using *UBQ10* as a positive control because the expression of this gene is stable throughout leaf development (Winter et al. 2007), as described in Section 2.9 (and Table 2-2) using the following thermal program; 94° C for 2 min. initial denaturation and a final extension for 7 min at 72° C while number of cycles was 40 (94° C, 1 min, 58° C, 1 min, 72° C, 1 min). Products were separated on 1.5 % agarose gels and visualized as described in Section 2.11.1. PCR products were then extracted and sequenced as described in Section 2.13.

### 3.2.4. RNA extraction and DNase treatment

RNA was isolated from frozen material using an RNeasy Plant Mini kit (QIAGEN) as described by the manufacturer (Section 2.6). Genomic DNA was then removed from RNA samples as described in Section 2.7. Subsequently, the first-strand cDNA was generated by reverse transcription of RNA as described in Section 2.8.

### 3.2.5. Estimation of mRNA by real time RT-PCR

Quantitative *CuAO* expression using real-time PCR was carried out according to Livak and Schmittgen (2001) as described earlier in Section 2.10. The transcript levels of each gene at each stage were expressed compared to the stage where its expression was the highest.

### 3.2.6. Chlorophyll determination

Chlorophyll content of leaves was determined according to Lichtenthaler (1987) as described in Section 2.15 using twelve individual leaves (of leaves 5 and 6) at each stage.

### 3.2.7. Analysis of GUS enzyme activity

Chemicals used for GUS assay, 4-methylumbelliferone (MU); 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid cyclohexyl ammonium (X-GlcA, CHA salt) and 4-Mu-*β*-D-Glucuronide Trihydrate (MUG), were purchased from Melford, UK.

#### 3.2.7.a) Histochemical GUS assay

For histochemical GUS assays, plant material, seedlings or leaves, were immersed in GUS staining solution (5 mg/ ml X-GlcA salt/ DMSO, 0.5 M sodium phosphate pH 7, 200 mM potassium ferricyanide, 20 mg/ml chloroamphenicol, 0.01 % Triton X-100) in flasks covered with aluminium foil to protect the solution from oxidation. Flasks were then vacuum infiltrated for 2-3 min and incubated overnight at 37° C. Following incubation, GUS staining solution was replaced by absolute ethanol for 3- 4 h to decolorize the leaves. This step was repeated as needed until plant tissues became completely white which means that all chlorophyll was removed. Ethanol was then replaced with 50 % sterilized glycerol solution in water and GUS-stained tissues were kept at 4° C. GUS staining was visualised under a light microscope and images of the stained tissues were captured as described in Section 2.15.

#### 3.2.7.b) Quantitative fluorogenic GUS assay

Proteins were extracted by grinding frozen plant tissues with liquid nitrogen in a micro-centrifuge tube with an Eppendorf grinder. This step was followed by the addition of 100 µl of GUS extraction buffer (50 mM sodium phosphate pH 7, 1 mM EDTA pH 8, 10 mM DTT, 0.1 % (w/ v) sarcosyl, 0.1 % (v/ v) Triton X-100). Tubes were then vortexed briefly and centrifuged in a Heraeus Fresco 17 centrifuge (Thermo scientific) at 13000 rpm, 4°C, for 5 min. The supernatant was aliquoted into small volumes and kept at -20° C until further use. BSA dilution standards in GUS extraction buffer (1-10 µg/ ml) were prepared and protein content was quantified using the Bradford assay (Bradford 1976) as follows: 5 µl of sample or BSA dilution standards were pipetted into wells of a flat bottom transparent 96 microplate (Greiner Bio-One, Germany). Bradford reagent (Biorad, 250 µl) was added to each well, mixed gently by pipetting up and down, and incubated at RT

for 10- 30 min. The absorbance was measured at 590 nm using an Infinite M200, quad4 monochromator™ detection system (Tecan Austria, Austria) and Magellan 701 software was used to analyse the output. A BSA standard curve was used to determine the concentration of unknown protein samples and the final unit was expressed as µg/ ml. A fluorogenic GUS assay was set up according to Jefferson (1987): Protein extract (5 µl) was added to wells of a Black-Opaque Optiplate-96 microplate (Perkin Elmer) filled with 50 µl GUS assay buffer (1 mM 4-MUG in GUS extraction buffer) and incubated at 37°C. Reaction mix (10 µl) was removed at 5, 10, 30 and 60 min of incubation, and the reaction was terminated by the addition of 90 µl of stop buffer (0.2 M $Na_2CO_3$). Fluorescence was measured with emission at 455 nm and excitation at 365 nm using Magellan 701 software and an Infinite M200, quad4 monochromator™ detection system (Tecan Austria, Austria). A freshly prepared MU dilution standard (ranging from 100 nM to 1 µM 4-MU in Stop buffer) was used to calibrate the instrument. With the assumption that 1µM 4-MU is equivalent to 1000 F.U, relative fluorescence units (F.U) produced by the MU, standards were plotted against concentration to calculate the activity in the unknown samples. The concentration of the unknown sample was determined from the standard curve, and the obtained values were further normalised against the incubation time (min) and total protein (µg) and expressed as nmol 4-MU/ min/ µg of protein.

## 3.3. Results

### 3.3.1. Sequence alignment of *AtCuAOs* and evolutionary relationships

Multiple sequence alignment of cDNA sequences revealed a low level of sequence homology when all members of *AtCuAO* were aligned (Appendix A), however, alignment of the *AtCuAO* family in groups showed a high level of sequence conservation. In particular, alignment of predicted amino acid sequences of the gene family excluding *AtCuAO3-SP*, *AtCuAO9*, as they are short proteins, and *AtCuAO3*, as it is a very long protein, showed a high level of sequence conservation.

Comparison of amino acid and nucleotide sequences among members of *AtCuAO* family suggested that they could be grouped into discrete subsets (Figure 3-1). Phylogenetic trees derived from these two types of sequence analysis, at protein and DNA levels, were

identical. In the resulting phylogram, two distinct groups can be discriminated; *AtAO1*/ *AtCuAO2*/ *AtCuAO8*/ *AtCuAO5* and *AtCuAO1*/ *AtCuAO3*-SP/ *AtCuAO4*/ *AtCuAO7*/ *AtCuAO9*. *AtCuAO3* was divergent and shared the lowest similarity to other members of *AtCuAO* family, which excludes it from either of the two groups. The arrangement of Arabidopsis *CuAO* gene family members in the resultant tree branches in the present work agrees with their arrangement in parts of the phylogenetic tree created in a previous study to compare the relationship of grapevine *CuAO* sequences with other known plant *CuAOs* such as Arabidopsis, pea, soybean, lentil and tomato (Agudelo-Romero et al. 2013).

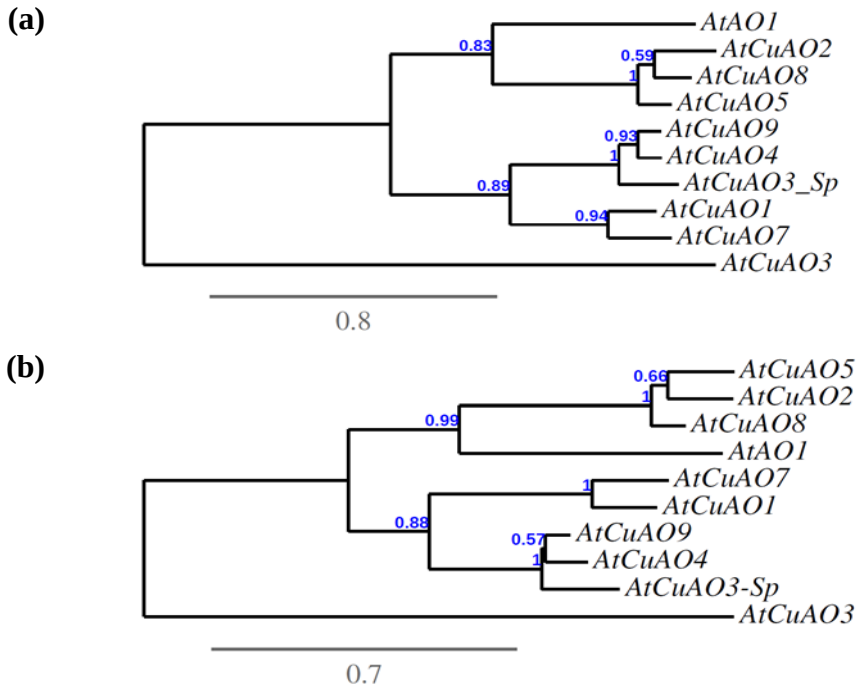



Figure 3-1 A phylogenetic tree of *AtCuAO* gene family. (a) Amino acid and (b) CDS nucleotide sequences showing the inferred evolutionary relationships between Arabidopsis *CuAO* genes.The phylogenetic trees were generated via Phylogeny.fr (http://www.phylogeny.fr) in "Advanced" mode. Multiple alignment of sequences was carried out by MUSCLE program (v3.7) using default parameters. The maximum likelihood method implemented in the PhyML program (v3.0) was used to reconstruct the phylogenetic tree. Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). The bootstrap values are indicated at the nodes. Accession numbers of *AtCuAO* genes are shown in Table 1-1.

### 3.3.2. Quantitative RT-PCR primer design, specificity, and RNA extraction

It was possible to design primers for all the *AtCuAO* family members such that at least one primer was from the 3'UTR zone except for *AtCuAO1* and *AtCuAO5* where the

3'UTR zone of these two genes was too similar to other genes. In addition, it was not possible to design primers for the short protein AtCuAO9 with similar criteria because no information was available about its 3'UTR sequence, and in addition homology with other genes was too high. Therefore, the first 300 nucleotides were used instead to design primers for *AtCuAO9* gene (Appendix A).

Testing the designed real-time PCR primers first on gDNA (Figure 3-2) showed that each pair of primers was efficient at amplifying a product of the predicted size (see Table 2-3). Amplification from the $H_2O$ control was detected when *AtAO1* primers were used but the product was clearly not the same size and probably nonspecific. Specificity was further confirmed by sequencing the PCR product and alignment of the sequence results with the entire gene sequences. However, for *AtCuAO9* sequencing results revealed lack of specificity of the primers designed for this gene. Two new pairs were designed one from the 3'UTR sequence following the last exon, which did not work, and the other one from the ORF, the first 300 nucleotides of the gene (Appendix A), which allowed amplification of the gDNA and showed high specificity based on sequencing and alignment results.

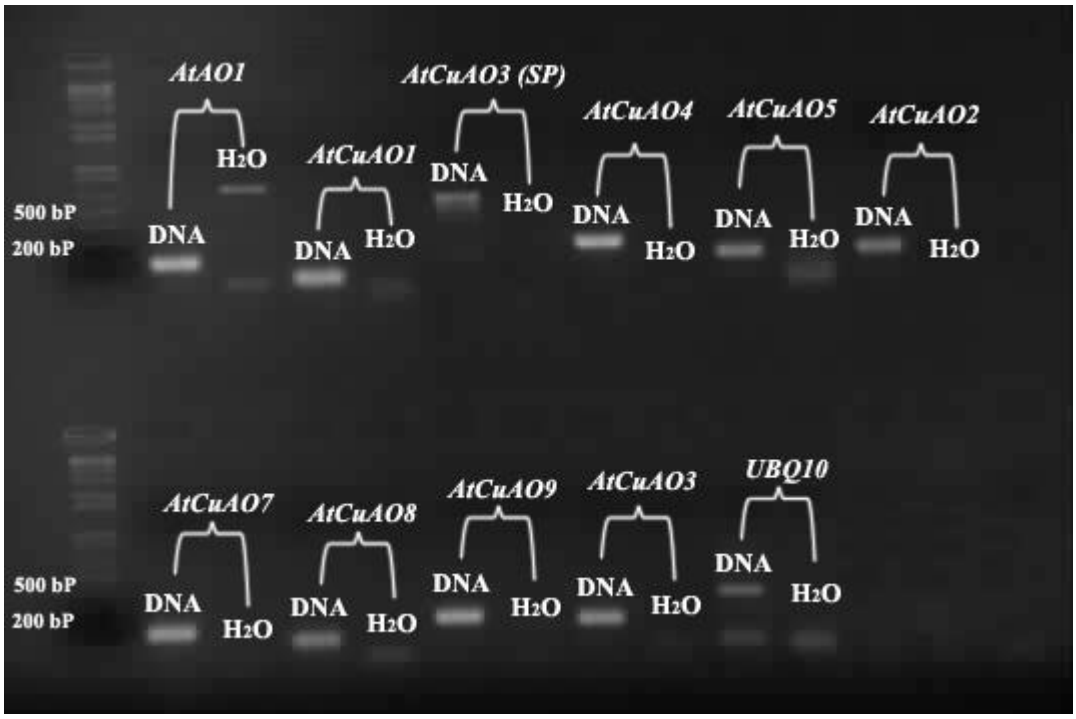



Figure 3-2 Test of *AtCuAO* primers on gDNA to verify the specificity. (Table 2-3, amplifying the correct gene). An ethidium bromide (EtBr) stained agarose gel was used to separate PCR products. *CuAO* genes are indicated by their symbols and *UBQ10* (Table 2-2) is used as a housekeeping gene.

RNA was extracted from plant leaves at different stages. Results showed that all RNA samples were of a good quality (Figure 3-3.a). DNA contamination was removed from RNA samples and then tested by PCR using 18S rRNA primers (Table 2-2) which revealed that all samples were free of genomic DNA (Figure 3-3.b). Quality of cDNA generated from the RNA was confirmed by amplification of *Actin2* (Table 2-2) by PCR using specific primers (Figure 3-3.c).

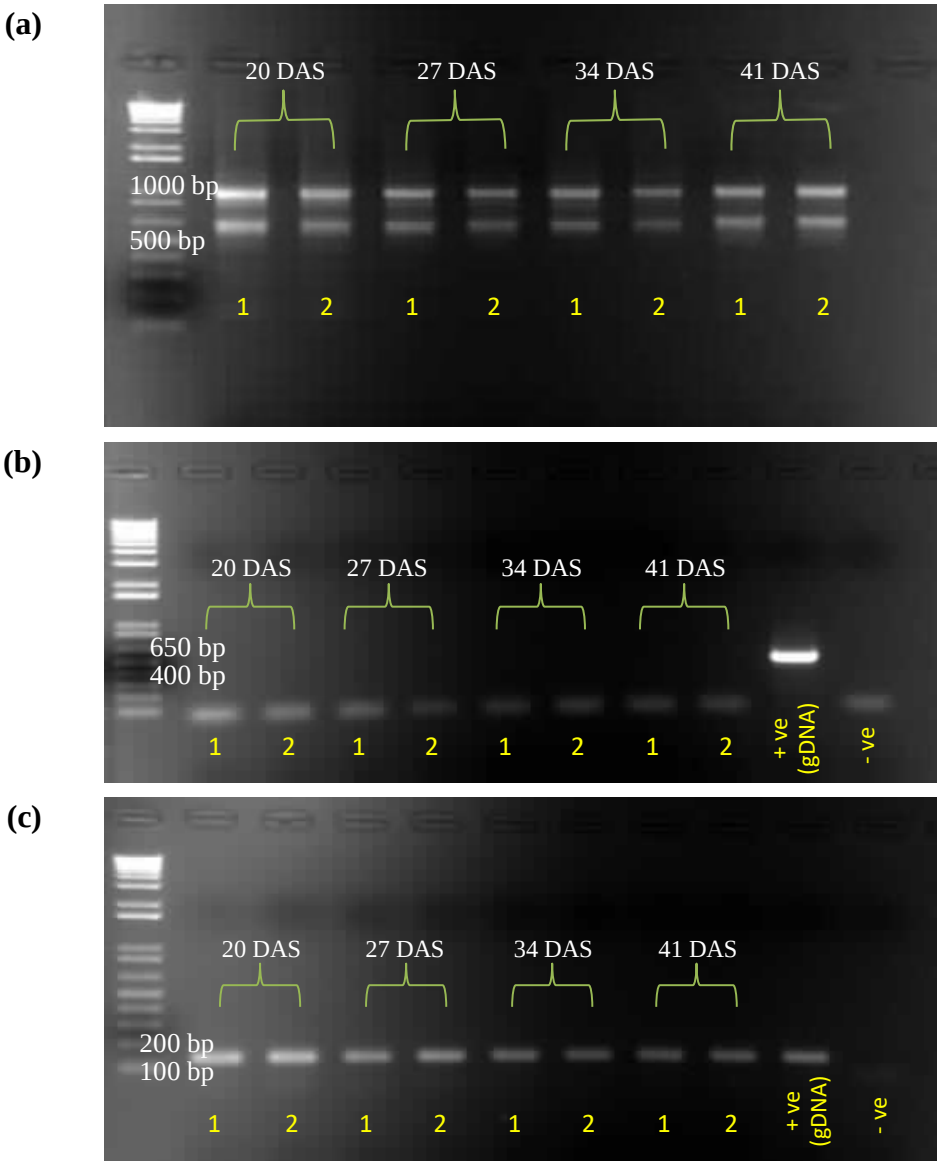



Figure 3-3 Quality control applied to isolated RNA from Arabidopsis leaves 5 and 6 before analysis by qRT-PCR. (a) Integrity of RNA was tested by agarose gel electrophoresis. (b) DNA digestion after DNase treatment was confirmed by PCR using 18S rRNA primers (Table 2-2). (c) Integrity of generated cDNA was tested by PCR reaction with *Actin2* primers (Table 2-2). Numbers 1 and 2 represent biological replicates at each stage. In both cases (b and c), gDNA was used as a positive control, 1Kb plus ladder (Invitrogen) was used as marker for PCR products, and electrophoresis performed on 1.4 % agarose.

As well as checking the qRT-PCR primers on gDNA, they were also tested using the single-strand cDNA generated from soil-grown Arabidopsis leaves no 5 and 6 at different stages during plant development, and the gDNA was used as a positive control. However, although amplification with *AtCuAO3*-SP, *AtCuAO5* and *AtCuAO9* was successful from gDNA after 40 PCR cycles, no amplicons were generated from the leaf cDNA (Figure 3-4.c, e, and i).

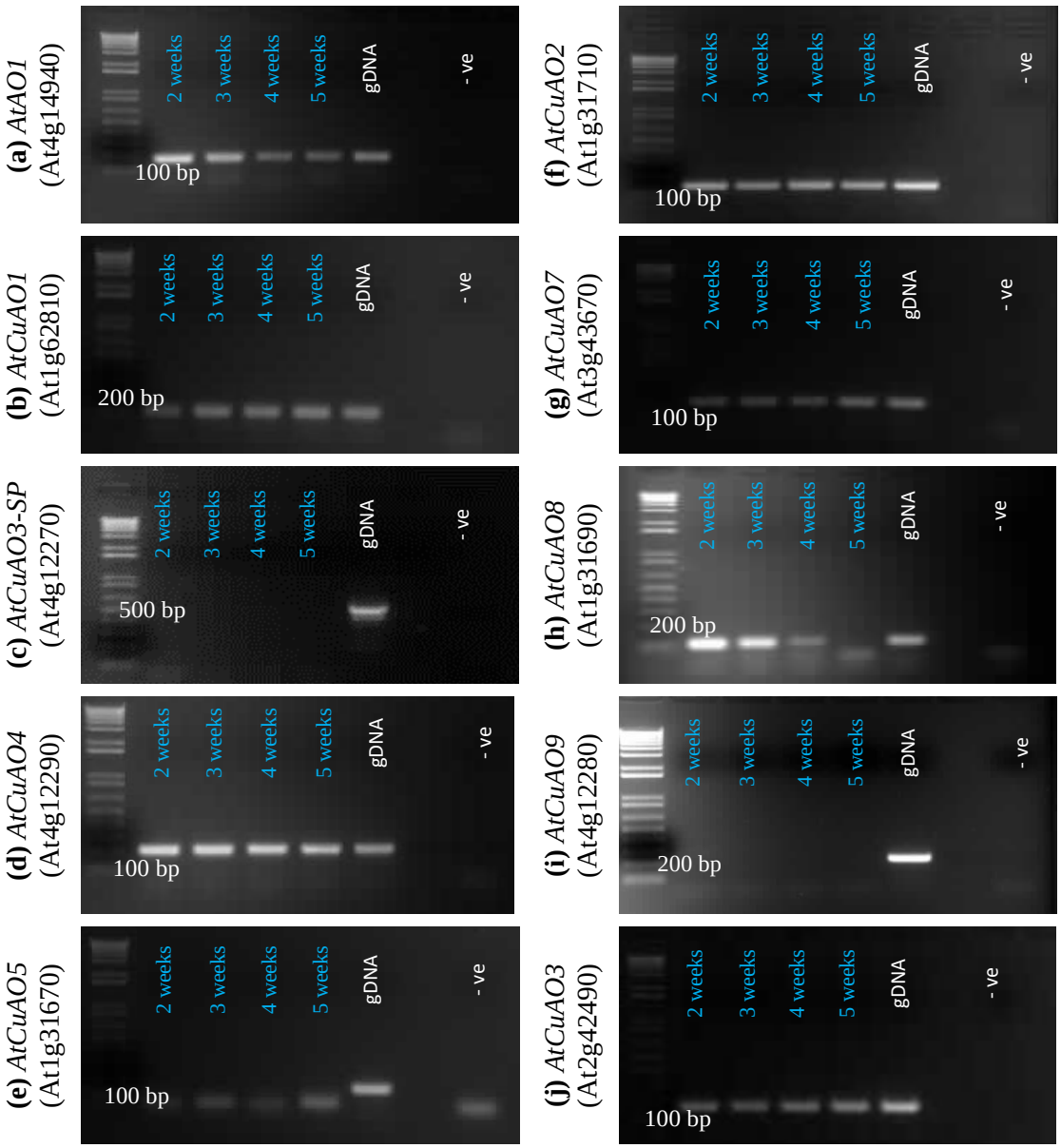



Figure 3-4 Test of the efficiency and specificity of *AtCuAO* primers on *Arabidopsis thaliana* leaf cDNA. Genomic DNA was used as a positive control. EtBr stained agarose gel of PCR products from gene specific primers (Table 2-3).

Both *AtCuAO3*-SP and *AtCuAO9* were analysed with other primer sets, which would amplify bigger fragments on the cDNA (an amplicon of 579 bp from *AtCuAO3*-SP and 645 bp from *AtCuAO9*) for more confirmation of the absence of these two transcripts. Results showed that neither of these two genes are represented in the leaf transcripts (Figure 3-5). Alternative primers for *AtCuAO5* were not sought as this gene from NCBI is a silique specific gene.

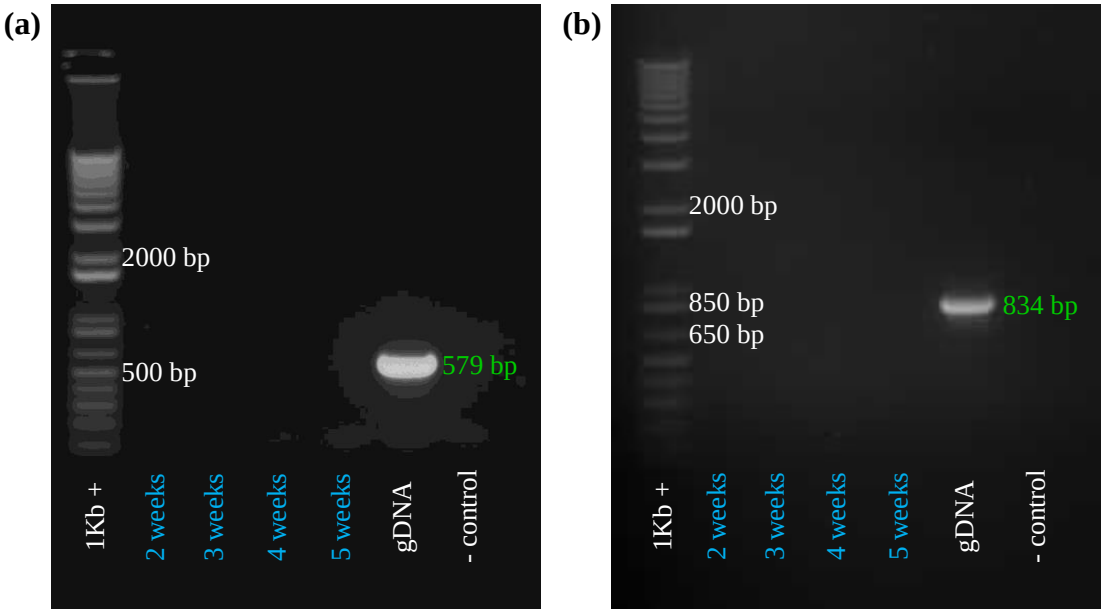
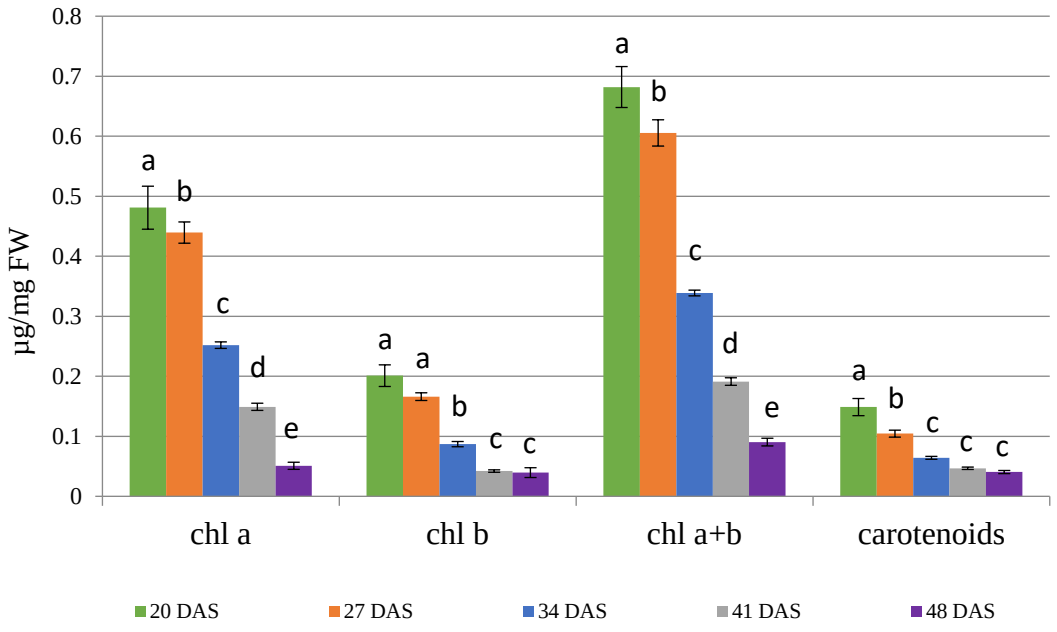



Figure 3-5 Confirmation of the absence of both *AtCuAO3-SP* and *AtCuAO9* from leaf transcripts using primers which are able to amplify bigger amplicons. (a) Arabidopsis leaf cDNA with alternative primers for *AtCuAO3-SP*. (b) Arabidopsis leaf cDNA with alternative primers for *AtCuAO9*. Genomic DNA (gDNA) was used as a positive control.

### 3.3.3. Chlorophyll content

The level of photosynthetic pigments was measured in leaves 5 and 6 of Arabidopsis rosettes as a marker for senescence progression (Figure 3-6). Total chlorophyll levels fell significantly at each time point compared with the amount at the previous measured stage until they reached the lowest level at 48 DAS. At this stage, leaves 5 and 6 were visibly yellow, and the total chlorophyll content was about 14 % of concentration recorded in the first studied stage (20 DAS). In contrast, carotenoid levels fell significantly as the plant developed up to 34 DAS, which was the stage at which yellowing commenced at the tip of leaves 5 and 6. After this, no variation in carotenoid content was observed.

**(a)**




**(b)** Representative at bolting

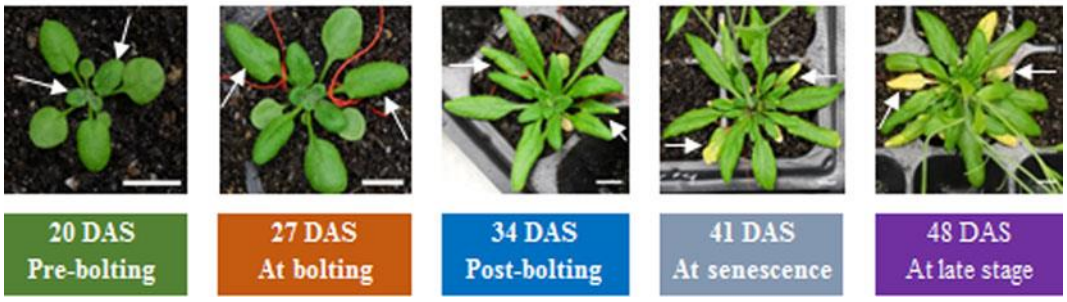


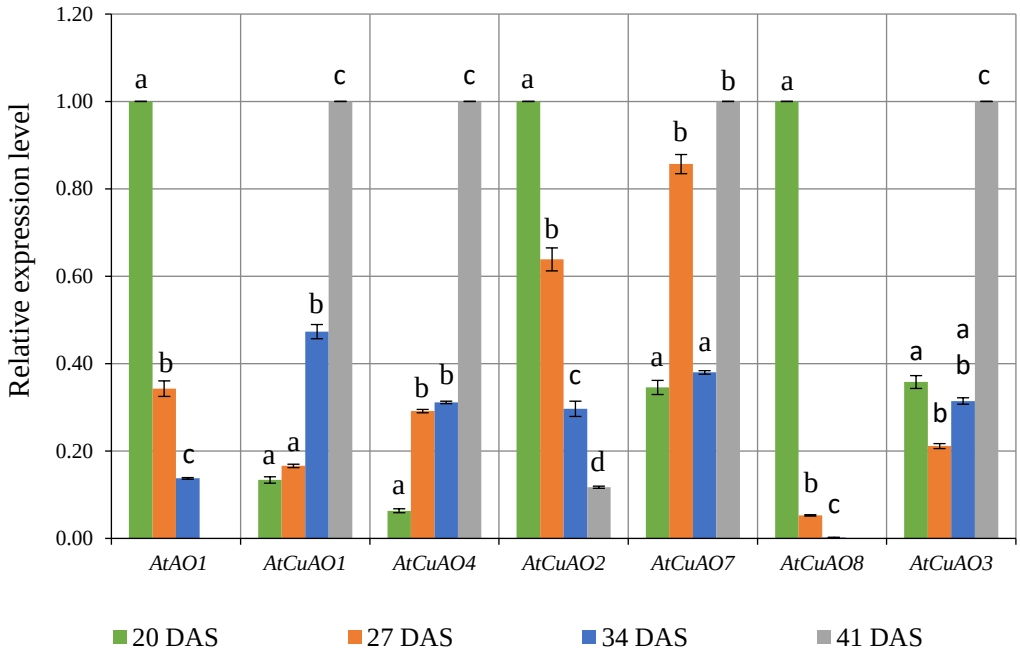

Figure 3-6 Progression of leaf senescence in soil grown *Arabidopsis thaliana* WT. (a) Photosynthetic pigment levels measured in leaves 5 and 6 at each stage of plant development. Different letters are used to indicate means that differ significantly (mean values ± SE; n=12; $P \leq 0.05$), and the results were statically tested using One-way ANOVA analysis. FW; fresh weight; DAS; days after sowing. (b) Images of the whole rosette (Scale bar = 1 cm).

### 3.3.4. Temporal expression of Arabidopsis *CuAO* genes

To understand more about the expression pattern of *AtCuAOs* in leaves during plant development, transcript levels of all the ten *AtCuAO* genes was examined by real-time RT-PCR using total RNA isolated from leaves 5 and 6 of *Arabidopsis thaliana* (Col-0) wild type at different stages of development (20, 27, 34, and 41 DAS). Results demonstrated that, seven out of ten genes of the *AtCuAO* gene family are expressed at

different levels during leaf development, whereas the expression level of three of them, *AtCuAO3-SP*; *AtCuAO5* and *AtCuAO9*, was below detection in leaves at all analysed stages during Arabidopsis growth (Figure 3-7).

**(a)**




**(b)** Representative at bolting

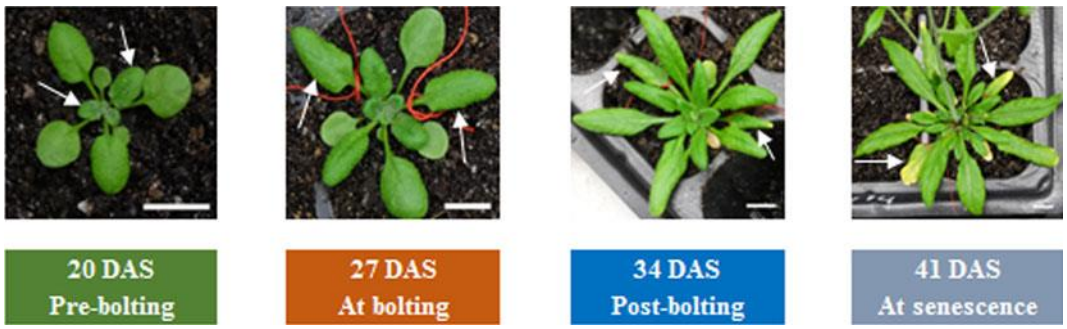



Figure 3-7 Expression pattern of the *AtCuAO* gene family in *Arabidopsis thaliana* WT. (a) Transcript levels of mRNA were estimated by qRT-PCR using SYBR green dye in leaves 5 and 6 at four different stages (20, 27, 34, 41 DAS) during WT Arabidopsis (Col-0) growth using gene specific primers designed for each member of *AtCuAO* gene family (Table 2-3). *Actin2* was used as a housekeeping gene to normalize target gene expression levels (see Table 2-3 for primer sequence). The transcript levels of each gene at each stage were expressed compared to the stage where its expression was the highest. Values are the mean ± SE of two biological replicates (20 leaves/ replicate), each with three analytical replicates. Bars of the graph represent standard errors, and significant differences in means are indicated by different letters ($P \leq 0.05$). The results were statically tested using One-way ANOVA analysis. (b) Images of the whole rosette (Scale bar = 1 cm).

The highest expression level of *AtAO1* and *AtCuAO8* was at early stages of leaf development (before bolting) and gradually declined until their expression was below detection in senescent leaves (Figure 3-7).

In the same manner, *AtCuAO2* was highly expressed in young leaves (20 DAS) and gradually declined upon growth progression until it reached its lowest level of expression during senescence (Figure 3-7).

In contrast, the transcript levels of *AtCuAO1*, *AtCuAO4* and *AtCuAO3* increased significantly in leaves of 41 day-old seedlings compared with earlier stages and the first two genes showed a very similar pattern of expression (Figure 3-7). Interestingly, *AtCuAO7* had a unique pattern of expression where the transcript of this gene accumulates at bolting, dropped post-bolting and then reached the highest level when leaves 5 and 6 were 41 days-old and almost senescent (Figure 3-7).

### 3.3.5. Spatial and temporal expression of *AtCuAO7* and *AtCuAO8*

The temporal and spatial expression pattern of selected *AtCuAO* genes was characterised in leaves at similar stages of plant growth (before bolting, at bolting, after bolting, and at senescence), by using *AtCuAO7*::GUS and *AtCuAO8*::GUS lines.

*SAG12*::GUS was used as a positive control since it is a senescence-specific gene (Gan and Amasino 1997), up-regulated during senescence in leaf, stem, and flowers (Grbić 2003), and is often used as a molecular marker for senescence (Weaver et al. 1998). WT plants were used as a negative control.

The first expression of *AtCuAO7*::GUS was detectable in leaves of 20 day old plants and occurred at specific sites in the leaf (Figure 3-8), such as the epithem of hydathodes (Figure 3-8.e), the epidermal hair trichomes (Figure 3-8.f), and axillary buds in the axils of leaves (Figure 3-8.g). However, during further leaf development, the expression of *AtCuAO7* was too low to be detected by *AtCuAO7*::GUS (Figure 3-9.d, Figure 3-10.c, and Figure 3-11.c). Furthermore, expression of *AtCuAO7*::GUS was high in roots during early stages of seedling development (Figure 3-8.c, Figure 3-9.d).

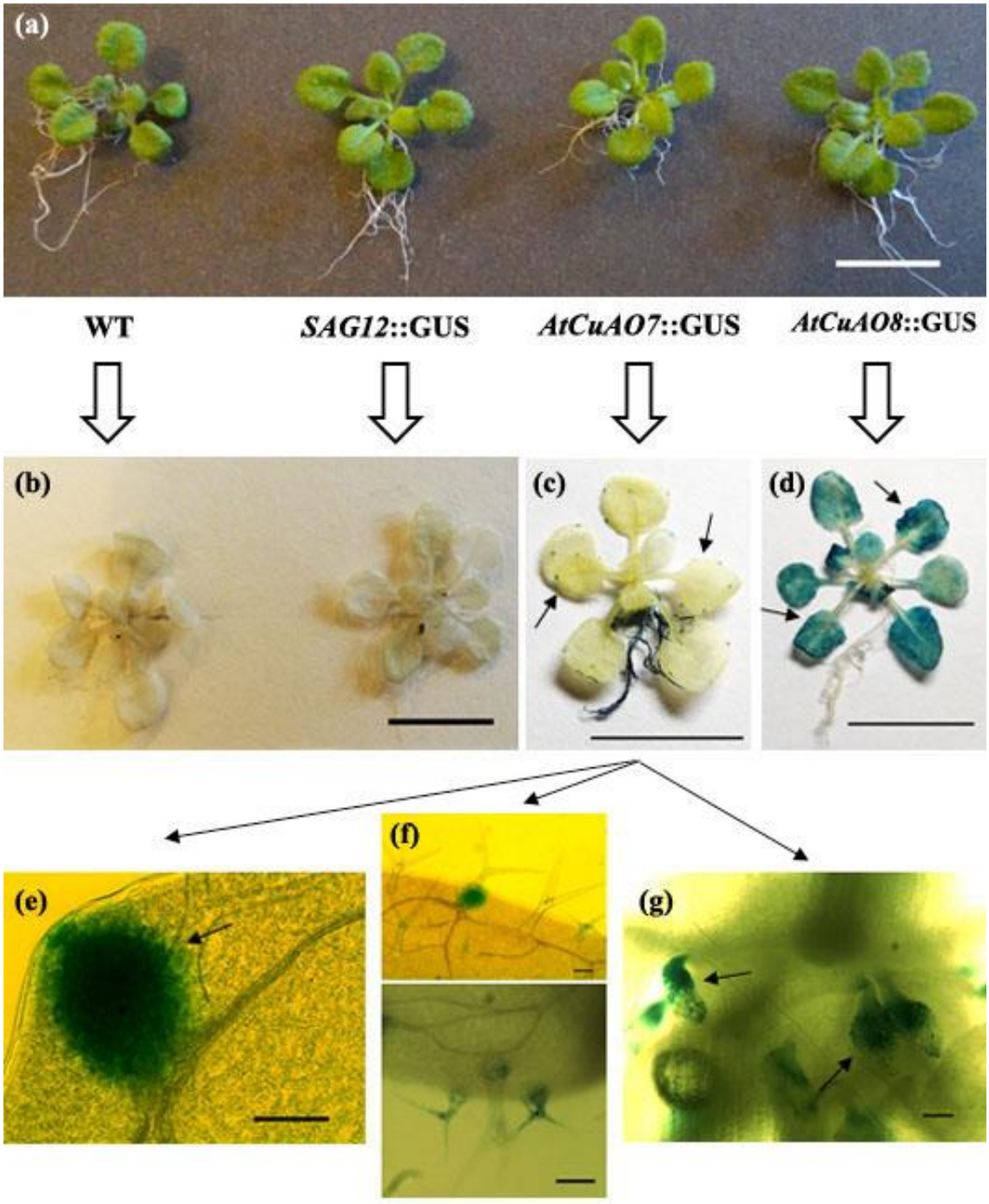



Figure 3-8 Histochemical GUS staining of *AtCuAO* transgenic *Arabidopsis thaliana* plants. (a) Pre-staining phenotype of *AtCuAO7*::GUS and *AtCuAO8*::GUS compared with WT and *SAG12*::GUS as negative and positive control plants respectively at 20 DAS (pre-bolting). (b-d) Post-staining phenotype of WT, *SAG12*::GUS, *AtCuAO7*::GUS and *AtCuAO8*::GUS respectively. Arrows in (c) and (d) indicate leaves number 5 and 6. (e-g); light microscopy analysis showing blue stain accumulation in different leaf sites of *AtCuAO7* transgenic plants, hydathodes, trichomes, and axillary vegetative meristems respectively. Scale bars of (a-d) = 1 cm and in (e-g) = 100 µm.

On the other hand, *AtCuAO8*::GUS was strongly expressed in leaves at all studied stages (Figure 3-8.d, Figure 3-9.e, Figure 3-10.d, and Figure 3-11.d and e). Strongest *AtCuAO8*::GUS expression was found in green leaves of young transformed plants, mainly in leaf blades (Figure 3-8.d), and the intensity of GUS staining declined as leaves aged and senesced (Figure 3-10.d and Figure 3-11.e). It can be observed that the onset of decline in *AtCuAO8* activity was associated with floral transition (Figure 3-9.e), and thereafter, expression appeared to depend on the level of leaf yellowing. It disappeared completely in senescent leaves (Figure 3-11.d) depending on leaf developmental stage, or remained at a low level of expression in greener leaves (Figure 3-11.e).

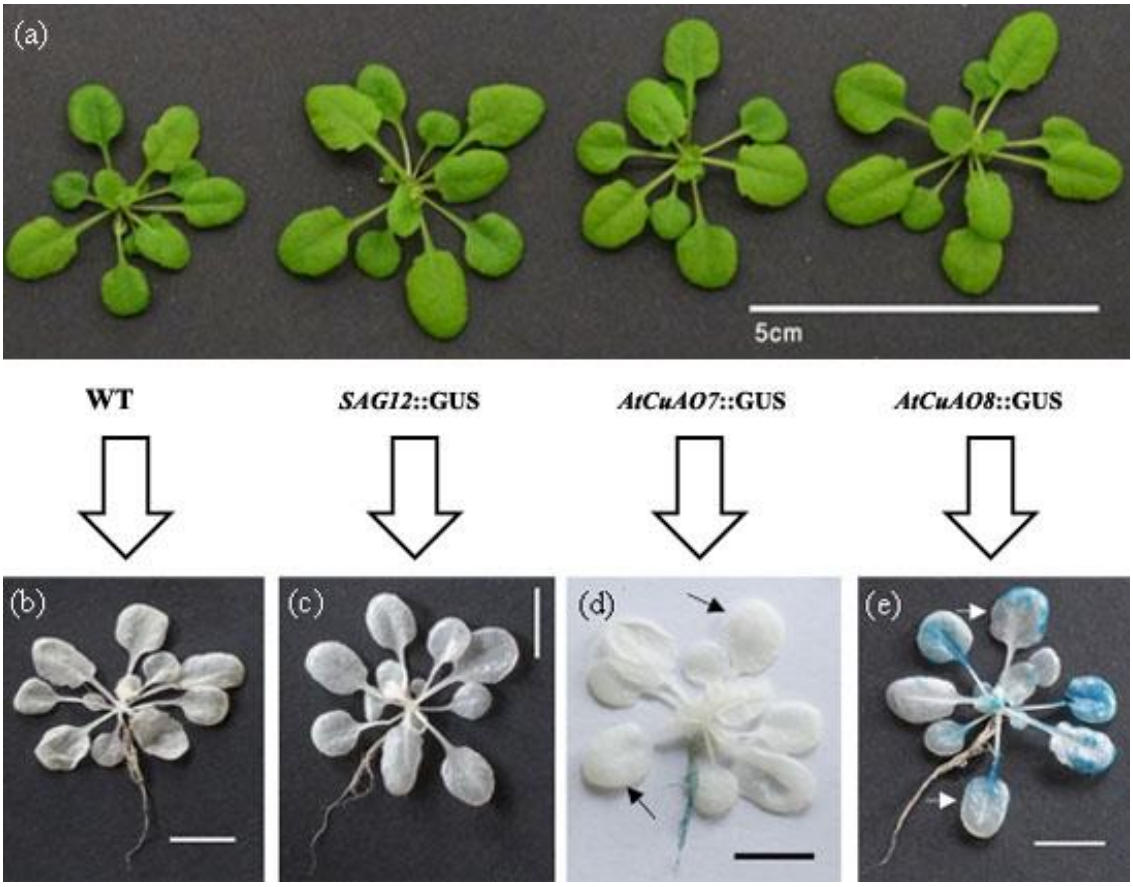



Figure 3-9 GUS expression pattern detecting *AtCuAOs* expression at bolting stage in 27-day old plants. (a) Shows plant phenotype at bolting before staining with the X-Gluc solution. (b-e) GUS histochemical staining of WT, *SAG12*::GUS, *AtCuAO7*::GUS and *AtCuAO8*::GUS respectively. Arrows in (d) and (e) indicate leaves number 5 and 6. Scale bars of (b-e) = 1 cm.

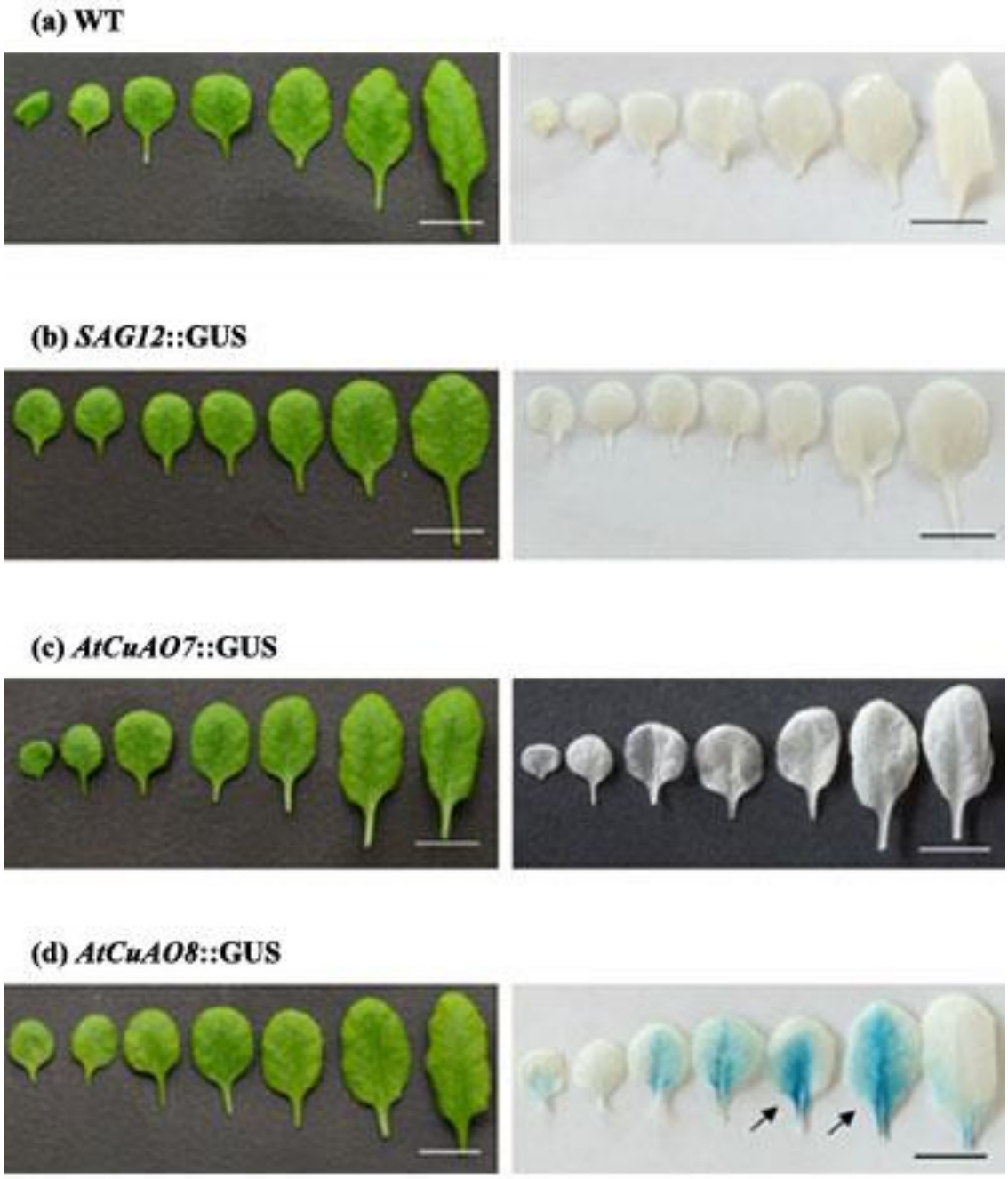



Figure 3-10 Expression of *AtCuAO7* and *AtCuAO8* in Arabidopsis transgenic plant leaves after bolting in 34-day old plants. Left panel shows leaf phenotype of the oldest seven leaves before staining starting from the oldest leaf on the left for different lines studied, grown under controlled conditions (16 h light/ 8 h dark). The right panel shows GUS activity after using X-Gluc as a substrate for histochemical staining. (a) WT, (b) *SAG12*::GUS, (c) *AtCuAO7*::GUS, and (d) *AtCuAO8*::GUS. Arrows indicate GUS expression pattern in leaves 5 and 6. Scale bars = 1 cm.

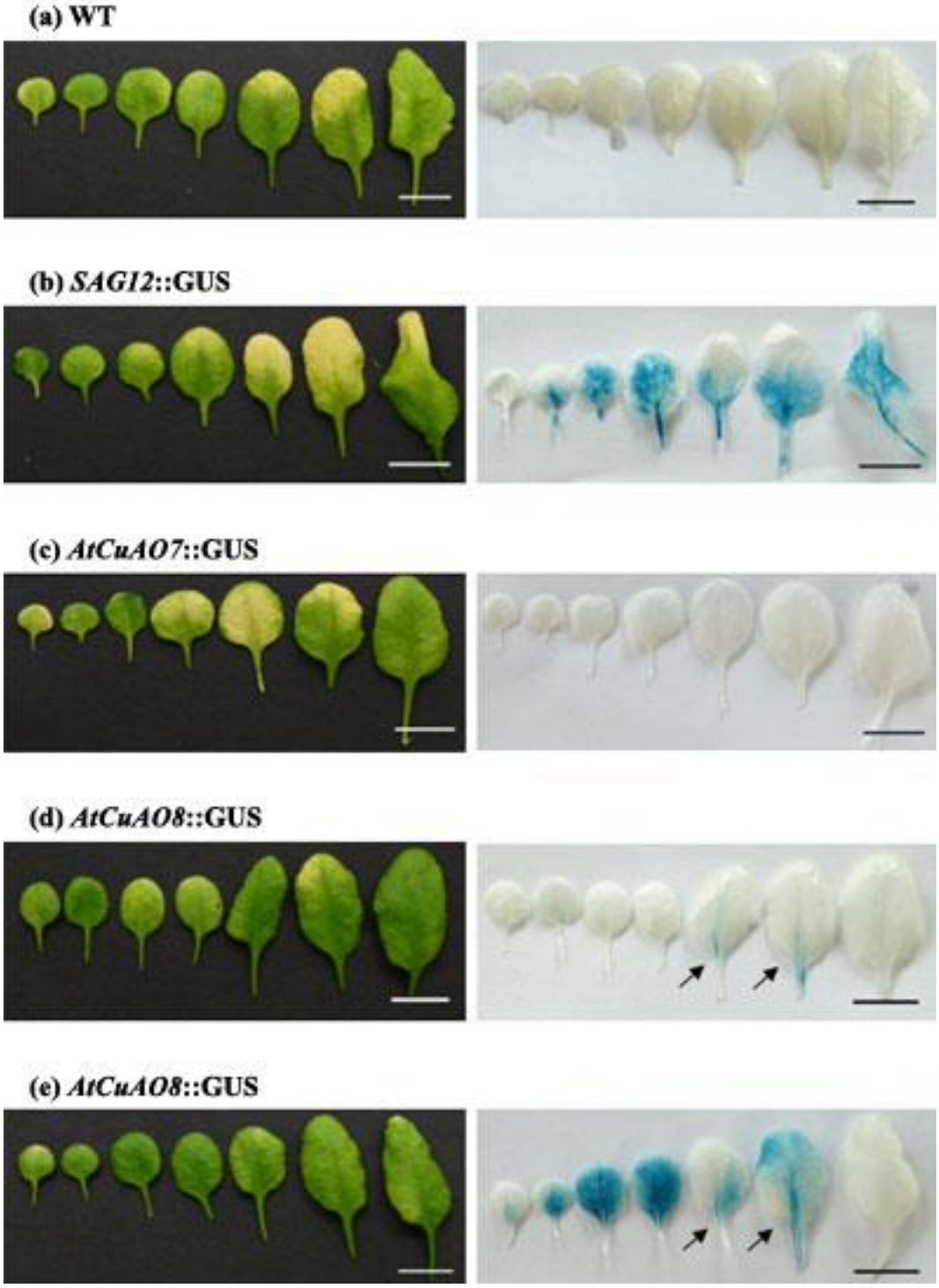



Figure 3-11 Expression of *AtCuAO7* and *AtCuAO8* in Arabidopsis transgenic plant leaves at late stage in 41-day old plants. Left panel shows leaf phenotype of the oldest seven leaves before staining, starting from the oldest one on the left for different studied lines grown under controlled conditions (16 h light/ 8 h dark). The right panel shows GUS activity after using X-Gluc as a substrate for histochemical staining. (a) WT, (b) *SAG12*::GUS, (c) *AtCuAO7*::GUS, (d and e) *AtCuAO8*::GUS transformed plants. Arrows indicate GUS expression pattern in leaves 5 and 6. Scale bars represent 1 cm.

GUS activity was further quantified by a fluorometric method (Figure 3-12). Levels of GUS activity in *AtCuAO8*::GUS plants was significantly high in leaves compared with WT and *SAG12*::GUS in all studied stages except the last stage (41 DAS), where its expression reduced to the lowest level (became undistinguishable from WT). This coincided with the onset of leaf yellowing, which was obvious at this stage, along with expression of the senescence marker gene *SAG12* which reflects the onset of late senescence processes in leaves.

GUS activity in the *SAG12*::GUS line was low up to 41 DAS where it suddenly rose and became significantly higher than the other plants. *AtCuAO7*::GUS activity was lower compared to *AtCuAO8*::GUS at all time points, and no significantly greater activity of *AtCuAO7*::GUS was detectable by the fluorogenic assay in any of the stages studied compared with the other lines tested including WT.

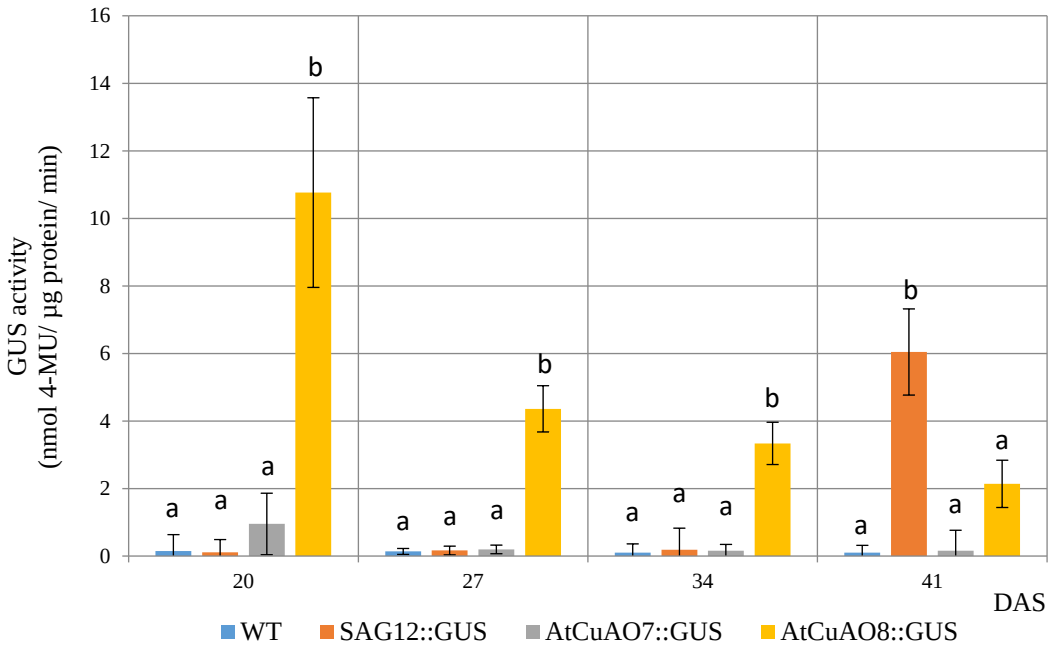



Figure 3-12 Fluorogenic assay of *AtCuAO8* and *AtCuAO7* driven GUS activity. Plants were first grown on MS medium with or without Kan and were then moved to soil. GUS activity was assayed quantitatively in leaves 5 and 6 at different stages under control conditions. Values are the mean ± SE of two biological replicates each with three analytical replicates. Bars of the graph represent standard errors. Significant differences in means between genotypes at each stage are indicated by different letters ($P \leq 0.05$). The results were statically tested using One-way ANOVA analysis.

## 3.4. Discussion

The results presented here provide, for the first time, a complete picture in leaves of the expression of seven of the ten *AtCuAO* genes together allowing a comparison in their patterns of expression throughout leaf senescence including before and after bolting.

Analysis of *CuAO* gene expression using real-time quantitative PCR revealed that the expression of three of them, *AtCuAO3-SP*; *AtCuAO5* and *AtCuAO9*, was below detection in leaves at all analysed stages. Results obtained by qRT-PCR about *AtCuAO5* expression in the present work are in good agreement with the microarray data on TAIR and PRESTA which showed that this gene is not expressed in leaves at any developmental stage, in addition to the information available on NCBI database which indicates that this gene is expressed in silique at specific stages. However, these two bioinformatic sources, TAIR and PRESTA, are not in full agreement regarding the expression information of *AtCuAO3-SP* and *AtCuAO9*.

According to TAIR, *AtCuAO3-SP* is expressed in young and senescent leaves while *AtCuAO9* is expressed only in senescent leaves; according to the microarray data from PRESTA the expression of these two genes did not clearly change during leaf development. Primer sets that were used to study the expression of all *AtCuAO* genes, including the three undetectable genes, by qRT-PCR in the present work were tested first on the gDNA and showed strong bands (Figure 3-2). In addition, the homology shown by multiple alignment between sequencing results of amplified fragments using gDNA and sequences of these genes was high.

To refute the doubt that primers used in the case of the two non-expressed genes, *AtCuAO3-SP* and *AtCuAO9*, were amplifying a small fragment which was below the limit of detection or that the sequence was in some way incorrect, another combination of primers which should amplify a bigger fragment were used. Both new sets of primers amplified the target sequence on the gDNA but nothing resulted by using them on the cDNA (Figure 3-5). These verification steps are probably sufficient to reflect the efficiency and specificity of these primers and hence I conclude that these genes are probably not expressed under normal conditions in leaves at the stages studied or they

may be expressed only under specific conditions. It is possible that array data may be giving a false positive result due to homology with other members of the gene family.

For some of the other genes studied here expression can be compared to the literature. Planas-Portell et al. (2013) examined the expression of three *CuAOs*, *AtCuAO1-3*, in the whole seedling at different time points (4-, 12-, 21-, and 28-day old plants) using real-time RT-PCR. They detected an increase in *AtCuAO1* and *AtCuAO3* by about 3-fold in 28-day old seedlings compared with young ones (4-day old) which fits with the increase with increasing age seen here for these two genes in leaves where *AtCuAO1* expression increased by about 7.5 fold while *AtCuAO3* increased by ~3 fold in leaves of 41-day old seedlings compared with younger ones of 20-day old plants by taking into account that comparison between transcript levels of each gene was performed at later stages in the present work. In contrast, expression analysis of *AtCuAO2* showed a decrease in its transcript accumulation in 28 day old seedlings compared with the 4 day old seedlings, although this change in gene activity was not significant (Planas-Portell et al. 2013). The significant decrease in *AtCuAO2* expression seen here between day 20 and day 41 (8.5 fold decrease) is thus in agreement with these previous data. Taking into consideration that using a specific organ (single leaves), as used in this study, may provide clearer results of gene expression change than utilizing RNA isolated from the whole seedling which is composed of different organs at different stages of development.

The expression results of *AtCuAO1*, *AtCuAO4* and *AtCuAO7* that showed an increasing trend with progressive stages of leaf development are consistent with results reported previously (Ghuge 2014). The developmental expression of three *CuAOs* in whole rosettes at four different time points (2-, 4-, 6-, and 8- week old plants) was investigated using qRT-PCR analysis, and results showed that the expression of *AtCuAO1* was induced by 3 and 9 fold (Ghuge 2014), which is consistent with what we found here where the expression of this gene increased about 8 fold in leaves of 41-day old seedlings as compared with those of 20-day old plants (Figure 3-7), while Ghuge (2014) reported an increase in *AtCuAO7* expression estimated by 2 fold, in 6 and 8 week old plants which is again in agreement with our results showing a significant increase by about 3 fold in 41-day old plant leaves as compared with those of 20-day old plants (Figure 3-7). The

expression of *AtCuAO4* increased dramatically with plant age and recorded a 21 fold increase in 6 and 8 week old plants relative to the transcript levels of these genes measured in 2 week old plants (Ghuge 2014). In addition, the effect of transition towards flowering on *AtCuAO4* transcript levels was tested in leaves of 22 day-old plants and of 52 day-old plants corresponding to before and after onset of flowering, and results showed a 21-fold induction in leaves *AtCuAO4* after flowering as compared to its transcript accumulation measured in leaves before flowering (Ghuge 2014). Again this agrees with results here showing a strong increase, by about 16 fold, in *AtCuAO4* gene expression in leaves following bolting, with the advantage that in the present study the use of single leaves gives a better representation of senescence progression than using the whole rosette since gene expression obtained from the whole rosette leaves results from leaves at different developmental stages.

There is no clear change shown by microarray data of the expression pattern of *AtAO1* in developing leaves, however, GUS staining analysis revealed high activity of this gene in young leaves of 4-5 day old seedlings (Ghuge 2014), and a previous study tested the expression of *AtAO1*-GUS in leaves at three different stages (primary, secondary and tertiary leaves) from a single Arabidopsis rosette and reported a high expression of *AtAO1*-GUS in the vascular tissue of immature developing leaves (tertiary leaves) which reduced in later stages of leaf development in primary and secondary leaves (Moller and McPherson 1998). These results support the expression presented here by qRT-PCR where the highest *AtAO1* expression was detected in young leaves and gradually reduced with plant age until it disappeared in senescent leaves.

To confirm the expression pattern of *AtCuAOs* at various stages of leaf growth obtained by real time RT-PCR analysis, the temporal leaf-specific expression patterns of two available GUS lines, *AtCuAO7*::GUS and *AtCuAO8*::GUS, were studied in leaves 5 and 6 at corresponding time points during plant development. Qualitative analysis of *AtCuAO7* promoter revealed GUS expression in leaves only at 20 DAS restricted to some specific sites as shown in Figure 3-8.c, but it was not significantly different from either *SAG12*::GUS or WT, as control plants, when it was analysed quantitatively by the fluorometric method. Furthermore, no induction of *AtCuA7*::GUS expression could be

detected in leaves beyond this time point. Results from the promoter-GUS lines were not therefore entirely consistent with expression data from transcriptional analysis. *AtCuAO7* was found to be expressed at early stages of leaf development and increase with plant age until it reached its highest level of expression at senescence (Ghuge, 2014; TAIR; https://www.arabidopsis.org/), which is in agreement with results obtained here by qRT-PCR. However, *AtCuA7* was expressed at low levels throughout early stages of leaf development (Winter et al. 2007). The reason for this underestimation of expression using the *AtCuAO7*::GUS reporter gene may include a higher sensitivity of real time PCR compared to GUS assays (Pistón et al. 2009), the low level of expression of this gene throughout early stages of leaf development, the length of the promoter used in the construct or the point of insertion in the genome of the transgenic plant. Another discrepancy between the GUS and TAIR data is *AtCuAO7* expression in root. According to TAIR, the expression of *AtCuA7* is very low in roots while it seems to be high in the *AtCuA7*::GUS plants during early stages of development (Figure 3-8.c, Figure 3-9.d). This further suggest a problem with the transgenic line. Variability in gene expression patterns can occur as a result of random integration of genes in the genome (Mertens 2008). Effects on expression have also been noted in Arabidopsis transgenic plants constructed with four different sizes of the *DRG2* promoter which resulted in undetectable GUS expression in case of shortest promoters while the longest promoters showed strong activity of GUS gene (Stafstrom 2008).

The results reported by *AtCuAO8*::GUS gene activity quantified qualitatively or quantitatively were in good agreement with previously reported microarray data (TAIR eFP Browser; http://bar. utoronto.ca/efp/cgi-bin/efpWeb.cgi) and with the real time PCR results presented in this work, showing a reduction in *AtCuAO8* expression as leaves age. In some cases *AtCuAO8* expression fell below detection or was very low (Figure 3-10.d and Figure 3-11.d and e). This variability may be due to variation in leaf development across different plants. The high variability of leaf development amongst *Arabidopsis thaliana* plants at a specific time after sowing has been reported previously (Granier et al. 2002; Leister et al. 1999), and the effect of variation in leaf emerging time and leaf position on levels of gene expression was indicated (Xie et al. 2012). Hence, differences in results of *AtCuAO8* expression produced by both methods may be attributed to the

variability in developmental stages of leaves used to perform the analysis due to variability in seed germination or plant developmental stage.

The evolutionary relationship resulting from the phylogenetic analysis using amino acid and CDS nucleotide sequences (Figure 3-1.a and b), which divided the *AtCuAO* gene family into two distinct groups, *AtAO1*/ *AtCuAO2*/ *AtCuAO8*/ *AtCuAO5* and *AtCuAO1*/ *AtCuAO3*-SP/ *AtCuAO4*/ *AtCuAO7*/ *AtCuAO9* maps very closely onto the distinct expression pattern seen here from real-time qRT-PCR. Amino acid alignment showed a high level of sequence conservation between *AtAO1*, *AtCuAO1* and *AtCuAO2* (Planas-Portell et al. 2013). *AtAO1* exhibited 60 and 65 % similarity to *AtCuAO1* and *AtCuAO2* respectively, while *AtCuAO1* and *AtCuAO2* showed a similarity of 63 %. However, the sequence similarity of *AtCuAO3* with *AtAO1*, *AtCuAO1*, and *AtCuAO2* was lower, 45%, 43%, and 44% respectively. Thus the differences in expression pattern may be reflected in differences in protein structure and hence distinct enzymatic roles during leaf development.

A variety of physiological, morphological and molecular changes take place during the different stages of leaf development which eventually lead to leaf senescence. One of the most obvious leaf senescence syndromes is the visible colour change as a result of chloroplast dismantling and chlorophyll degradation (Bleecker and Patterson 1997). In the present work, chlorophyll contents were analysed along with *AtCuAO* gene family expression analysis during leaf development to act as a marker for leaf development and senescence. The expression pattern of one group, *AtCuAO1*, *AtCuAO4*, *AtCuAO7* and *AtCuAO3*, was negatively associated with total chlorophyll contents suggesting that these genes could have a more relevant role in leaf senescence. In contrast, the expression of the other group of genes, *AtAO1*, *AtCuAO2* and *AtCuAO8*, was positively correlated to the total chlorophyll concentrations suggesting a relation between the activity of these genes and chlorophyll content. The present results of the expression patterns of *AtCuAO1*, *AtCuAO4*, *AtCuAO7* and *AtCuAO3* are supported by the information available about microarray analysis of *CuAOs* at TAIR, the Arabidopsis genome database (Winter et al. 2007), which showed that these four genes are included within the six members of *CuAOs* that are highly expressed at late stages of plant development.

In fact it may not be surprising that some *AtCuAOs* are expressed at early stages of leaf development, due to the increasing evidence that suggests the contribution of some of them, *AtAO1* and *AtCuAO1*, to vascular tissue differentiation, via hydrogen peroxide ($H_2O_2$) generated by CuAO activity, which is a key process in young leaves (Cona et al. 2006; Paschalidis and Roubelakis-Angelakis 2005a). Arabidopsis AtAO1 protein was detected in the tracheary elements of root vascular tissue, root caps, and cells subject to PCD (Moller and McPherson 1998). However, the high level of the other *CuAO* transcripts at late stages, during leaf senescence, are less clear. The final developmental phase of leaf is the senescence program (Schippers et al. 2007) which includes many changes in gene expression (Lim et al. 2007). Towards the end of leaf senescence, once most remobilization of nutrients has taken place, cells start to undergo PCD (Gan 2007). This is also often associated with a rise in reactive oxygen species (Woo et al. 2013). The fall in chlorophyll levels post-bolting indicates that remobilisation has been initiated, which will ultimately lead to PCD. Hydrogen peroxide ($H_2O_2$) is one of the products produced by PAs catabolism via the action of CuAOs (Moschou et al. 2012). It is possible that *AtCuAO* genes which were highly expressed during leaf senescence (*AtCuAO1*, *AtCuAO4*, *AtCuAO7* and *AtCuAO3*) may contribute as sources of reactive oxygen species during leaf senescence but this would need further investigation.

# Chapter Four

## Manipulation of *AtCuAO4*

# 4. Effects of mutation and over-expression of *AtCUAO4* on Arabidopsis growth and development

## 4.1. Introduction

Of the ten *AtCuAO* genes in the Arabidopsis copper binding diamine oxidase gene family, *AtCuAO4* (At4g12290) (Figure 4-1) is one of the *CuAO* genes known to be highly expressed in ageing leaves (Arabidopsis genome database; TAIR eFP browser; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). It was therefore selected here for further study. Ghuge (2014) further verified its expression in whole rosettes and using promoter-GUS lines, and characterised the seedling phenotypes of two mutant lines and several over-expressor lines which were made available to us by Prof. Alessandra Cona, University of Rome (Roma Tre) for a whole plant phenotypic analysis.

According to the data on the eFP browser, AtCuAO4 is located in vacuoles and the gene is most highly expressed in maturing seeds and in senescent leaves (Winter et al. 2007). A more detailed expression analysis using qRT-PCR revealed that under normal conditions the expression of *AtCuAO4* increases dramatically (21 fold) in leaves of 52-day old plants as compared to its expression in leaves of 22-day old plants, whereas GUS-staining analysis in five-day old seedlings showed that *AtCuAO4* transcript is abundant in the new emerging leaves and tips of cotyledons (Ghuge 2014). Furthermore, *AtCuAO4* was identified as a senescence associated gene [SAG], by suppression subtractive hybridisation (Gepstein *et al.* 2003) confirmed by northern blotting.

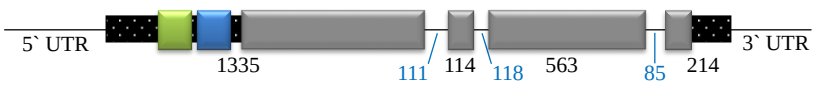



Figure 4-1 Schematic representation of the full length *AtCuAO4* (At4g12290) gene. Gene size = 2540 bp on the genomic DNA, and 2226 bp on CDS-ORF sequence. The domains were identified using the NCBI conserved domain search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and they are shown in different colours [green, *CuAO* N2 domain with a length of 250 bp starts from 250 to 500; blue, *CuAO* N3 domain with a length of 300 bp starts from 525 to 825]. Function of both N2 and N3 domains is uncertain. The rest of the protein coding region contains the active site and copper binding residues. The enzyme catalyses the oxidative deamination of primary amines to the corresponding aldehydes, with concomitant reduction of molecular oxygen to hydrogen peroxide.

Recently, Ghuge (2014) analysed the expression of the *AtCuAO4* gene quantitatively using qRT-PCR in twelve-day old seedlings following treatment with phytohormones

and abiotic stresses. Application of the defence related hormone, salicylic acid (SA) induced the expression of the gene indicating a possible role for this gene during pathogen infections. Treatments with abscisic acid (ABA; 10 and 100 µM) as well as treatment with NaCl also induced *AtCuAO4* expression suggesting the involvement of *AtCuAO4* in abiotic stress responses related to regulation of water balance such as salt or water stress. However, cytokinin down-regulated the expression of *AtCuAO4*, while auxin and mannitol treatments were not effective in modulating the expression of the gene. Furthermore, as *AtCuAO4* mRNA transcript is abundant in guard cells (TAIR eFP browser; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi, and PRESTA; http://www2. warwick.ac.uk), Ghuge (2014) investigated the involvement of AtCuAO4-induced polyamine (PA) oxidation in guard cells in the ABA-mediated stomatal closure. While no differences in the stomatal closure between WT and *AtCuAO4* mutants (BIS#4 and C#4) under normal conditions, a significant reduction in the stomatal closure was observed in the two *AtCuAO4* mutants as compared to WT plants after treatment with 10 and 100 µM ABA. Treatment of WT plants with the hydrogen peroxide ($H_2O_2$)-scavenger dimethylthiourea (DMTU) reversed the ABA-induced stomatal closure (~75%) while treatment with DMTU alone did not affect stomatal closure. Treatment with the substrate of AtCuAO4, putrescine (Put) at 100 µM, induced stomatal closure in both *AtCuAO4* mutants and WT plants to a different extent (~56% in WT, ~27% in BIS#4 and ~33% in C#4). These results provided new evidence that PA degradation via AtCuAO4 activity is an essential source of $H_2O_2$, the component involved in signalling pathways leading to ABA-induced stomatal closure in leaves of *Arabidopsis thaliana*.

In this chapter in order to test the hypothesis that *AtCuAO4* (At4g12290) may be an important gene in plant senescence, its functional role was investigated by studying the aerial phenotype and senescence of two lines with T-DNA insertions in the gene of interest (C#4 and BIS#4), and three lines (P9, P17 and P27) constitutively overexpressing the same gene under optimal conditions of growth in comparison with WT plants. In addition, the two over-expression lines P17 and P27 and the two mutant lines C#4 and BIS#4 were assessed for their response to dark-induced leaf senescence. Expression of the transgene at the protein level in the over-expression lines was verified in Prof. Cona's lab through western blots using an antibody targeting a His tag incorporated into the

coding region of the construct. Real time PCR was also used to verify that total transcripts of *AtCuAO4* were increased in the over-expressing lines using gene-specific primers, Appendix B). To understand whether PA titres were altered by manipulating the expression of *AtCuAO4*, levels of free polyamines [S-PA], Put, spermidine (Spd), and spermine (Spm), were also evaluated in two specific leaves, leaves 5 and 6, at two critical time points (before and after bolting). In addition, I tested the hypothesis that effects of the mutations in *AtCuAO4* on flowering time might be mediated by the growth hormone gibberellic acid (GA).

## 4.2. Materials and methods

### 4.2.1. Plant materials and growth conditions

Three transgenic Arabidopsis lines (P9, P17 and P27) over-expressing the copper amine oxidase gene *AtCuAO4* (At4g12290), under the control of the constitutive promoter 35SCaMV (Figure 4-2.a), and two homozygous mutant lines with a T-DNA insertion in the first exon of *AtCuAO4* gene, SALK_072954.55.00.x (line C#4) and GK_011C04-013046 (line BIS#4) (Figure 4-2.b) were kindly donated by the lab of Prof. Alessandra Cona, University of Rome (Roma Tre). The background ecotype of these manipulated seeds was Columbia (Col-0). Seeds and a WT line was also kindly donated by Prof. A. Cona.

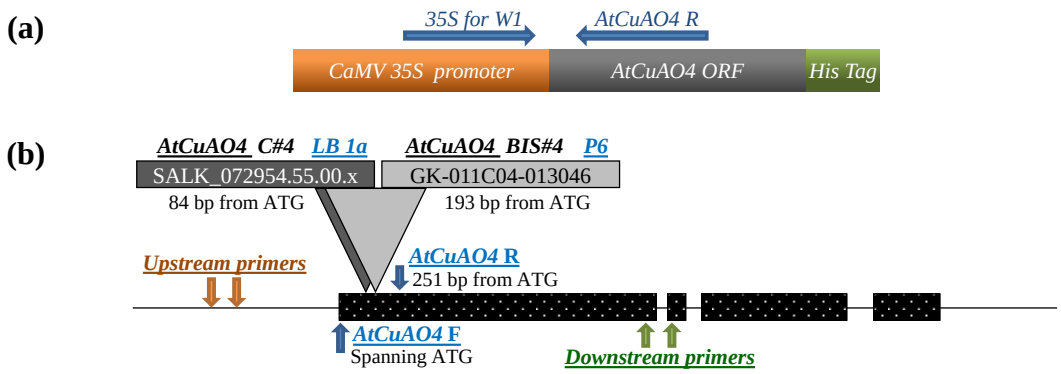



Figure 4-2 *Arabidopsis thaliana* transgenic and mutant lines used to study *AtCuAO4* gene. (a), Schematic representation of the construct used for generating the transgenic lines (P17 and P27 and P9). 35S CaMV::*AtCuAO4*-His tag cloned first into pDONR 221 (entry vector) and then into pK2GW7 (destination vector) and then transformed into plants. (b), Locations of the two T-DNA insertions on *AtCuAO4* in the two lines used (C#4 and BIS#4). Location of primers used to genotype both over-expression and mutant lines are shown in blue. Primers for upstream and downstream regions of the T-DNA insertion site are shown in brown and green respectively in (b).

Homozygous T-DNA insertion lines were grown alongside WT on soil (as described in Section 2.1), while heterozygous over-expression lines with their control (WT plants) were grown on MS medium in Petri dishes supplemented with kanamycin (Kan) at 50 µg/ ml for overexpression lines or without for WT seeds (as described in Section 2.1).

### 4.2.2. Isolation of Plant DNA, PCR, and Agarose gel electrophoresis

For genotyping purposes, DNA was extracted as described by Edwards et al. (1991) (Section 2.5). PCR reactions using extracted DNA were performed as described in Section 2.9. All primers are listed in Table 2-2. The PCR reaction products were then electrophoresed on an agarose gel and then visualized under UV light as described in Section 2.11.1.

### 4.2.3. RNA extraction and DNase treatment

RNA was isolated from frozen material using an RNeasy Plant Mini kit (QIAGEN) as described by the manufacturer (Section 2.6). Genomic DNA was then removed from RNA samples (Section 2.7), and subsequently the first-strand cDNA was generated (Section 2.8).

### 4.2.4. Estimation of mRNA by real time RT-PCR (qRT-PCR)

Quantitative expression of selected genes involved in GA metabolism and of two floral genes was carried out using real-time PCR according to Livak and Schmittgen (2001) as described in Section 2.10. All primers used for qRT-PCR are listed in Table 2-3. The transcript level of each analysed gene in each mutant line was expressed compared to the transcript level of the same gene in WT plants.

### 4.2.5. Backcrossing mutant lines

Plants were grown on soil as described in Section 2.1. The first stalks were removed and plants were ready after 6-7 weeks when they had developed 5-6 inflorescences. Branches with the largest buds were selected on the mother plant of mutant lines, and marked with

a small piece of micro-pore tape. The mature flowers and siliques were then removed from the selected branches.

Using a dissecting microscope (Section 2.16) and fine forceps, unopened buds were opened and sepals, petals and stamens were removed leaving the mature stigmas. Fresh fully opened flowers of WT plants with sticky and yellow pollen were removed from plants and squeezed with fine forceps to expose the anther. The end of stigmas of the mutant lines were then touched with the anthers of WT flowers until they were covered with pollen grains.

Plants with pollinated flowers were then allowed to grow in optimal conditions for 2-3 weeks until siliques were completely developed. When siliques were a little yellow, they were cut individually and allowed to dry fully in opened Eppendorf tubes labelled with the plant name and date.

### 4.2.6. Growth and Physiology

All phenotypic traits were carefully visualized daily starting when the first set of true leaves had emerged.

The rate of plant development was characterized by the number of days from sowing to emergence of leaves numbers 1- 9 which was scored when each leaf was initiated and become visible at the vegetative shoot apical meristem. In addition, bolting day, first open flower day and first silique day were assessed as described in Section 2.4.

Plant growth was estimated by the following quantitative characters: number of rosette leaves at bolting, total number of rosette leaves and number of yellow leaves at four weeks post-bolting, length of the primary stalk at four weeks post-bolting (measured from the base of inflorescence stem at the point of attachment to plant rosette to the tip using a cotton thread), rosette fresh weight at four weeks post-bolting, and rosette diameter at four weeks post-bolting (measured using electronic digital calipers, Maplin electronics, UK). Productivity of plants was assessed as described in Section 2.4.

The measurement of inflorescence elongation rate was carried out on six replicates of each plant as follows: a wooden stick was fixed in the soil next to the plant post bolting day when the length of the bolt was ~1- 2 cm. The height of the inflorescence was marked on the wooden stick every day over a two week period. Marks on the stick were then measured to determine a daily stem extension rate.

Before bolting (-1 week), 2 weeks after bolting, and 4 weeks after bolting (late stage), leaf numbers 5 and 6 were harvested, flash frozen in liquid nitrogen and stored at -80 for evaluation of chlorophyll content (Section 2.15).

### 4.2.7. Dark treatment of rosettes perturbed in the expression of the polyamine catabolic gene *AtCuAO4*

#### 4.2.7.a) Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) were used in this experiment as a control. The two homozygous mutant lines (C#4 and BIS#4) were stratified and sown on soil as described in Section 2.1. Two segregating populations of over-expression lines (P17 and P27) were surface sterilized, stratified, and sown on MS medium containing Kan (50 µg/ ml) as described in Sections 2.1 and 2.2. Survivors (which would be homozygous or heterozygous for the transgene) were transferred to soil as described previously in Section 2.1. In both cases, plants were grown under short day conditions at 21° C (Section 2.1) for 30 days then rosettes with at least nine leaves were harvested before bolting to study dark induced leaf senescence. Leaves 5 and 6 were sampled (6 leaves/ time point) at 0 and 8 days of dark treatment for chlorophyll analysis as described in Section 2.15

#### 4.2.7.b) Dark-induction protocol for Arabidopsis rosettes

Harvested Arabidopsis rosettes were rinsed briefly using sterile water. Rosettes were placed on a 9 cm wet filter paper in the lid of a Petri dish ensuring that rosettes would not interfere with each other or the side of the dish. Dishes were then incubated at 22° C in complete darkness inside a thick walled plastic box for quantification of dark-induced senescence.

#### 4.2.7.c) Quantitation of senescence by image analysis

Plants were photographed every day using a web-cam (Microsoft LifeCam) attached to a computer until the rosettes became completely yellow. Photographs were analysed using the colour histogram function in Image J software to obtain the RGB score for a defined rectangle from the centre of leaf number 5. RGB intensities were normalized using a white-background reference point (Figure 1 in Appendix E), and average green/ red ratios provided a quantitative measure of leaf yellowing where a ratio of around 0.8 indicates the initiation of senescence. Data were further analysed using an R script programme to produce values which could be then analysed statistically by SPSS as described in Section 2.17.

### 4.2.8. Seed germination assay

Seeds used in this experiment, were harvested at approximately the same time from individual plants grown in identical environmental conditions. Seeds of different genotypes used in the Arabidopsis phenotype experiment were surface-sterilized and stratified as described in Section 2.3. Imbibed seeds were then plated in batches of 30-50 individuals in each Petri dish containing wet 3MM filter paper (Whatman, Maidstone, UK) (5 dishes/ line). Petri dishes were then incubated under long day conditions (Section 2.1) and seed germination was assessed regularly by monitoring radicle emergence using a dissecting microscope (Section 2.16). The number of germinated seeds on each day was noted and the percentage of germination was calculated as follows:

$$Percentage\ of\ germination = \frac{number\ of\ daily\ germinated\ seeds\ x\ 100}{total\ number\ of\ sown\ seeds}$$

### 4.2.9. Analysis of Polyamine in *Arabidopsis thaliana* plants

High-performance liquid chromatography (HPLC) was used as a method for the quantification of putrescine (Put), spermidine (Spd) and spermine (Spm) in their free form. Leaf numbers 5 and 6 were collected from ten Arabidopsis plants grown on soil as described in (Section 2.1) at two specific stages of growth, the vegetative stage (pre-

bolting) and the reproductive stage (post-bolting). This analysis was repeated two times (2 biological replicates), each with 3 analytical replicates.

#### 4.2.9.a) Extraction of polyamines from Arabidopsis leaves

Sample preparation was carried out according to the method of Marcé *et al.* (1995) as follows: 150-200 mg of leaves were ground to a fine powder in liquid nitrogen using a plastic pestle and a micro-centrifuge tube. They were then homogenized in 5 % (v/ v) cold perchloric acid (PCA; Fisher Scientific, UK) at a ratio of 100 mg fresh weight/ 300 µl PCA in an ice bath. After 30 min of incubation on ice, homogenates were centrifuged at 17,000 g, 4° C, for 25 minutes in a Heraeus Fresco 17 centrifuge (Thermo scientific) and then the supernatant, containing the free polyamines, was stored at -20° C until dansylation was performed.

#### 4.2.9.b) Dansylation

Prior to separation by HPLC, amino groups of polyamines were derivatised using the derivatization reagent dansyl chloride (to enhance the detection of PAs by UV absorbance or fluorescence) as described by Marcé et al. (1995).

In a glass tube, 200 µl of perchloric acid extracts were mixed with 200 µl of a saturated solution of sodium carbonate $Na_2CO_3$ (Fisher Scientific, UK) and 400 µl of dansyl chloride (5 mg/ ml in acetone, Sigma Aldrich, UK). Next, 40 µl of 0.05 mM 1,7 diamino heptane (DIA; Sigma Aldrich, UK) were added as an internal standard as it resolves well from the derivatives of endogenous polyamines. The resulting mixture was vortexed briefly for 30 s and stored in the dark in a water bath at 70° C for 10 min.

To stop the reaction and react with excess dansyl chloride, 100 µl of a proline solution (100 mg/ ml water, Fisher Scientific, UK) was added, vortexed briefly for 30 s, and then stored in the dark for 30 min at room temperature. Following incubation, 500 µl of toluene (Fisher Scientific, UK) was added in order to extract dansylated polyamines, vortexed intensively for 30 s, and then the milky layer was removed to be discarded.

Toluene was then evaporated from each tube under a stream of nitrogen gas and the dry polyamines were re-suspended in 400 µl of acetonitrile (Fisher Scientific, UK). Tubes were then vortexed for 1 min to thoroughly dissolve the polyamines. Alongside, a blank sample using dansylated 5 % PCA was run.

Polyamine standard stock solutions (putrescine (Put), spermidine (Spd), spermine (Spm), cadaverine (Cad), and diamino heptane (DIA), Sigma Aldrich, UK) at a concentration of 10 mM were made in water and then diluted to obtain different concentrations of standards (ranging from 1.25 µM to 16.7 µM containing DIA at 8.3µM) and they were also dansylated following the same procedure described above.

Finally, the dansylated samples were passed through a 0.45 µm pore size syringe filter (PVDF membrane, GE Healthcare Life Sciences) into a dark vial prior to injection into the HPLC (20 µl of sample was injected into the HPLC for each analytical replicate).

#### 4.2.9.c) Measurement of free polyamines by HPLC

Reversed-phase HPLC was carried out using a Thermo Separation Products HPLC system consisting of SCM 1000 Degasser, P4000 Pump, AS300 Autosampler, and UV-6000 LP photodiode array detector (measurement of absorption was at two wavelengths 216.5-217.5 and 249-251 nm). The derivatised PAs were separated on a Phenomenex Max-RP column (250 mm x 4.6 mm with Phenomenex C18 security guard).

Dansylated polyamines were separated with the HPLC method optimized by Marcé et al. (1995). The procedure was carried out as follows: 20 µl of sample or standard were injected into the HPLC system, and then they were subjected to a gradient elution in acetonitrile and water over 15 minutes (Figure 4-3).

The initial conditions were 70 % acetonitrile and 30 % water pumping at a flow-rate of 1.5 ml/ min. The mixture was pumped for 4 min; then the acetonitrile concentration was increased to 100 % and was kept constant at this concentration for another 4 min, and finally returned to the initial conditions. After each cycle, the column was re-equilibrated in the remaining 6 min.

Elution was performed at room temperature (22° C), and quantification of the different amines was performed using the HPLC system's Thermo Xcalibur software (V 1.2). Areas of each sample peak were integrated and compared to those of the standards of known concentration. Polyamine final concentrations were expressed as nmol/ gm FW.

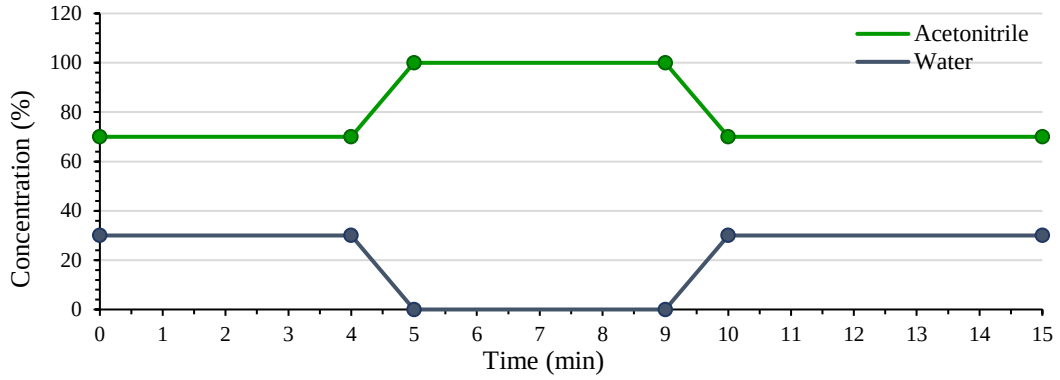



Figure 4-3 Diagram of the gradient used in the HPLC method. Percentage concentrations of acetonitrile (green line) and water (blue line) in the mobile phase vs. time in minutes.

### 4.2.10. Treatment of Arabidopsis plants with $GA_3$

Homozygous seeds of C#4 and BIS#4 along with WT seeds were sown on soil and grown under LD conditions (Section 2.1). Beginning at the 6-7 leaves stage, a solution of $GA_3$ (dissolved in ethanol and made up to 50 µM, Sigma, UK) containing 0.02 % Tween-20 was applied to plants by spraying on aerial parts (plant rosettes) on six alternate days until the onset of bolting. The concentration 50 µM of $GA_3$ was chosen based on Devaiah *et al.* (2009); a higher concentration of 100 µM was tested and gave severe abnormalities in plant growth.

Twelve replicates of each mutant line and WT were used for $GA_3$ treatment along with a similar number of the same plants used as control and sprayed with water at the same time intervals. The effect of $GA_3$ application on initiation of the inflorescence stem (bolting), number of leaves at this stage and day of the first flower were evaluated in the two mutant lines and WT plants with or without $GA_3$ treatment. In addition, two weeks after sowing (before bolting), five rosettes of each untreated mutant line and WT were collected, immediately frozen in liquid nitrogen, and stored at -80° C until further use for RNA extraction.

## 4.3. Results

### 4.3.1. Screening of *AtCuAO4* T-DNA mutant plants to check the lines were homozygous

Plants of mutant lines used as a source of seeds in the phenotyping experiment were genotyped to verify that they were homozygotes and therefore carried the T-DNA insertion on both alleles.

Extracted DNA was screened firstly with gene specific primers which span the insertion site to ensure the absence of the WT gene then genotyped using mutant specific primers for each line (Table 2-2) to verify the presence of the T-DNA insertion (Figure 4-4). No product was produced from any of the mutant plants, when gene specific primers spanning the T-DNA insertion region were used (using Hot Star Taq polymerase), compared with the WT gene (positive control) indicating that the WT allele was not present in the mutant plants and they were therefore indeed homozygous mutants (Figure 4-4.a).

The PCR results presented bands with a size of 150 bp when P6 and *AtCuAO4*-R were used with DNA extracted from BIS#4 plants (Figure 4-4.b). Whereas LBa1 and *AtCuAO4*-F amplified a product of 400 bp from the DNA of C#4 plants (Figure 4-4.c) indicating in both cases that the T-DNA insertion was present.

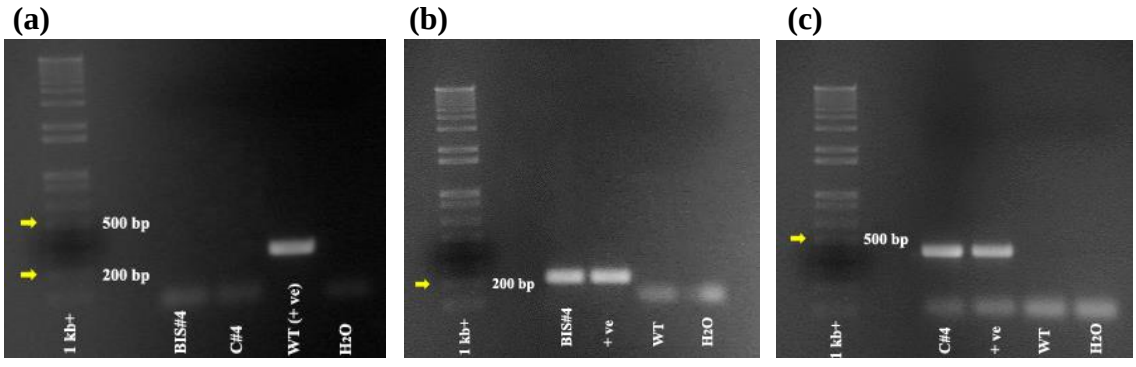



Figure 4-4 Screening *AtCuAO4* mutant plants used as a source of seeds. Ethidium bromide (EtBr) stained agarose gel of PCR products from gene specific primers: (a) *AtCuAO4*-F + *AtCuAO4*-R primers, (b) P6 + *AtCuAO4*-R primers, and (c) *AtCuAO4*-F + LBa1 primers. gDNA of WT plant, gDNA of known BIS#4 mutant, and gDNA of known C#4 mutant were used as a control in (a), (b) and (c) consecutively.

### 4.3.2. Verification of *AtCuAO4* expression in T-DNA insertion mutant lines

RNA was extracted from leaves of five week old mutant (C#4 and BIS#4), and WT plants (leaves 5 and 6 of 5 rosettes/ line) and was analysed by PCR. Five week old plants were chosen as at this stage of development the *AtCuAO4* transcripts should be abundant.

First, the integrity of extracted RNA was checked on an agarose gel (Section 2.11.1) and results revealed that all extracted RNAs were of a good quality (Figure 4-5.a). DNA in RNA samples was digested and then RNA samples were tested by PCR using 18S rRNA primers (Section 2.9 and Table 2-2) to confirm the absence of any contamination with DNA traces. This showed that all samples were free of genomic DNA (Figure 4-5.b). Quality of cDNA generated from the RNA was confirmed by amplification of *Actin2* by PCR using actin specific primers (Section 2.9 and Table 2-2) (Figure 4-5.c).

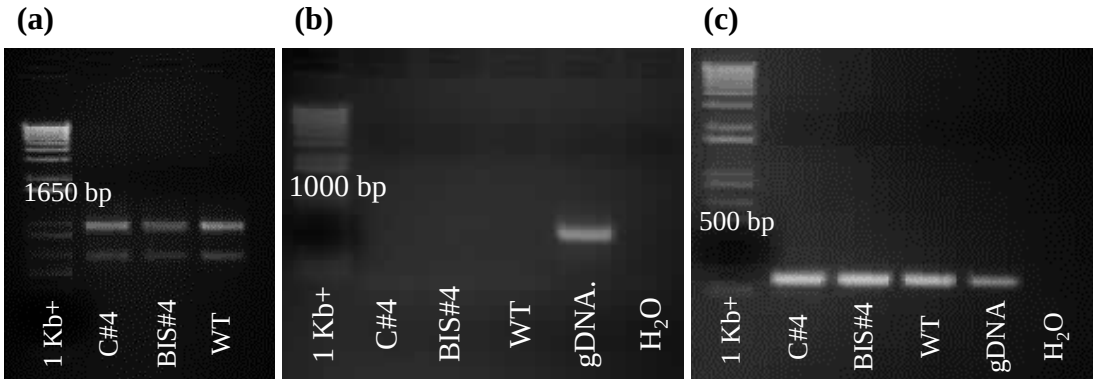



Figure 4-5 Quality control applied to isolated RNA from Arabidopsis leaves 5 and 6 of 5-week old WT and *AtCuAO4* mutant plants. (a) Integrity of RNA was tested by agarose gel electrophoresis. (b) DNA digestion after DNase treatment was confirmed by PCR using 18S rRNA primers (Table 2-2). (c) Integrity of generated cDNA was tested by PCR reaction with *Actin2* primers (Table 2-2). Genomic DNA (gDNA) was used in both b and c as a positive control while water was used as a negative control to detect any contamination. Electrophoresis was performed on EtBr stained 1 % agarose gels.

To confirm the lack of expression of *AtCuAO4* mRNA as a result of T-DNA insertions in the mutant lines, three different combinations of primers were used to amplify three regions within the *AtCuAO4* mRNA in WT and mutant plants, up-stream (in the 5` UTR zone), flanking (to span the T-DNA insertion site), and down-stream of the T-DNA insertion site (flanking an intron between the 1$^{st}$ and 2$^{nd}$ exons) (Figure 4-2.b and Table 2-2).

Primers upstream of the T-DNA insertion site amplified a fragment of 200 bp on cDNAs of all analysed mRNAs as predicted from the sequence (Table 2-2, Figure 4-6.a). When primers flanking the T-DNA insertion site were used for PCR, the amplification product of 283 bp from the *AtCuAO4* transcript was absent in both mutants (C#4 and BIS#4) and present only in cDNA of WT plants (Figure 4-6.b). When primers downstream of the T-DNA insertion site were used, both mutant lines produced only a very low amount of amplification product and only cDNA of WT plants produced an abundant fragment of the predicted size (88 bp), while a fragment of 199 bp was amplified from gDNA of WT (Figure 4-6.c).

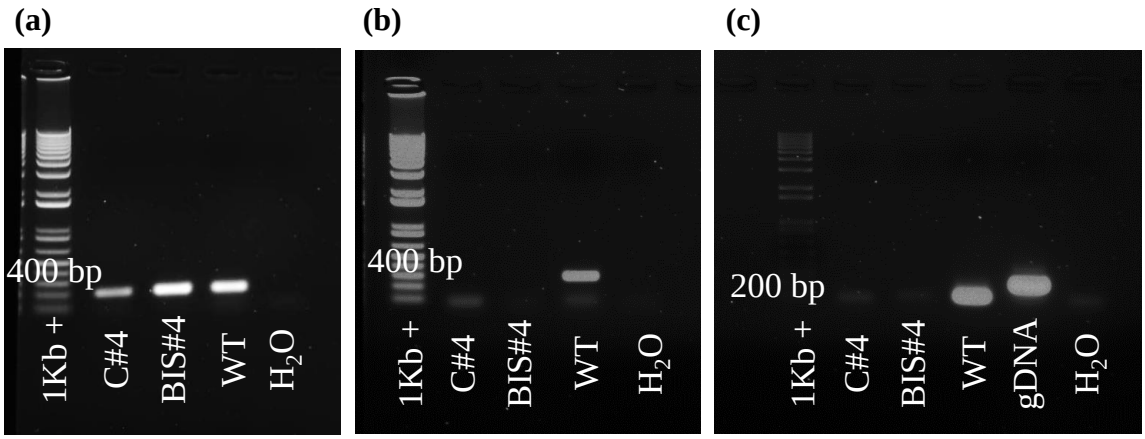



Figure 4-6 PCR analysis of *AtCuAO4* T-DNA insertional lines C#4 (SALK_072954.55.00.x) and BIS#4 (GK_011C04-013046). Three different primer pairs were used to amplify three regions on *AtCuAO4* mRNA (Figure 4-2.b, Table 2-2): (a) primers for upstream of the T-DNA insertion site, (b) primers spanning the T-DNA insertion site, (c) primers for downstream of the T-DNA insertion site. Wild type mRNA (WT) was used as a positive control, and $H_2O$ as a negative control. Since downstream primers span an intron, genomic DNA (gDNA) was used in (c) as another positive control. EtBr stained 1 % agarose gels were used to separate PCR products.

### 4.3.3. Seed germination in T-DNA insertion and over-expressor lines of *AtCuAO4*

Homozygous T-DNA insertion lines for both C#4 and BIS#4 were tested to see if their germination was affected. At the beginning of the experiment, radical protrusion of both T-DNA lines was considerably slower compared to WT (Figure 4-7.a).

On the first day, less than 50 % of both mutant seeds (C#4 and BIS#4) germinated while more than 80 % of WT seeds had done so. On the second day and the following days of

the experiment period, no clear differences were observed between the germination of either mutant and WT seeds.

On the other hand, radical emergence rate of both homozygous over-expression lines (P17 and P27) was similar to that of the WT seeds and almost 100 % of seeds of each tested plant germinated within 24 h (Figure 4-7.b). Note that overexpression seed batches used for evaluating the germination were tested on MS medium in Petri dishes supplemented with Kan at 50 µg/ ml.

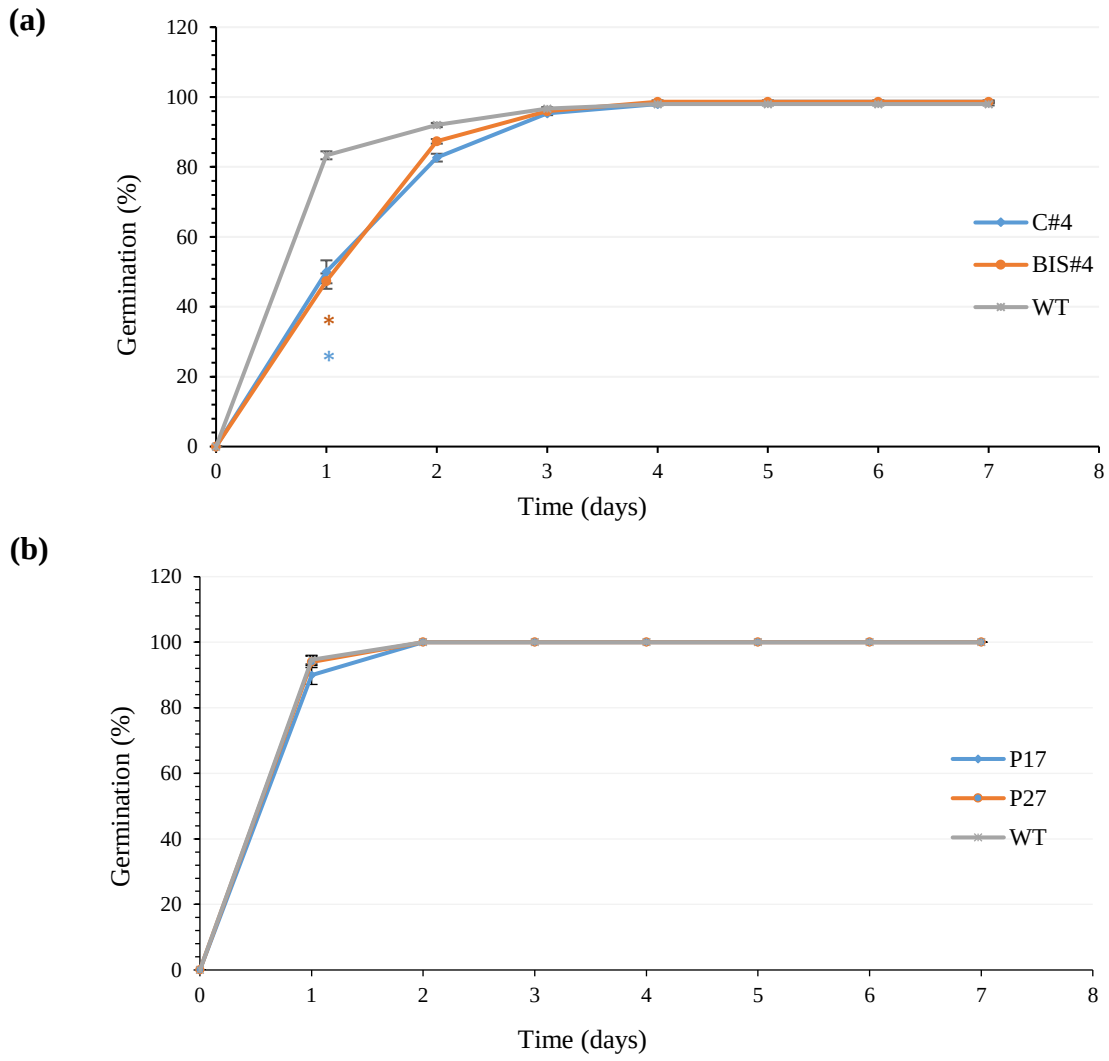



Figure 4-7 Effect of *AtCuAO4* manipulation on seed germination. (a) Mutant lines (C#4 and BIS#4) and (b) over-expressor lines (P17 and P27) compared to WT seeds. Surface sterilized seeds were sown on filter paper saturated with sterile distilled water in Petri dishes and monitored regularly for radical protrusion. Values are the mean ± SE of five biological replicates in (a) (30 seeds/ replicate) and three biological replicates in (b) (50 seeds/ replicate). Significant differences in means are indicated by * at $P \leq 0.05$ based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

### 4.3.4. Growth and development of *AtCuAO4* over-expressor and T-DNA mutant lines

Emergence of leaves 1-9 was delayed significantly in the C#4 mutant line and emergence of leaves 4, 5, 6, 8 and 9 was also delayed in BIS#4 compared with WT (by about 2 d) (Figure 4-8.a). However, the overexpression lines did not show clear differences in time of emergence of their leaves (Figure 4-8.b).

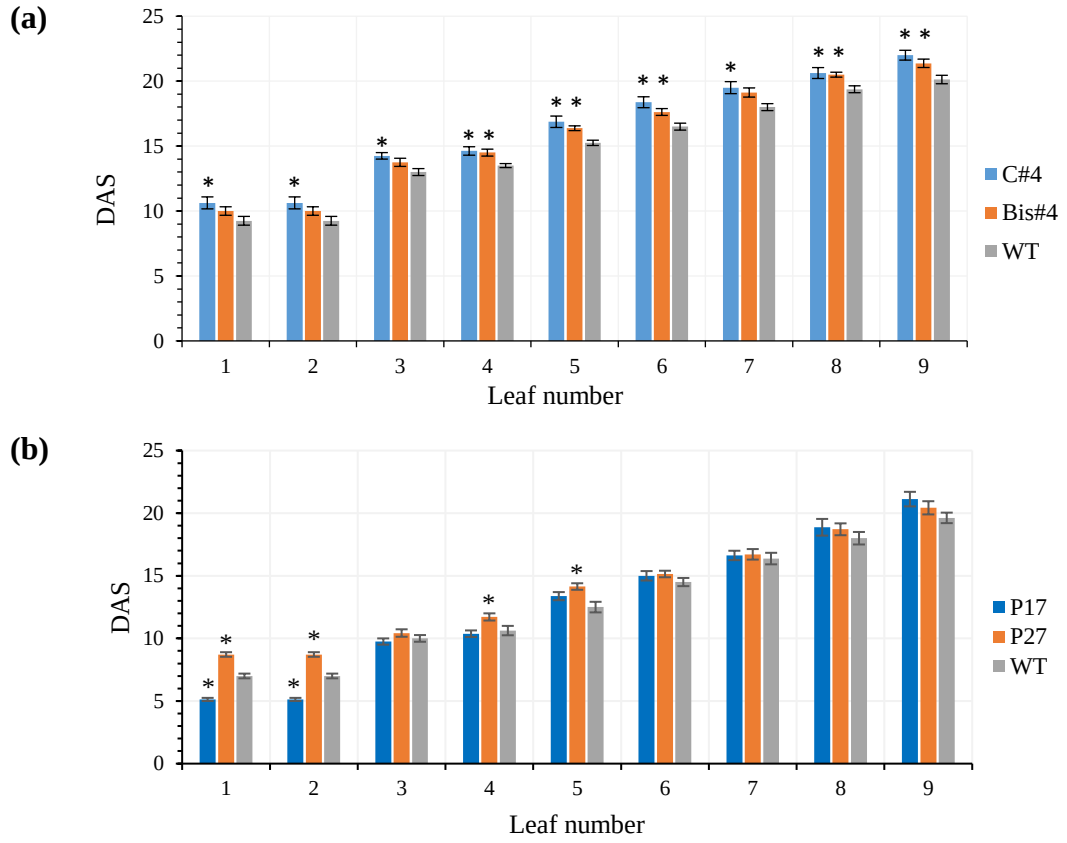



Figure 4-8 Comparison of emergence time of individual leaves of *AtCuAO4*-mutant plants and *AtCuAO4*-overexpressing lines with wild type Arabidopsis. (a) Mutant plants (C#4 and BIS#4) and (b) overexpressing plants (lines P17 and P27). Mean ± SE; n = 8; significant differences are indicated by asterisks ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

Plants from both mutant lines (C#4 and BIS#4) bolted and flowered later and they produced a greater number of leaves before bolting relative to WT (Figure 4-9.a). Conversely in transgenic lines (P17 and P27) bolting and flowering occurred sooner compared with WT (Figure 4-9.b). However, the number of leaves produced before bolting were only significantly lower in P17 but not P27 compared to WT.

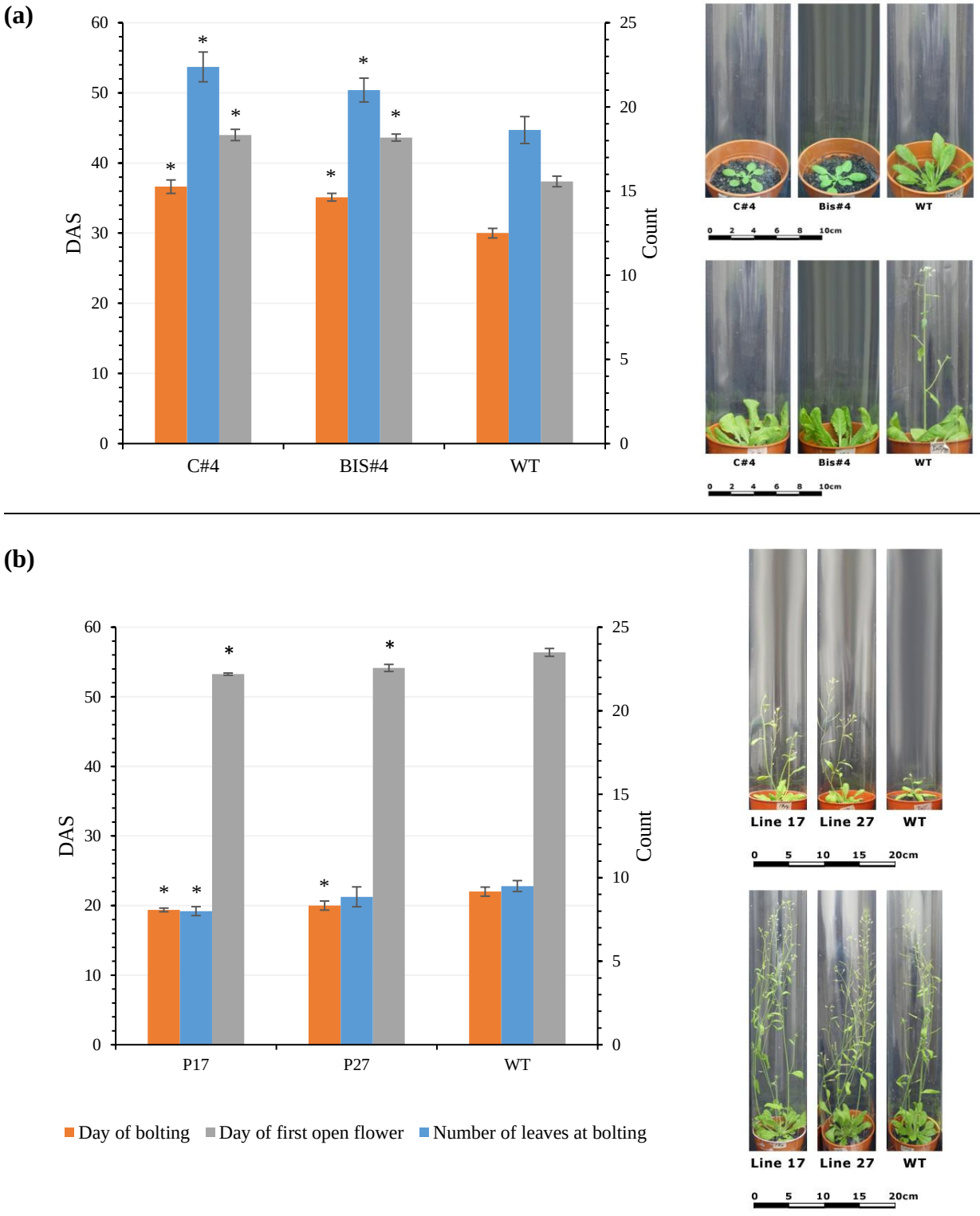



Figure 4-9 Developmental stages of wild type Arabidopsis, *AtCuAO4* mutants and *AtCuAO4* over-expressors. (a) C#4 and BIS#4 mutant lines and (b) P17 and P27 overexpression lines. Right-hand images show phenotype of representative plants at different stages of development: (a) 33-day old plants at the top and 48-day old plants at the bottom, (b) 26-day old plants at the top and 42-day old plants at the bottom. Mean ± SE; n=8; asterisks indicate significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

At four weeks post-bolting there were fewer yellow leaves in both mutant lines (C#4 and BIS#4). However, the number of total leaves was only lower in BIS#4 plants compared to WT, and rosette biomass was not significantly different from WT plants in either mutant line (Figure 4-10.a). These three parameters in the two over-expression lines (P17 and P27) were not significantly different to WT plants (Figure 4-10.b).

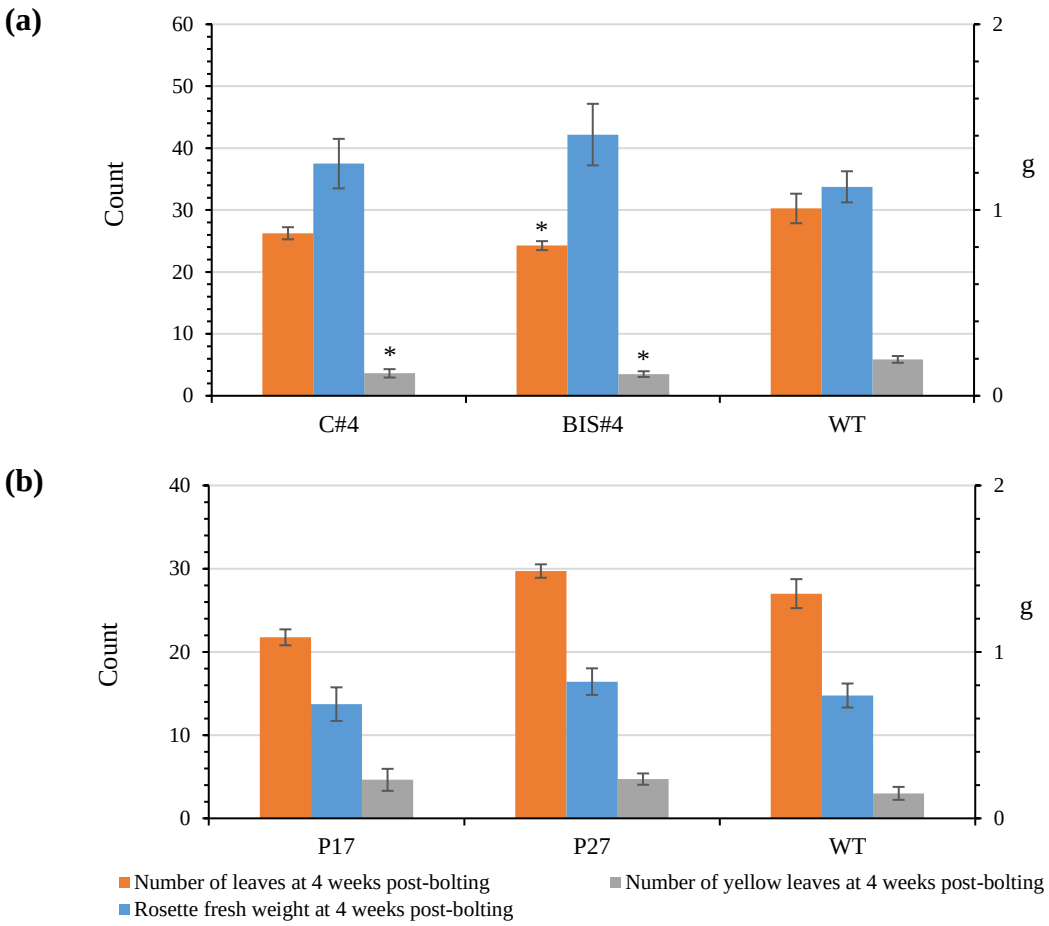



Figure 4-10 Effect of *AtCuAO4* mutation or overexpression on number of leaves at 4 weeks post-bolting, number of yellow leaves and fresh weight of rosettes. (a) C#4 and BIS#4 mutant lines and (b) P17 and P27 overexpression lines. Mean ± SE, n = 8, asterisks indicate significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

Four weeks post-bolting, the height of the primary stalk and number of siliques produced by the two T-DNA insertional lines (C#4 and BIS#4) were both significantly lower than WT (Figure 4-11.a). However, there were no clear significant differences in these parameters in the overexpression lines compared to WT plants (Figure 4-11.b). Rosette diameter was also unaffected by perturbation of *AtCuAO4* expression.

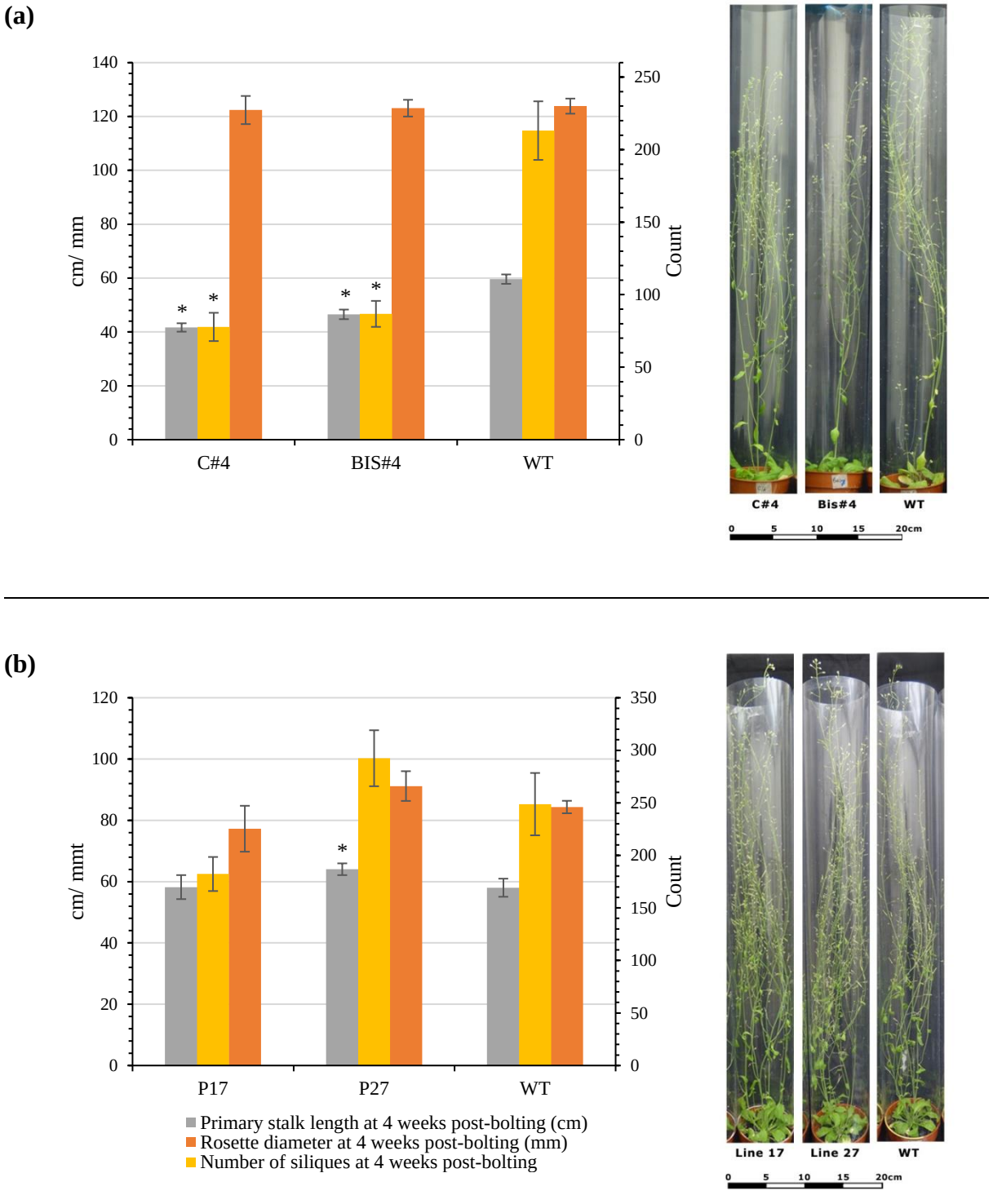



Figure 4-11 Length of primary inflorescence shoot, rosette diameter and number of siliques produced by the plant at 4 weeks after bolting in plants perturbed in *AtCuAO4* expression either via mutation or over-expression compared to WT plants. (a) *AtCuAO4* mutated lines C#4 and BIS#4. (b) *AtCuAO4* overexpression lines P17 and P27. Total number of siliques was scored on the primary inflorescence, secondary inflorescence branches, and inflorescence branches (Section 2.4). Right-hand images show plant phenotype at 4 weeks post-bolting corresponding to left-hand graphs. Mean ± SE; n =8; asterisks indicate significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

The concentration of photosynthetic pigments was measured in leaves 5 and 6 of mutants and WT rosettes as a marker for senescence progression (Figure 4-12). There were no clear differences in the concentrations of photosynthetic pigments between mutant lines and WT plants at an early stage of growth (pre-bolting). However, knock-out of the expression of *AtCuAO4* in the two mutant lines (C#4 and BIS#4) resulted in significantly higher total chlorophyll levels compared with WT at both measured stages, 2 and 4 weeks post-bolting (Figure 4-12.a). In contrast, no variation in the total chlorophyll content was observed in either of the over-expression lines (P17 and P27) compared to WT plants (Figure 4-12.b).

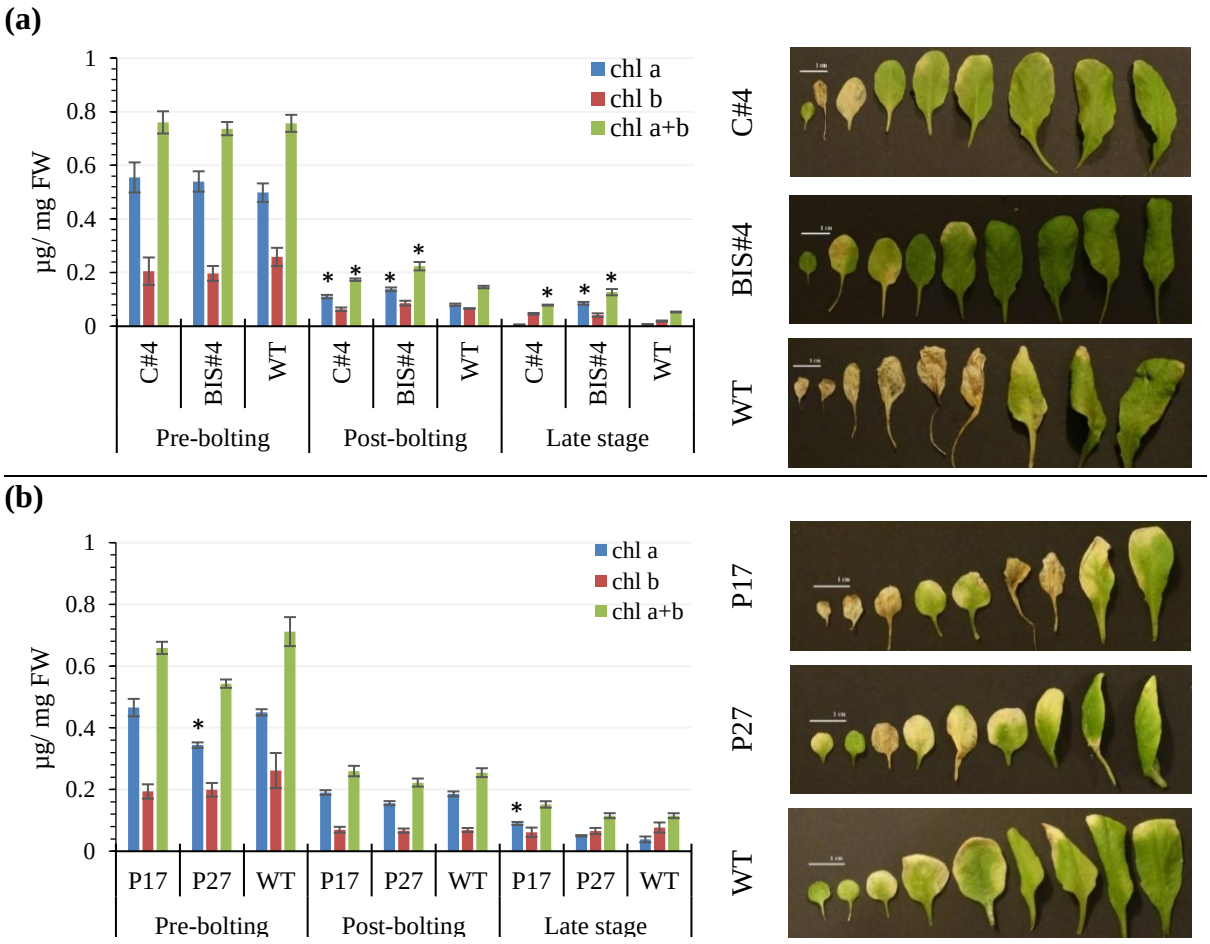



Figure 4-12 Chlorophyll contents of the two T-DNA insertional lines and the two over-expression lines relative to WT plants. (a) C#4 and BIS#4 mutant lines and (b) P17 and P27 overexpression lines. Different chlorophyll levels were analysed in leaves number 5 and 6 of different lines at three different stages. Pre-bolting indicate 1 week pre-bolting, post-bolting indicate 2 weeks after bolting and late stage indicates 4 weeks post-bolting. The right panel shows differences in leaf yellowing of the nine oldest leaves (oldest to youngest, left to right) from mutants and overexpression lines grown under 16 h light/ 8 h dark for 8-9 weeks. Values are mean ± SE (n = 6). Asterisks indicate statistically significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

Since the two over-expression lines were not always consistent in their phenotype, a third line, P9, was analysed phenotypically to confirm the results from the two other lines. In general, results of the P9 phenotyping showed no clear differences relative to WT except in bolting and flowering days, length of the primary stalk and chlorophyll contents at four weeks post-bolting (Figure 4-13).

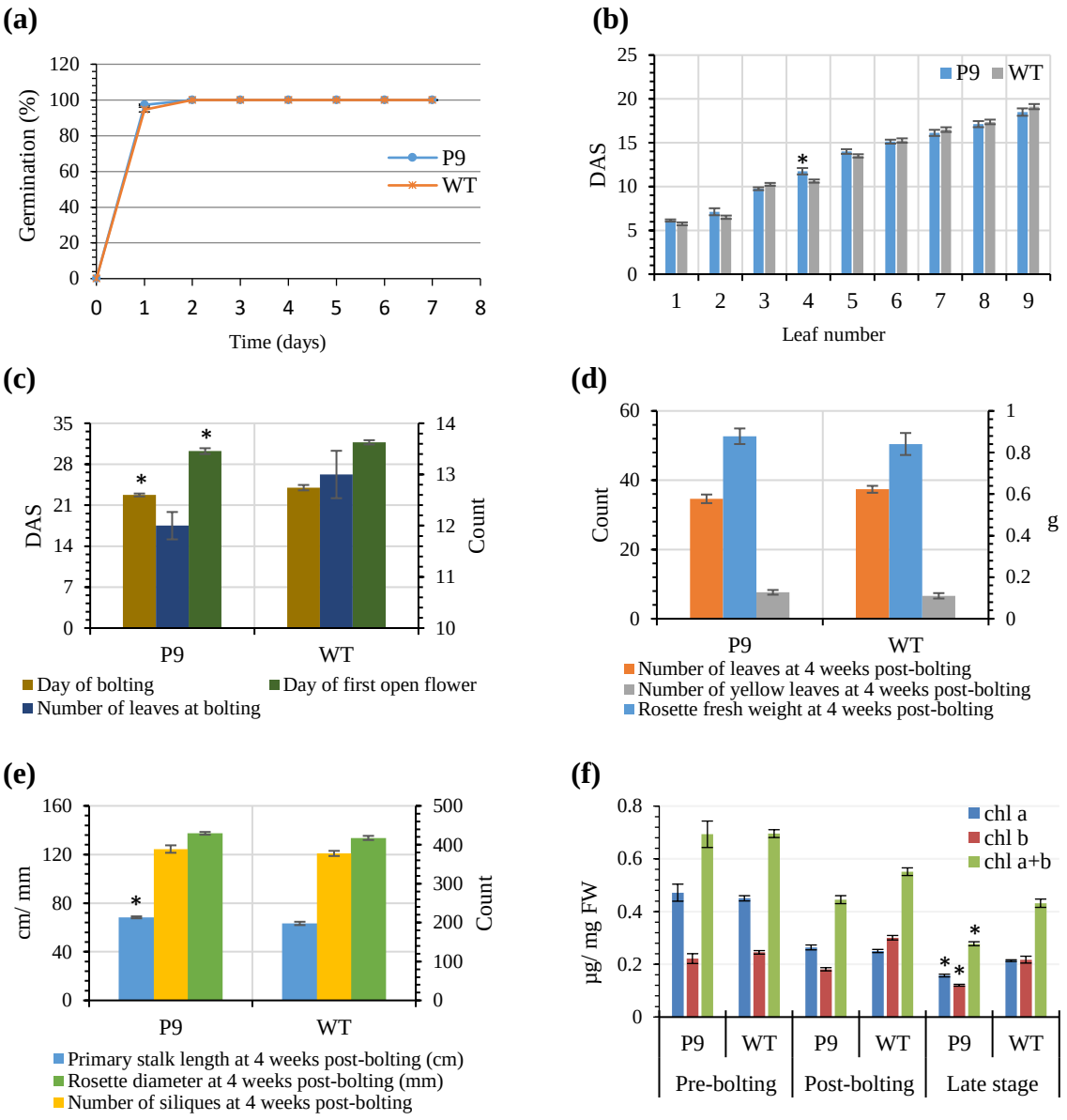



Figure 4-13 Phenotypic analysis of the *AtCuAO4* over-expression line P9 compared to wild type plants. (a) Germination rate, (b) emergence time of individual leaves, (c) early morphological and developmental traits, (d) and (e) late morphological and developmental traits, and (f) chlorophyll levels at three different stages of plant development, pre-bolting indicate 1 week pre-bolting, post-bolting indicate 2 weeks after bolting and late stage indicates 4 weeks post-bolting. n = 8 in a-e, while in f, n = 6. Mean ± SE, asterisks indicate significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

Overexpressing *AtCuAO4* in P9 did not affect germination rate or leaf emergence (Figure 4-13.a and b) but accelerated bolting and flowering times (Figure 4-13.c), although not the number of leaves at bolting.

Numbers of leaves, rosette fresh weight and number of yellow leaves at four weeks post-bolting were, however, unaffected (Figure 4-13.d). *AtCuAO4* over-expression in the P9 line did increase the primary stalk length but not rosette diameter or numbers of siliques at four weeks post-bolting weeks (Figure 4-13.e).

Total chlorophyll levels were reduced in leaves of the P9 line at four weeks post-bolting weeks compared to WT (Figure 4-13.f).

Images of the whole plant of line P9 and WT at different stages of development are shown in Figure 4-14.

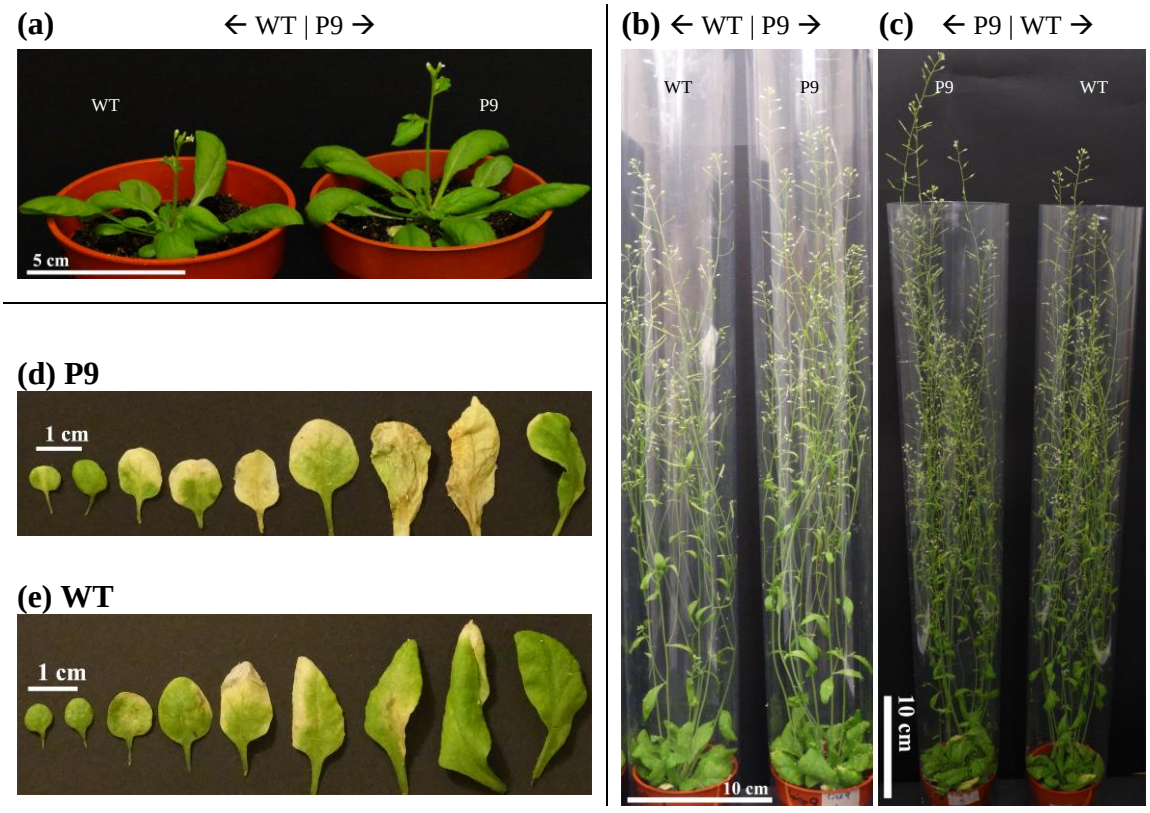



Figure 4-14 Phenotype of the over-expression line P9 relative to wild type plants. (a) 26-day old plants (~1 week post-bolting), (b) 42-day old plants (2 weeks post-bolting), and (c) plant phenotype at 4 weeks post-bolting. The differences in leaf yellowing of the nine oldest leaves, oldest to youngest (left to right), from the over-expression line P9 (d) and WT (e) grown under 16 h light/ 8 h dark for 8 weeks.

Storage of detached *AtCuAO4* rosettes in the dark affected the progression of leaf senescence, however, there was no consistent response between the two mutant or the two over-expressor lines (Figure 4-15). Overexpression of *AtCuAO4* accelerated senescence significantly in the dark in one line (P27) as indicated by the higher RGB values (an indication of yellowing) which was clear at early time points (day 2, 3, 4, 5 and 6) compared with control plants. In contrast, in the other line (P17), darkness delayed yellowing significantly at early stages (day 1, 2 and 3) and then no clear differences were obvious between WT and overexpression lines. On the other hand, both mutant lines were late in their senescence during the dark period but this retardation was significant only on the first day for C#4 and in the last three days of incubation in the dark for BIS#4 when compared to WT plants.

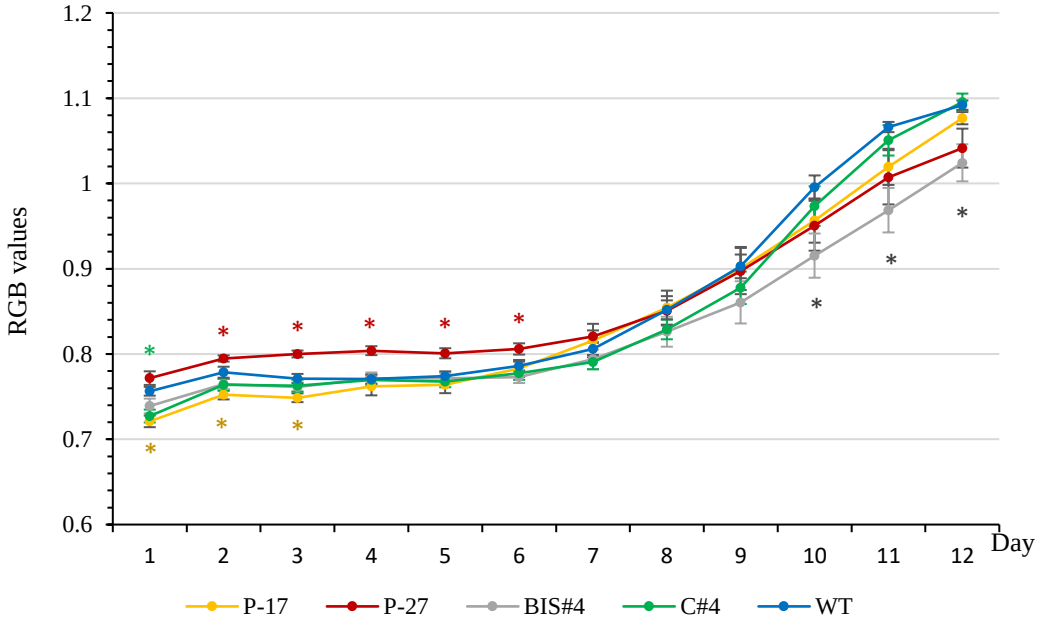



Figure 4-15 Comparison of leaf senescence in *AtCuAO4* mutant and overexpressor lines with wild type during dark-induced senescence. 30-day old rosettes grown on soil under short-day conditions, with at least nine leaves, were used in this experiment. Change in leaves color was examined daily during dark incubation by obtaining RGB score. Data represent mean values ± SE (n=9). * $P \leq 0.05$.

The fifth and sixth leaves were sampled after 0 and 8 days in the dark for chlorophyll analysis (Figure 4-16). Values of the concentration of chlorophyll confirmed that there were no significant differences between WT and *AtCuAO4* manipulated plants in the progression of dark-induced leaf senescence.

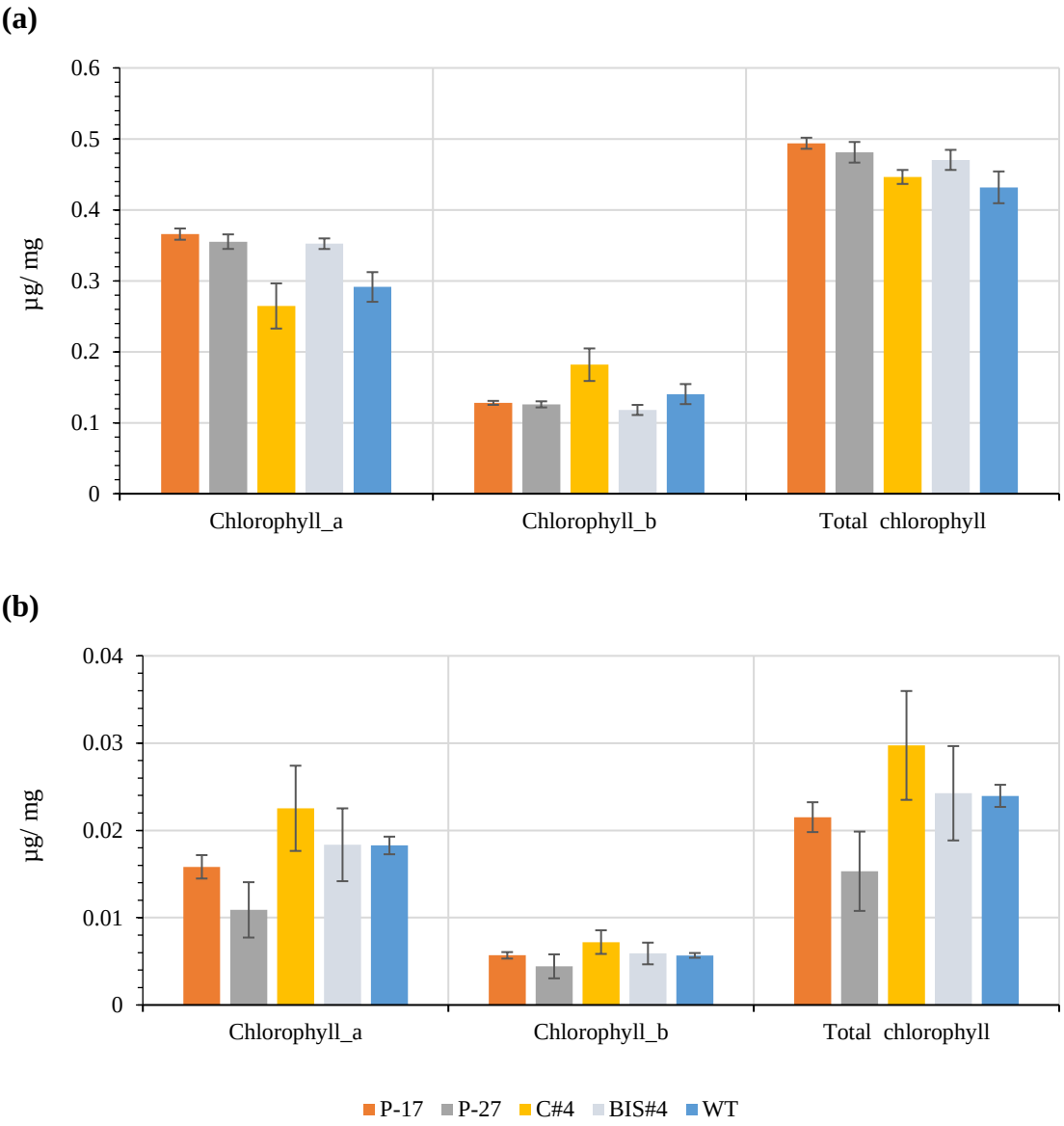


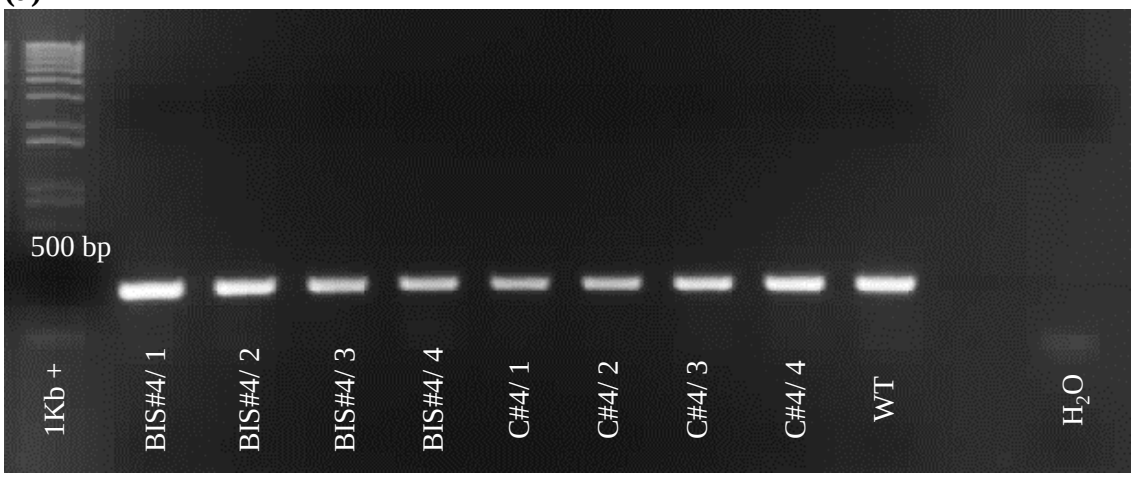

Figure 4-16 Chlorophyll content of *AtCuAO4* manipulated leaves incubated in the dark to induce senescence. (a) Chlorophyll content of leaves at the beginning of treatment. (b) Chlorophyll levels in leaves incubated in the dark for 8 days. Data represent mean values ± SE (n=6). * $P \leq 0.05$.

### 4.3.5. Dominance of *AtCuAO4* T-DNA insertion mutant alleles

To verify that the *AtCuAO4* alleles in the two mutant lines C#4 and BIS#4 were indeed recessive, the mutant lines were backcrossed to WT and the phenotype of heterozygote mutant plants were compared to WT. Seeds of siliques produced by pollination of homozygous mutant plant stigmas with WT flower pollen were sown on soil. Genotyping of randomly selected plants using both WT *AtCuAO4* gene primers and T-DNA insertion

specific primers showed that the plants were heterozygous bearing both mutated and WT alleles (Figure 4-17).

**(a)**




**(b)**

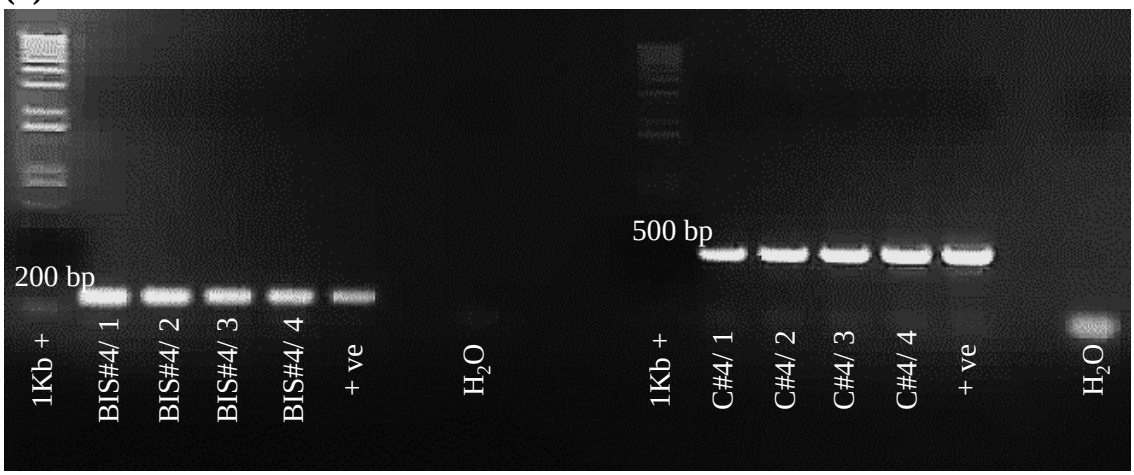



Figure 4-17 Genotyping plants produced by backcrossing mutated plants with WT. (a) PCR products from gene specific primers *AtCuAO4*-F + *AtCuAO4*-R (Table 2-2). (b) PCR products from mutant specific primers P6 + *AtCuAO4*-R (on the left), and *AtCuAO4*-F + LBa1 (on the right). Genomic DNA (gDNA) and DNA from homozygous mutant plants were used in (a) and (b) respectively as positive controls while water was used as a negative control to detect any contamination. Electrophoresis was performed on EtBr stained 1 % agarose gels.

Heterozygous plants from the backcrossed seed were monitored for bolting day relative to WTs to check the dominance of mutant alleles (Figure 4-18). Results showed a significant retardation in bolting day of the heterozygous C#4 mutant plants compared

with WT, while heterozygotes of the other mutant line (BIS#4) were similar in their bolting day to WT plants.

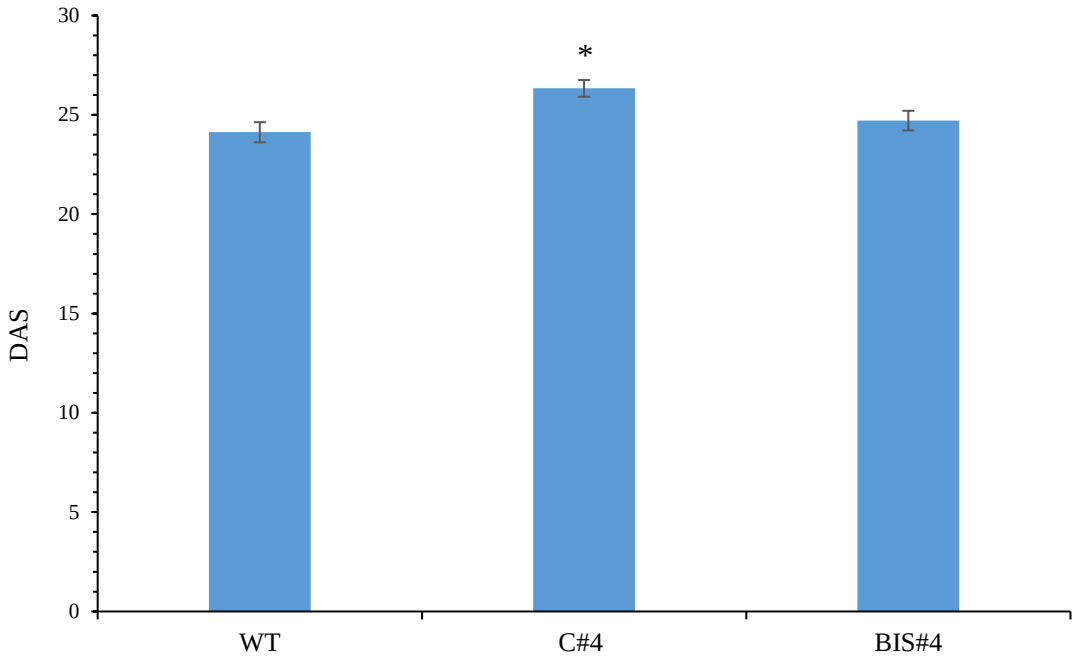



Figure 4-18 Bolting day of heterozygous mutants compared to wild type plants. Error bars represent standard error of the mean, values are means ± SE (n = 24), asterisk indicates significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not. The experiment was repeated twice with similar results.

### 4.3.6. The effect of mutation of *AtCuAO4* on stem elongation, plant productivity and silique phenotype

Since the *AtCuAO4* mutant lines (C#4 and BIS#4) both showed shorter stems and fewer siliques at four weeks post-bolting weeks, a more detailed analysis of growth and silique phenotype was made. When the floral (primary) inflorescence of the two mutant lines (C#4 and BIS#4) and WT reached ~1-2 cm in length, measurement of stem extension rate was started (Figure 4-19). During the first four days WT stalks elongated much faster than the inflorescence stems of mutant lines (Figure 4-19.a). The stem elongation rate of the mutants remained lower until they reached a length of ~15 cm (Figure 4-19.b). After this point the extension rate of both mutants increased which compensated for the shorter stem length, but stem length remained significantly shorter until the end of the experiment.

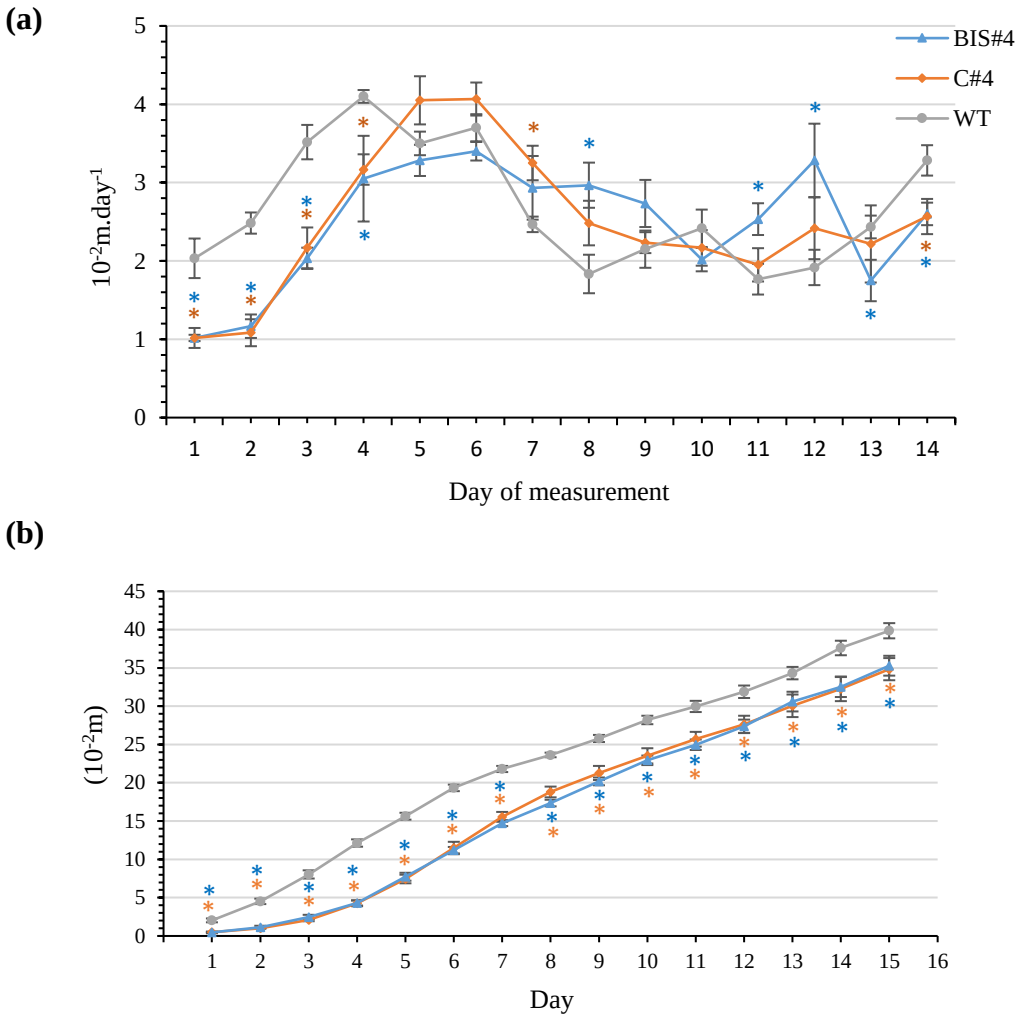



Figure 4-19 Effect of knocking out the expression of *AtCuAO4* in BIS#4 and C#4 lines on the growth of the primary stem compared to WT. (a) Elongation rate of the primary stalk over two weeks (b) Length of the primary stalk over two weeks. Error bars represent standard error of the mean, values are means ± SE (n = 6), asterisks indicate significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not.

The activity of the reproductive system after bolting in mutant lines C#4 and BIS#4 compared with WT plants was analysed by scoring the number of siliques produced over a week on the primary inflorescence, secondary inflorescence branches (at the axils of the cauline leaves) and inflorescence branches (grown out from the axillary buds subtended by rosette leaves) starting from the day of formation of the first silique (Figure 4-20.a). Results show that abolishing the expression of *AtCuAO4* has no effect on plant productivity: no clear difference in number of siliques per week was observed relative to WT plants. However, both mutant lines generated significantly longer siliques compared to WT siliques (Figure 4-20.b and c).

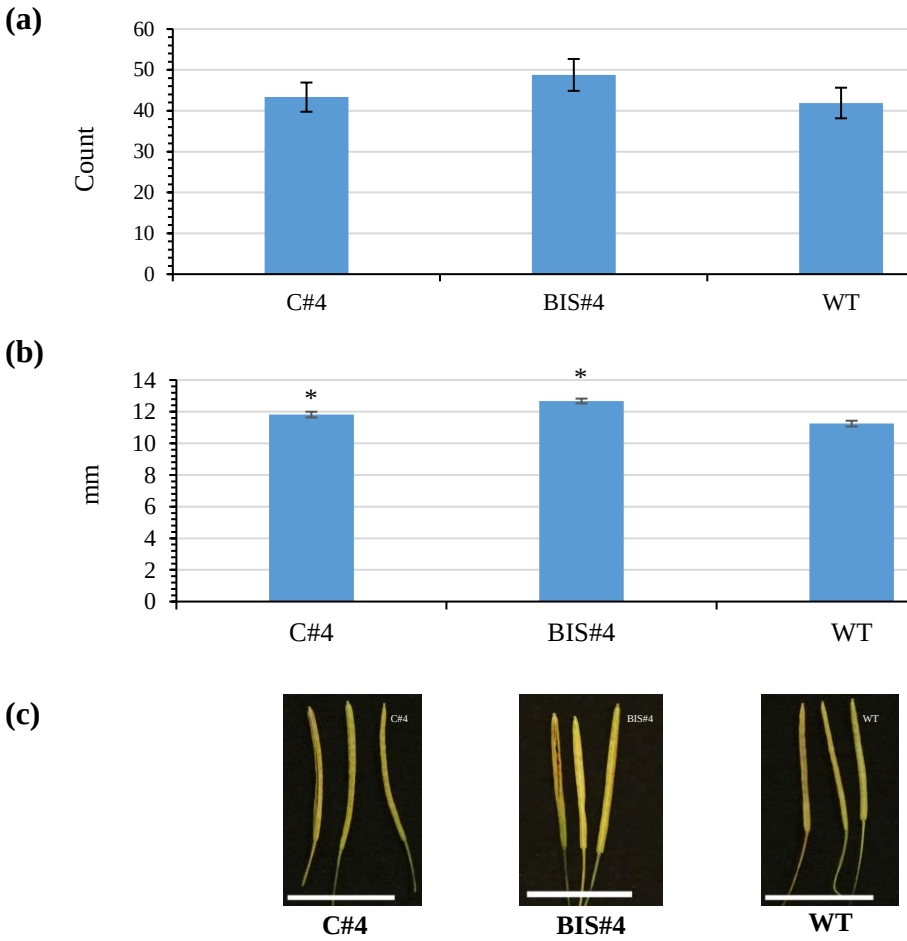



Figure 4-20 Effect of knocking out the expression of *AtCuAO4* in the two mutant lines C#4 and BIS#4 on plant productivity and silique phenotype as compared to WT plants. (a) Total number of siliques produced by the plant on the primary inflorescence, secondary inflorescence branches, and inflorescence branches over a week starting from formation of the first silique (n = 30-33). (b) Mature silique length of 8-week old plants (n = 100). Values are mean ± SE, asterisks indicate statistically significant differences to WT ($P \leq 0.05$) based on a T-test where data were normally distributed or Mann-Whitney test where data were not. (c) Mature siliques at 4 weeks post-bolting, scale bar = 1 cm

### 4.3.7. The effect of manipulation of the expression of *AtCuAO4* on free polyamine content in Arabidopsis single leaves

To determine whether the phenotypic effects due to perturbation of *AtCuAO4* expression were mediated by a change in the concentrations of PAs, relative concentrations of Put, Spd and Spm were analysed in rosette leaves 5 and 6 before and after bolting. The elution order of the different dansylated standard polyamines (Figure 4-21) was as follows: Put (RT; 6.54 min), Cad (RT; 6.96 min), DIA (RT; 7.63 min), Spd (RT; 8.18 min), and Spm (RT; 8.92 min). The standard deviations of these values ranged between +/- 0.25 of a minute.

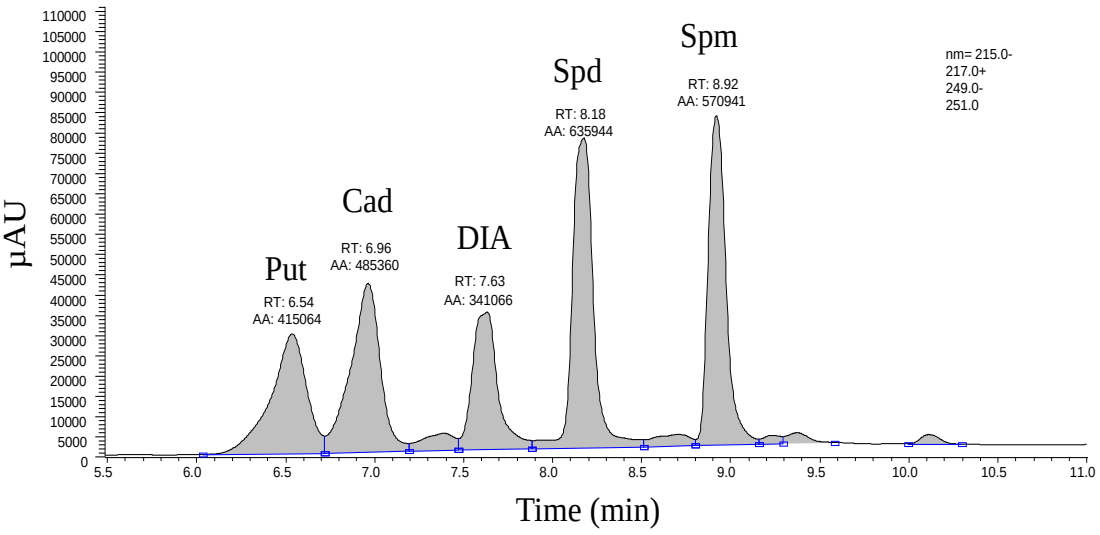



Figure 4-21 Chromatogram of dansylated standard polyamines. Peaks: 6.54 (Put), 6.96 (Cad), 7.63 (DIA), 8.18 (Spd) and 8.92 (Spm).

Analysis of free polyamine content before bolting in leaves 5 and 6 of two week old mutant plants (Figure 4-22.a) showed a 1.7 and 1.36 fold increase in the free Put content in leaves of BIS#4 and C#4 respectively compared to the WT. No differences in content of the free form of the tetra-amine Spm were observed pre-bolting between mutants and WT leaves. Put content increased in all tested plants during reproduction compared with the vegetative stage but this increase was not significantly different among the three tested lines. Conversely, Spm content in leaves declined during reproductive development relative to the vegetative stage but its content in leaves of both mutants was significantly higher and almost double (1.75 fold) that in WT leaves. An increase in the tri-amine Spd content in its free form was seen pre-bolting in leaves of mutant plants with suppressed *AtCuAO4* expression, but this increase was only significant in leaves of C#4 compared with wild type (Figure 4-22.b). Free Spd decreased in leaves of all studied plant lines post-bolting relative to their titres in the pre-bolting stage. However, free Spd content was significantly higher in leaves of mutant plants (BIS#4 and C#4) compared with leaves of WT plants showing a ~1.5 and ~1.7 fold increase in BIS#4 and C#4 leaves respectively compared to WT leaves at the same stage. Total PA content was significantly high in both mutant plants pre-bolting as compared with WT, however, total PAs reduced post-bolting in all plants including WT relative to its level pre-bolting with no clear difference between mutants and WT at this stage (Figure 4-22.c).

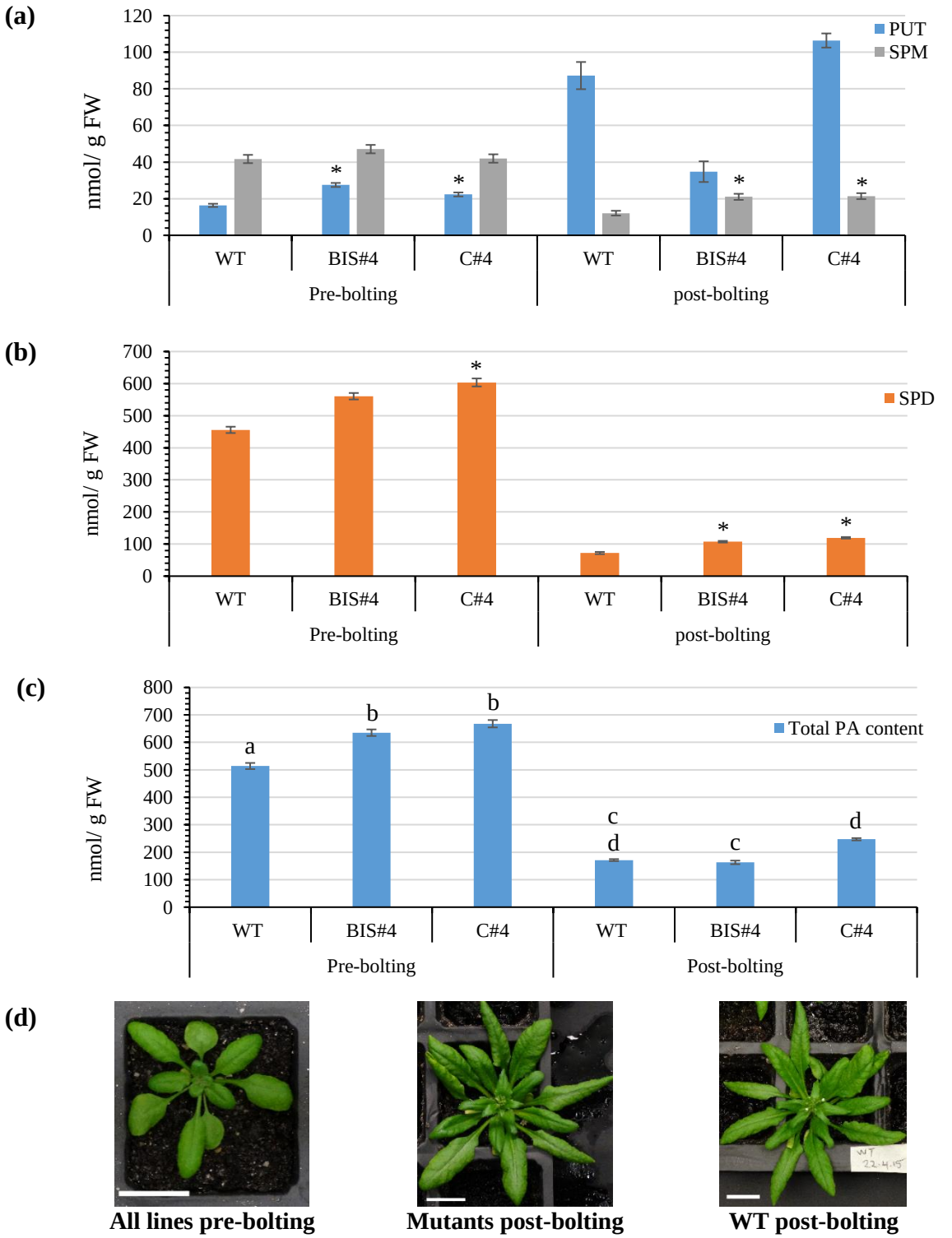



Figure 4-22 Free polyamine contents in leaf 5 and 6 of the two mutant lines (C#4 and BIS#4) and WT plants at two critical stages, pre-bolting and post-bolting. (a) Content of the di-amine Put and the tetra-amine Spm. (b) Content of the tri-amine Spd. (c) Content of the total free polyamines. Asterisks in (a) and (b) indicate values significantly different from the corresponding values of the wild type (WT) plants at $P \leq 0.05$ based on a T-test where data were normally distributed or Mann-Whitney test where data were not. Significant differences in means between genotypes at both stages in (c) are indicated by different letters ($P \leq 0.05$), statistically tested using One-Way ANOVA. Mean values ± SE, n = 6. (d) Images of plant stage at sampling, pre-bolting for all, mutants post-bolting and WT post-bolting respectively (Scale bars = 2 cm).

### 4.3.8. Effect of exogenous $GA_3$ on phenotype of *AtCuAO4* mutant plants

Following early treatment (at 6-7 leaves stage) with $GA_3$ at 50 µM, the effect of $GA_3$ on initiation of the inflorescence stalk (bolting), number of leaves at bolting and day of the first open flower were evaluated in the two mutant lines, C#4 and BIS#4, compared to WT plants (Figure 4-23). Results revealed that the delayed bolting and flowering and increased number of leaves at bolting seen in the two mutants (Figure 4-9.a) were reversed in the two mutant lines sprayed with $GA_3$ as compared to plants of the same mutant lines treated with water while at this concentration of $GA_3$ WT development was not affected, in measured parameters, by $GA_3$ treatment (Figure 4-24).

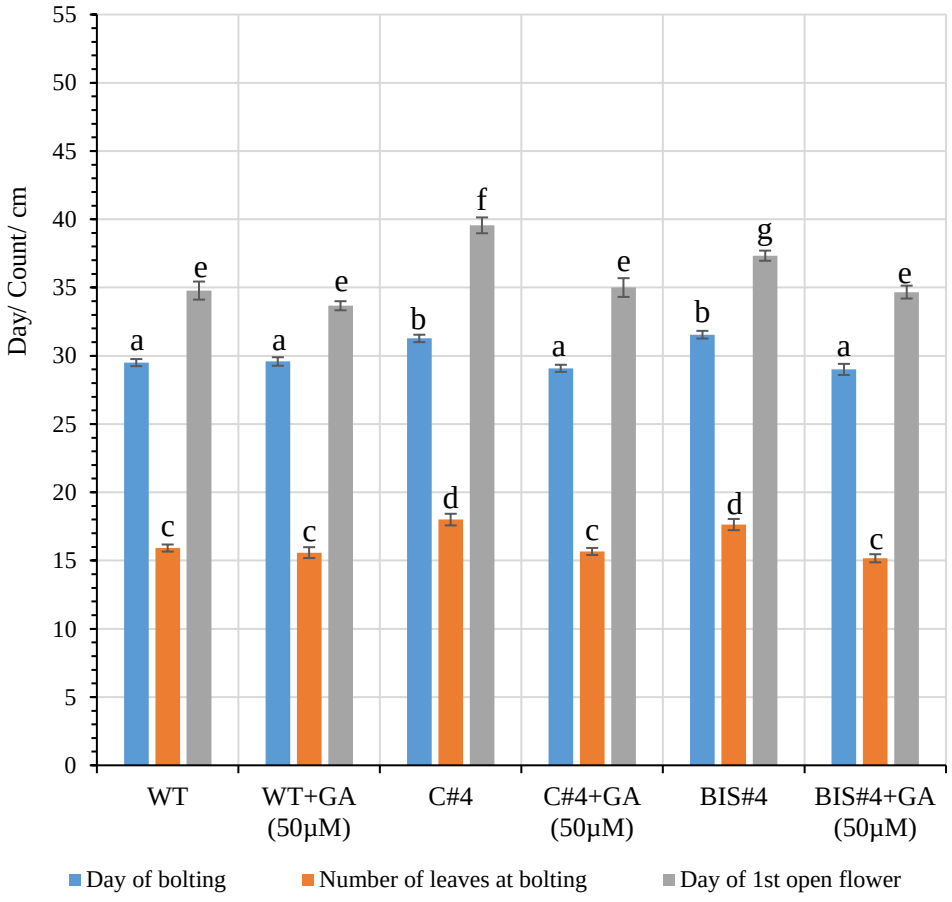



Figure 4-23 Effect of exogenous application of $GA_3$ on bolting day and number of leaves at bolting of the two mutant lines (C#4 and BIS#4) and wild type plants. Error bars represent standard error of the mean. Letters indicate a statistically significant difference (n = 12, One-way ANOVA, $P \leq 0.05$) between genotypes as a result of different treatments. The experiment was repeated twice with similar results.

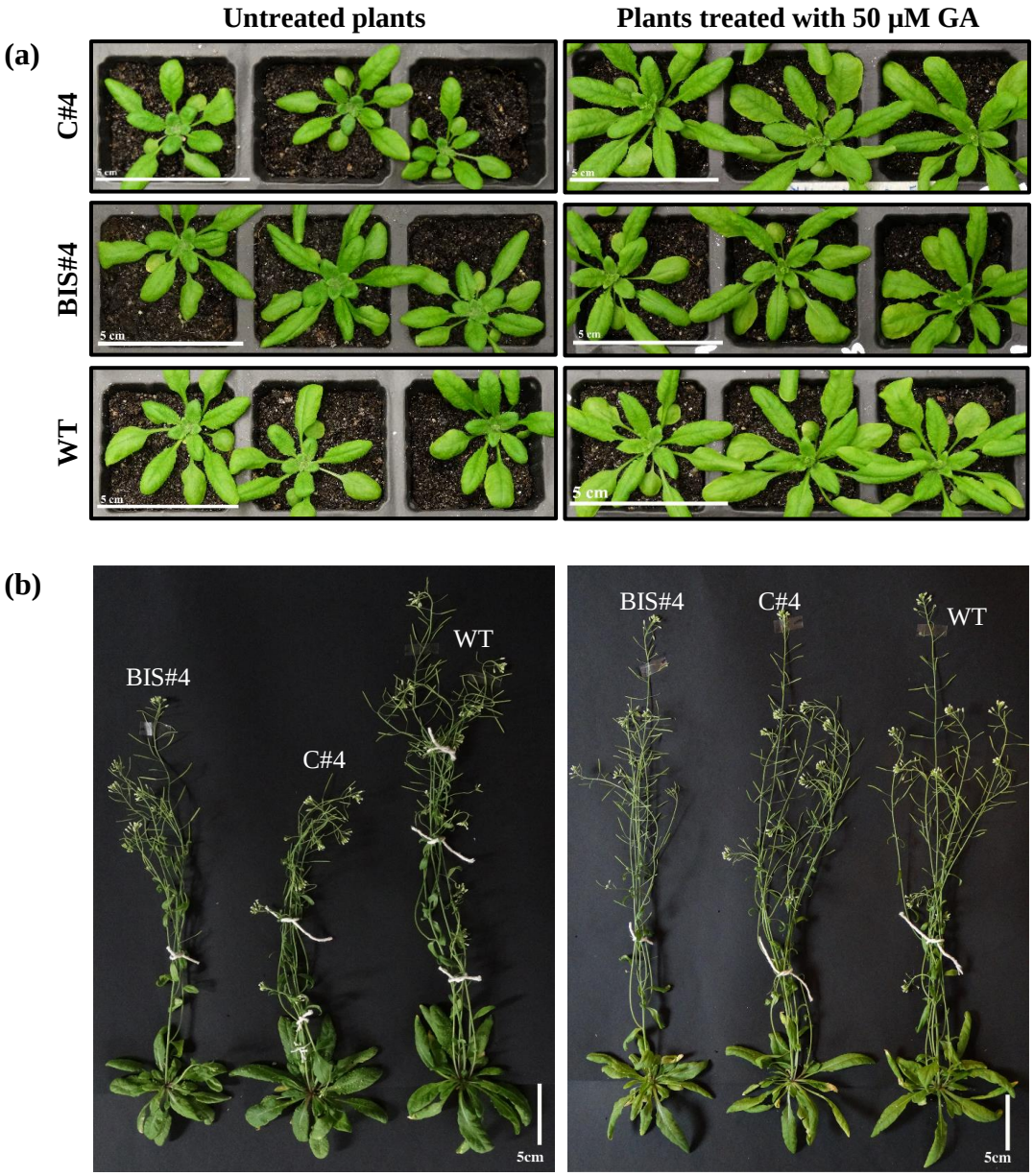



Figure 4-24 Exogenous application of GA rescues the effects of *AtCuAO4* mutation. (a) Phenotype of untreated and $GA_3$-treated homozygous *AtCuAO4* mutant lines and WT plants, grown under LD conditions as described in Section 2.1, at the stage of bolting of untreated WT plants. (b) Phenotype of homozygous mutants and WT plants at ~6 weeks with or without $GA_3$ application. Scale bars = 5 cm.

RNA was extracted from two week old rosettes of the two insertion lines, C#4 and BIS#4, and WT (5 rosettes/ genotype), and all the RNA samples were of a good quality (Figure 4-25.a). DNA contamination was removed from the RNA samples and then they were tested by PCR using 18S rRNA primers (Table 2-2) which revealed that all samples were free of genomic DNA (Figure 4-25.b). Quality of cDNA generated from the RNA

was confirmed by amplification of *Actin2* (Table 2-2) by PCR using specific primers (Figure 4-25.c).

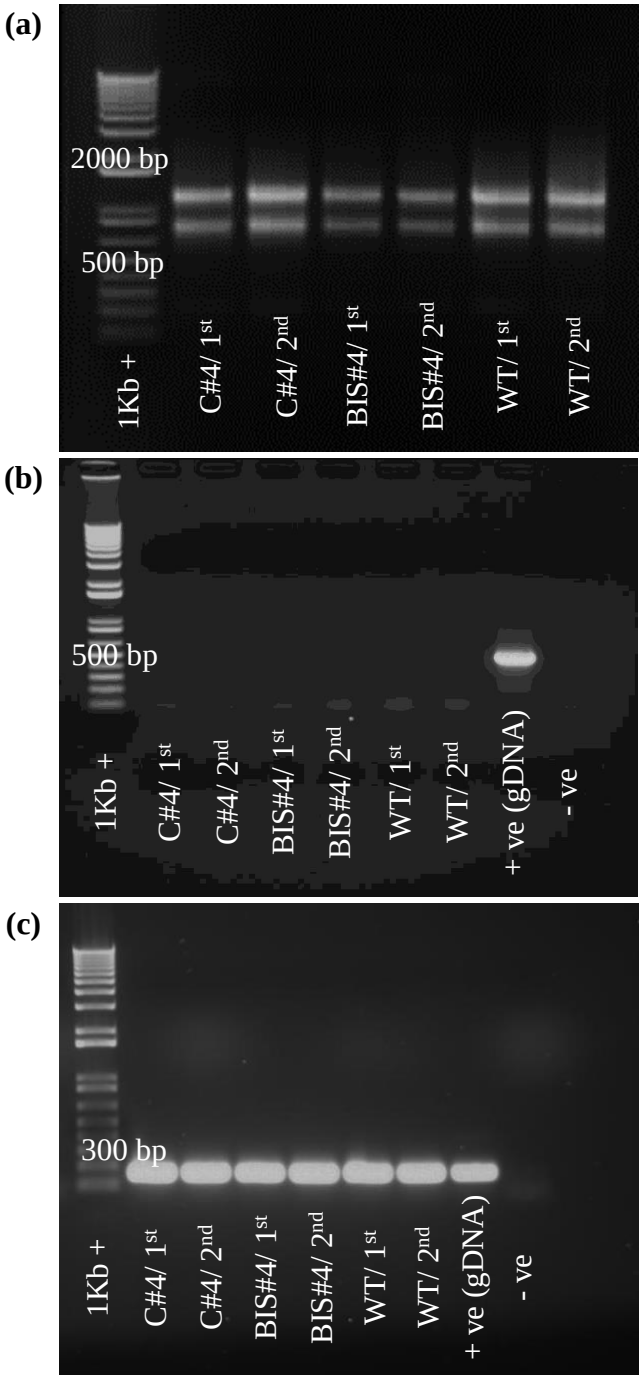



Figure 4-25 Quality control applied to isolated RNA from the two Arabidopsis T-DNA insertion lines (C#4 and BIS#4) and wild type before qRT-PCR analysis. (a) Integrity of RNA was tested by agarose gel electrophoresis. (b) DNA digestion after DNase treatment was confirmed by PCR using 18S rRNA primers (Table 2-2). (c) Integrity of generated cDNA was tested by PCR reaction with *Actin2* primers (Table 2-2). All steps were performed on two biological replicates (1st and 2nd) from each mutant line and WT plant. In both cases (b and c), genomic DNA (gDNA) was used as a positive control, and electrophoresis performed on 1.4 % agarose.

The relative transcript abundance of genes encoding GA biosynthetic enzymes as well as of selected floral genes as markers for floral development was evaluated in two week old rosettes of WT and *AtCuAO4* mutant plants (C#4 and BIS#4) grown under LD conditions as described in Section 2.1 (Figure 4-26). This stage was chosen because growing the plant for two weeks under long day conditions activates flowering genes and thus induces plant to flowering. For example, expression of *SOC1* is one of the earliest markers for floral induction in the meristem (Borner *et al.* 2000). The GA biosynthesis genes tested included *AtCPS1*, *AtKS1*, *AtGA20ox1*, *AtGA3ox1*, and *AtGA2ox1* along with the two floral genes *SUP* and *SOC1*. Apart from *AtCPS1* which was below detection in all tested plants including WT, results showed that all analysed GA biosynthetic genes were significantly down-regulated to different extents in both mutant lines relative to WT plants. Expression of the floral gene *SUP* was not detected in 2-week rosettes of either mutant or WT plants, however the transcript of the other floral gene *SOC1* was detectable and its abundance was significantly reduced in the two T-DNA insertional lines as compared to WT plants.

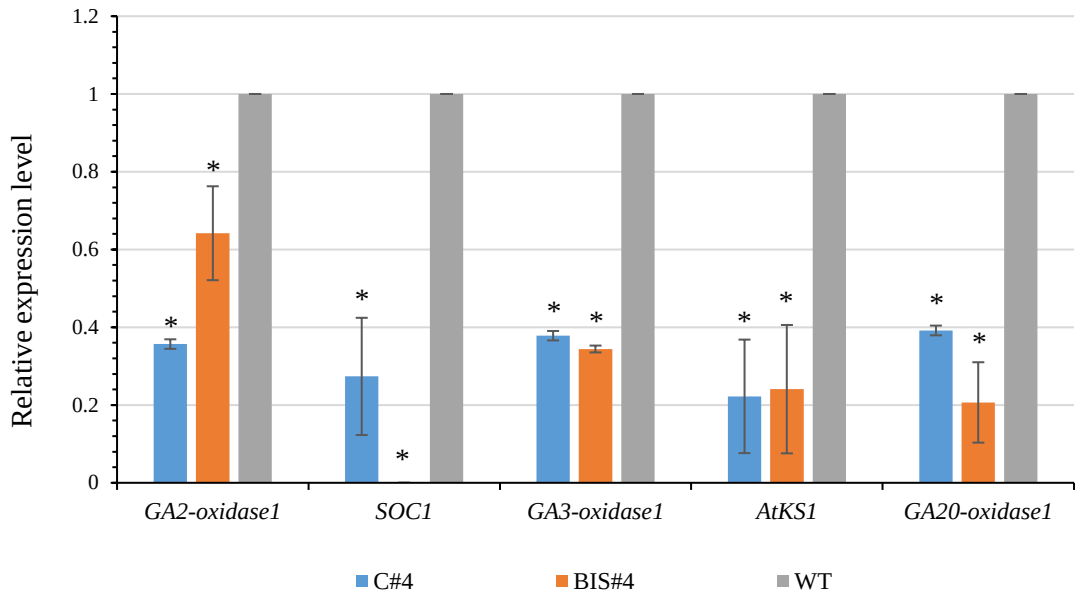



Figure 4-26 The effect of repression of *AtCuAO4* on the expression of the floral gene *SOC1* and GA biosynthetic genes in mutant lines (BIS#4 and C#4) and wild type (WT) plants. Two week old seedlings grown under LD conditions (Section 2.1) were used for real-time RT-PCR analysis. *Actin2* was used as a housekeeping gene to normalize target gene expression levels (see Table 2-3 for primer sequence). Transcript levels are expressed relative to the level of transcripts in WT plants. Values are the mean ± SE of two biological replicates each with three analytical replicates. Bars of the graph represent standard errors, and significant differences in means are indicated by different letters ($P \leq 0.05$). The results were statically tested using One-way ANOVA analysis.

## 4.4. Discussion

PCR screening confirmed that both T-DNA insertions for the *AtCuAO4* gene were homozygous (Figure 4-4), hence seeds could be sown directly onto soil for phenotyping experiments. Verification of mutant lines using three different combination of primers (upstream, flanking, and downstream; Figure 4-6) showed that both mutants, C#4 and BIS#4, did not produce transcripts flanking the T-DNA insertion site, while faint bands were detected when downstream primers were used which could be primer dimers or possibly due to a very weak expression of the mutant allele driven from the T-DNA insertion (Wang 2008). However, a product was obtained from the 5` end, upstream of the insertion point. It is assumed therefore that although truncated transcripts are produced, the gene is essentially knocked out by both insertions and no functional *AtCuAO4* protein can be produced by either of them.

Analysis of heterozygous mutant lines generated by the backcross of homozygous T-DNA insertional lines with WT (Figure 4-18), revealed that C#4 is probably a dominant or semi-dominant mutant (since homozygous mutant lines were not phenotyped alongside, it is not possible to be sure) while BIS#4 gives a first evidence that the insertion confers a recessive trait. Most T-DNA insertion mutants are recessive (Shu *et al.* 2012a), however, a dominant effect could arise if, for example, a truncated transcript or protein is produced from the mutant allele resulting in interference with the transcription or enzymatic activity of the WT allele (Lodish *et al.* 2000). Results of free PA content may support the suggestion that C#4 could be a dominant mutant as C#4 plants showed high accumulation of the higher polyamine Spd pre-bolting (Figure 4-22.b).

Since, wild type Arabidopsis plants from different regions vary in terms of shape, development and physiology (TAIR, About Arabidopsis: https://www.arabidopsis.org/ portals /education/aboutarabidopsis.jsp), great care was taken in this part of the project to use WT as a source of seed that was directly comparable to the mutant and transgenic lines. Transgenic over-expressor and mutant lines were compared to WT which was received from the Cona lab in Italy where overexpression lines were originally generated. However, for the mutant lines, it might have been better to have chosen different WT sources due to small differences observed in some studied traits (for example:

productivity, Figure 4-20.a) which might have been more evident had isogenic WT seed been used. So, for the future experiments it would be useful either to source WT lines directly from NASC (the source of mutant seeds) or to obtain WT seeds from the segregation of mutant heterozygous lines.

Although previous results with barley leaves indicated that endogenous levels of the polyamines Spd and Spm did not change during dark-induced senescence, the concentration of Put rose dramatically (Legocka and Zajchert 1999). Moreover, although the rise in Put in barley leaves did not appear to delay senescence, expression of genes related to PA metabolism were positively affected in barley (Sobieszczuk-Nowicka et al. 2015). To determine whether changing the balance of PAs through manipulation of *CuAO* genes affects the progression of dark-induced leaf senescence in Arabidopsis, the senescence symptoms of *AtCuAO4* mutant and over-expressor lines were examined in which it is presumed the balance of PAs may be altered. Results indicated no obvious phenotypic difference between studied lines and WT (Figure 4-15). Thus *AtCuAO4* is probably not required for darkness-induced leaf senescence. Lack of correlation between PA content and dark-induced senescence was also noted previously as inhibitors of polyamine biosynthetic enzymes did not affect the progression of cereal leaf senescence, suggesting that endogenous PA levels may not have an important role in regulating dark-induced senescence (Birecka et al. 1991; Chen and Kao 1991).

Since the effects of PAs in regulating plant growth and development are mediated by the regulation of cellular PA levels (Planas-Portell et al. 2013), an obvious hypothesis is that the delay in flowering and senescence in both of the mutant lines as a result of the *AtCuAO4* knockout was due to a change in the concentration of free polyamines. The oxidative deamination action of CuAOs contributes to down-regulation of free PA levels in plants (Moschou et al. 2008), and specifically Put is the substrate of AtCuAOs (Walters 2003). For example, high CuAO activity in tobacco leaves resulted in lower Put levels (Cona *et al.* 2014). Hence, my hypothesis was that down-regulation of *AtCuAO4* may result in increased levels of Put. However, *AtCuAO4* is not the only *AtCuAO* gene in Arabidopsis as it belongs to a gene family of 10 members (Planas-Portell et al. 2013). Therefore, there may be compensatory up-regulation of other *AtCuAO* genes due to the

disruption of *AtCuAO4* expression such that the PA contents would not be not affected. This hypothesis was tested by analysing the levels of free polyamines at two critical stages, pre- and post-bolting in both mutants and WT.

Similar concentrations of different PAs were found in leaves of WT plants during vegetative and reproductive stages as compared to previous work (Bagni *et al.* 2006). Although mutation of *AtCuAO4* in the present work repressed only one gene involved in Put catabolism, it was found to be enough to produce a clear accumulation of Put in leaves before bolting, and also increased both Spm and Spd after bolting. Before bolting, there appeared to be no clear increase in the tri-amine Spd and the tetra-amine Spm, however, a significant increase in both of them was observed in both mutant lines compared to the WT after bolting. This might also be attributable to the inhibition of *AtCuAO4* activity.

In many plant systems, high concentrations of PAs are associated with rapid cell division (Heimer and Mizrahi 1982; Montague *et al.* 1978; Slocum and Galston 1985). However, an imbalance of PAs can also have negative effects. The balance in PA distribution in different parts of the plant depends on the stage of development and is an important factor controlling plant development (Urano et al. 2003). Excess accumulation of polyamines is toxic to plant cells (Bouchereau et al. 1999), and high levels of Spd and Spm detected in leaves of both mutant rosettes, and associated with normal titres of Put post-bolting relative to WTs, could be a mechanism for regulating the cellular contents of polyamines to reduce the toxicity of high Put levels. The higher content of Spm could be due to elevated concentrations of its precursor Spd which was detected before bolting (Figure 4-22.b), although this was only significant in the C#4 plants.

The effect on seed germination could also be due to PA imbalance although the levels of PAs in seeds of the mutant lines were not tested. Several studies have previously related the PA [(Spm + Spd) : Put] ratio with seed germination indicating the importance of Spm and Spd in the period prior to radicle protrusion and the fundamental role of Put at later stages when the division rate is high (Dias *et al.* 2009; Pieruzzi *et al.* 2011). Another possible explanation for the initial delay in germination of *AtCuAO4* knockout seeds is by affecting $H_2O_2$ production, the product of Put oxidation by CuAO (Cona et al. 2006).

This is essential for seed dormancy breaking (Bailly *et al.* 2008), and acts as a promoter of seed germination (Ogawa and Iwabuchi 2001). In Arabidopsis, seed germination is promoted by $H_2O_2$ through promoting GA biosynthesis and up-regulating ABA catabolism (Liu *et al.* 2010). Treatment of pea seeds with $H_2O_2$ increased germination rate and seedling growth and this was attributed to the key role played by $H_2O_2$ in regulating these processes via mediating specific changes at the proteome, transcriptome and hormonal levels (Barba-Espín *et al.* 2011).

On the other hand, seed germination, root protrusion and other growth processes need energy for the growing tissues (Gallardo *et al.* 2001). As another product of the CuAO enzymatic activity is $\gamma$-aminobutyric acid (GABA), an important compound for energy production (Bouche and Fromm 2004), another hypothesis is that the mutation of *AtCuAO4* down-regulated GABA biosynthesis pathways derived from oxidation of PAs via CuAOs (Kuznetsov and Shevyakova 2007) resulting in reduced energy levels with consequent effects on germination. However, the delay in germination time does not seem sufficient to account for the later effects. For example, day of bolting was delayed by over 5 days while germination was only delayed by 1 day. While other studies have shown that flowering time and seed germination are co-regulated (Chiang *et al.* 2009), the present results suggest that the down-regulation of this gene affects development and flowering time independently of seed germination.

Mutant lines appeared to be delayed in several characteristics compared to WT (summarised in Table 4-1): firstly in germination, then in leaf emergence, bolting and flowering and in the size of the rosette before bolting occurred. At four weeks post-bolting, mutant lines were also delayed in senescence (as determined by number of yellowed leaves) although differences in total leaf number were no longer significant. Higher total chlorophyll content found at late stages (6 and 8 weeks) in both mutant lines is another piece of evidence for senescence retardation as a result of knocking out the expression of *AtCuAO4*. Stalk height and number of siliques produced were also lower in the mutant lines. These data suggest that suppression of the expression of *AtCuAO4* significantly delays growth including flowering and senescence (Figure 4-9.a, Figure 4-10.a, Figure 4-11.a, Figure 4-12.a, and Figure 4-27).

The high level of Put observed in rosette leaves was associated with retardation in the mutant lines in their transition from vegetative to reproductive stage, indicating that levels of free Put over this specific threshold may disturb normal development of the plant. It was reported that high Put accumulation as a result of overexpressing arginine decarboxylase (*ADC2*), the gene involved in Put biosynthesis, in Arabidopsis resulted in late flowering (Alcazar et al. 2005). However, similar observations were recorded in Arabidopsis plants with reduced Put due to simultaneous silencing of the two *ADC* by amiRNA:*ADC* (Sánchez-Rangel *et al.* 2016). In transgenic tobacco plants, overexpression of oat *ADC* accumulated Put and showed an early-flowering phenotype in one line while the rest of the transgenic plants which showed higher or lower Put levels did not exhibit any changes from control plants (Masgrau *et al.* 1997). These results emphasize that flowering time is affected by changes in PA levels.

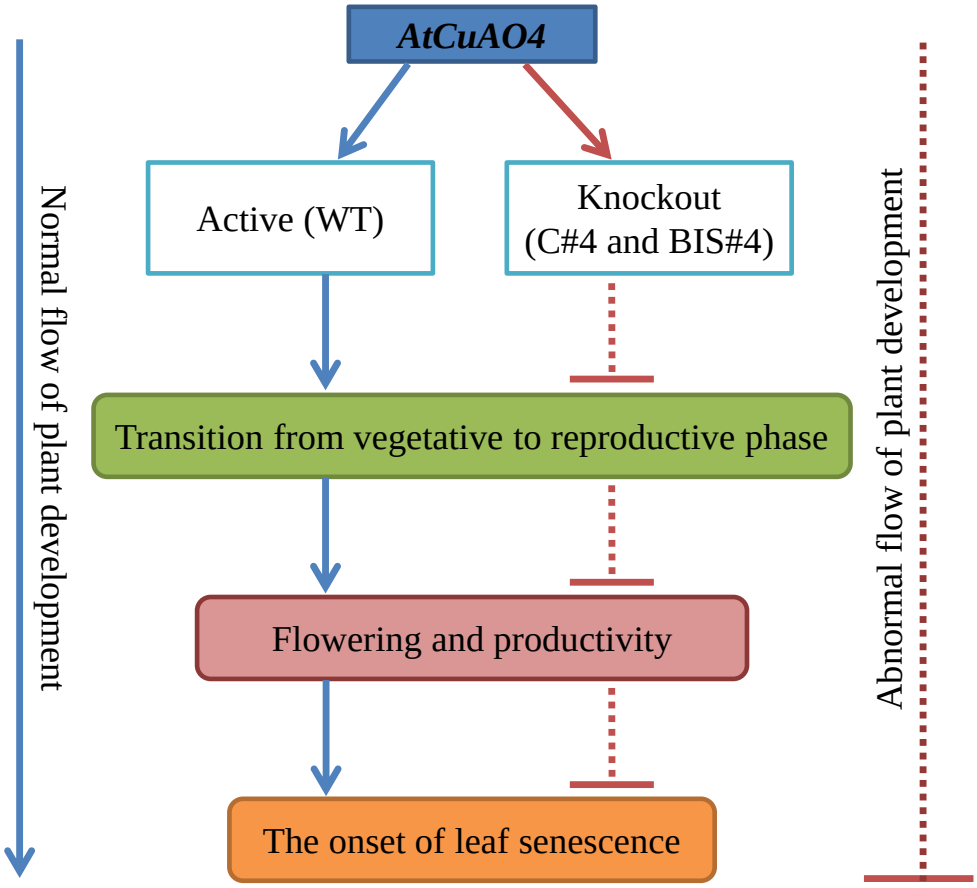



Figure 4-27 Flowchart depicting the impact of *AtCuAO4* (At4g12290) gene on the natural progression of growth in *Arabidopsis thaliana*. The pointed arrows indicate activation, and repression is represented by blunt arrows.

Table 4-1 Phenotype of the two mutant lines C#4 and BIS#4 and the three over-expression lines P9, P17, and P27 as compared to WT plants.

| Phenotype | C#4 | BIS#4 | P17 | P27 | P9 |
|---|---|---|---|---|---|
| Emergence of leaves | Emergence of leaves 1-9 was **delayed** significantly | Emergence of leaves 4, 5, 6, 8 and 9 was **delayed** | Leaves no. 1 and 2 were **earlier** in their emergence while the rest were similar to WT | Leaves no. 1, 2, 4 and 5 were **later** in their emergence while the rest were similar to WT | Leaf no. 4 was **later** in its emergence while the rest were similar to WT |
| Bolting day | Later | Later | Earlier | Earlier | Earlier |
| No. of leaves at bolting | Greater number of leaves | Greater number of leaves | Less leaves | - | - |
| 1st flower day | Later | Later | Earlier | Earlier | Earlier |
| No. of leaves at 4 weeks post-bolting | - | Lower | - | - | - |
| No. of yellow leaves at 4 weeks post-bolting | Fewer | Fewer | - | - | - |
| Rosette's fresh weight | - | - | - | - | - |
| Primary stalk's length | Shorter | Shorter | - | Taller | Taller |
| Rosette's diameter | - | - | - | - | - |
| No. of siliques at 4 weeks post-bolting | Lower | Lower | - | - | - |
| Total chlorophyll level (a+b) | Higher at 2 and 4 weeks post-bolting | Higher at 2 and 4 weeks post-bolting | - | - | Lower at 4 weeks post-bolting |

Endogenous PA levels were perturbed in transgenic plants which were engineered using sense arginine decarboxylase (*ADC*), a key enzyme involved in the biosynthesis of Put in plants (Masgrau et al. 1997) or antisense *S*-adenosylmethionine decarboxylase (*SAMDC*), which is necessary for the synthesis of Spd and Spm (Kumar *et al.* 1996) and this affected plant development, including stem elongation, due to defects in cell elongation. Therefore, I tested the hypothesis that knockouts in the expression of *AtCuAO4* with altered PA levels may also be affected in stem length. This was tested by assessing stem elongation rate of mutant plants compared with WT and primary stalk length 4 weeks after bolting. Results showed an initial temporary lower stem expansion rate which seemed to recover afterwards (Figure 4-19), indicating no continued defect in stem cell elongation, however the overall stem length was reduced in the mutant lines. This effect could also be due to the elevated levels of PAs.

Previous studies also reported that high Spd concentrations may act as a growth suppressor (Vuosku *et al.* 2012), and treatment of Arabidopsis with Spd under LD conditions had an inhibitory effect on flowering (Applewhite et al. 2000). Moreover, high levels of Put can alter the plant phenotype including reduced stem growth and leaf chlorosis to different degrees depending on the level of accumulated Put (Alcazar et al. 2005; Masgrau et al. 1997). The early increase in Spd observed here in mutant leaves (though not significant in both) might be another possible cause for delaying bolting in the mutant lines. An important molecular mechanisms of polyamine action in cell proliferation and cell division has been suggested based on their ability to modulate DNA conformation and interactions between DNA and protein by their direct binding to DNA (Stes *et al.* 2011). Further investigations are required to understand whether cell proliferation and/ or differentiation was affected by accumulation of Put or Spd before bolting.

In the present work, both mutant lines display a significantly postponed leaf senescence phenotype. This could be attributed to the indirect effect of delayed bolting (Nooden and Penney 2001; Sharabi-Schwager *et al.* 2010) or directly to the effect of high levels of PAs seen post-bolting (Figure 4-22). The result of dark treatment of both mutants (Figure 4-15) would seem to support this first hypothesis, i.e. that effects on senescence

are indirect. It has been reported that treatment with PAs prevents or delays senescence progression (Sood and Nagar 2003). Levels of polyamines have been correlated to senescence suggesting that reduction in polyamine levels may be a step in initiating senescence while treatment with polyamines can prevent senescence (Shama and Alderson 2005). The anti-senescence action of PAs may be due to potential repression of ethylene synthesis and to their effect in stabilizing and protecting membranes by combining with negatively charged phospholipids (Galston and Sawhney 1990). Postponement in bolting day is another possible contributor in the shift observed in the successive developmental processes including senescence.

Effects of amine oxidases (AOs) on plant growth and development are also mediated by their oxidation reaction products including aminoaldehydes, GABA ($\gamma$-aminobutiric acid) and markedly, $H_2O_2$ (Planas-Portell et al. 2013). Therefore, another possible explanation for the observed alterations in the mutants' phenotype is due to the temporary repression of one of the pathways for $H_2O_2$ production (by inhibition of PA-terminal oxidation by AtCuAO4). This may then be later compensated by other members of the family since the genetic redundancy by gene family members is common to compensate for each other's loss (Kafri *et al.* 2009). In general, the polyamine catabolic product $H_2O_2$ has well-established roles in plant growth and development (Lim *et al.* 2006). $H_2O_2$ produced in particular from oxidation of PAs by CuAOs is an important element in the biosynthesis of lignin and cell-wall cross-linking reactions and cell expansion and thus has been suggested as a fundamental factor in regulating growth and development processes (Lim et al. 2006; Tavladoraki *et al.* 2012). A burst of $H_2O_2$ is also associated with bolting (Zimmermann *et al.* 2006), thus a reduction in CuAO-generated $H_2O_2$ may account for the delayed bolting in the *AtCuAO4* mutant lines.

Another effect noted in transgenic plants which were manipulated to produce higher levels of PAs was an effect on GA biosynthesis. Transgenic Arabidopsis plants overexpressing *ADC2* accumulated high concentrations of Put and the expression of GA biosynthetic genes involved in early and latter parts of the GA biosynthetic pathway were down-regulated, resulting in reduced levels of bioactive GA which in turn resulted in a severe dwarf and late-flowering phenotypes (Alcazar et al. 2005). GA promotes cell

division and expansion thus both processes contribute to the elongation of internode in the pith rib meristem region (Achard *et al.* 2009; Cowling and Harberd 1999). Thus a dwarf phenotype is consistent with a reduction in GA biosynthesis through an indirect effect of elevated PAs.

The phenotype observed in *AtCuAO4* mutant plants (late bolting, late flowering, and short stalks), although not as severe as reported by Alcazar et al. (2005) is consistent with GA-deficiency. Hence, the effects of exogenous GA application were tested. The response of the mutants to exogenous $GA_3$ application (Figure 4-23), suggested a role for *AtCuAO4* in regulating GA biosynthetic genes. Therefore, the relative transcript levels of selected genes encoding the primary enzymes involved in GA metabolism as well as two GA-controlled floral genes, *SOC1* and *SUP*, were analysed in 2-week old rosettes of WT and *AtCuAO4* mutant plants grown under normal conditions. The results obtained showed that expression of GA biosynthesis genes as well as the flowering gene *SOC1*, a transcription factor associated with floral homeotic genes (Moon et al. 2003) was affected. Again this would be consistent with the high level of Put in Arabidopsis rosettes before bolting, as a result of the *AtCuAO4* knockout. However, GA biosynthesis is regulated mainly by light, temperature, and feedback inhibition (Hedden and Kamiya 1997), and the developmental transition from vegetative to reproductive stage in Arabidopsis is controlled by several pathways including the photoperiodic pathway and response pathway to the growth regulator gibberellin (Fornara et al. 2010). Hence, it would be necessary to measure GA content in the mutant lines before bolting to verify whether down-regulation of GA production in mutant plants may be a cause of the mutant phenotypes.

As well as analysing the phenotype of knockout mutants, analysis of over-expressors can be useful in understanding the function of genes and gene families (Bolle *et al.* 2011). However caution must be exercised in the interpretation of over-expression lines especially when the transgene is driven by a different promoter to the native gene, as was the case in the *AtCuAO4* over-expression lines. In this case the gene will be expressed at different times and locations compared to the native gene and thus phenotypic effects may be due to ectopic expression rather than true over-expression.

Three *AtCuAO4* over-expressor lines were analysed, all of which expressed the transgene at high levels, with expression in P17 > P9 > P27. Phenotypes differed between the lines (Table 4-1), however the relationship between level of *AtCuAO4* expression and effects on the phenotype were not directly proportional. For instance, P17 which had the highest level of *AtCuAO4* expression (372 x relative to WT) showed early emergence of some leaves, however, other lines (P9 and P27) with lower expression levels of *AtCuAO4* showed taller flowering stalks. None of the three transgenic lines over-expressing *AtCuAO4* presented any obvious differences in germination as compared to WT seed. In addition, no consistent phenotypic differences in vegetative growth or development were noted relative to WT plants. The only phenotypic characters that were consistently altered in all three over-expressor lines were shortening of the time to bolting and first open flower. The lack of a correspondingly opposite phenotype in the over-expressor lines could be explained in several ways. Compensatory mechanisms may prevent a drop in PAs for example by down-regulation of other catabolic genes, or by increasing PA biosynthesis (Rea *et al.* 2004). Since PAs are essential for many cellular functions (Kusano *et al.* 2007), it is possible that the plant is less able to tolerate their reduction. For example, a reduction in PA concentrations (Put, Spd and Spm) in transgenic tobacco plants due to silencing ornithine decarboxylase (ODC, the PA biosynthetic enzyme) resulted in a negative impact on plant growth and vigour (Dalton *et al.* 2016). Further studies are important in order to elucidate whether in fact PA contents are affected in the transgenic over-expressor lines and what homeostatic mechanisms might be activated to compensate for the increased *AtCuAO4* expression.

# Chapter Five

## Silencing of *AtCuAOs*

# 5. The effect on *Arabidopsis thaliana* development of expressing artificial microRNA targeted to *AtCuAO* gene family members

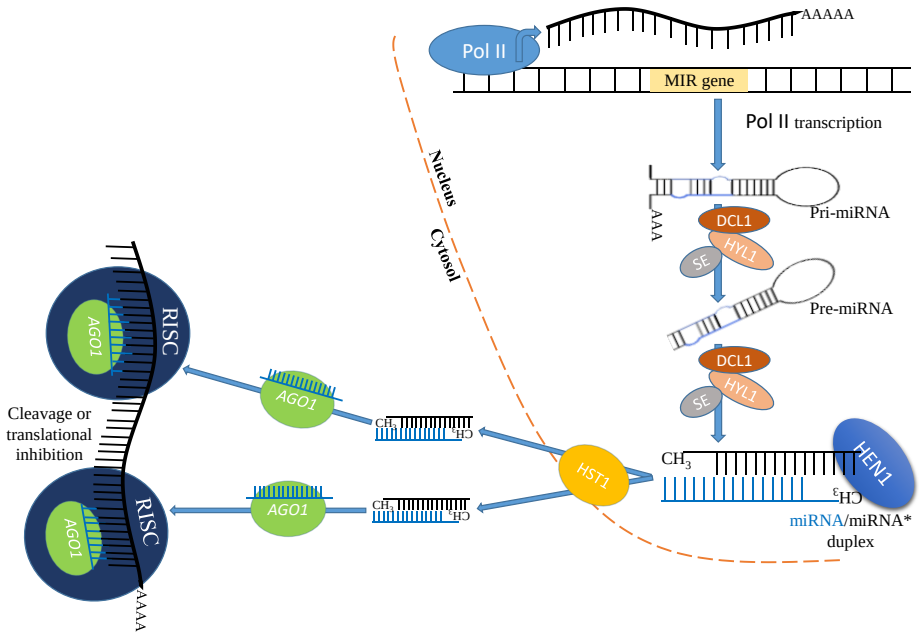
## 5.1. Introduction

In various organisms, it is common that large multi-gene families comprise genes with identical or extremely similar sequences, and this phenomenon is more prevalent in plants including *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000). Genetic redundancy includes partial overlap in functions of genes amongst family members to compensate for each other's loss (Kafri et al. 2009). The occurrence of the functional redundancy of genes within family members makes the analysis of single gene functions more complicated (Jover-Gil *et al.* 2014). Owing to the phenotypic similarity between many single mutants and wild type, resulting from the compensation of the lack of function of the mutated gene by the overlapping function of one or more paralogs, in most cases there is a requirement for double, triple, or quadruple mutant combinations of loss of function mutations to affect paralogous genes and show increasing severity of phenotypes (Martienssen and Irish 1999; Pérez-Pérez *et al.* 2009). To overcome the difficulties in using analysis of mutants for genes that overlap functionally, different techniques have been applied such as overexpression methods (Tautz 2000; Weigel *et al.* 2000), and RNA interference based techniques (Abbott *et al.* 2002; Ott *et al.* 2005; Wagner 2005). However, these techniques may have some disadvantages represented by pleiotropic and neomorphic phenotypes caused by overexpression which make interpretation difficult (Strabala 2008), and off-target effects as a result of using RNA interference methods. This is due to the generation of numerous small RNAs from one double stranded RNA which potentially affect undesired targets (Jackson *et al.* 2003). Alternatively, artificial micro RNA precursors can be computationally designed in plants to form mature amiRNAs which are able to target a specific group of possibly redundant genes (Ossowski *et al.* 2008; Schwab *et al.* 2006).

MicroRNAs (miRNAs), small non-coding RNAs with a length of ~21 nucleotides, are described in eukaryotes as important endogenous regulators of gene expression through their involvement in the process of post-transcriptional gene silencing (Bartel 2004). They are derived from hairpin RNA precursors to produce one predominant miRNA

alongside an imperfectly complementary antisense miRNA* (Figure 5-1.a) (Axtell 2013). Micro RNAs in plants induce cleavage of the target mRNA at a position opposite to nucleotides10 and 11 of the miRNA (Llave *et al.* 2002) (Figure 5-1.b).

**(a)**




**(b)**


Figure 5-1 Biogenesis and function of miRNAs. (a)A single strand RNA is transcribed from nuclear encoded *MIR* gene via RNA polymerase II (POLII) and then folds back forming a double stranded stem-loop structure (Pri-miRNA). Pri-miRNA is further converted into pre-miRNA via the action of DCL1with assistance of HYL1 and SE proteins. Pre-miRNA is then converted into a 20-22 nucleotide miRNA/miRNA* duplex in the presence of the three proteins (DCL1, HYL1and SE). A methylation catalysed by HEN1 at the 3` terminus of duplex takes place before its exportation to the cytoplasm by HST1. One strand of the duplex (miRNA) incorporates into AGO1 protein the stimulator and guide of RISC. RISC binds to the target transcript by sequence complementarity to control mRNA cleavage and translation inhibition (Khraiwesh *et al.* 2012). (b) Alignment between mRNA target site and miRNA, the expected miRNA-based cleavage site is indicated by a red arrow.

Recently, experiments and bioinformatic predictions identified many putative miRNAs in plants (Sunkar and Jagadeeswaran 2008; Zhang *et al.* 2006), but the biological roles of these miRNAs in plant functional genomic networks and their target genes are mainly still obscure (Chen *et al.* 2015). MicroRNAs were first discovered in Arabidopsis and since then studies on plant miRNAs have become one of the most important fields in

plant biology which has made substantial progress in the past few years (Sun 2012). By employing direct cloning technology, more than one hundred miRNAs were identified in Arabidopsis in 2002 by several independent research groups, some of them are highly conserved in other plants (Llave et al. 2002; Reinhart *et al.* 2002).

The regulation of gene expression by miRNAs in plants is carried out via different modes of action that have yet to be fully understood (de Alba *et al.* 2013). The common mechanism for miRNA-mediated gene regulation in plants is by guiding RISC, the RNA-induced silencing complex, in the presence of the RNA slicer protein AGO1 to a complete or near-complete binding to their mRNA targets, which causes RNA cleavage or degradation and thus repress the expression of the gene (Figure 5-1.a) (Baumberger and Baulcombe 2005; Rhoades *et al.* 2002; Schwab *et al.* 2005). In addition, in some cases miRNAs direct DNA methylation (Bao *et al.* 2004), or block mRNA translation when they bind imperfectly to it (Brodersen *et al.* 2008). Mismatches between miRNA and their targets are mostly found towards the 3` end suggesting that pairing to the 5` and central part of the miRNA is most important (Mallory *et al.* 2004).

Experimentally, artificial micro RNAs (amiRNAs) can be designed to analyse functions of single or multiple target genes of interest by efficient silencing of endogenous gene expression at a post-transcriptional level (Ossowski et al. 2008; Schwab et al. 2006). This approach was first used to silence individual genes in human cells (Zeng *et al.* 2002), and later in Arabidopsis plants (Parizotto *et al.* 2004). In plants, amiRNAs can be designed to effectively down-regulate the expression of specific endogenous genes leading to loss of function changes in phenotype (Hauser *et al.* 2013), and they are effective when expressed under the control of a constitutive promoter (Li *et al.* 2014). The amiRNA approach employs the precursor of a natural miRNA as a backbone by replacing the natural miRNA/miRNA* duplex with the artificial one to gain new targeting ability (Ossowski et al. 2008; Vaucheret *et al.* 2004), and allows processing of the amiRNA in a similar way to the natural one producing mature functional amiRNA (Figure 5-2) (Hu *et al.* 2009). High level of miRNA accumulation of the desired sequence results when both sequences (miRNA/miRNA*) are changed without altering structural features like bulges or mismatches (Ossowski et al. 2008). The effectiveness of the amiRNAs has been

underlined by the fact that they have high specificity as with the endogenous miRNAs, which facilitates optimizing their sequences to silence one or multiple target mRNAs without disturbing the expression of other transcripts (Schwab et al. 2006; Schwab et al. 2005). The mechanism of amiRNA action does not require a perfect complementarity with its targets and up to five mismatches are accepted (Ossowski et al. 2008).

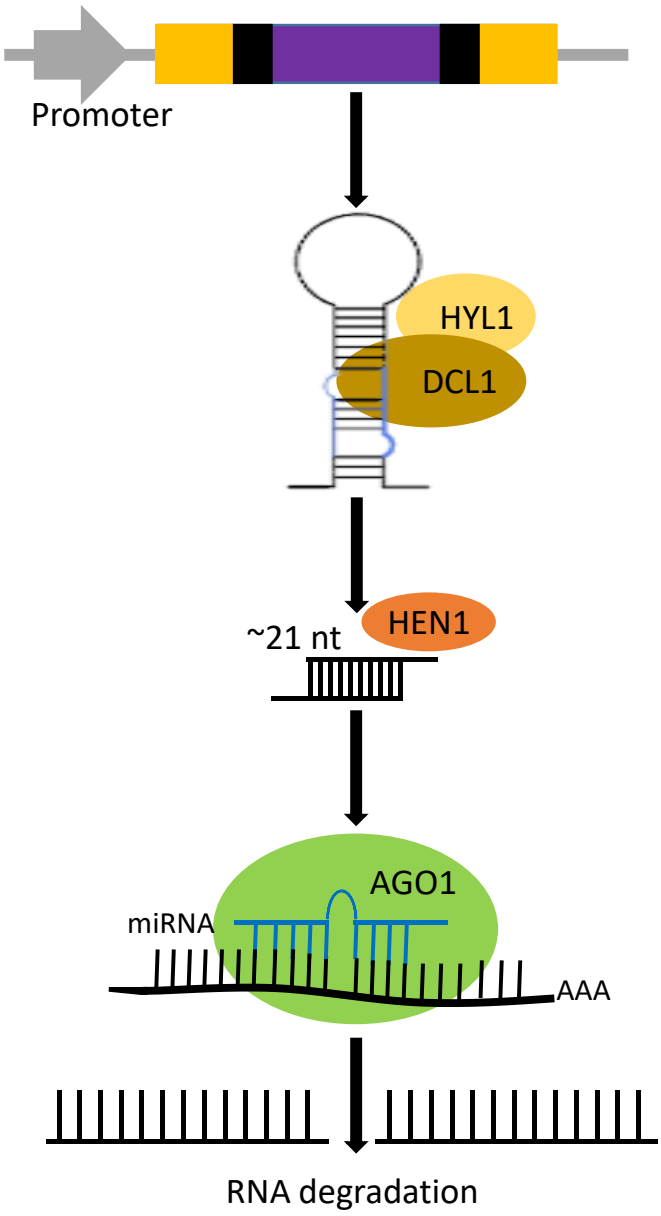



Figure 5-2 amiRNA pathway mediating transgene-induced gene silencing in plants. From non-coding genes, amiRNA are transcribed and then processed to 21 nucleotide miRNA via the action of DCL1 which interacts with the double stranded RNA binding protein HYL1. HEN1 catalysis methylation of the miRNA duplex at the 3` end. The 21-nucleotide miRNA guides cleavage of target transcript via AGO1. Black bars represent sequences that the amiRNA is derived from (Ossowski et al. 2008).

This technique has been efficiently applied to dicotyledonous plants including *Arabidopsis thaliana*, and it has proven to be more specific in mediating gene silencing and has less off-target effects compared with other techniques of gene knockdown (Ossowski et al. 2008; Schwab et al. 2006). AmiRNAs can be designed to target in a highly specific way one or several genes of interest to induce post-transcriptional gene silencing (PTGS) (Ossowski et al. 2008; Schwab et al. 2005).

To test the effect of amiRNA on multiple target genes, Schwab et al. (2006) designed three types of amiRNA with multiple possible targets, the first was amiR-trichome which was designed to target three *MYB* genes involved in trichome patterning, the second was including amiR-*mads-2* and amiR-*mads-1* to silence genes that have a role in regulating floral patterning and flowering time respectively, and the third was including amiR-*yabby-2* and amiR-*yabby-1* which target several members of the *YABBY* gene family, with one predicted target for amiR-yabby-2 and two for amiR-yabby-1. Their results demonstrated that multiple mRNAs can be successfully targeted by designed amiRNAs, however, the level of down-regulation of different targets varied with no clear correlation of targeting effectiveness either with levels of target expression in the WT or level of complementarity between designed amiRNAs and their target mRNAs (Schwab et al. 2006). Interestingly, while several amiRNAs can perform translational inhibition of their target gene, a small decrease in the abundance of target mRNA is produced (Yu and Pilot 2014). Hence, testing the effectiveness of amiRNA at the level of the target protein accumulation is more suitable than at the level of target mRNA (Li *et al.* 2013a).

Given their effectiveness in silencing other Arabidopsis genes, and as protein alignment of AtCuAOs (Figure 1-3) shows that the conservation of functional domains is almost high amongst different AtCuAOs which implies that there can be a functional redundancy between them, use of amiRNAs seemed a good tool to use to understand the role of different members of the *AtCuAO* gene family. Two specific artificial miRNAs (CSHL_058443 and CSHL_017399) were selected due to their homology to several members of this gene family. My hypothesis was that the two amiRNA constructs would generate transgenic plants with differing phenotypes that would provide useful insight into the functions of the *AtCuAO* family members during development and senescence.

## 5.2. Materials and Methods

### 5.2.1. Artificial microRNA constructs

Two artificial microRNA (amiRNA) clones (CSHL_017399 and CSHL_058443) were requested and received from the Arabidopsis Biological Resource Centre (ABRC). These amiRNAs were designed by WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) (Schwab et al. 2006) to resemble natural plant miRNAs in the following criteria: (1) to start with uridine which is overrepresented in most plant miRNAs, (2) to include adenine as the 10th nucleotide which is most favourable for artificial siRNA, and (3) between one and three mismatches to the target genes were introduced in the 3`of the amiRNA to minimize the probability that the amiRNA works as a primer for RNA-dependent RNA polymerase and thereby trigger secondary RNAi.

According to the PHANTOM Database (Hauser et al. 2013) (http://phantomdb.ucsd.edu/amioverview/):

amiRNA CSHL_058443 was specifically designed to match the *AtAO1* (AT4G14940) gene sequence and targets the following genes: *AtAO1* (AT4G14940), *AtCuAO2* (AT1G31710), *AtCuAO4* (AT4G12290), *AtCuAO5* (AT1G31670), *AtCuAO7* (AT3G43670), and *AtCuAO9* (AT4G12280).

amiRNA CSHL_017399, was specifically designed on the *AtCuAO5* (AT1G31670) gene sequence and targets the following genes: *AtAO1* (AT4G14940), *AtCuAO2* (AT1G31710), *AtCuAO3* (AT2G42490), *AtCuAO4* (AT4G12290), *AtCuAO5* (AT1G31670), *AtCuAO8* (AT1G31690), and *AtCuAO9* (AT4G12280).

The amiRNAs received had been previously cloned into a pAmiR vector (derived from pGreenII, Appendix C) and transformed into *Escherichia coli*. On receipt, each clone was streaked onto LB medium (1 % tryptone, 0.5 % yeast extract, 1 % sodium chloride, 1.5% agar adjusted to pH 7) containing the antibiotic spectinomycin (Spec, 50 µg/ ml) for selection. Petri dishes were incubated overnight at 37° C in a Heratherm incubator (Thermo scientific). A single colony was used then to inoculate 5 ml of liquid LB medium

supplemented with antibiotic (50 µg/ ml Spec) in a 50 ml Falcon tube (2 tubes per clone) and incubated at 37° C, 200 rpm, overnight (14-16 h) in a Gallenkamp Orbital incubator. Glycerol stocks were prepared by centrifuging 1ml of the bacterial cultures at 8,000 rpm, at RT, for 3 min in an Eppendorf MiniSpin® microcentrifuge. The supernatant was then removed and the resulting pellet was re-suspended in 4:1 LB medium: sterile glycerol (800 µl LB and 200 µl glycerol), mixed gently and stored at -80° C.

### 5.2.2. Plasmid DNA purification

Purification of plasmid DNA was performed at room temperature using the QIAprep Spin miniprep kit (QIAGEN) following the manufacturer's protocol. An LB overnight culture (2.5 ml of the bacterial host cell cultures, 3 replicates/ clone) was pelleted by centrifugation in a microcentrifuge tube at 13,000 rpm for 1 min in an Eppendorf MiniSpin® microcentrifuge. The cells were re-suspended in 250 µl of Buffer P1, followed by the addition of 250 µl of buffer P2 and mixed gently by inversion 4 to 6 times. Buffer N3 (350 µl) was then added and the tube was immediately inverted gently 4 to 6 times. The lysate was centrifuged at 13,000 rpm for 10 min as above and the supernatant was transferred to the QIAprep Spin column for further centrifugation for 30 seconds. The flow-through was discarded and the column was washed with 750 µl of buffer PE with centrifugation at 13,000 rpm for 1 min as above. The centrifugation was repeated to remove any residual wash buffer and then the flow-through was discarded. The column was transferred to a clean microcentrifuge tube, 40 µl of sterile distilled water was added and left to stand for 2 min before centrifugation twice at 13,000 rpm for 1 min as above. The isolated DNA was stored at -20° C before further use. To increase the concentration of the plasmid, replicates of each clone were precipitated by adding 1:10 (v/ v) sodium acetate and 2 volumes of 100 % ethanol. After overnight incubation at -20°C, the mixture was then centrifuged at 4° C, 13,000 rpm for 20 min. The supernatant was then removed and pellet washed with 70 % cold ethanol. Following a further centrifugation at 13,000 rpm, 4° C for 10- 15 min as above, the supernatant was removed and the pellet dried at RT and finally dissolved in 20 µl sterile distilled water and stored at -20° C. All centrifugation steps at 4° C were performed using Heraeus Fresco 17 centrifuge (Thermo scientific) (Figure 5-3).

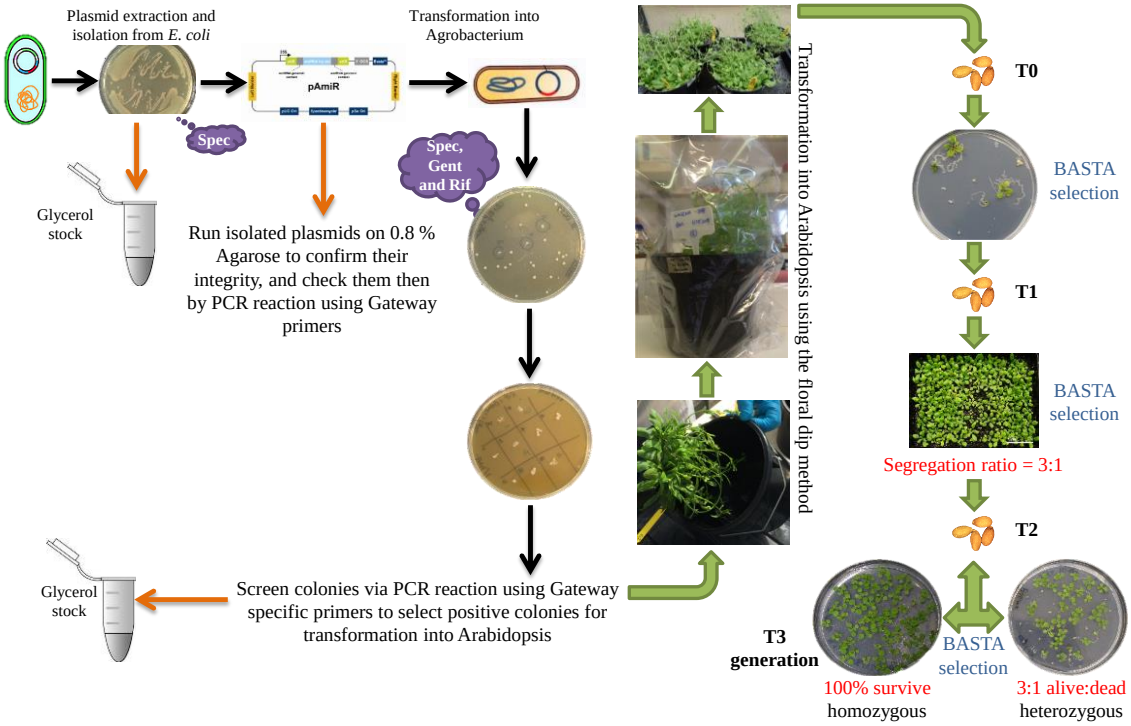



Figure 5-3 Scheme to summarize steps followed in this work to transform *Arabidopsis thaliana* with amiRNA clones and to generate Arabidopsis plants containing homozygous amiRNA in their genome in the T3 generation.

### 5.2.3. Plasmid DNA quantification

Plasmid DNA concentration expressed as ng/ µl was determined as described in Section 2.11.2. In order to confirm the identity of both artificial microRNAs, CSHL_058443 and CSHL_017399, both clones, were subjected to different analysis prior to the transformation into *Agrobacterium tumefaciens*.

To check the presence (integrity) and size of plasmids, 4 µl of the purified plasmid were mixed with 2 µl of 5x PCR green buffer and were then run on a 0.8 % [w/ v] agarose gel (Section 2.11.1). Subsequently, plasmids were amplified by the PCR reaction using Gateway primers (Table 2-2) as described in Section 2.9. PCR products were then electrophoresed on a 1% agarose gel (Section 2.11.1). PCR products were extracted from the gel and sequenced as described in Sections 2.12 and 2.13 respectively. Sequencing results were used to design primers as described in Section 2.14 and Table 2-2 to be used for insertion verification (Figure 5-3).

### 5.2.4. Preparation of the competent *Agrobacterium tumefaciens* cells

*Agrobacterium tumefaciens* strain GV3101 from a -80° C glycerol stock was streaked onto LB medium supplemented with 100 µg/ ml rifampicin (Rif) and 25 µg/ ml gentamicin (Gent) (2 replicates). Following incubation at 28-30° C for 3 days in a Vindon scientific incubator (Diggle Oldham, England), a single antibiotic-resistant colony was selected to inoculate 5 ml of LB supplemented with the same antibiotics and incubated overnight at 28-30° C, in a Gallenkamp orbital incubator set at 200 rpm. Then 1 ml of the overnight culture was used to inoculate a 250 ml conical flask containing 100 ml LB medium with the same antibiotics (Rif and Gent), and incubated overnight at 28-30° C with shaking at 200 rpm for 1-2 days until high cell density was attained (~0.8 optical density). Cultures were then divided into 25 ml aliquots and cells were harvested by centrifugation at 4,000 rpm, 4° C for 10 min using JA-20 rotor at in a Beckman Coulter Avanti® J-E centrifuge. The supernatant was removed and the cells were gently re-suspended in 1 ml of ice cold 20 mM CaCl2 (sterilized by filtration). Suspended cells were then divided into 100 µl aliquots in pre-cooled Eppendorf tubes which were frozen in liquid nitrogen and stored at -80° C.

### 5.2.5. Transformation of *Agrobacterium tumefaciens* (GV3101) competent cells

Competent *Agrobacterium tumefaciens* cells were transformed as follows: 0.3-1 µg of plasmid DNA were added to an aliquot (100 µl) of cells that had been thawed on ice prior to transformation, and mixed gently. The cells were then frozen in liquid nitrogen for several seconds, and then thawed again at 37° C for 5 min. LB medium (1 ml) was added and tubes were incubated at 28-30° C for 4 hours at 100 rpm in a Gallenkamp cooled orbital incubator.

To harvest transformed cells, cultures were then centrifuged at 8,000 rpm for 1 min at RT using an Eppendorf MiniSpin plus® microcentrifuge. The supernatant was removed and 100 µl of LB were added and gently flicked. The cells were spread onto LB agar dishes containing Rif (100 µg/ ml), Gent (25 µg/ ml), and Spec (50 µg/ ml), which were then incubated at 28-30° C for 3 days and the appearance of colonies was monitored.

### 5.2.6. Colony screening of *Agrobacterium tumefaciens* (GV3101)

Single colonies were randomly selected from each clone and plated on LB agar in Petri dishes containing appropriate antibiotics (Rif 100 µg/ ml, Gent 25 µg/ ml, and Spec 50 µg/ ml) and incubated at 28-30° C for 2 days in a Vindon scientific incubator (Diggle Oldham, England). The resulting colonies were used as template for PCR colony reactions (Section 2.9) with Gateway specific primers (ABB1 and ABB2, Table 2-2) to select colonies for Arabidopsis transformation. Of each clone, two positive colonies were used for glycerol stock preparation as described in Section 5.2.1 (Figure 5-3).

### 5.2.7. Transformation of Arabidopsis

*Arabidopsis thaliana* (Col-0) plants were transformed with *Agrobacterium tumefaciens* strain GV3101 harbouring the construct by the floral dip method (Clough and Bent 1998) as adapted by Logemann *et al.* (2006). For each clone of the amiRNA, 3-4 pots of soil-grown Arabidopsis plants were prepared (4-6 plants/ pot). The primary inflorescence stems were removed in order to encourage proliferation of secondary stems, and plants were ready after approximately 10-15 days (7 weeks after germination) when they had only few fertilized siliques and many immature flower clusters.

Positive colonies of transformed Agrobacterium (Section 5.2.6) were plated on LB agar dishes containing 100 µg/ ml Rif and 50 µg/ ml Spec and grown at 28-30° C until they formed a thick bacterial layer (for about 4 days). Using a glass spatula, bacteria were collected from dishes by scraping and then they were re-suspended in 20 ml LB in a sterile Falcon tube. Fresh LB media was further added to the tube of bacterial suspension until the optical density at OD600 reached 2. A solution containing sucrose 5 % [w/ v], and Silwett 77 (0.03 % [v/v], surfactant, Lehle Seeds) was prepared for each clone to be transformed and poured into an appropriate container. Immediately before transformation into the plants, bacteria were added to the Silwett mix and the inflorescences of Arabidopsis plants were dipped in the solution for 5-10 seconds with gentle agitation.

Dipped plants were then laid on their sides in an appropriate tray, covered with a plastic bag to maintain humidity and placed away from excessive light overnight. Subsequently,

the transformed plants were watered and grown under optimal growth conditions for about 3 weeks until they set seeds. Plants were allowed to dry and T0 seeds were harvested (Figure 5-3).

### 5.2.8. Screening transformed plants

#### 5.2.8.a) Screening on MS agar medium for resistance to BASTA

Approximately 400-500 mg of T0 seeds were surface sterilized, along with positive control seeds (BASTA resistant), and negative control seeds (WT) in sterile 50 ml Falcon tubes using the following protocol: 5 min in 70 % ethanol followed by 5 min in sterilizing solution (2.1 % sodium hypochlorite, 0.05 % Tween-20) and then rinsed (3-5 times) with sterile distilled water for 5 min each. Remaining water was removed carefully and 25 ml of sterile 0.8 % agarose at 45° C was added and mixed with the seeds. Immediately, using a sterile pipette, 5 ml of the seed/ agarose suspension was spread onto the surface of ½ MS 1 % agar in each Petri dish, supplemented with BASTA (Glufosinate ammonium, Sigma Aldrich) at 5 µg/ ml for selection and segregation analysis. Dishes were tipped gently to spread agarose evenly across the surface of the medium, and they were then left in a laminar flow hood for ~20 min to solidify the 0.8 % agarose. Dishes with the seeds were then sealed carefully with micro-pore tape and stratified in the dark at 4° C for 48 h to promote uniform germination, and finally transferred to a controlled growth chamber with LD conditions (Section 2.1). Dishes were checked periodically, and each BASTA resistant seedling (T1 transformant) with 4- 6 leaves was transferred to a single pot filled with autoclaved soil, covered to control humidity until it adapted to the new conditions, and placed in controlled conditions to continue growth.

#### 5.2.8.b) Screening on soil for resistance to BASTA

BASTA resistant seeds were also screened on soil which allows screening of larger numbers of seeds compared with MS screening. For an even distribution of Arabidopsis seeds, seeds were mixed with fine sand at a ratio of 0.5:10 gm and sprinkled using a sieve onto the surface of wet sterilized soil (Section 2.1) in planting trays. Trays were covered with transparent plastic lids, placed at 4° C for 72 h for stratification to synchronize

germination, and then moved to an incubator maintained at 22° C under a 16/ 8 h day/ night regime. As the germination of seeds started, lids were removed to prevent excessive humidity.

For selection of Arabidopsis seedlings harbouring the BASTA resistance gene, the commercial herbicide BASTA (13.52 % w/w, Glufosinate-ammonium, Kaspar, Aventis CropScience, UK Limited) was sprayed onto plants in a fume hood at 120 µg/ ml three times, a week between each, started at seedling cotyledon stage. After approximately 3 weeks of growth in the incubator, BASTA resistant seedlings were distinguished from untransformed plants by the presence of true green leaves, while untransformed plants presented bleaching leaves or failed to grow. Each positive T1 plant was transplanted to a single pot, picked out using a spatula with some soil surrounding the roots to avoid any damage to the root system of the seedling.

Positive T1 Arabidopsis seedlings selected by either screening method were allowed to grow to maturity and produce seeds of the T2 generation. For PCR verification of insertions, a leaf of each positive plant was collected in an Eppendorf tube, snap frozen in liquid nitrogen and stored at -80° C until further use.

### 5.2.9. Study of the segregation ratios (Mendelian ratio) in successive generations

The segregation of T2 transgenic Arabidopsis progenies was tested by sowing ~100 seeds of each chosen line on soil and spraying them at cotyledon stage with BASTA as described in Section 5.2.8.b), while T3 segregation analysis was done by sowing ~100 seeds of the studied lines on MS supplied with BASTA as described in Section 5.2.8.a); (Figure 5-3).

### 5.2.10. Molecular analysis of transgenic plants

Genomic PCR analysis of plants that presented resistance to BASTA treatment was performed as described in Section 2.9 as follows: total genomic DNA was isolated from the leaves of resistant plants as described in Section 2.5. The quality of the DNA was checked by amplifying the 18S rRNA using PUV2 and PUV4 primers (Table 2-2). Two

pairs of primers were chosen for PCR analysis to confirm the presence of the inserted gene, CSHL_058443-F or CSHL_017399-F with ABB2 and 35S with ABB2 (Table 2-2). The amplification products were electrophoresed on 1% agarose gels and then visualized as described in Section 2.11.1.

### 5.2.11. Phenotypic characterization of transgenic Arabidopsis plants

#### 5.2.11.a) Measurement of growth parameters

Plants were inspected every day starting from the day of bolting. Plant phenotyping for bolting time, number of leaves at bolting and day of first flower was performed as described in Section 2.4. Productivity rate was evaluated as described in Section 2.4. Mature siliques were harvested 2 weeks after the formation of the first silique to analyse silique length and seed number per silique. For this purpose, 10 siliques from each plant were fixed in ethanol with acetic acid (3:1 v/ v) overnight, then hydrated in 70 % [v/ v] aqueous ethanol. Seed number per silique and silique length were scored under the microscope as described in Section 2.15.

#### 5.2.11.b) Microscopy

Stems of approximately 8 week old plants, ~15 cm in length, were hand sectioned using a razor blade at the base of the inflorescence and resulting cross sections were mounted in a drop of water on glass slides. Observations were carried out with a fluorescence microscope (BH-2 Olympus) under UV excitation, and fluorescent images were captured using a Tucsen Camera (Tucsen imaging technology Co, Ltd. TCA-5.0 C). Measurements of stem sections were carried out using ImageJ software.

## 5.3. Results

### 5.3.1. Plasmid and cloning verification

In order to verify the identity of both clones (CSHL_058443 and CSHL_017399), they were subjected to various analyses prior to transformation into *Agrobacterium tumefaciens*. Following miniprep purification, both purified amiRNA-constructed

plasmids were checked by gel electrophoresis and results showed a band of approximately 5-6 kb as expected (Appendix C). Gel analysis showed a higher concentration of CSHL_017399 plasmid compared to CSHL_058443 plasmid (Figure 5-4.a) as expected from the concentrations measured by spectrophotometry (376.7 ng/ µl and 281.7 ng/ µl respectively).

To identify the insert, 1 µl of each purified plasmid was used as a template for a PCR reaction using Gateway primers which resulted in a strong band from each template (Figure 5-4.b) of the correct size. PCR products were purified, run on an agarose gel to check their integrity (Figure 5-4.c), and sent for DNA sequencing.

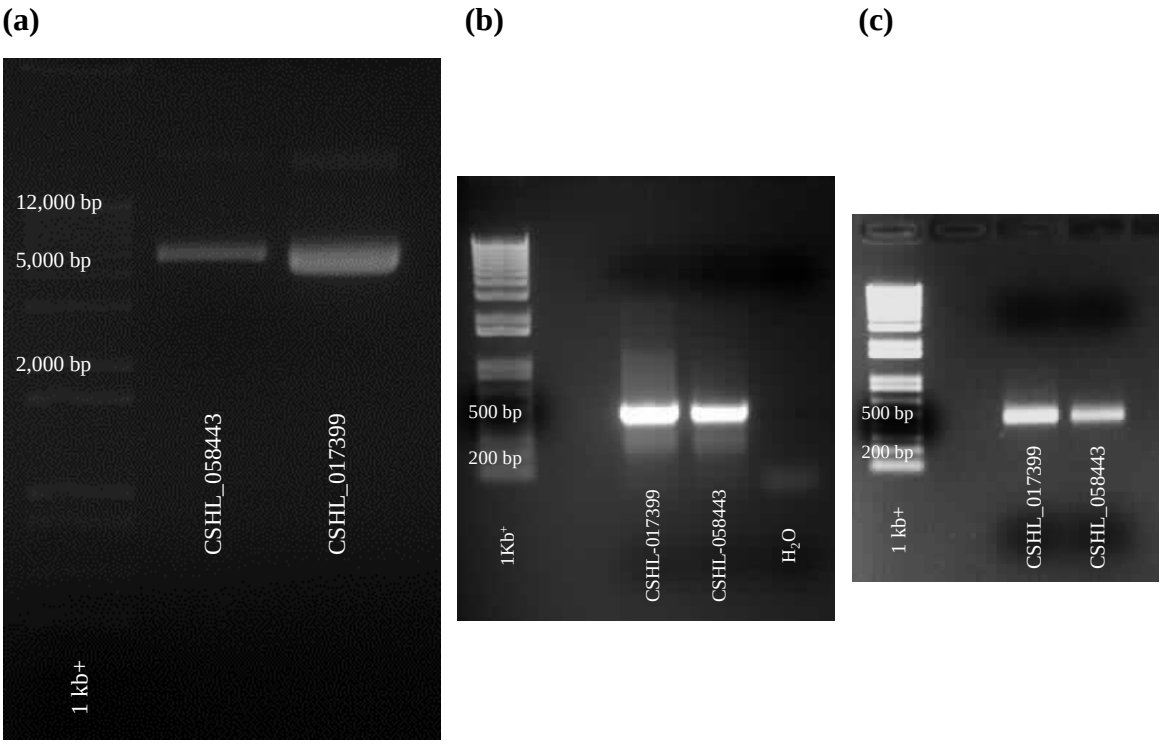
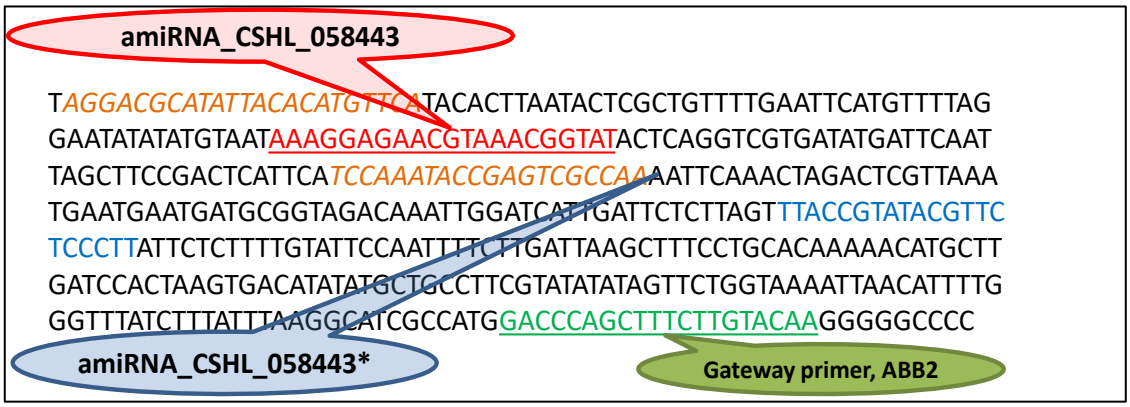



Figure 5-4 Analysis of quality and integrity of extracted CSHL_017399 and CSHL_058443 plasmids and verification of amiRNA insertion. (a) Purified plasmids after isolation from the bacterial host *E coli*. (b) Identification of the amiRNA insertion in both plasmids using Gateway primers. (c) Gel electrophoresis of PCR purified products. Ethidium bromide (EtBr) stained agarose gels were used to separate DNA fragments.

ClustalW analysis of the sequences from the PCR products and amiRNA sequences confirmed the presence of each designed amiRNA sequence in the purified plasmids (Figure 5-5).

**(a)**

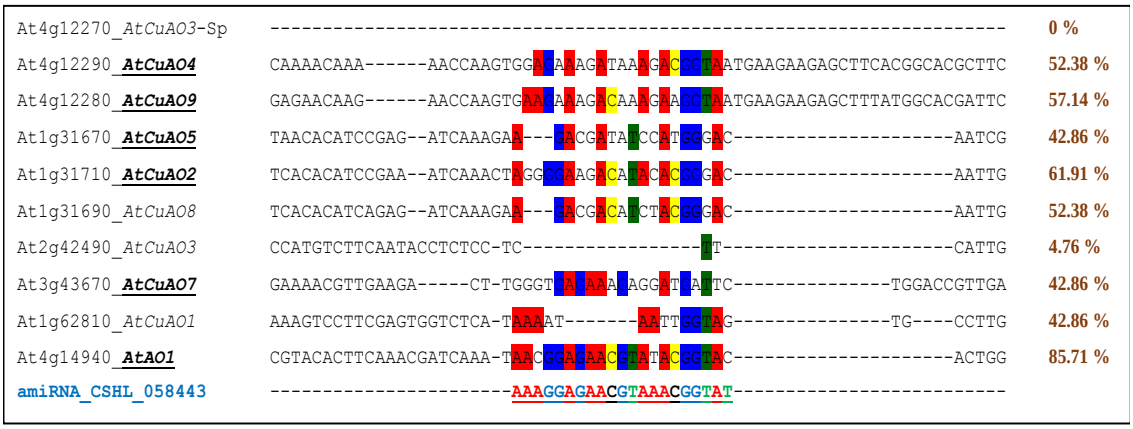



**(b)**


Figure 5-5 Confirmation of the presence and sequence of amiRNAs. Both PCR amplicons were amplified using Gateway primers (Table 2-2), purified, sequenced and finally sequencing results were aligned with known sequences of amiRNAs. (a) CSHL_058443. (b) CSHL_017399. Red colour shows sequence of the amiRNA, green colour shows sequence of Gateway_ABB2 primer, blue colour shows sequence of the amiRNA*, and orange shows primers designed using the sequences.

Alignment between the amiRNA CSHL_058443 and the CDS of the ten members of *AtCuAO* gene family revealed a homology of ~86 % with the gene it was designed from, *AtAO1*, and a homology which ranged between ~5 and ~62 % with the other members of the *AtCuAO* gene family (Figure 5-6.a). The amiRNA CSHL_017399 showed a high homology (between ~67 and ~91 %) with five members of the gene family (*AtCuAO2*, *AtCuAO4*, *AtCuAO5*, *AtCuAO7*, *AtCuA8*) including the gene used to design it, *AtCuAO5*. Homology ranged between ~14 and ~62 % with the other members of *AtCuAO* gene family (Figure 5-6.b). *AtCuAO3-SP* did not match either of the two amiRNA sequences.

**(a)**




**(b)**

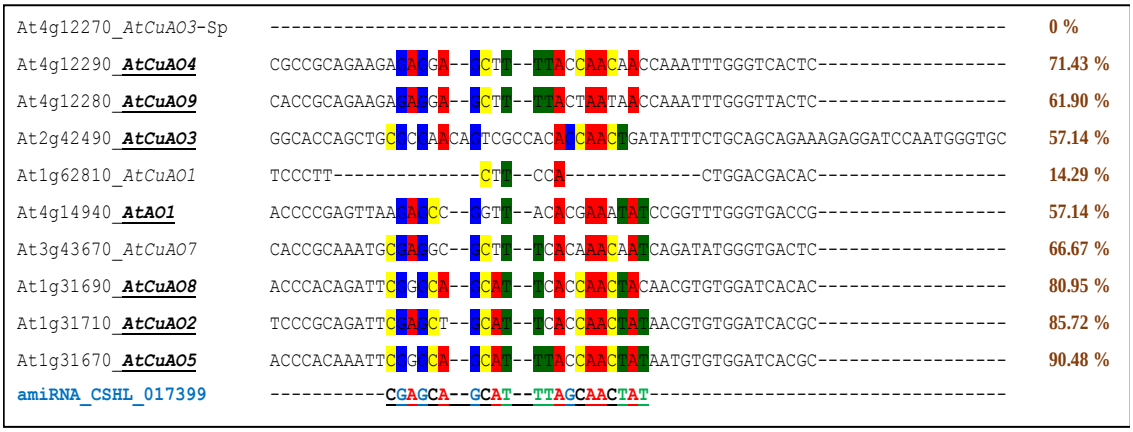



Figure 5-6 ClustalW alignment of *AtCuAO* CDS sequences with amiRNA sequences. Genes in bold are targets of the amiRNA used in the alignment as indicated in Section 5.2.1. Nucleotides on *AtCuAO* genes homologous to those of amiRNA are highlighted. Right panels show the percentage of homology between each gene and aligned amiRNA.

### 5.3.2. Confirmation of the transformation into *Agrobacterium tumefaciens*

Initially, the efficiency of competent Agrobacterium cells was confirmed by transforming a positive control plasmid pTA 7003. After transformation of each amiRNA clone into *Agrobacterium tumefaciens*, successful transformants were selected first through the use of appropriate antibiotics on Petri dishes, and then through PCR on 8- 10 colonies to confirm the presence of the insertion (Figure 5-7). A collection of 16 randomly selected colonies (8 each from either CSHL_058443 or CSHL_017399) were tested by PCR using GateWay primers (Table 2-2). PCR results showed that two of eight colonies of the

amiRNA CSHL_058443 and four of eight colonies of the amiRNA CSHL_017399 were positive and amplified a fragment of 500 bp (Figure 5-7).

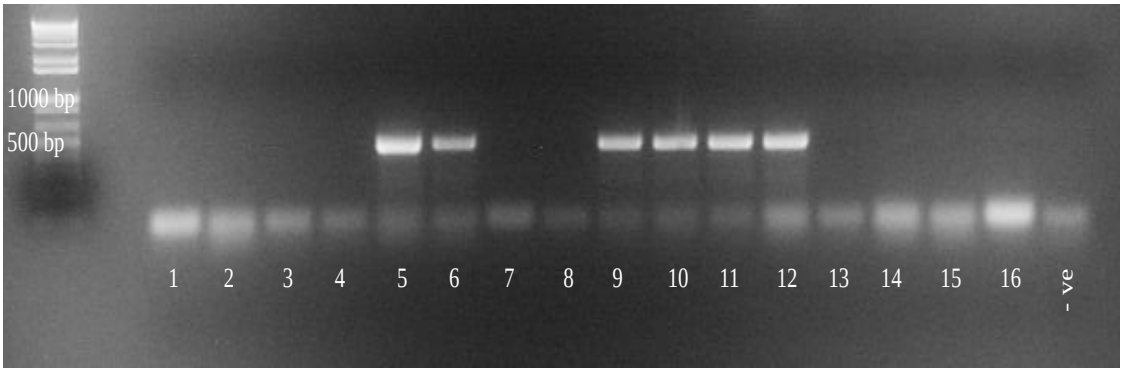



Figure 5-7 Screening colonies using Gateway specific primers to select positive colonies from the transformation of the plasmids into *Agrobacterium tumefaciens*. Numbers 1-8 refer to colonies from the amiRNA CSHL_058443, while numbers 9-16 refer to amiRNA CSHL_017399 transformations. An EtBr stained agarose gel was used to separate PCR products.

### 5.3.3. Strategy for screening transformed Arabidopsis lines

In order to choose PCR primer sets appropriate for detection and amplification of the inserted amiRNAs in CSHL_058443 and CSHL_017399 constructs, five primer combinations were used (Figure 5-8.a). First, since the vector contains the Gateway® recombination sites, the universal primers ABB1 and ABB2 (Gateway F and R) were used to amplify a fragment of 500 bp that flanked the insertions. As the expression of the construct is driven by the cauliflower mosaic virus 35S promoter, another set was chosen including 35S and the ABB2 (Gateway R) primers which amplifies a fragment of 600 bp. A set of primers, spanning the amiRNA-guided cleavage site, was also designed based on sequencing results (Figure 5-5) to amplify a fragment of 158 bp. The fourth combination used the amiRNA sequence as a forward primer in combination with ABB2 to amplify a fragment of 350 bp. Finally, because the used vector contains the bar gene that confers BASTA resistance for selection of transformants carrying the vector, amiRNA sequences were used also with 229Bar gene to amplify a fragment of 1,000 bp. All primer combinations produced the expected amplicon sizes with both amiRNAs, CSHL_058443 and CSHL_017399 (Figure 5-8.b), clearly confirming that both plasmids harboured the inserted amiRNA constructs.

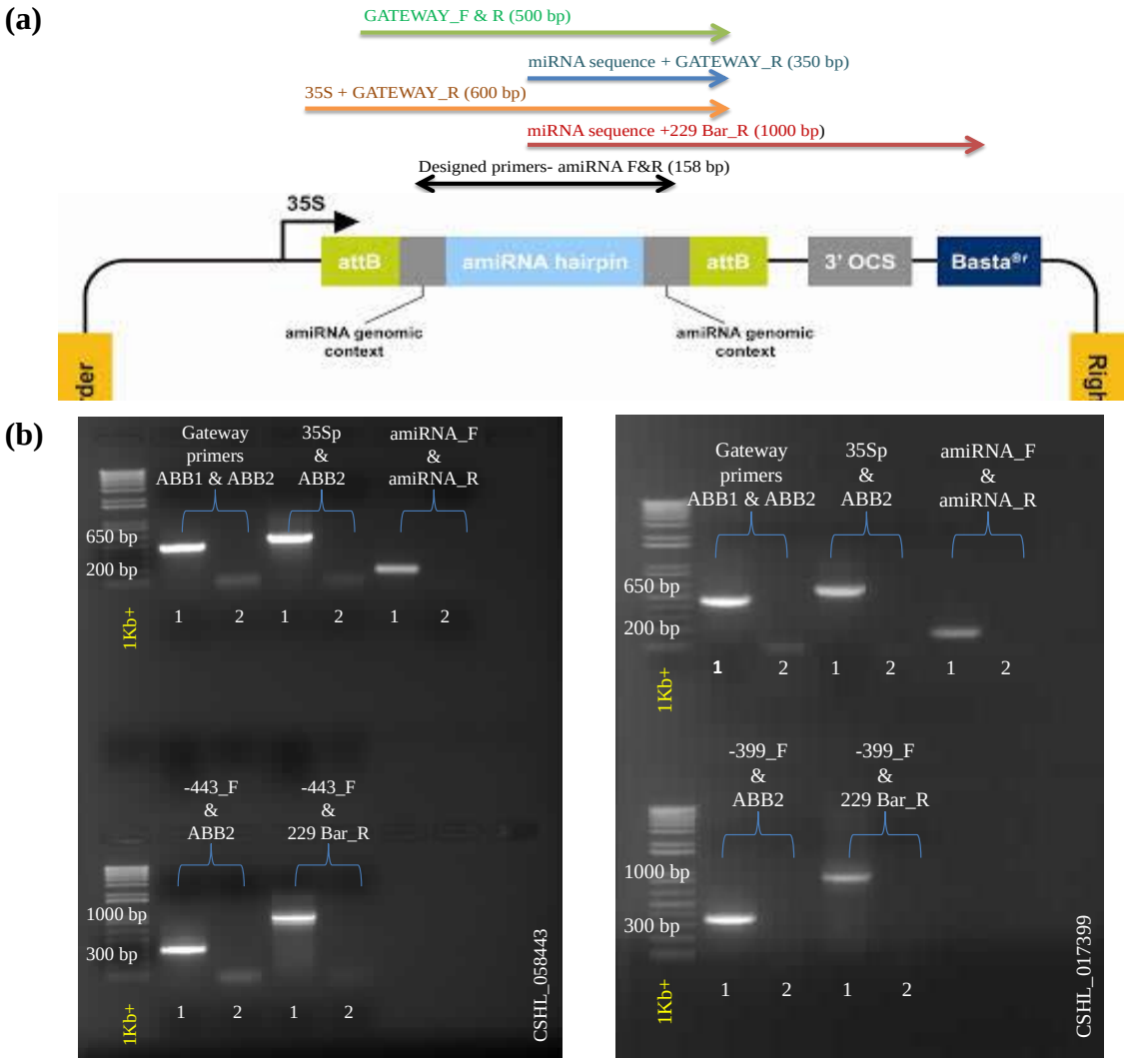



Figure 5-8 PCR analysis of amiRNAs. (a) Scheme of the five primer combinations chosen for verification of the amiRNA insertions. (b) PCR products using different sets of primers confirmed the presence of amiRNA in both plasmids, CSHL_058443 on the left and CSHL_017399 on the right. 1 amiRNA plasmid used as a template and 2 $H_2O$ as a negative control.

Three primer combinations were chosen for insertion verification of the Arabidopsis transgenic plants. These combinations of primers were first tested on the genomic DNA of WT plants using plasmids (CSHL_058443 or CSHL_017399) as positive controls. The first pair were amiRNA_F and amiRNA_R which give a PCR product of 158 bp. The second set included the amiRNA sequence as a forward primer (referred to -399_F or -443_F) with the Gateway reverse primer (ABB2) that amplify a fragment of 350 bp. The third set comprised 35S, as a forward primer, and ABB2 as reverse primer which amplify a fragment of 600 bp. Results showed that primers designed on the PCR product were able to amplify a fragment from the WT gDNA represented in a 158 bp PCR product

similar to the one using the positive control. None of the other two primer combinations (amiRNA sequence with ABB2 or 35S with ABB2) produced any PCR products when wild type gDNA was used as a template (Figure 5-9).

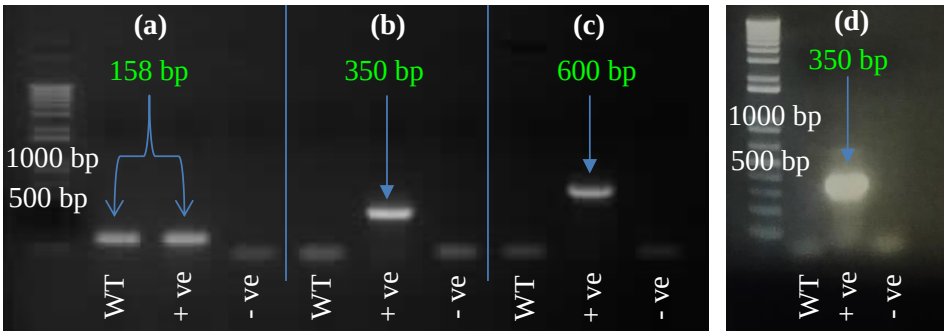



Figure 5-9 PCR results using WT genomic DNA as template with three different combinations of primers. (a) amiRNA_F and amiRNA_R primers (targeting both amiRNAs). (b) amiRNA sequence as a forward primer with ABB2 as a reverse (specific for the amiRNA CSHL_017399). (c) 35S with ABB2 (spanning both amiRNAs). (d) amiRNA sequence as a forward primer with ABB2 as a reverse (specific for the amiRNA CSHL_058443). CSHL_017399 plasmid was used as a positive control (+ve) in (a), (b) and (c), CSHL_058443 plasmid was used as a positive control (+ve) in (d), while $H_2O$ was used as a negative control (-ve). An EtBr stained agarose gel (1 %) was used to separate the PCR products.

### 5.3.4. Transformation into *Arabidopsis thaliana* plants

Flowering Arabidopsis plants were dipped in the *Agrobacterium tumefaciens* suspensions carrying either amiRNA CSHL_058443 or CSHL_017399. The resulting T0 seeds were screened with the herbicide BASTA (Figure 5-10). At first, screening was performed on MS medium supplied with BASTA at 5 µg/ ml which produced seven positive T1 plants that should carry the amiRNA CSHL_017399.

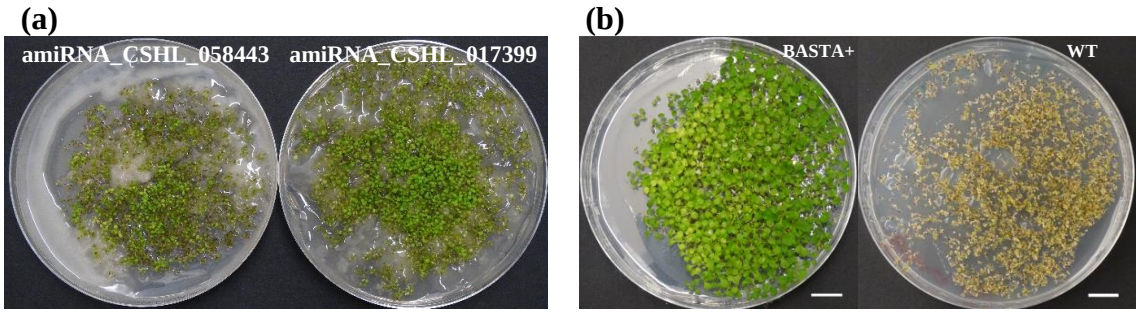



Figure 5-10 Screening of T0 seeds of *Arabidopsis thaliana* (Col-0) transformed with amiRNA on MS medium containing 5 µg/ ml BASTA. (a) 24-day old T1 BASTA-resistant seedlings obtained from plants subjected to floral dip transformation using *Agrobacterium tumefaciens* strain GV3101 harbouring the binary plasmid pAmiR. (b) As controls, both BASTA-resistant and wild type plants were grown alongside on selection media under identical conditions. Size of bars = 1 cm.

To check that the selected plants were transgenic, genomic DNA was isolated from all surviving plants which were resistant to BASTA, and screened for the transgene CSHL_017399 by PCR using two sets of primers (35S with ABB2 and -399_F with ABB2, Table 2-2). Results revealed that only three of the seven positive plants (plants 2, 4 and 5) were harbouring the transgene CSHL_017399 in their genome (Figure 5-11). Plants selected using BASTA which did not carry the transgene may have arisen from WT escaping through the BASTA screen or rearrangement of the construct.

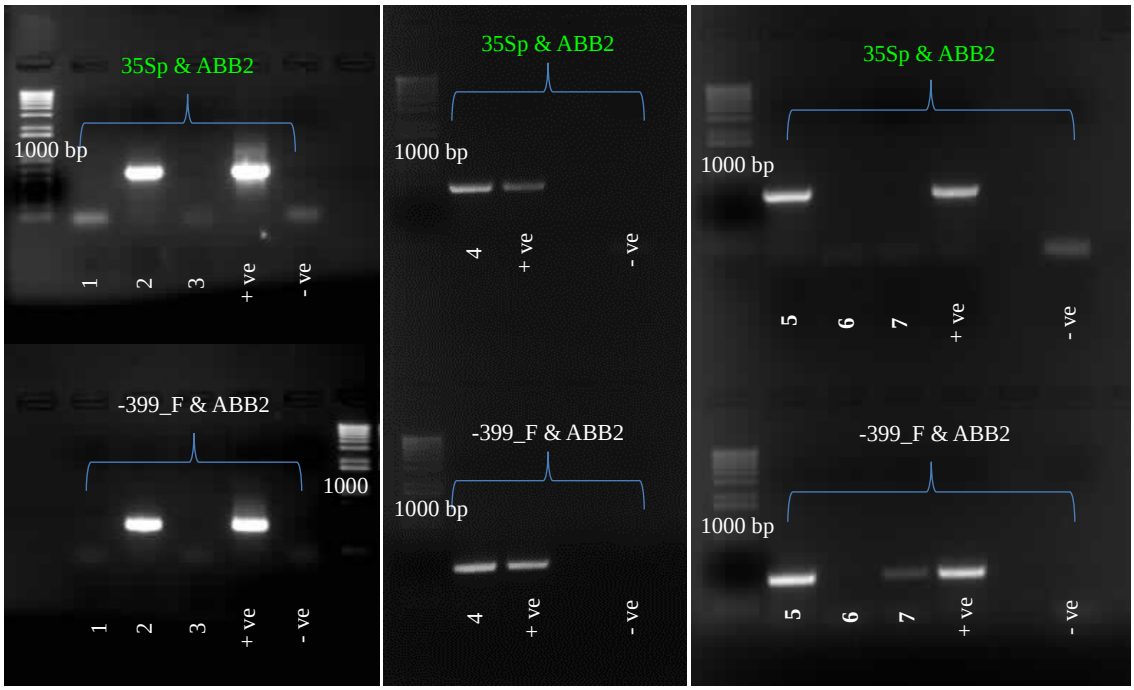



Figure 5-11 Identification of three positive plants carrying the amiRNA CSHL_017399. Verification of the seven positive plants produced from screening on MS medium supplied with the herbicide BASTA (5 µg/ml) through PCR with two primer combinations (indicated on gel images). Numbers refer to plants resistant to the herbicide BASTA. CSHL_017399 plasmid was used as a positive control (+ ve) and $H_2O$ as a negative control (- ve).

As shown in (Figure 5-10.a), screening on MS agar medium produced high level of contamination likely to be *Agrobacterium tumefaciens*. In addition, screening on MS produced only three lines of positive transformed plants carrying the amiRNA CSHL_017399 (Figure 5-11) while no plants carrying the amiRNA CSHL_058443 were identified. Therefore, an alternative method for screening was used (screening of transformed seeds on soil). Before screening T0 seeds on soil, the efficiency of 120 µg/ml BASTA was tested on wild type and BASTA-resistant plants (Figure 5-12). Results

showed that BASTA resistant plants germinated and grew normally, whereas the growth of the wild type was repressed as a result of treatment with BASTA. This confirmed the effectiveness of both the concentration of BASTA used and the treatment of growing plants in soil by spraying.

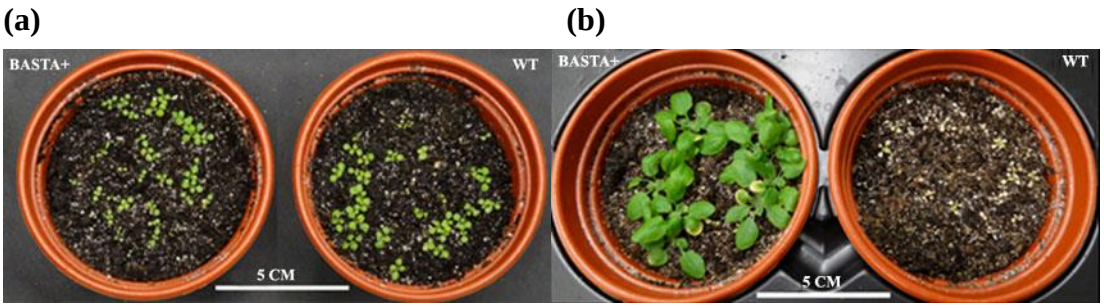



Figure 5-12 Testing the efficiency of the commercial BASTA at 120 µg/ ml on BASTA-tolerant and WT plants grown in soil. (a) Two week old plants before treatment. (b) Four week old plants post treatment with BASTA. BASTA tolerant plants on the left and WT on the right.

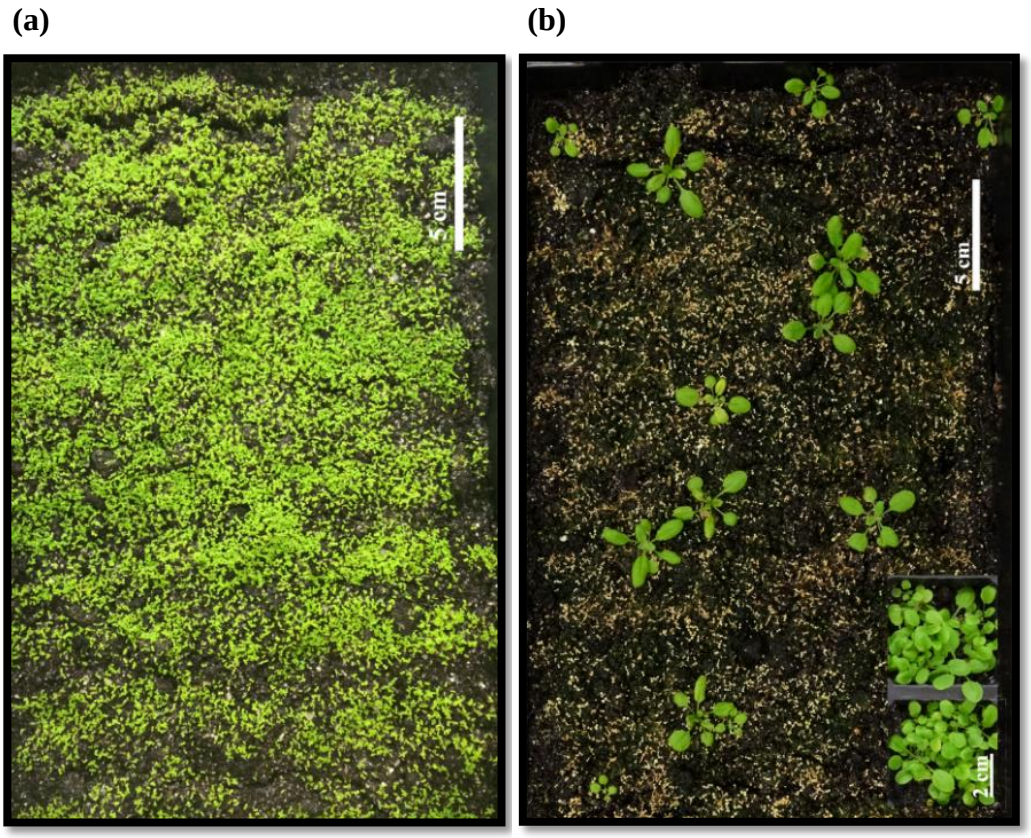



Figure 5-13 Screening of T0 transformed seeds on soil using BASTA at 120 µg/ ml. (a) Seedlings at cotyledon stage before spraying with BASTA solution. (b) Four week old plants after the third spray. The insert in (b) shows the positive control BASTA resistant plants.

Screening of T0 seeds on soil resulted in the selection of 25 individuals of the amiRNA CSHL_017399 and 35 plants of the amiRNA CSHL_058443, all of which were resistant to BASTA treatment (Figure 5-13). To confirm the presence of the amiRNA insertion within the genome of resulting T1 plants, genomic DNA was isolated from all of them and the two combinations of PCR primers were used as described previously in Section 2.5 and Section 2.9. PCR results revealed that 23 lines of the amiRNA CSHL_017399 and 34 of the amiRNA CSHL_058443 contained the constructs (Figure 5-14 and Figure 5-15).

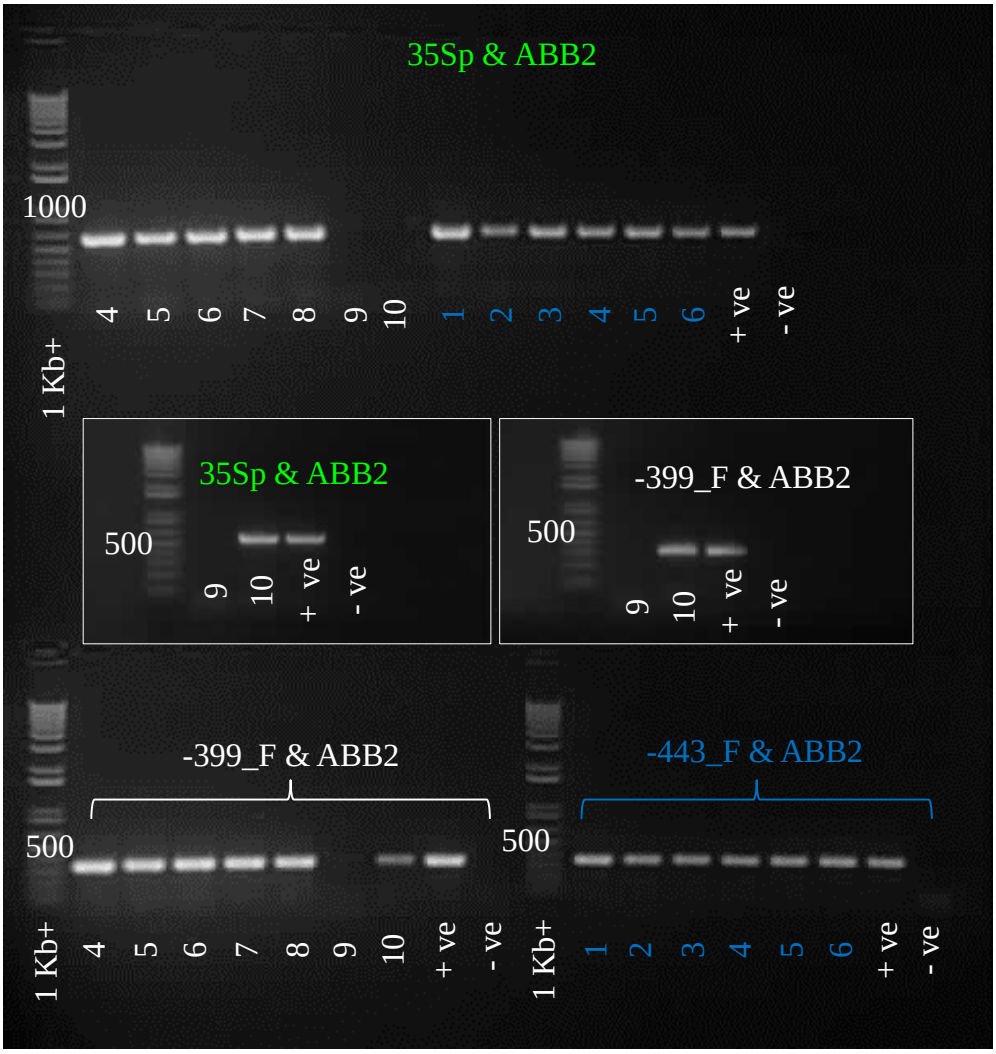



Figure 5-14 Detection of positive plants produced from screening with BASTA at 120 µg/ ml on soil. Surviving plants were screened with two primer combinations (indicated on gel results). Numbers refer to plants resistant to the herbicide BASTA, numbers 4-10 in white refer to T1 plants screened for the amiRNA CSHL_017399, while numbers 1-6 in blue refer to T1 plants screened for the amiRNA CSHL_058443. The plasmids CSHL_017399 and CSHL_058443 were used as positive controls (+ ve) with 4-10 and 1-6 respectively. Negative control (- ve) was $H_2O$. Inserts show repeated PCR with plants number 9 and 10 of the amiRNA CSHL_017399.

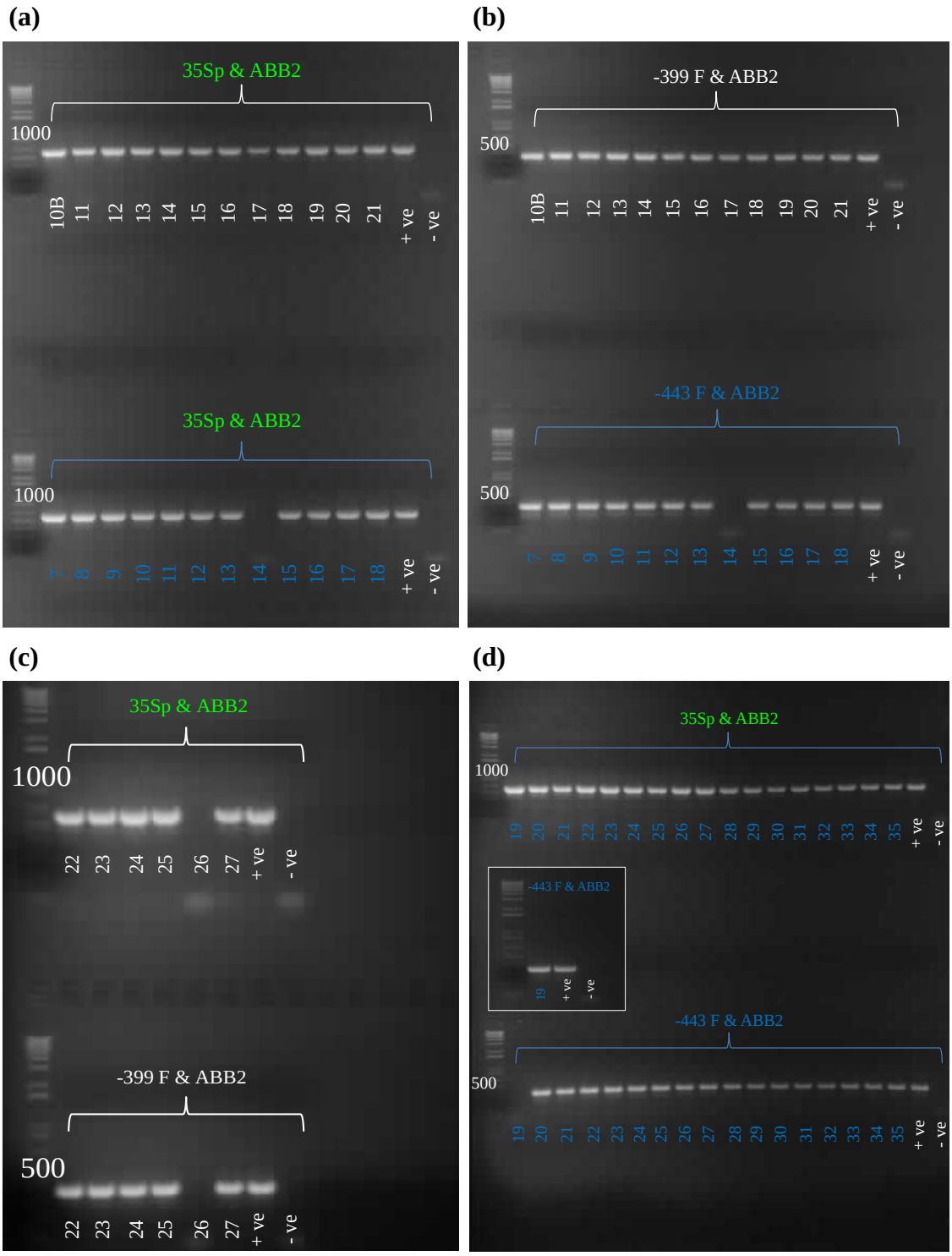
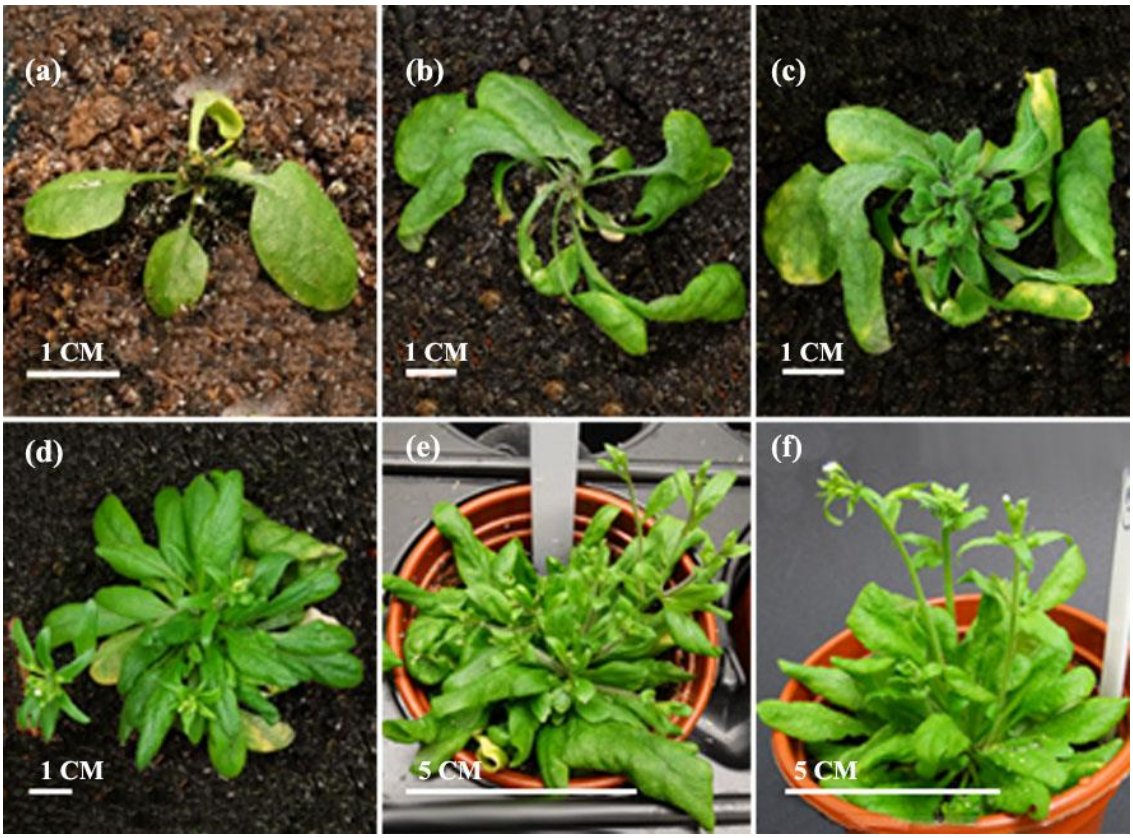



Figure 5-15 PCR screening of positive plants treated with BASTA at 120 µg/ ml, using two primer combinations. Used primers are indicated on gel images. Numbers 10- 27 in white refer to T1 screened plants carrying the amiRNA CSHL_017399. Numbers 7- 35 in blue refer to T1 screened plants carrying the amiRNA CSHL_058443. The plasmids CSHL_017399 and CSHL_058443 were used as positive controls (+ ve) with 10- 27 and 7- 35 respectively. The insert in (d) shows repeated PCR of the positive plant number 19 from the CSHL_058443 transformation.

### 5.3.5. Phenotyping Arabidopsis transformed plants

#### 5.3.5.a) T1 generation phenotype

Twenty eight of the amiRNA CSHL_058443 and 20 of the amiRNA CSHL_017399 transformed T1 transgenic plants which were verified as positive by PCR were monitored for the presence of visible abnormal phenotypes compared with WT plants. Phenotyping T1 transformed plants from both constructs showed a wide range of phenotypes with both constructs showing the full range of phenotypes in various combinations across the different lines. These included misshapen rosette leaves mixed with normal ones, floppy stems, long or short siliques with more or fewer seeds respectively, more or fewer siliques (some plants produced mostly sterile siliques), early or late development (based on time of bolting), absence of the primary flowering stalk and presence of multiple stalks, and formation of a wide flat stem with enlarged inflorescence meristems (Figure 5-16). The phenotype of each selected line is detailed in Table 5-1 and Table 5-2.



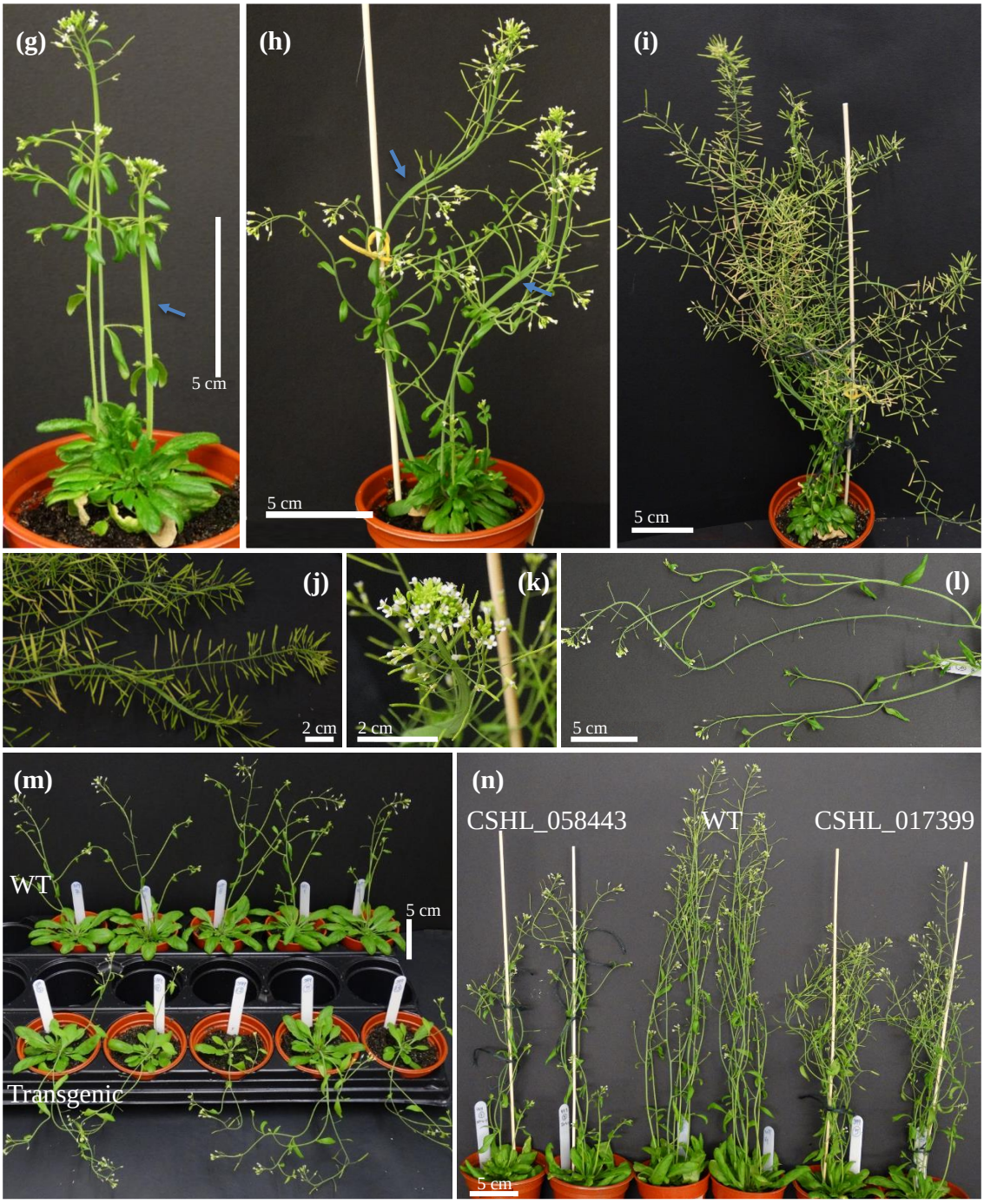



Figure 5-16 The morphological phenotypes of T1 transgenic plants. (a and b) At four and five weeks respectively transgenic plants showed irregular growth of plant leaves. (c) The early arrested period in leaf development was followed by the restoration of leaf production in 6 week old plants. (d, e and f) Seven week old plants with multiple stalks and no clear primary stalk amongst them. (g and h) At four and five weeks respectively transgenic plants showed flattened inflorescence stalks. (i and j) Morphology of plants with more frequent siliques. (k) Top view of a developing inflorescence from a plant that produces more siliques. (l) Branches of sterile plants. (m) Transformed plants with floppy stems compared to WT. (n) A comparison between WT and transformant plants showing reduced height. Scale of images is shown.

Table 5-1 Phenotypic alterations in development of selected T1 *Arabidopsis thaliana* lines carrying the amiRNA CSHL_058443.

| Lines of the amiRNA CSHL_ 058443 | floppy stem | long siliques | short siliques | more frequent siliques | Late development | multi stalks (no primary stalk) | flat wide stem | too many leaves | mis-shapen rosette leaves | notes |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | | √ | | √ | | | | | | |
| 8 | | √ | | | | √ | | | | |
| 9 | | | | | | | | | | |
| 10 | | | | | | | | | | |
| 11 | | | | | | | | | | |
| 12 | | | | | | | | | √ | was not able to set seeds |
| 13 | | √ | | | | | | | | |
| 15 | | | | | | | | √ | | |
| 16 | | | | | | | | | | |
| 17 | | | | | √ | √ | | √ | √ | |
| 18 | | | | | | | | | | |
| 19 | | | | | | | | | | |
| 20 | | √ | √ | | | | √ | | | |
| 21 | | | | √ | √ | | √ | | | |
| 22 | | | | √ | | | √ | | | |
| 23 | | | | | √ | | | | | died before setting seeds at 8w |
| 24 | | √ | | | √ | | | | | |
| 25 | | | | | | | | | | |
| 26 | | | | | | | | | | normal fat siliques |
| 27 | | √ | | | | | | | | |
| 28 | √ | √ | √ | | | | | | | |
| 29 | | √ | √ | | | | | | | |
| 30 | | | | √ | √ | | √ | | | |
| 31 | | √ | √ | | √ | | √ | | | |
| 32 | | √ | | | | | | | | |
| 33 | | √ | | √ | | | | | | |
| 34 | | √ (fat) | | | √ | | | | | |
| 35 | | | | | √ | | | | | |

Table 5-2 Phenotypic alterations in development of selected T1 *Arabidopsis thaliana* lines carrying the amiRNA CSHL_017399.

| Lines of the amiRNA CSHL_017399 | floppy stem | long siliques | short siliques | more frequent siliques | late development | multi stalks (no primary stalk) | flat wide stem | too many leaves | mis-shapen rosette leaves | notes |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | √ | √ | | | | | | √ | | |
| 2 | | | √ | √ | √ | √ | √ | √ | | |
| 3 | √ | √ | | | | | | | | |
| 10B | | | √ | | | | | √ | √ | |
| 11 | | | √ | | | √ | | | | |
| 12 | | | | | | | | | | |
| 13 | √ | | | | | | | | | |
| 14 | | | √ (fat) | | | √ | | | | |
| 15 | | | | | | √ | | | √ | |
| 16 | | | | | | √ | | | √ | normal fat silique |
| 17 | | | √ | | | √ | | | | |
| 18 | | √ | √ | | | | | √ | √ | |
| 19 | | | √ | | | | | | | |
| 20 | √ | | √ | | | √ | | | | |
| 21 | | | √ | | | | | | | |
| 22 | | | √ | | | | | | | |
| 23 | | √ | | | | | | | | |
| 24 | | | | | | | | | √ | |
| 25 | √ | | | | | | | | | |
| 27 | | | √ | | | | | | | |

As shown in Figure 5-17, both amiRNAs affected some important stages of plant growth and development. All lines of the amiRNA CSHL_058443 and most of amiRNA CSHL_017399 lines showed delay in the bolting day, day of first open flower and in production of the first silique. A clear change in plant productivity was represented by the number of siliques produced within one week. Four plants from each amiRNA transformation showed an extraordinary increase in the number of siliques produced, while the majority of lines showed a reduction in this respect (17 of the amiRNA CSHL_058443 and 10 of the amiRNA CSHL_017399). Transformed plants that were late in bolting day, flowering day and thus day of producing the first siliques also showed delayed senescence although this phenotype was not scored.

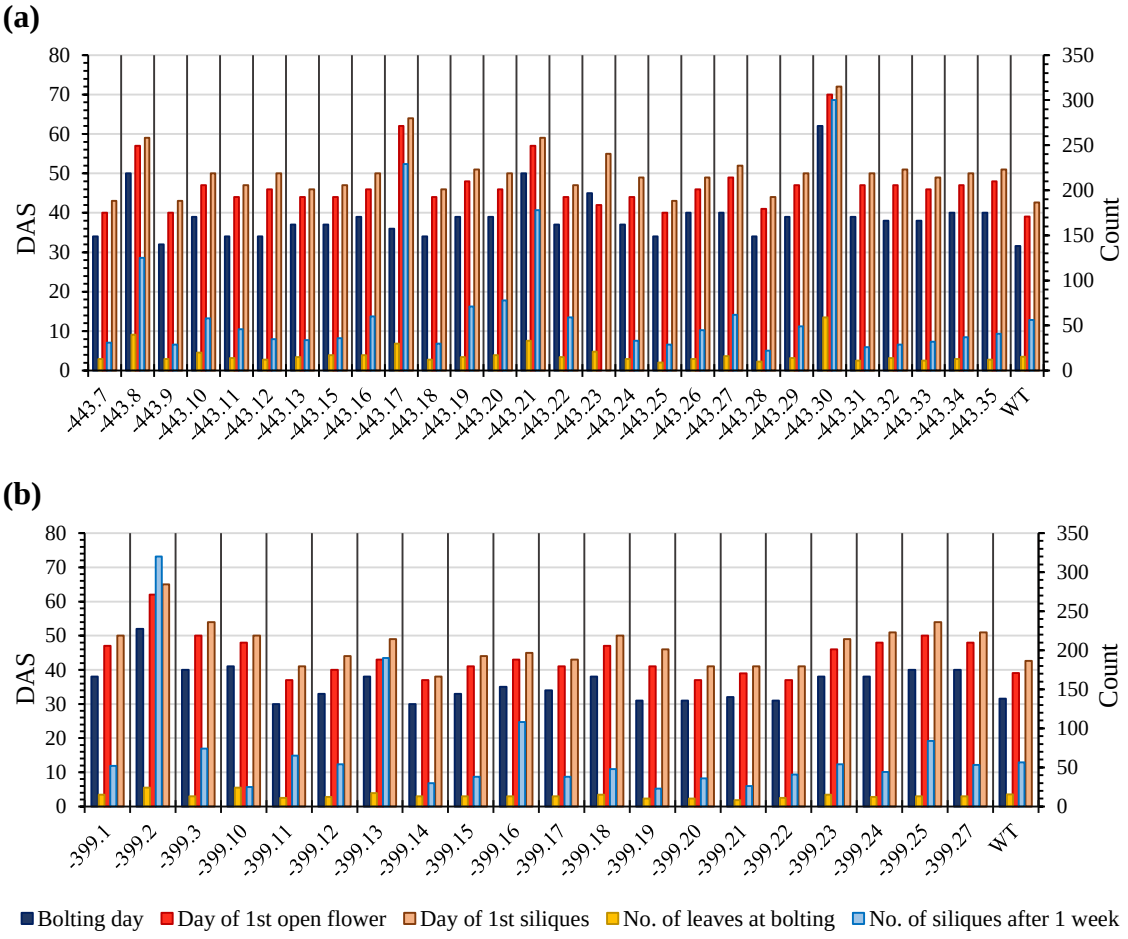



Figure 5-17 Morphological and developmental alteration in T1 transgenic plants. (a) Phenotype of T1 plants (single lines) transformed with the amiRNA CSHL_058443 compared with WT. (b) Phenotype of T1 plants (single lines) transformed with the amiRNA CSHL_017399 compared with WT. Number of siliques was scored on the primary inflorescence, secondary inflorescence branches, and inflorescence branches after one week following formation of the first silique. DAS; days after sowing. Since there were no replicates at this stage (T1 generation), no error bars are shown.

Silique length and number of seeds per silique in 17 randomly selected lines of both amiRNA transformants were strongly affected in different ways and to different extents (Figure 5-18). Seven of the nine (78 %) CSHL_058443 lines (line number 13, 15, 24, 29, 32, 33, and 34) had longer siliques, six of them with an increased number of seeds although one plant (no. 24) had fewer seeds despite the longer siliques, one plant (no. 26) had normal silique length with increased number of seeds, and one line (no. 27) showed no differences to WT. CSHL_017399 lines showed similar silique phenotypes but in different proportions: five out of eight plants (63 %) had short siliques two of them (no. 10 and 20) were with fewer seeds, one had long siliques with more seeds (no. 3), one had normal siliques with more seeds (no. 18) and one was not significantly different to WT (no. 23).

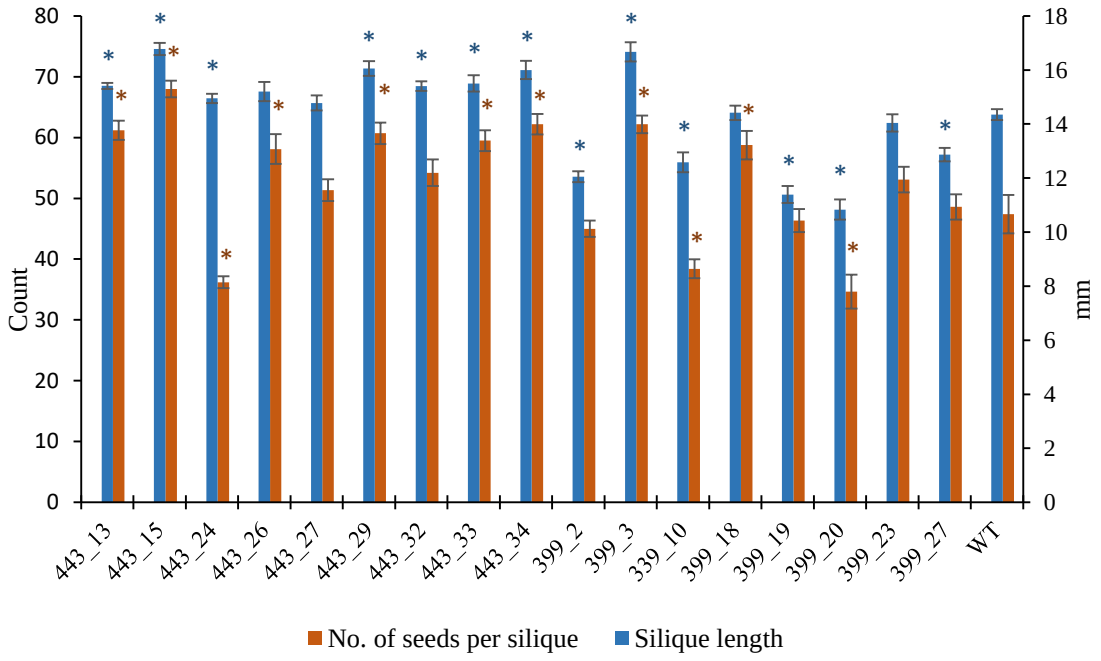



Figure 5-18 Silique length and number of seeds per silique of randomly selected plants from the T1 generation of both amiRNAs. (n = 10; means ± SE; asterisks indicate significant differences to WT ($P \leq 0.05$) based on T-test when data was normally distributed or Mann-Whitney test when data was not normal).

When dissecting T1 transgenic shoots that showed flat wide stems either carrying the CSHL_058443 or CSHL_017399 transgene, unusual types of shoot development were noted. Hand sectioning showed that these shoots had more vascular bundles, but tissue organization appeared normal (Figure 5-19).

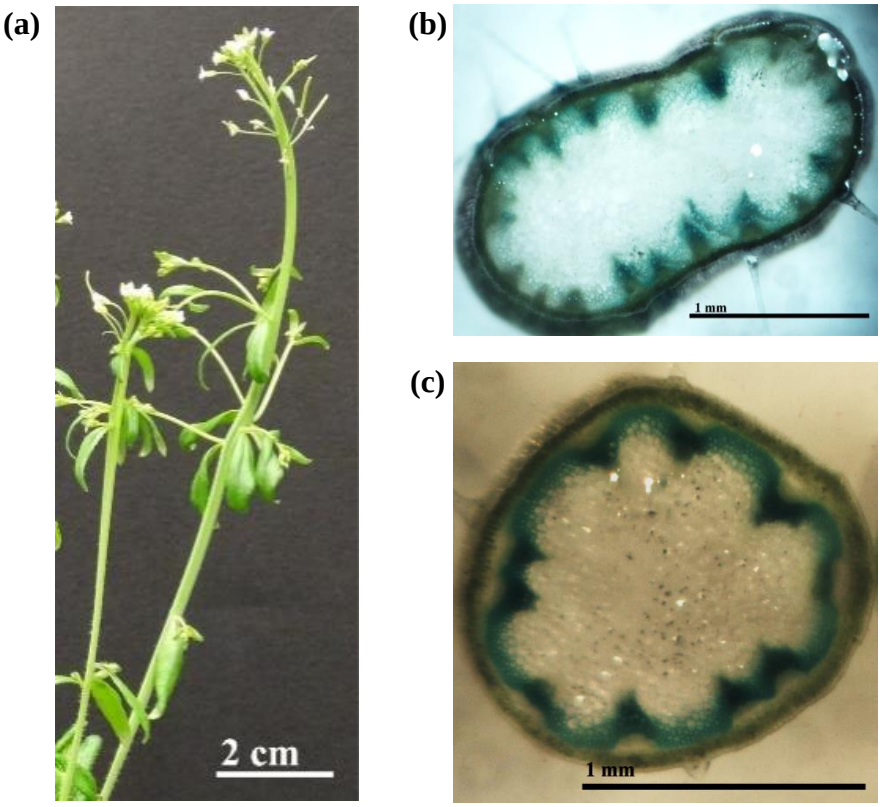



Figure 5-19 Abnormal stem phenotype observed in T1 transformed plants. (a) Flattened inflorescences observed in some individuals of transgenic plants. (b) Transverse sectioning in abnormal flattened stem. (c) Transverse sectioning in Wild type stem. Size of scale bars are shown on images. In (b) and (c) hand-cut sections in the middle of the stem were observed with a fluorescence microscope under UV excitation.

### 5.3.5.b) T2 generation phenotype

Three T1 lines of the amiRNA CSHL_058443 (line 2, 4 and 6), and another three of the amiRNA CSHL_017399 (line 1, 2 and 3) were chosen for the study of the phenotype in the T2 generation. T1 seeds were initially screened to verify segregation ratios. Following BASTA treatment, the number of surviving seedlings was counted and statistically analyzed for a 3:1 alive: dead ratio using a Chi-square ($\chi 2$) test. The null hypothesis was that the observed alive: dead ratio would not deviate from the expected Mendelian ratio of 3: 1 (alive: dead, when selected with BASTA), predicted using the number of seedlings alive before BASTA treatment. The Chi-square value was not significant ($P > 0.05$) in one line of the amiRNA CSHL_058443 (line 2), and two lines of the amiRNA CSHL_017399 (lines 1 and 2) indicating that these lines were in fact hemizygous and probably carrying a single transgene insertion (Table 5-3). However, the Chi-square value

was significant ($P < 0.05$) in the remaining lines. Ten T2 individuals of each of the three selected T1 lines from each construct were transferred to single pots to phenotype them.

Table 5-3 T2 Transformants segregated in a 3:1 alive: dead ratio following selection with BASTA at 120 µg/ ml. Seeds of each T1 line were sown on soil and sprayed with BASTA as described in Section 5.2.8.b). The numbers of seeds were counted at sowing and number of surviving seedlings were counted after spraying. A $\chi^2$ statistical test was used to check whether these lines followed the expected 3:1 ratio for a stably-integrated single insert.

| Construct | Line number | Expected | | Observed | | $\chi^2$ p value | T1 phenotype |
|---|---|---|---|---|---|---|---|
| | | Alive | Dead | Alive | Dead | | |
| amiRNA CSHL_058 443 | Line2 | 90 | 30 | 90 | 30 | 1.000 | NT* |
| | Line4 | 90 | 30 | 77 | 43 | 0.006 | NT* |
| | Line6 | 90 | 30 | 79 | 41 | 0.020 | NT* |
| amiRNA CSHL_017 399 | Line1 | 90 | 30 | 84 | 36 | 0.206 | See Table 5-2 |
| | Line2 | 90 | 30 | 88 | 32 | 0.673 | See Table 5-2 |
| | Line3 | 90 | 30 | 105 | 15 | 0.002 | See Table 5-2 |

* NT; not tested

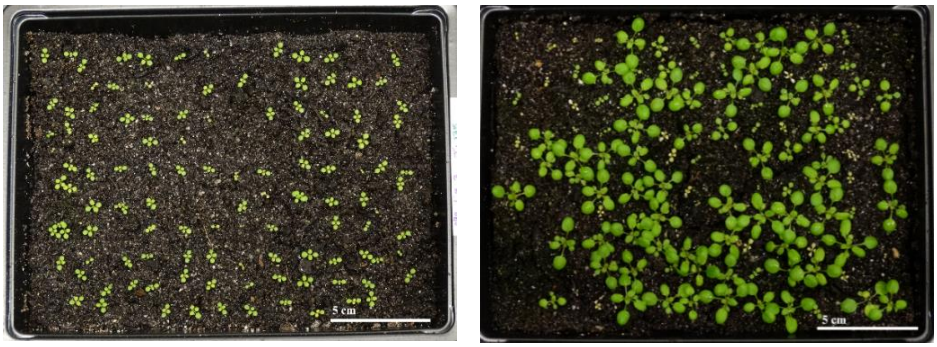



Figure 5-20 Transgene segregation in T2 generation of line 3 of the amiRNA CSHL_017399 as a representative. Left tray shows one week old seedlings. Tray on the right shows surviving plants following the first spray with BASTA at 120 µg/ ml.

The T2 generation plants from the three T1 lines of each construct showed a range of phenotypic abnormalities in plant development similar to those observed among transformants of the T1 generation (Figure 5-21). Four of the lines showed delayed production of siliques, accompanied by delayed bolting and flowering in most of them. Number of leaves at bolting was only affected in one line and number of siliques produced over a week was significantly lower in two lines compared to WT.

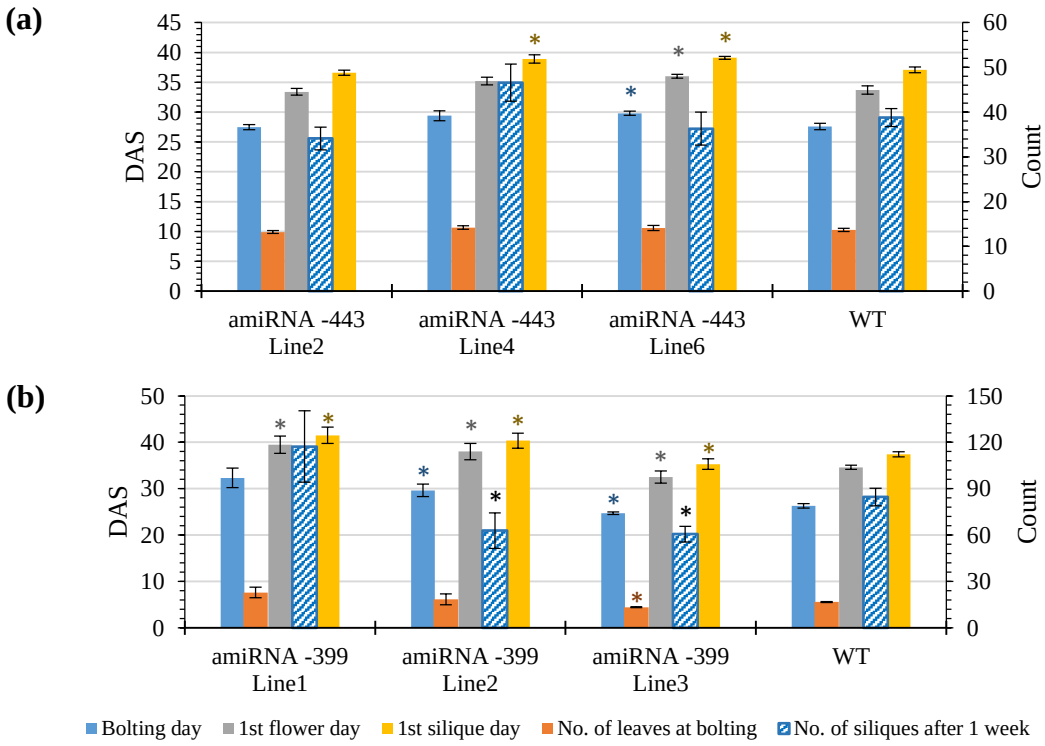



Figure 5-21 The effect of the transgene on the growth and morphology of T2 plants during different stages of development. (a) Transgene CSHL_058443. (b) Transgene CSHL_017399. Number of siliques was scored after one week following formation of the first silique on the primary inflorescence, secondary inflorescence branches, and inflorescence branches. (n = 10; means ± SE; asterisks indicate significant differences to WT ($P \leq 0.05$) based on T-test when data was normally distributed or Mann-Whitney test when data was not normal).

Silique length and number of seeds per siliques in T2 generation plants were also affected by both amiRNAs (Figure 5-22.a). Plants transformed with the amiRNA CSHL_058443 (line 2, 4, and 6) showed a significant increase in silique length and number of seeds per silique in all studied lines, however, investigation of only one line carrying the amiRNA CSHL_017399 (line 3), which showed visible increase in silique length in T1, revealed a significant reduction in silique length but without affecting the number of seeds produced per silique.

One line from each amiRNA was selected for more detailed investigation on the effect of the transgene on silique phenotype (Figure 5-22.b and c). The majority (70 %) of line 2 plants carrying the amiRNA CSHL_058443 showed a significant increase in silique length while only one plant (2.4) showed a significant decrease in this trait. In addition, 57 % of line 2 plants which produced significantly longer siliques also showed an increase

in the number of seeds produced per silique (Figure 5-22.b). Line 3 plants carrying the artificial micro RNA CSHL_017399 showed more variation in these characters. Two plants (20 %) showed longer siliques with increased number of seeds produced compared with WT plants (3.1 and 3.2), while the rest (80 %) produced shorter siliques with no significant change in seed number per silique compared to WT (Figure 5-22.c).

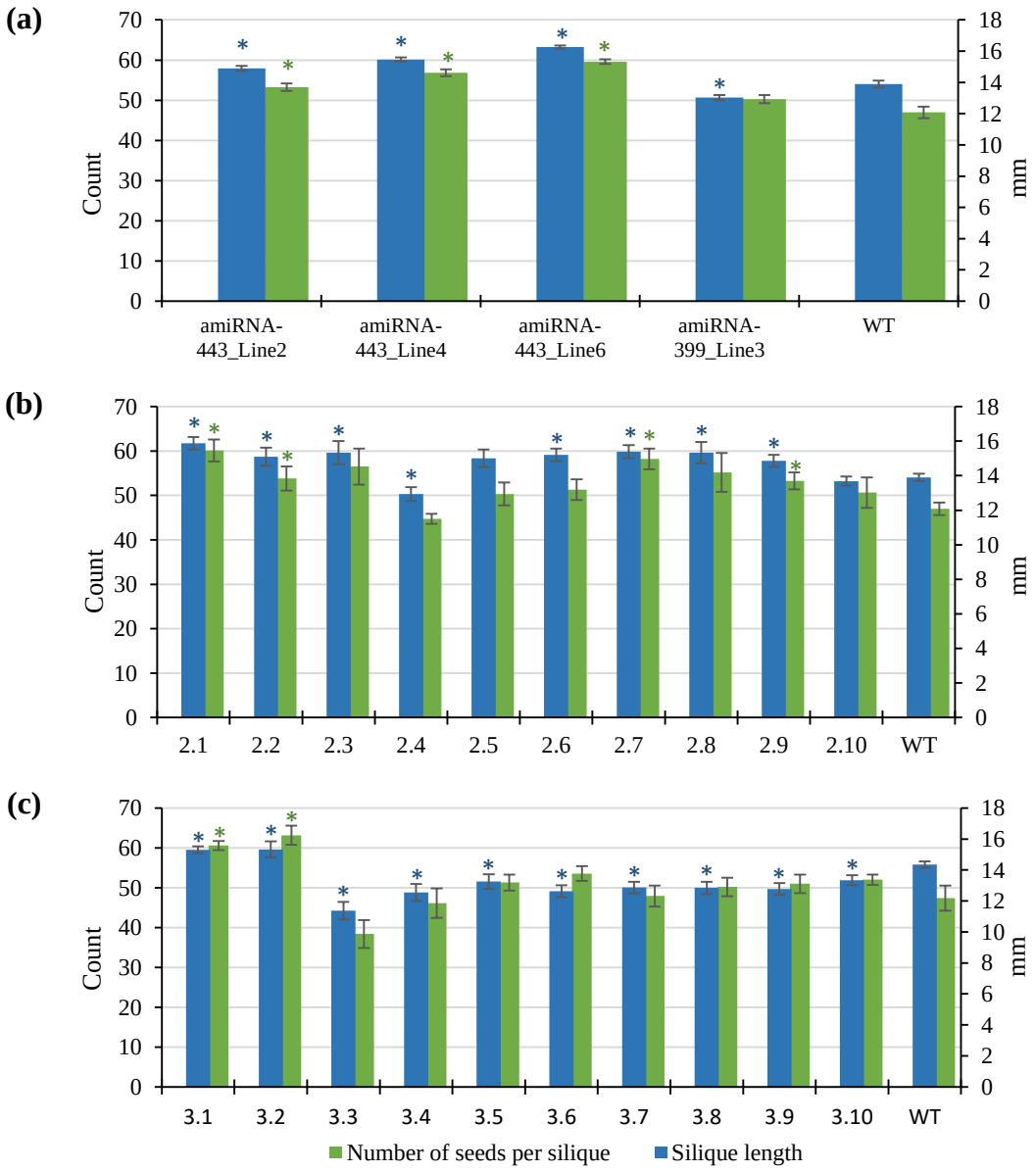



Figure 5-22 Productivity assessment of the T2 generation plants by scoring silique length and number of seeds per silique. (a) Productivity of selected T2 transformed lines (n = 10-30 for WT and ~100 for other lines). (b) Productivity in individual T2 line 2 transformant plants carrying the amiRNA CSHL_058443. (c) Productivity in individual T2 line 3 transformants carrying the amiRNA CSHL_017399. Mature siliques were collected randomly from plant inflorescences at 56 DAS. Means ± SE; n = 10 in b and c; asterisks indicate significant differences to WT ($P \leq 0.05$) based on T-test when data was normally distributed or Mann-Whitney test when data was not normal.

### 5.3.5.c) T3 generation phenotype

Silique length and number of seeds per silique were one of the most interesting altered phenotypes observed in T1 and T2 generations of the amiRNA transgenic lines. Thus, one line of the amiRNA CSHL_058443 (line 2) that showed long silique and one of the amiRNA CSHL_017399 that showed short siliques in the T2 generation (line 3) were selected to be investigated in the subsequent generation (T3), in order to get better understanding of the heritability of this trait across the successive generations. Firstly, the Mendelian ratio of T3 seeds from three T2 CSHL_058443 line 2 and three CSHL_017399 line 3 individuals was checked and results showed that two of the three selected plants of each amiRNA were homozygous as indicated by the 100 % survival of seedlings under BASTA selection (Table 5-4) (CSHL_058443 plants 2.7 and 2.8, and CSHL_017399 plants 3.3 and 3.5).

Table 5-4 T3 Transformant line segregation following selection with BASTA at 5 µg/ ml. Seeds were sown on MS medium containing BASTA as described in Section 5.2.8.a). The numbers of seeds were counted at sowing and number of survived seedlings were counted after 2 weeks.

| Construct | Studied plant | Alive | Dead | Percentage of survival |
|---|---|---|---|---|
| amiRNA- CSHL_058443 | 2.7 | 142 | 0 | 100 % |
| | 2.8 | 145 | 0 | 100 % |
| | 2.9 | 110 | 35 | 75.86 % |
| amiRNA CSHL_017399 | 3.1 | 75 | 43 | 63.56 % |
| | 3.3 | 123 | 0 | 100 % |
| | 3.5 | 117 | 0 | 100 % |

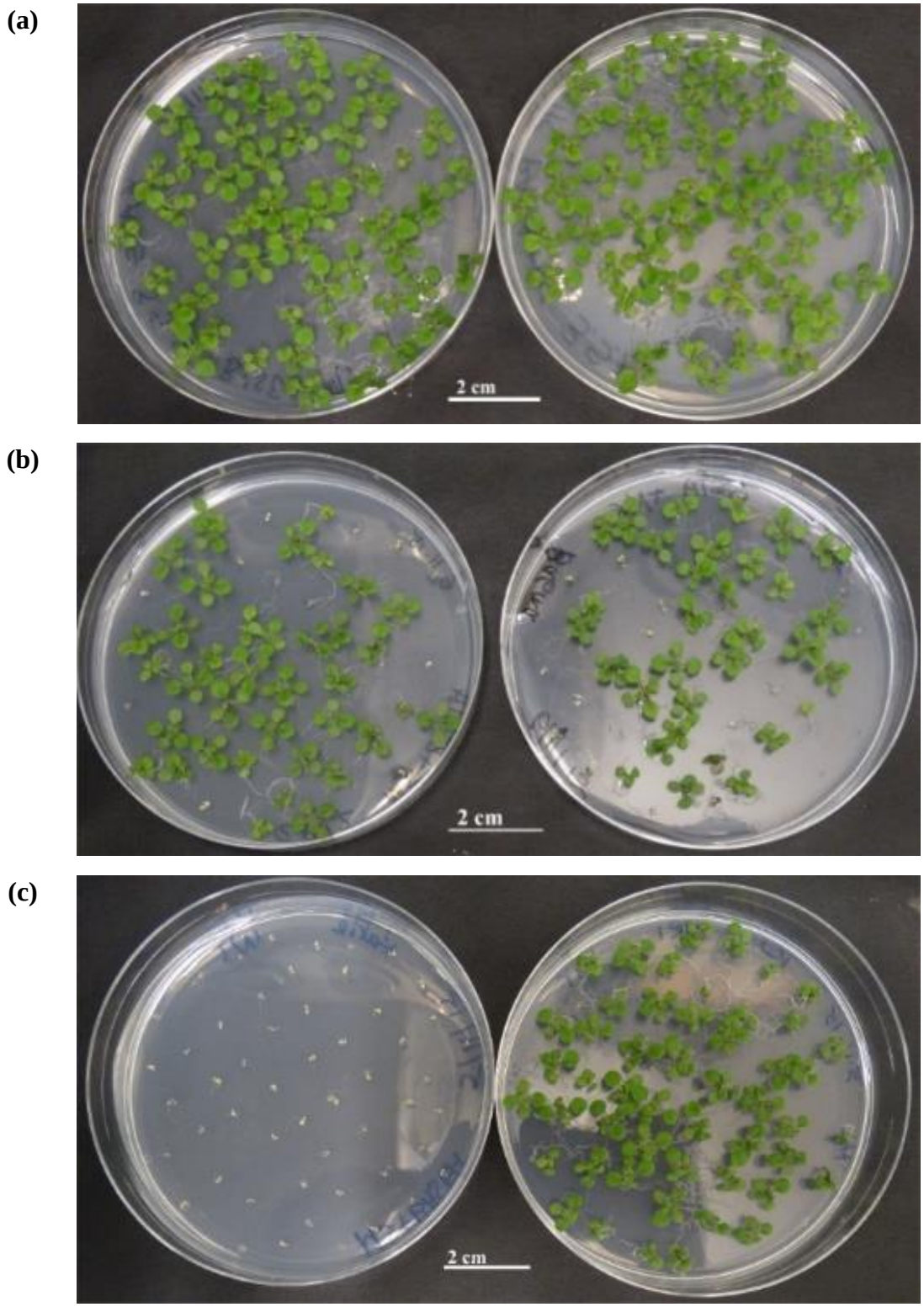



Figure 5-23 Transgene segregation in T3 generation of selected amiRNA T2 individuals from two T1 lines: line 2 from the amiRNA CSHL_058443 and line 3 from the amiRNA CSHL_017399. (a) Homozygous plants, (b) hemizygous plants and (c) control plants; WT as a negative control on the left and BASTA resistant plants on the right, three weeks after sowing.

Ten plants from each T2 line (CSHL_058443 plants 2.7, 2.8 and 2.9; CSHL_017399 plants 3.2, 3.3 and 3.5) were grown to maturity and growth parameters were recorded for the T3 generation from these lines. T3 progeny from the two heterozygous plants (2.9 and 3.1 Table 5-4) were first genotyped by PCR as described previously in Section 02.9 before including in the analysis of the phenotypes.

The transgenic amiRNA plants from the T3 generation also showed a range of phenotypic deviations and abnormalities in plant growth and development similar to those observed among the transformants in T1 and T2 generations, in addition to some new phenotypes such as organ fusion in a few rare plants. Mean time of bolting was delayed in T3 progeny from one of the amiRNA CSHL_058443 T2 lines (2.9) (Figure 5-24.a), while bolting was significantly accelerated in T3 progeny from the two homozygous lines (3.3 and 3.5) of the amiRNA CSHL_017399 (Figure 5-24.b). T3 progeny from two of the amiRNA CSHL_058443 lines (2.8 and 2.9) were significantly late in their flowering while T3 progeny from homozygous plants carrying the other amiRNA (CSHL_017399) flowered significantly earlier (lines 3.3 and 3.5). Alterations in flowering time, as a result of the amiRNA transgenes, in turn affected the day of producing the first silique which followed a similar pattern of differences compared with WT plants being delayed in T3 progeny of all three amiRNA CSHL_058443 T2 lines and accelerated in T3 progeny from two of the amiRNA CSHL_017399 lines (3.3 and 3.5). Number of rosette leaves at bolting was significantly less in T3 progeny of all three amiRNA CSHL_017399 and one of the amiRNA CSHL_058443 lines (2.7) compared to WT plants, the exception being the T3 progeny from the heterozygous 2.9 line and the homozygous 2.8 line where there was no significant change or increased number of leaves respectively as compared to WT plants.

Productivity of transformed plants, represented by rate of silique production in a week, was higher in T3 progeny from both homozygous T2 plants carrying the amiRNA CSHL_058443. One T2 line (3.1) transformed with the amiRNA CSHL_017399 showed a significant reduction in this parameter. In addition, around half (53.3 %) of plants carrying the amiRNA CSHL_017399 had the floppy stem phenotype, whereas flat wide stems and multi-stalks with no distinguished primary stalk phenotypes occurred at 10 % and 13.3% respectively within transformants carrying the amiRNA CSHL_058443.

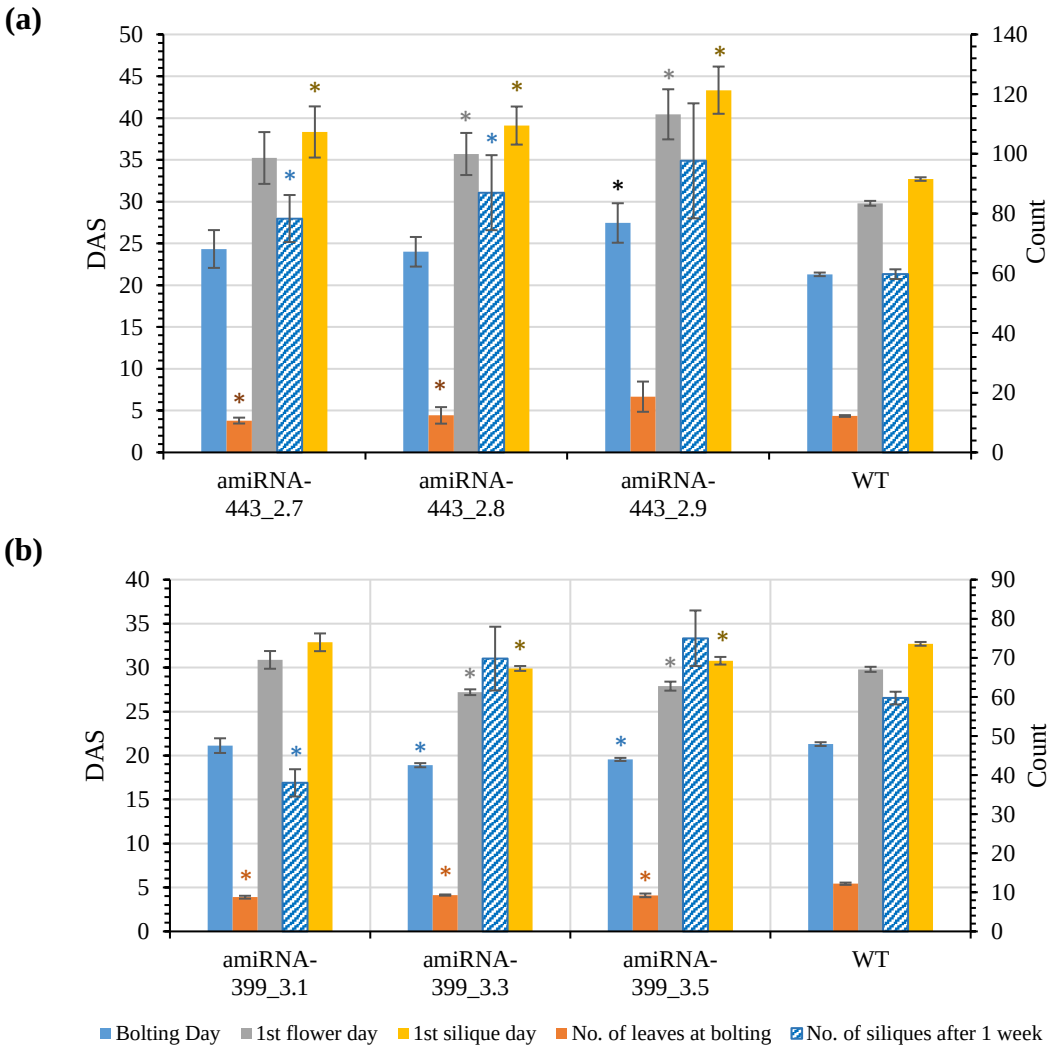



Figure 5-24 Morphological and developmental alterations in T3 generation amiRNA plants during different stages of growth. (a) T3 plant phenotype affected by the amiRNA CSHL_058443. (b) Phenotype of T3 plants transformed with the amiRNA CSHL_017399. Number of siliques was scored on the primary inflorescence, secondary inflorescence branches, and inflorescence branches after one week following formation of the first silique. (n = 10; means ± SE; asterisks indicate significant differences to WT ($P \leq 0.05$) based on T-test when data was normally distributed or Mann-Whitney test when data was not normal).

The effect of the amiRNA transgenes on the silique length and number of seeds produced per silique in the T3 generation was also investigated. Although only one line (CSHL_058443, 2.9) showed a significant difference in silique length (shorter siliques), both homozygous T3 plants carrying the amiRNA CSHL_058443 (2.7 and 2.8) produced a significantly high number of seeds compared to seed number of WT plants (Figure 5-25). No clear differences in these parameters were observed in T3 progeny of the amiRNA CSHL_017399.

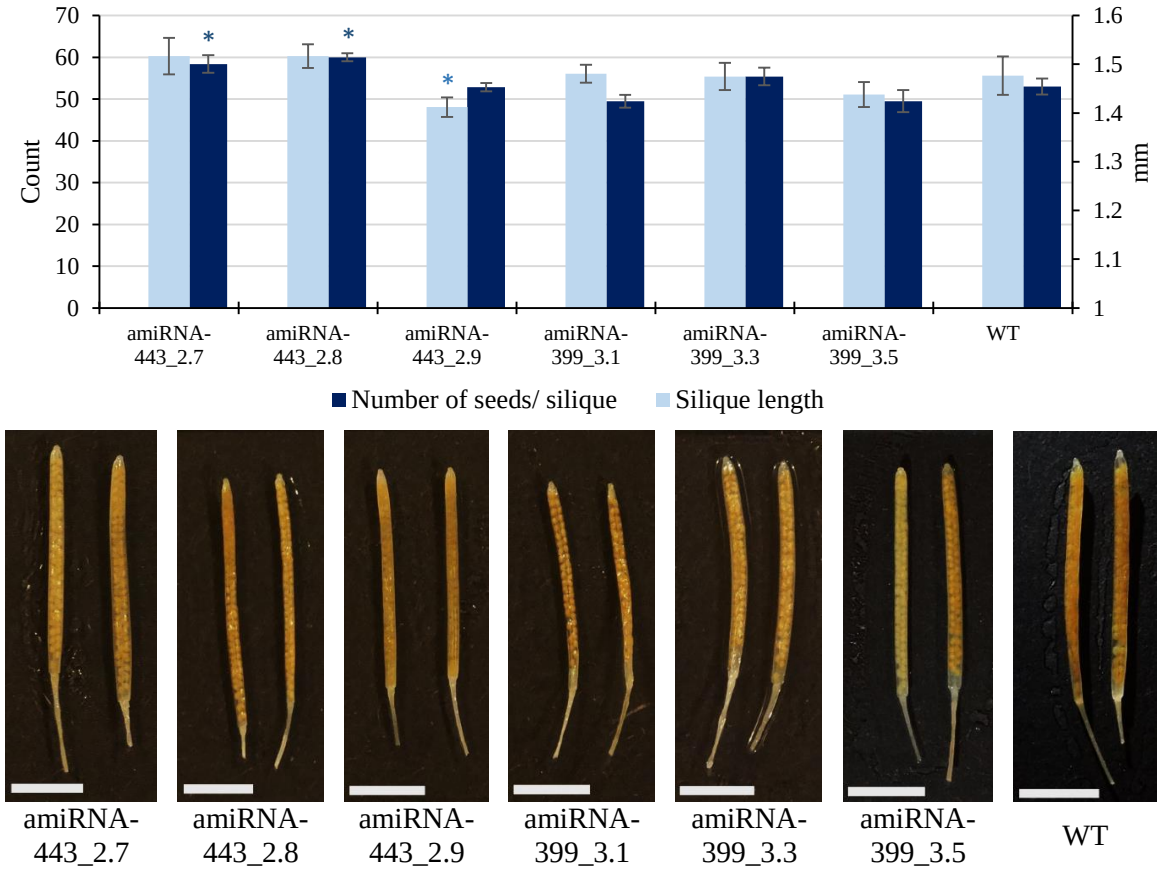



Figure 5-25 Productivity of transformed amiRNA plants in the T3 generation of three T2 plants from each amiRNA construct by scoring silique length and number of seeds per silique. Images show phenotype of mature siliques, scale = 5 mm. (n = 30; means ± SE; asterisks indicate significant differences to WT ($P \leq 0.05$) based on T-test when data was normally distributed or Mann-Whitney test when data was not normal).

Since transformation with both amiRNAs resulted in floppy stem phenotypes, the vascular tissues were investigated in T3 generation plants (Figure 5-26) to determine whether the floppy stems could be explained by alterations in arrangement or size of the vascular tissues. All studied plants had abnormal vasculature. A significant reduction in the width of vascular bundles was observed in all studied plants, which was associated with a decrease in vascular bundle depth in all studied plants carrying the amiRNA CSHL_017399 (3.1, 3.3 and 3.5), and one plant carrying the amiRNA CSHL_058443 (2.8). Interestingly, homozygous plants carrying the amiRNA CSHL_058443 (2.7 and 2.8) showed a clear increase in the depth of the interfascicular fibre reaching almost double its thickness compared to WT stems, while other transformed plants did not. In contrast, interfascicular width was not affected in the majority of the transgenic lines tested. Alteration in cortex thickness was variable among plants of each amiRNA clone.

Area of stem was also reduced in some of the transformed plants but not always consistently. Bundle number did not show any statistically significant differences.

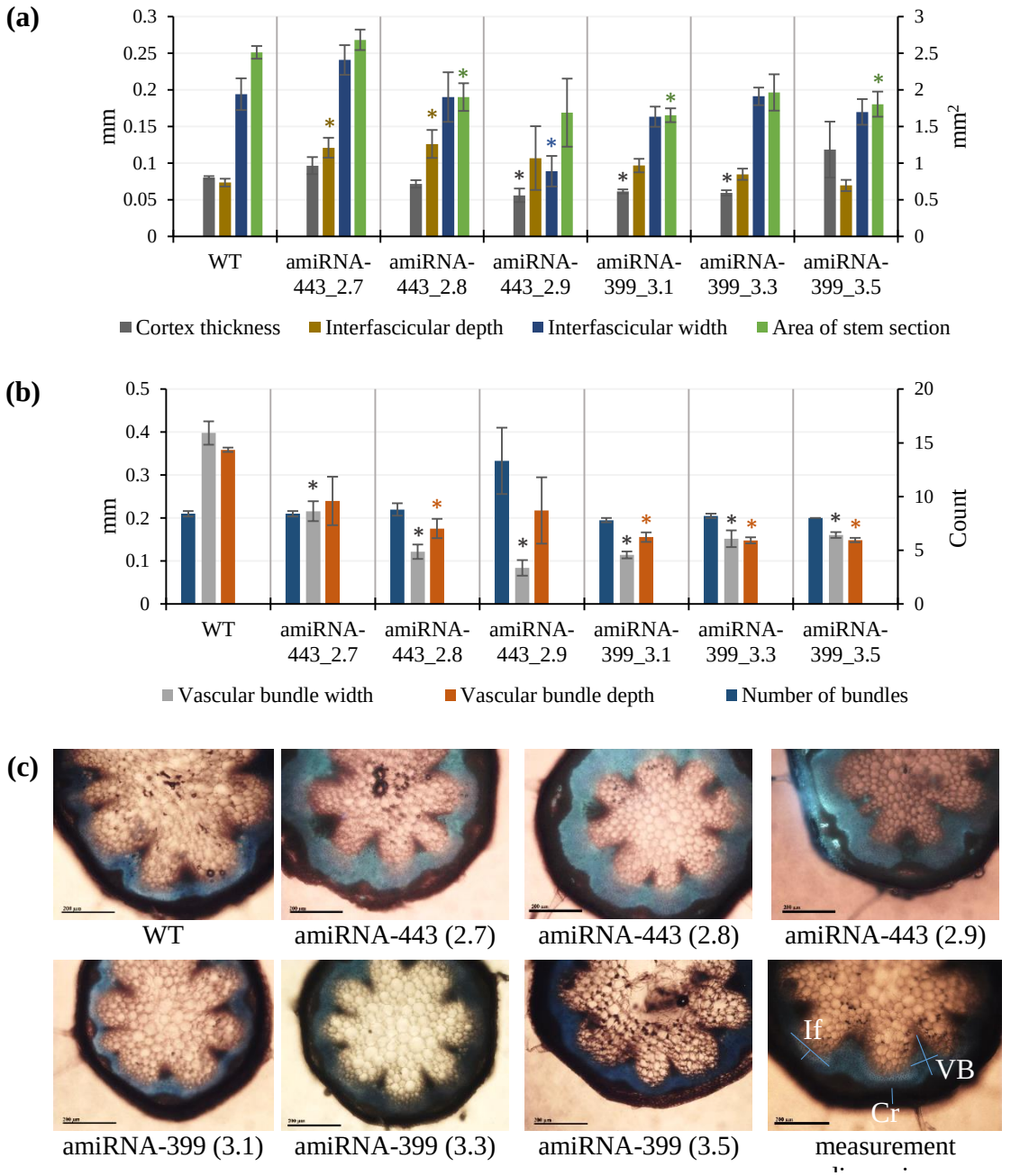



Figure 5-26 Analysis of stem architecture and anatomy of stems in WT and selected T3 transformed plants carrying the amiRNAs CSHL_058443 or CSHL_017399. Stems of approximately 8 week old plants, ~15 cm in length, were hand sectioned using a razor blade at the base of the inflorescence. (a and b) Measurements of vascular structure (n = 5 for number of bundles and area of stem section and 15 for the rest; means ± SE; asterisks indicate significant differences to WT ($P \leq 0.05$) based on T-test when data was normally distributed or Mann-Whitney test when data was not normal). (c) Hand cut sections of the basal portion of the inflorescence stem of mature plants (8 weeks) observed under UV light. Scale of the microscopic images = 200 µm. Abbreviations on the bottom right hand image are interfascicular (IF), vascular bundle (VB), and cortex (Cr).

Plants from a single line showed a wide range of pleiotropic phenotypes. For example, progeny from line 2.9 of the amiRNA CSHL_058443, which was heterozygous, showed considerable variation in the deposition of phenolic compounds and in stem shape (Figure 5-27).

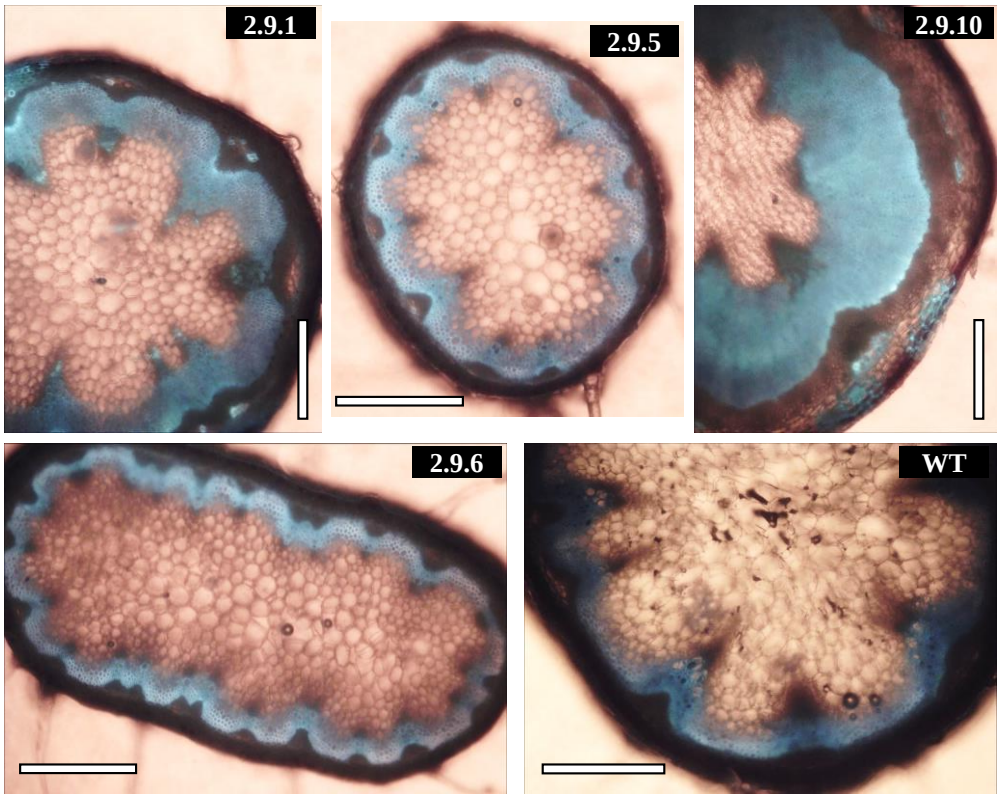



Figure 5-27 Multiple phenotypes within plants of one line (2.9) of amiRNA CSHL_058443. Plant names are indicated. Scale of the microscopic images = 200 µm.

## 5.4. Discussion

Verification steps for the plasmids received, confirmed their integrity. Percentage of homology produced by alignment of both amiRNA sequencing results with the CDS sequences of all the ten members of the *AtCuAO* gene family confirmed the presence of homologous amiRNA-binding sites on the mRNA of target genes but with different levels of homology which may affect their activity in altering expression of target genes. However, note, importantly that verification of the down-regulation of the *AtCuAO* genes was not carried out and is needed for a full interpretation of the results. Although each amiRNA potentially down-regulates different members of the *AtCuAO* gene family, a

similar range of phenotype alterations were noted within transformed plants of both constructs. This might be attributed to the fact that in both amiRNAs *AtAO1*, *AtCuAO2*, *AtCuAO4*, *AtCuAO5*, and *AtCuAO9* are target genes. These common targets might be involved in prevalent roles mediating regulatory processes in organs which showed the more frequent phenotypic changes among transformed plants. Moreover, *AtCuAO1* homology is low with both amiRNAs: 43 % with amiRNA CSHL_058443 and 14.3 % with the amiRNA CSHL_017399. Likewise, the percentage of *AtCuAO3* homology is also low: 5 % with amiRNA CSHL_058443 and 57.14 % with the amiRNA CSHL_017399. Transcript levels of both genes, *AtCuAO3* and *AtCuAO1*, progressively increased with Arabidopsis development and reaches the highest levels in 28 day old seedlings compared with younger ones (4 days old), suggesting a possible role in plant development (Planas-Portell et al. 2013). Since redundancy within gene family members is common (Kafri et al. 2009), one explanation for the similar range of phenotypic alterations shown early by both transgenes might be through a compensation of the down-regulation of target genes by an increase in AtCuAO1 and AtCuAO3 activity, assuming that they are not able to completely complement the down-regulation of the other family members as redundancy can be partial or complete between genes (Zhang *et al.* 2012). miRNAs direct DNA methylation (Bao et al. 2004), or block mRNA translation when they bind imperfectly to it (Brodersen et al. 2008), this may provide another possible explanation for the anomalous phenotype in both amiRNA plants through perturbing mRNA of genes with low homology.

Results showed that overexpression of both amiRNAs led to significant biological alterations in Arabidopsis vegetative and floral development represented in a wide range of phenotypic traits. Abnormalities observed during the development of *Arabidopsis thaliana* plants consisted in formation of multi-stalks, flat stems, floppy stems (fasciated stems), misshapen leaves, changes in number of leaves produced, number of siliques formed, size of siliques, time of bolting, time of flowering, time of first silique, and time of senescence.

The wide range of phenotypic changes reflects perhaps the large numbers of functions that polyamines (PAs) perform during plant development. Although in this work levels

of the different PAs were not measured in the transgenic plants and nor was the expression of the *AtCuAO* gene family, the hypothesis is that the amiRNAs would down-regulate some or all of the *AtCuAO* gene family at critical stages of plant development resulting in the perturbation of PA homeostasis and also potentially changes in the production of hydrogen peroxide ($H_2O_2$) necessary as a signalling molecule (Ghuge *et al.* 2015c; Neill *et al.* 2002).

The flat stem phenotype resembles the fully fasciated (Fuf) mutant in Arabidopsis that showed a dramatic increase in size of the vegetative apical meristem without causing any alteration in the meristem zonation or the shape and differentiation of vegetative organs but produced club-shaped siliques (Medford *et al.* 1992). The flattened stem phenotype in different Arabidopsis fasciation mutants has been attributed to allelism and different loci (Clark *et al.* 1993; Leyser and Furner 1992) this could be the case in the amiRNA plants which showed this phenomenon.

The misshapen leaves shown by some of the amiRNA plants (Figure 5-16.b and c) could perhaps due to epinasty. Leaf epinasty is a differential cell growth in leaf surfaces as a result of a greater expansion in cells of the adaxial surface cells as compared to cells in the abaxial surface leading to the downward bending of the leaf (Pazmiño *et al.* 2014). The common substrate *S*-adenosylmethionine (SAM) acts as a precursor for the biosynthesis of ethylene and as a source of aminopropyl moiety in the synthesis of the higher polyamines spermidine (Spd) and spermine (Spm), see Figure 1-1 (Moschou et al. 2012). High accumulation of Spd-Spm in transgenic tomato fruits led to higher rate of ethylene biosynthesis (Mehta *et al.* 2002). The epinastic phenotype observed in some amiRNA plant leaves might be due to accumulation of PAs as a result of manipulating the level of *AtCuAO* expressions which in turn would allow SAM to be available for ethylene biosynthesis, although further experimental evidence is needed.

Both amiRNAs led to abnormalities in vascular system formation. These alterations in stem architectures affected the flexibility of stems and thus their consistency leading to floppy or straight stem phenotypes. Since effects on the stem rigidity may be attributable to effects of PAs on xylem differentiation (Tisi et al. 2011a) and PA-derived $H_2O_2$, via

AtAO1 activity, is involved in vascular tissue differentiation (Ghuge et al. 2015a), observed abnormalities in vascular tissue differentiation may attributed to perturbation of PA levels or their catabolite titres as a result of silencing some of the *AtCuAOs* in transformed plants.

Lignin formation during vascular differentiation is associated with $H_2O_2$ (a product of PA catabolism) generation and cell wall bound peroxidases (de Marco and Roubelakis-Angelakis 1996). ROS play a key role in vascular differentiation, lignin polymerization, and cell wall expansion and growth (Grant and Loake 2000). In plants, the high expression of *AtCuAOs* in vascular tissues, epidermis and wound periderm, where processes of lignification and cell wall stiffening are active, suggested their role in $H_2O_2$ production for cell wall rigidity and strengthening (Cona et al. 2006; Ghuge 2014; Paschalidis and Roubelakis-Angelakis 2005a).

The involvement of PAs in development of vascular tissues has been demonstrated (Vera-Sirera *et al.* 2010), however, alterations in the formation of vasculature has been reported due to unbalanced levels of PAs. In this regard, the dwarf mutant *bud2* defective in *S*-adenosylmethionine decarboxylase (*SAMDC4*) affected PA biosynthesis leading to expanded vascular system, with increased bundle size and lignin content, in different parts of Arabidopsis plant (Ge *et al.* 2006). Likewise, loss of function of the xylem specific gene *ACL5*, which encodes a protein involved in the synthesis of the PA thermospermine, negatively affected the secondary growth of the vascular tissue leading to a dwarf phenotype due to abnormalities in the structure of stem's vascular bundles such as lack of xylem fibres and morphologically altered vessel elements (Kakehi *et al.* 2008). This could be the case in the present results (Figure 5-26), where stem anatomy showed clear changes in vascular differentiation that might be ascribed to changes in PA and/ or $H_2O_2$ levels.

Another possibility for floppy stem might be changes in cellular turgor due to changes in lignin deposition. Lignin provides rigidity and mechanical strength to the tissues of the plant and renders the cell walls hydrophobic and impermeable (Campbell and Sederoff 1996). As lignin reduces the water permeability of the secondary cell walls (Raven *et al.*

1999), tightening cell walls by lignin biosynthesis in response to stresses is often accompanied by a decrease in cell wall extensibility and/or turgor pressure (Le Gall *et al.* 2015). Hence, alterations in lignin deposition in amiRNA plants may also affect turgor pressure of the cell through changes in water and solute permeability which in turn will affect the mechanical support of plant stems leading to straightness or floppiness.

Notably, there was a transgenerational inheritance of the pleiotropic developmental defects caused by the two amiRNAs, however, they were not highly stable over subsequent generations of each line. Similar phenotypic alterations in plant development were consistently detected at different levels over generations of different lines. In addition, some new traits which were not scored in the parental generations were detected in the T3 generation. The wide range of pleiotropic phenotypes observed in stem sections of progeny from line 2.9 of the amiRNA CSHL_058443 (Figure 5-27) could be due to variations in the effectiveness of the amiRNA in reducing the expression of target genes which in turn will affect levels of PAs and their catabolic products.

Transformation of Arabidopsis plants with either CSHL_017399 or CSHL_058443 also affected plant productivity. This effect displayed different degrees of altered flowering day, fruit setting time, and silique phenotype (silique length and number of seeds per silique) as compared to WT plants. Previous studies showed the requirement of PAs as essential factors during the reproductive stage of plant development as well as the importance of the balance in PAs distribution in different organs of the plant depending on the stage of development, for instance, putrescine (Put) and Spd biosynthesis is increase at early stages of *Citrus sinensis* flowering and during anthesis (Kushad *et al.* 1990). Likewise, quantification of PA levels by HPLC in 6-week-old bolted *Arabidopsis thaliana* (Columbia ecotype) plants under normal conditions showed high levels of the PAs Put, Spd and Spm accumulated in reproductive organs, such as buds, flowers and siliques compared with their levels in leaves and roots (Urano et al. 2003). Furthermore, exogenous treatment of olive with different PAs accelerated floral initiation and fruit set (Rugini and Mencuccini 1985). However, exogenous treatment of the nonflowering cultivars of *Spirudela* with Spm or Spd did not induce flowering (de Cantú and Kandeler 1989), indicating that PA on its own is not sufficient for flowering induction but

homeostasis in the floral PA levels along with other factors are critical for this process. Since CuAOs may act as modifiers of PA levels by influencing PA metabolism (Rea et al. 2004), the early flowering showed by plants transformed with the amiRNA CSHL_017399 and late flowering observed in plants transformed with the amiRNA CSHL_058443 might be due to unbalanced PA levels as a result of disturbing the expression of several members of the *AtCuAO* gene family which in turn affected different aspects of reproductive development.

Previous reports indicated that overexpression of oat arginine decarboxylase (*ADC*) in tobacco plants resulted in high accumulation of Put which led to a 28- 55 fold increase in seeds and the severity of these alterations was correlated with putrescine concentration (Masgrau et al. 1997), however, unbalanced PA levels, low Spd and high Put, in the double mutant Arabidopsis plants affected in the expression of spermidine synthase, *SPDS1* and *SPDS2*, led to abnormally shrunken seeds and arrested embryos at the heart-torpedo transition stage (Imai et al. 2004b). Since germination of pollen and growth of their tubes are key events in the sexual reproduction of plants (Wu *et al.* 2008), another possible explanation for productivity alterations (fruit setting times and silique phenotypes) could be a defect in pollen grain germination and pollen tube growth that may occur due to irregular PA levels as a result of manipulating the expression of *AtCuAOs*. In this regard, high levels of PA contents and increased activity of the biosynthetic enzyme SAMDC have been reported in tomato pollen (Song *et al.* 2002). Treatment of pollen with SAMDC inhibitor prevented pollen germination (Antognoni and Bagni 2008), while non active SAMDC in the anther resulted in male sterility (Falasca et al. 2010). Furthermore, all *WOX* genes are involved in maintaining a balance between cell division and differentiation (Gallois *et al.* 2004). Mutation of *WOX1* gene in *wox1-D* plants modulates the activity of SAMDC1 thus altering PA homeostasis which disturbed cell proliferation resulting in male sterility, and anther dehiscence failure (Zhang *et al.* 2011). Thus, these results highlight the importance of balance of the higher PAs, Spd and Spm, for plant fertility which might be affected by perturbing the expression of *AtCuAOs* as Put (the substrate of AtCuAOs) is a precursor of both Spd and Spm (Bhatnagar *et al.* 2002).

As catabolism of PAs via polyamine oxidases (PAOs) and CuAOs is essential for modulating pollen cell wall rigidity, through regulation of cell wall component deposition, (Aloisi *et al.* 2016) which is essential for normal growth of the pollen tube and thus successful fertilization (Tanaka *et al.* 2013), and the role of PAs in ovary development was proved (Alburquerque *et al.* 2006), another possible explanation for the changes in seed number that could be due to a defect in the fertilization process or in the generation of the ovules. Levels of PA catabolism products might be potential contributor to observed irregular reproductive phenotype. Oxidation of Put by CuAOs generates $\Delta^1$-pyrroline which can be further catabolized into $\gamma$-aminobutyric acid (GABA) (Rea et al. 2004). Variations in GABA levels have been observed during Arabidopsis development and high GABA levels were correlated with formation of siliques with reduced size resulting in production of significantly fewer seeds (Mirabella *et al.* 2008). Since GABA has been suggested as an agent that guides pollen tube growth in Arabidopsis via maintaining the concentration gradient along the pollen tube path in the pistil (Palanivelu *et al.* 2003), perturbing PA catabolism by manipulating *AtCuAO* expression may influence pistil GABA levels leading to defects in pollen tube growth and guidance.

On the other hand, high activity of CuAO upon Put treatment in *Vicia faba* has been positively correlated with $H_2O_2$ production (An et al. 2008), while CuAO loss of function disrupted $H_2O_2$ generation (Qu *et al.* 2014), indicating the possibility of perturbing $H_2O_2$ levels as a result of manipulating *AtCuAO* expression in amiRNA plants. Pollen tubes have a tip specific $[Ca^{2+}]$cyt gradient which is fundamental for their elongation (Malho *et al.* 1995), and PA-derived $H_2O_2$ has been reported as an activator of $Ca^{2+}$ current in the pollen tube plasma membrane (Wu *et al.* 2010). Exogenous treatment of pollen grains with high concentrations of Spd triggered a $Ca^{2+}$ influx beyond the optimum which led to defect in the $Ca^{2+}$ gradient and inhibition of pollen tube growth (Wu et al. 2010). In addition, loss of function of the other source of PA-derived $H_2O_2$, *atpao3* mutant, disturbed $H_2O_2$ levels and led to decrease in fertility as a result of reduction in pollen tube growth (Wu et al. 2010).

Collectively, these results indicate the critical role of PAs and their catabolites in plant fertility and may help to understand the wide range of alterations in the productivity of

transformed plants observed in the present work which again could be due to irregular levels of PAs and/ or their catabolites. However clearly effects on pollen and hence fertilization would only affect seed number and potentially silique length but not the number of siliques. This effect on productivity therefore must be due to other effects induced by the amiRNA presumably linked to changes in PA homeostasis.

Both transgenes, CSHL_058443 and CSHL_017399, affected other obvious developmental aspects such as plant length and timing of leaf yellowing. This might be again due to perturbed levels of PAs or their catabolites. Results of previous studies may support this suggestion, for example, overexpression of *ADC2* in *A. thaliana* resulted in elevated Put contents, the product of Arginine decarboxylation via ADC, which led to dwarfism and delayed flowering (Alcazar et al. 2005). Likewise, overexpression of oat *ADC* in tobacco plants showed phenotypic abnormalities, included short internodes, and this was attributed to the enhanced level of Put (Panicot *et al.* 2002). Changes in endogenous PA levels in the transgenic plants with engineered sense *ADC* (Masgrau et al. 1997) or antisense *SAMDC* genes (Kumar et al. 1996) affected leaf morphology and stem elongation. Furthermore, elevated levels of accumulated Put as a consequence of overexpressing ornithine decarboxylase (*ODC*) led to wrinkled leaves in tobacco plants (DeScenzo and Minocha 1993). In contrast, failure in PA homeostasis as a result of mutation in the Arabidopsis *WOX1* gene led to a significant reduction in polyamine content which affected the natural progression of plant development and resulted in dwarfism (Zhang et al. 2011). Also, loss-of-function mutants of one of the two spermine synthases, *ACL5*, in Arabidopsis showed a severe defect in stem elongation (Hanzawa et al. 2000). These results indicate the fundamental role of balanced PAs in regulating plant development. On the other hand, $H_2O_2$ generated via polyamine catabolism in the cell wall has been suggested to be implicated in peroxidase-mediated processes such as suberization, lignification, and cross-linking of cell wall components (Moller and McPherson 1998; Rea et al. 2004). Taking into account these results, the wide range of phenotypes seen in transgenic plants carrying the two amiRNAs might be also attributable to variations in the severity of affecting the expression of target genes which in turn will affect levels of PAs and their catabolic products. The mechanism by which

*AtCuAOs*, which are targets of both amiRNAs, can affect plant development is still unclear, and this question merits further investigation.

Based on findings in the present chapter, assuming that the amiRNAs are indeed down-regulating one or more *AtCuAO* genes and that this is affecting PA homeostasis we can conclude that PA catabolism plays an important role in plant growth and development along with its indirect role in delaying plant senescence. Manipulation of expression of genes involved in PA catabolism pathways could indeed be utilized as a tool for generating interesting phenotypes such as increased seed production. In order to get better understanding of the observed phenotypes and to be able to draw a conclusion regarding the effectiveness of these two amiRNAs in altering *AtCuAO* expression, more investigations are required to analyse the expression of target genes of both amiRNAs at different stages of plant development, taking into consideration stages where AtCuAO were highly expressed (Figure 3-7), and to determine PA and their catabolic metabolite levels in transformed plants.

# Chapter Six

## General Discussion

## 6. Discussion and final conclusion

Polyamines (PAs) are implicated in almost all aspects of plant growth and development including cell division and elongation, embryogenesis, organogenesis, flowering, and are well known for their anti-stress and anti-senescence effects (Sagor *et al.* 2016). Some of PA's physiological effects are related to regulation of PA homeostasis (Zhang et al. 2011), and some others are linked to biologically-active reaction products that form as a downstream consequence of PA catabolism by amine oxidases (AOs) (Cona et al. 2006; Planas-Portell et al. 2013), which are also responsible, along with the biosynthetic enzymes, for maintaining cellular PA homeostasis (Angelini et al. 2010). One of the two AOs in plants are copper-containing amine oxidases and *Arabidopsis thaliana* contains a gene family of ten members (*AtCuAOs*) encoding these enzymes (see **Chapter 1**, Section 1.1.2). Owing to their dual function, participation in the production of key metabolites and preservation of PA cellular balance, the studies described in the present thesis were driven mainly by an attempt to understand roles of *AtCuAOs* as potential mediators of PA actions in plants and whether one or more members of the *AtCuAO* gene family might have a role in plant senescence.

One aspect of the present work was aimed at tracking the temporal expression pattern of *AtCuAOs* in specific leaves during *Arabidopsis thaliana* development under optimal-controlled conditions using two techniques (**Chapter 3**). Available information about the expression levels of the whole *AtCuAO* gene family was based on analysis of specific leaves using microarray analysis (TAIR eFP browser; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi, and PRESTA; http://www2.warwick.ac.uk). However, quantitative real time PCR (qRT-PCR) has become the method of choice for accurate measurements of transcript abundance of genes (Gachon *et al.* 2004) with higher sensitivity when compared with microarray analysis (Lewis *et al.* 2013). The qRT-PCR technique was therefore adopted here to detect RNA expression of all *AtCuAO* family members in single leaves of wild type Arabidopsis plants at four critical time points (young leaf, before bolting, after bolting, senescent leaf) to confirm and extend the results from the microarrays. GUS staining was the other method used to confirm the qRT-PCR expression profiling of two selected *AtCuAOs* in transgenic plants allowing an analysis of both temporal and spatial expression in whole plants.

For further elucidation of mRNA functions in the *AtCuAO* gene family, the other aspect of this research was dedicated to study different physiological responses to the genetic manipulation of selected members of the *AtCuAO* family. First, a detailed functional analysis of one gene of the family was performed in plants knocked-out in the expression of *AtCuAO4* by the insertion of T-DNA (**Chapter 4**). The second approach was by transforming Arabidopsis wild type plants with artificial microRNAs (amiRNAs) complement to selected genes of the family and tracking the resulted phenotypic changes as a response to alterations in their expression (**Chapter 5**).

The main findings in the different chapters are summarized and discussed in the following sections, including future prospects.

## 6.1. Expression patterns of the *AtCuAO* gene family during *Arabidopsis thaliana* leaf development

One important clue to the possible functions of the *AtCuAO* gene family in leaves was their expression pattern (Chapter 3, Section 3.3.4). It is interesting to note that the expression of all seven detected *AtCuAOs* was persistent throughout various stages of Arabidopsis leaf life-span until early senescence, but with selectively variable mRNA levels and there was expression of at least four of the genes at all stages tested including mid/late-senescence (Figure 6-1) indicating a possible functional specialization of the respective enzymes.

Several interesting insights on the regulation of *AtCuAOs* expression in Arabidopsis developing leaves were observed. The expression of three members of the family (*AtCuAO3*-SP, *AtCuAO5* and *AtCuAO9*) was not detected in any of the studied leaf stages which is consistent with other bioinformatic sources, TAIR and PRESTA, for the silique-specific gene *AtCuAO5* (NCBI; http://ncbi.nlm.nih.gov/pubmed), but not for the other two genes, *AtCuAO3*-SP, *AtCuAO9 (TAIR and PRESTA*). As verification steps confirmed the efficiency of primers used (Chapter 3 Section 3.3.2), I concluded that these genes are not expressed in leaves, suggesting that their proteins might have more specialized functions in other plant organs at least under the growth conditions used in this study. Furthermore, recent reports indicate that only eight out of ten *CuAO* genes in *Arabidopsis*

*thaliana* encode putative functional CuAOs, and *AtCuAO3*-SP, and *AtCuAO9* may have other functions (Tavladoraki et al. 2016) which reinforces the present findings.

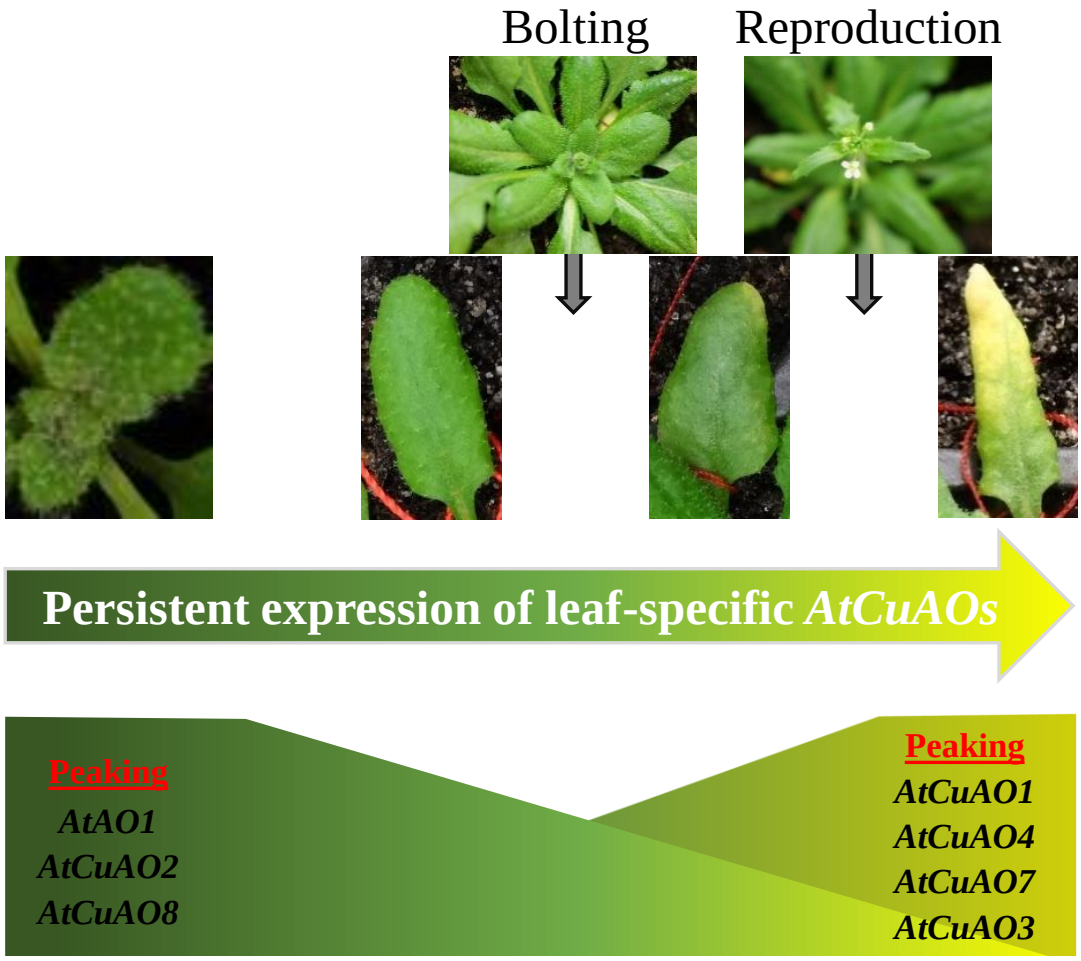



Figure 6-1 Leaf-specific expression of the *AtCuAO* gene family throughout the life of Arabidopsis leaves. Different levels of *AtCuAO* expression were detected at all stages of leaf development.

The present qRT-PCR results revealed that leaf-specific *AtCuAOs* can be divided into two groups with a contrasting expression patterns: the first consists of three genes (*AtAO1*, *AtCuAO2* and *AtCuAO8*) positively associated with total chlorophyll contents showing the highest level of expression at the earliest time point and that seem to be more specific for young leaves, whereas the second set includes four genes (*AtCuAO1*, *AtCuAO4*, *AtCuAO7* and *AtCuAO3*) negatively correlated to the total chlorophyll concentrations reaching peak of up-regulation at the latest time point and seems to be more related to processes that happen during leaf senescence (Figure 6-1). Expression patterns of *AtCuAO1*, *AtCuAO2*, *AtCuAO3*, *AtCuAO4* and *AtCuAO7* is in full agreement

with previously documented qRT-PCR results either in the whole seedlings or rosette leaves (Ghuge 2014; Planas-Portell et al. 2013). Likewise, the expression of *AtCuAO8* with previously reported microarray data (TAIR eFP Browser; http://bar.utoronto.ca/efp /cgi-bin/efpWeb.cgi). Previous studies on *AtAO1* did not show its expression in developing leaves, however, Moller and McPherson (1998) reported high *AtAO1*-GUS expression in the vascular tissue of young leaves which became irregular in the older ones of the same Arabidopsis rosette. Thus, all of the previous *AtCuAO* expression studies support qRT-PCR results displayed in the present work (discussed in details in Chapter 3 Section 3.4). However, the data was fragmentary and expression patterns of all ten members of the gene family had not previously been analysed simultaneously to enable a direct comparison amongst them.

What was perhaps most striking was the distinctive expression pattern of *AtCuAO7* gene during leaf development in which the transcript of this gene accumulates dramatically just before bolting time (the floral transition) as compared with previous and following stages before it peaks at senescence. Recently, the role of hydrogen peroxide $H_2O_2$ as a potential flowering initiation factor has been highlighted and a distinct increment in its levels concomitant with bolting and flowering was reported in Arabidopsis (Bieker *et al.* 2012; Miao *et al.* 2004; Zimmermann et al. 2006). Thus, based on the known role of *AtCuAOs* in PA degradation which yields $H_2O_2$, the hypothesis that *AtCuAO7* might be implicated in this fundamental process can be proposed as its transcript increased dramatically just before bolting. This should be examined experimentally in future work by testing lines genetically modified in *AtCuAO7* expression to determine bolting and flowering times and the related level of endogenous $H_2O_2$ in leaves at these three stages (before-, at- and after-bolting).

The increased expression levels of the three *AtCuAO* genes (*AtAO1*, *AtCuAO2* and *AtCuAO8*) observed at early stage in young leaves, where processes of cell division, cell expansion and cell differentiation into stomata, trichomes and vascular tissue are still in progress (Kalve et al. 2014), might be explained as an essential step for generating fundamental compounds such as $H_2O_2$ and $\gamma$-aminobutyric acid (GABA) that contribute to the regulation of these important processes. In plant cells, there are several pathways

of $H_2O_2$ production and amine oxidases (AOs), including CuAOs and polyamine oxidases (PAOs), are part of one of these pathways through PA catabolism (Niu and Liao 2016). $H_2O_2$ plays important roles in regulating lignin formation in plant cells (Kärkönen and Koutaniemi 2010), and in enhancing cell expansion by increasing the region of pectin synthesis and thus pectin content (Xiong et al. 2015). In particular, $H_2O_2$ produced under normal conditions by putrescine (Put) degradation, via the activity of cell-wall localized CuAOs, is suggested to be an important factor involved in lignification and cross-linking reactions (Gill and Tuteja 2010). The high expression of *AtCuAOs* in plant vascular tissues, epidermis and wound periderm, suggested their contribution to $H_2O_2$ production for cell wall rigidity and strengthening where processes of lignification are active (Cona et al. 2006; Ghuge 2014; Paschalidis and Roubelakis-Angelakis 2005a). Furthermore, the role of $H_2O_2$ generated by Put degradation via AtAO1 in vascular tissue differentiation of immature developing leaves was documented, and the high expression of *AtAO1*-GUS in vascular tissue preceding lignification led to the suggestion that *AtAO1* is a good marker for vascular development (Moller and McPherson 1998). Another important signalling molecule, GABA, can be generated from the oxidation of 4-aminobutanal, the product of Put catabolism by CuAOs, via aminoaldehyde dehydrogenase (AMADH) activity (Xing et al. 2007). Several functions in plants have been attributed to GABA (Bouche and Fromm 2004). For instance, it enhances the morphological growth (Li *et al.* 2016), functions of the photosynthetic machinery, chlorophyll biosynthesis, gas exchange capacities and membrane stabilization (Luo *et al.* 2011).

Increased expression of *AtCuAO* genes during senescence suggests that these enzymes may have a role in the regulation of progression of senescence processes and these four *AtCuAOs* might be considered as senescence associated genes. Leaf senescence is under direct nuclear control: a considerable number of genes that are expressed in green leaves are down-regulated while genes known as senescence associated genes (SAGs) are up-regulated (Gan and Amasino 1997). Through the tight temporal and spatial regulation of its generation and scavenging, $H_2O_2$ plays a role in regulating plant growth and morphogenesis (Nathues *et al.* 2004; Niu and Liao 2016; Wojtyla *et al.* 2016). However, at high levels, it contributes to cell degradation at late stages of senescence (Zimmermann et al. 2006). A peroxisomal localization has been reported for the AtCuAO3 (At2g42490)

protein, while AtCuAO1 (At1g62810) is predicted to be an extracellular (apoplastic) protein (Planas-Portell et al. 2013). AtCuAO4 (At4g12290) protein is located in vacuoles while AtCuAO7 (At3g43670) is an apoplastic protein (TAIR; https://www.arabidopsis .org/). Peroxisomes are important sites of $H_2O_2$ production (Rubinstein 2000), and the peroxisomal ROS have been involved in leaf senescence (Palma *et al.* 2002; Pastori and del Rio 1997). Autophagy, the important mechanism for chloroplast degradation during leaf senescence, takes place in vacuoles (Wada and Ishida 2013), and can be induced by $H_2O_2$ (Xiong *et al.* 2007). The leaf apoplast is involved in signal recognition and nutrient remobilization during senescence (Martínez and Guiamet 2014). Therefore, all the *AtCuAO* genes that are up-regulated in senescent leaves (*AtCuAO1*, *AtCuAO*4, *AtCuAO7* and *AtCuAO3*) encode proteins localized in cell compartments which are involved in senescence related processes. Thus an increase in their expression might be a mechanism of $H_2O_2$ production during developmental leaf senescence.

The different expression pattern observed between the two gene groups in the present study reveals the different roles played by *AtCuAOs* during two fundamentally opposite processes in leaf development, active growth and death. However, their precise function in these processes is as yet largely unknown. More studies are required to understand the role of each encoded protein in these two crucial stages: generating plants in which single, double, or triple genes of each groups are knocked out would be helpful to discover effects on development and senescence. It would also be interesting to discover whether these mutants can be complemented by other members of the *AtCuAO* gene family or whether there is specificity at the protein level. Alternatively, CRISPR/Cas system also would be a useful tool here as it is highly efficient in silencing multiple genes (Feng *et al.* 2013; Li *et al.* 2013b).

To confirm and extend results obtained by qRT-PCR, two available GUS lines were analysed (*AtCuA7*::GUS and *AtCuA8*::GUS), but comparison of the results from the promoter-GUS lines was not entirely consistent with expression data from transcriptional analysis. This might be due to the length of the promoter used in the construct or the point of insertion in the genome of the transgenic plant (Mertens 2008; Stafstrom 2008). Future work should be undertaken to examine more GUS-lines for each member of the *AtCuAO*

family and to use longer promoter fragments. GUS lines that reflect the expression pattern of the endogenous genes would be useful for exploring in more detail spatial and temporal expression of the genes as well as the effects of stresses on their induction. In addition, as these lines includes the two reporter genes, GUS and GFP (Appendix B,c), it would be interesting to investigate GFP activity as it could be more accurate in assessment of the spatial and temporal activity of promoters (Kavita and Burma 2008).

## 6.2. The effect of knocking-out the expression of one member of *AtCuAO* gene family on Arabidopsis growth and development

In order to better understand the regulatory function of *AtCuAO* genes during plant development and senescence, one of the highly expressed genes in senescent leaves (*AtCuAO4*) was selected to investigate in more detail the effect of the genetic modulation of *AtCuAO* expression on different aspects of growth, reproduction and senescence during various stages of *Arabidopsis thaliana* development under optimal growth conditions. Several phenotypic deviations from wild type (WT) plants were observed in plants knocked-out in the expression of *AtCuAO4* by T-DNA insertion. These changes can be summarized as follows:

- Significant delay in day of bolting and thus first open flower.
- Early biomass enhancement represented by comparatively more rosette leaves at bolting time.
- Reduction in the length of the inflorescence stems.
- Marked increase in leaf contents of the total chlorophyll (a and b) and a clear retardation in leaf senescence.

Repressing the expression of *AtCuAO4* perturbed the catabolic pathway of PAs which resulted in Put accumulation in mutant leaves during the vegetative stage (pre-bolting). Accumulation of Put in *AtCuAO4* mutants is consistent with the reported higher affinity of AtCuAO enzymes toward Put (Planas-Portell et al. 2013). However, no comparable changes in Put contents were recorded during the reproductive stage (post bolting), whereas leaves of both mutants showed a significant increase in the two higher PAs,

spermidine (Spd) and spermine (Spm), at this stage. Since balance of PAs is an important agent in regulating plant growth and development (Urano et al. 2003), and as turnover to higher PAs is one of the mechanisms employed by plants to achieve homeostasis of intracellular PA levels (Angelini et al. 2010; Kusano et al. 2008), the later increase in the higher PAs (Spd and Spm) might be attributed to the activity of enzymes involved in the conversion of excess Put to Spd and Spm as a regulatory mechanism to control Put levels within the non-toxic range for survival, however, analysing the transcript levels of genes encoding proteins involved in Spd and Spm synthesis, such as spermidine synthase (SPDS), spermine synthase (SPMS), and *S*-adenosylmethionine decarboxylase (SAMDC), is required to confirm this suggestion. Generally, high levels of Put are associated with active cell division, and enhancing shoot growth (Aragão *et al.* 2016; Parimalan *et al.* 2011) while increased Spd and Spm contents are more linked to cellular differentiation processes (Matilla 1996). Increased numbers of leaves in the mutant plants at bolting could perhaps therefore be related to the high Put levels.

The phenotypic characteristics of both mutants (BIS#4 and C#4) include traits typical to known gibberellic acid (GA)-deficient (*ga1*–*ga5*) mutants (Koornneef and Van der Veen 1980), namely shortened inflorescence stems and late-flowering. The effect of Put accumulation on perturbing GA metabolism was already documented (Alcazar et al. 2005). Experiments were therefore undertaken to ascertain whether changes in *AtCuAO4* mutant phenotype were due to defects in GA metabolism, or were merely a consequence of unbalanced levels of PAs and their catabolites which are crucial in regulating several physiological and developmental processes in plants (Kusano et al. 2007; Martin-Tanguy 2001; Paschalidis and Roubelakis-Angelakis 2005a). Mutants were treated with gibberellin and expression of selected dioxygenase genes involved in GA metabolism (Figure 1-6) was analysed in rosette leaves pre-bolting by qRT-PCR. The phenotypic deviations from WT in bolting day, first open flower day and number of leaves at bolting were counteracted by exogenously applied $GA_3$ and plant stems elongated normally (Figure 4-24.b), indicating that the GA signalling pathway functions normally in the mutant lines and that the *AtCuAO4* mutation is indeed affecting GA metabolic genes. The repression of GA biosynthetic genes *AtKS1*, *AtGA20ox1* and *AtGA3ox1* suggests that the effect of raised Put levels in the *AtCuAO4* mutants is in repressing GA biosynthesis, with

consequent effects on shoot growth, a reduction in *SOC1* expression and hence delayed flowering time (Moon et al. 2003). *SOC1* loss-of-function mutation delays flowering significantly and shows increased rosette leaf number at flowering time, as well as increases in Arabidopsis lifespan which was suggested to be independent of flowering time (Melzer *et al.* 2008). In the *AtCuAO4* mutants the effect on leaf senescence appears to be linked to the flowering time as dark induced senescence was not significantly different to WT. However, these results suggest *SOC1* as one of the candidate genes that is involved in the *AtCuAO4* mutant phenotype observed in the present study.

Genetic alterations in expression of gibberellin metabolic genes can modulate flux of the intermediates in the later stages of the pathway (Hedden and Phillips 2000). To determine whether GA metabolism was altered as a result of modification in the expression of GA-metabolic genes, the levels of selected endogenous GAs in both mutants and WT were quantified in 20 day old rosettes grown under control growth conditions by collaborators at the University of Pisa (Appendix D). $GA_1$, $GA_3$, $GA_4$ and $GA_7$ are the most biologically active gibberellins in plants (Randoux et al. 2012), and predominant presence of $GA_1$ and $GA_4$ in several plant species suggests that these two GAs are the functionally active forms of GAs in plants (Hedden and Thomas 2012; Nelson and Steber 2016), so production of active gibberellins will be discussed here.

AtGA20ox catalyses the three consecutive oxidation steps of active-GA precursors, $GA_{12}$ and $GA_{53}$, to produce $GA_9$ and $GA_{20}$ respectively as immediate precursors of active GAs (Lange 1997), and further activation of $GA_9$ and $GA_{20}$ by oxidation via GA3ox activity yields biologically active GAs (Figure 1-6) (Hedden and Phillips 2000). In both active-GA biosynthesis pathways, starting with $GA_{12}$ or $GA_{53}$ as initial precursors, both mutants, C#4 and BIS#4, showed a significant decrease in the two immediate active-gibberellin precursors $GA_9$ and $GA_{20}$ (Appendix D), which is consistent with the reduced expression of *AtGA20ox1* that was scored in both mutant lines (Figure 4-26).

The level of AtGA3ox1 activity in C#4 and BIS#4 generated the two bioactive gibberellins $GA_4$ and $GA_3$, by $GA_9$ and $GA_{20}$ oxidation respectively, at similar levels as WT, however, contents of mutant plants of the other two active gibberellins $GA_7$ and

$GA_1$, via oxidation of the same immediate precursors respectively, were significantly reduced as compared to WT plant contents. AtGA3ox is encoded by a gene family consisting four members that are functionally redundant (Mitchum et al. 2006). Expression of only one member was analysed here so it is possible that all four members are not affected by the *AtCuAO4* mutation (putatively mediated by the increase in Put) and hence the unaffected $GA_3$ and $GA_4$ contents in both mutants may be due to functional redundancy within *AtGA3ox* family members. It is also possible that $GA_7$ and $GA_1$ were decreased significantly due to low *AtGA3ox* gene expression and for the sake of forming optimal concentrations of the other active gibberellins, $GA_4$ and $GA_3$, in the relevant pathway (Figure 1-6).

Active gibberellins play multiple roles in plant development: for example, $GA_1$ is responsible for stem elongation by enhancing division and elongation of cells (Davies 2012), while $GA_4$ is the most biologically active gibberellin in regulating shoot extension and flower initiation (Eriksson et al. 2006) and in promoting the FLOWERING LOCUS T (*FT)* expression in wild-type (Col-0) Arabidopsis plants under long-day conditions thus promoting flowering (Hisamatsu and King 2008). Treatment of late flowering Arabidopsis mutant *fca* with $GA_7$ accelerates flowering (Chandler and Dean 1994), and treatment of Arabidopsis WT grown in short-day conditions with $GA_3$ reduces expression of *SVP*, the repressor of *SOC1* gene expression, and thus activates expression of floral pathway integrators (Li et al. 2008).

Decreased levels of $GA_1$ and $GA_7$ scored in both mutant lines here seem to be responsible for defects in their phenotype as alterations in GA contents influence plant growth (Biemelt *et al.* 2004) and GAs stimulate flowering through activating *LFY* and *SOC1* expressions (Blázquez et al. 1998; Moon et al. 2003). Both $GA_4$ and $GA_7$ are important in restoring WT phenotype in dwarf and late flowering Arabidopsis mutants defective in either starch synthesis or degradation (Paparelli et al. 2013). Accumulation of Put in Arabidopsis transgenic plants overexpressing *ADC2*, one of the two genes encoding arginine decarboxylase, led to severe dwarfism and late flowering which was a result of reduced contents of both bioactive gibberellins $GA_4$ and $GA_1$ as compared to wild type plants (Alcazar et al. 2005). This may imply that different active gibberellins have

complementary regulatory roles and act synergistically in controlling different processes during plant development. Severity of defects in mutant phenotype seen here was less pronounced and thus it is possible that perturbing concentrations of active GAs led to deviation of mutant plants from WT growth pattern.

Content of $GA_4$ increases dramatically in the Arabidopsis shoot apex shortly before floral initiation (Eriksson et al. 2006) and the delay in flowering seen here was only of a few days, thus it is also possible that significant changes in $GA_4$ and $GA_3$ in the mutants would be seen if shoot apices were analysed in isolation rather than whole seedlings. In addition because only *AtGA3ox1* expression was analysed in this work, analysis of other family members may make a greater contribution to understanding the control of contents of active gibberellins $GA_1$ and $GA_4$.

Dwarfism is the distinctive phenotype of Arabidopsis plants defective in either gibberellin synthesis or signalling (Hedden and Thomas 2012; Yamaguchi 2008), and lower differences in active $GA_4$ content after bolting reported previously in dwarf Arabidopsis plants, which accumulated higher Put levels due to arginine decarboxylase (*ADC2*) overexpression, was suggested to be due to reduction in $GA_4$ contents of WT with age (Alcazar et al. 2005). Verifying the growth of the primary stems of both *AtCuAO4* mutants (Figure 4-19) showed only a minor delay in the extension of mutant stems which was sufficient to reduce the final stem length of both mutants significantly relative to WT stems. This indicates that the effect of Put accumulation on the gibberellin biosynthetic pathway possibly was transient. By controlling the excess Put levels observed before bolting through conversion to other forms of PAs, as it is evidenced in Figure 4-22, the defect in GA metabolism as a result of elevated Put was perhaps mitigated. Alternatively, gibberellins in WT plants are actually reduced with age explaining the late identical rate of stem extension in both WT and mutants (Figure 4-19.b). This can be verified experimentally in future work by estimating cell division rate in the pith rib meristem region where GAs promote cell division and cell expansion contributing to internode elongation (Achard et al. 2009; Cowling and Harberd 1999), and altering endogenous gibberellin contents affects the size of the division zone leading to changes in rates of organ growth (Nelissen *et al.* 2012).

As shown by qRT-PCR results (Chapter 3, Section 3.3.4) which are in agreement with findings from other studies (Ghuge, 2014; TAIR; https://www.arabidopsis.org/), the transcript abundance of *AtCuAO4* is high in senescent leaves. During age-dependent natural senescence under normal growth conditions, repression of the expression of *AtCuAO4* gene resulted in delayed senescence symptoms in both mutant lines (C#4 and BIS#4) relative to WT plants by retaining higher chlorophyll contents and delaying the visible yellowing. However, the possibility of *AtCuAO4* being directly involved in all types of leaf senescence was refuted by testing leaves of WT plants and mutants through dark treatment (Chapter 4, Section 4.3.4), indicating that the relation between AtCuAO4 activity and leaf senescence is not straight forward.

The most likely explanation is that the effect on leaf senescence is due to the delay in flowering. Arabidopsis is a monocarpic plant and leaf senescence is closely linked to the onset of flowering. The late flowering Arabidopsis mutant *gigantea* showed a long-lived phenotype (Kurepa *et al.* 1998), however in another late flowering mutant (*co-2*) leaf longevity was not affected (Hensel *et al.* 1993). The direct influence of flowering time on senescence in Arabidopsis still appears to be unresolved (Levey and Wingler 2005; Thomas 2013) although there is a clearly strong link between delayed flowering and delayed senescence. Nevertheless, it is possible that changes in PA metabolism in the *AtCuAO4* mutants affect senescence processes directly as well as indirectly through an effect on flowering time. Although dark-induced senescence activates a shared pool of genes with developmental senescence, it is distinct (Buchanan-Wollaston et al. 2005). Senescence of the *AtCuAO4* mutants under short day conditions where flowering is inhibited was not tested, this would be an important experiment to establish whether natural leaf senescence is affected by *AtCuAO4* mutation in the absence of flowering. Using an *in situ* non-destructive assessment such as using the chlorophyll meter SPAD (Cerovic *et al.* 2012) would be useful here as indicator of changes in chlorophyll content and thus senescence progression.

As discussed above the most likely effect on delayed senescence through delayed flowering is through the increased Put which affects, in some way as yet unknown, GA biosynthesis and hence the expression of key flowering genes. It could however also be

mediated by an effect on $H_2O_2$ generation. This might perturb the peak of $H_2O_2$ seen at bolting (Zimmermann et al. 2006) and which may play a regulatory role in promoting flowering. Elevation in the $H_2O_2$ production at this critical point was described as a communication mechanism between leaves and the floral meristem either for flowering induction or for alteration in leaf metabolism to recycle their contents supporting the newly developing reproductive organs leading ultimately to leaf senescence (Bañuelos *et al.* 2008). Since high $H_2O_2$ levels are required for Arabidopsis bolting and flowering (Bieker et al. 2012; Miao et al. 2004), blocking *AtCuAO4* activity possibly affected $H_2O_2$ production in both mutant lines which in turn delayed bolting and thus flowering and eventually retarded leaf senescence. Yet another route may be via changes in GABA generation which may affect the senescence programme through its effect on ethylene synthesis (Kathiresan *et al.* 1997).

It is also possible that some aspects of senescence are affected directly by the changes in PA concentrations noted after bolting: specifically the increase in Spm and Spd. Spm has reported anti-senescence properties in plants (Del Duca et al. 2014; Moschou and Roubelakis-Angelakis 2014; Serafini-Fracassini et al. 2010), and exogenous Spd was able to inhibit RNase activity, chlorophyll decline and protein degradation from thylakoid membranes in barley leaf discs in the dark (Legocka and Zajchert 1999). This may be due to Spd and Spm exceeding Put in their efficiency in the elimination of reactive oxygen species (Kuznetsov and Shevyakova 2007). In accordance, it was found recently that inhibition of Spd and Spm catabolism via inactivation of PAO activity drastically delayed barley leaf senescence in the dark, and this was associated with reduced Put, increased Spd and Spm levels, decreased $H_2O_2$, and slowing down of the senescence-associated chlorophyll loss (Sobieszczuk-Nowicka et al. 2015). Whether increased levels of leaf Spd and Spm observed in leaves of both *AtCuAO4* mutants after bolting contributed to the delayed senescence in these plants compared with WT leaves remains to be established though, since apparently they did not affect dark-induced senescence.

According to the above explanations for the resulted BIS#4 and C#4 phenotype, a tentative model suggesting a potential involvement of different response pathways to AtCuAO4 inactivation in the regulation of different physiological processes in the plant

is proposed in Figure 6-2. Confirmation of $H_2O_2$ involvement with these candidates and quantification of its levels in mutant lines using cHyper reporter (Cheng *et al.* 2013; Costa *et al.* 2010) and DAB (3,30-diaminobenzidine) assay (Augeri *et al.* 1990) will contribute to a better understanding of its role in regulating several processes in the plants.

These results emphasize the physiological importance of PA catabolism as modulated by the AtCuAO4 enzyme and thus the significant impacts of perturbing the expression of *AtCuAO4* (At4g12290) on different aspects of plant growth and development as well as plant longevity, and led to the suggestion that the catabolic activities played by this gene are fundamental throughout plant life as its mutation perturbed the endogenous levels of PAs and deviated the plant from the normal pattern of growth.

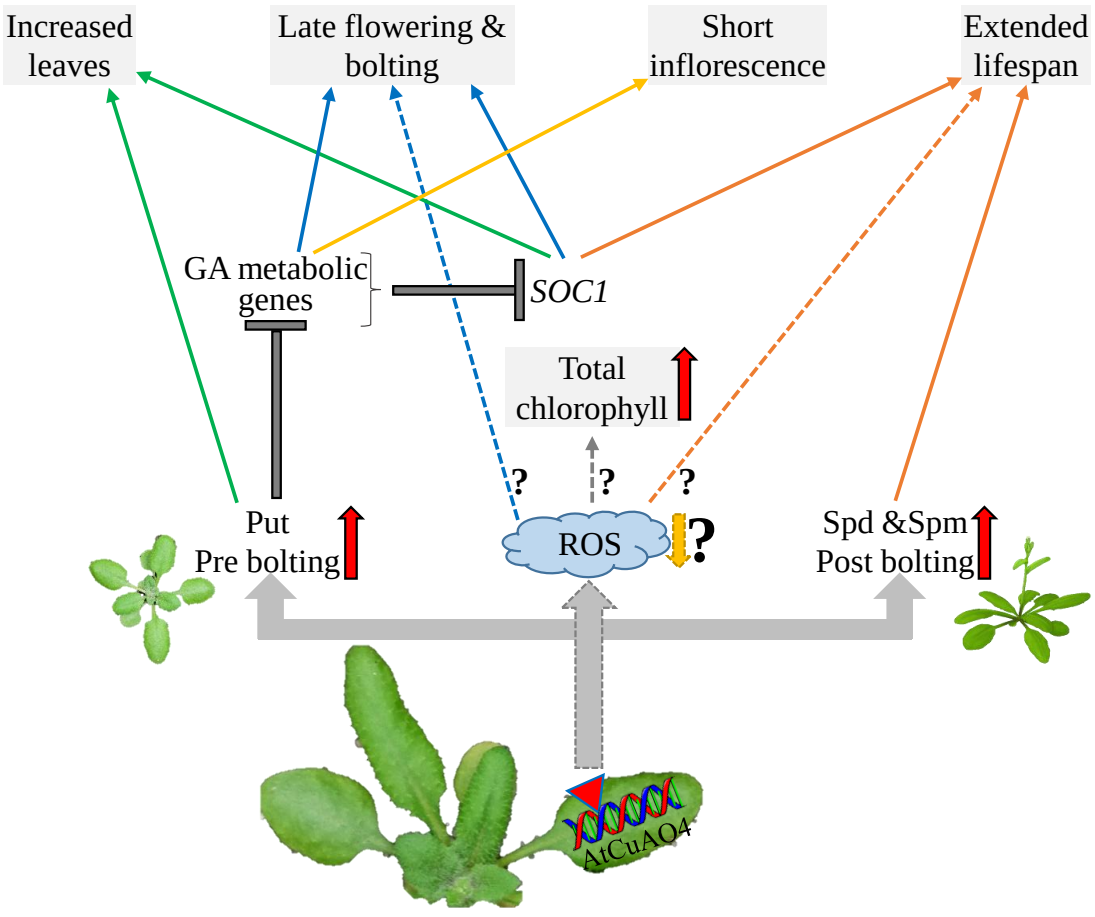



Figure 6-2 Proposed regulation of phenotype of the mutant lines BIS#4 and C#4 by different pathways upon inactivation of *AtCuAO4* gene by T-DNA insertion mutagenesis. Dotted arrows indicate possible regulation based on other studies stated above, while solid arrows are based on analysis performed in this work and correlations between changes seen in gene expression, PA content and changes in growth and development. Colour of arrows varies depending on the observed phenotype.

Based on the analysis of the transcription levels of *AtCuAOs* in developing leaves conducted in this work by qRT-PCR, the *AtCuAO* gene family is expressed in leaves concurrently with critical processes in plant life: maturation of young leaves, at the transition to flowering, at reproduction stage and at senescence. In Arabidopsis, evidence for the functional diversity of the *AtCuAO* genes was provided based on the expression of three *AtCuAOs* in response to different exogenous treatments together with the different cellular localization of their corresponding proteins (Planas-Portell et al. 2013). Bearing in mind that the enzymes encoded are all performing a similar function, catabolizing PAs, such diversified expression pattern of *AtCuAO* family members during undisturbed development in leaves suggests functional diversity between *AtCuAO* genes. Hence, it will be interesting to dissect in detail the effects of insertional mutants in each individual *AtCuAO* family member on regulation of PA levels and the influence of repressing their activity on different aspects of plant growth and development and plant senescence as well as their influence on GA metabolism. This work is in progress in the Rogers lab and elsewhere and its results may give further information about the exact role played by each *AtCuAO* gene and thus the function performed by the whole family.

In addition, as roles orchestrated by AtCuAOs are triggered mainly by catabolising PAs and releasing metabolites that are important in different physiological processes in plant life (Martin-Tanguy 2001; Paschalidis and Roubelakis-Angelakis 2005a), it is worth detecting product formation as a result of their activity, notably $H_2O_2$ that plays important roles during different processes of plant growth and development (Carucci et al. 2014). Histochemical staining and quantitative assays using DAB (3,30-diaminobenzidine) which reveals levels of $H_2O_2$ accumulation (Augeri et al. 1990) could be used to assess whether perturbation of *AtCuAO* expression affects $H_2O_2$ at critical points in plant development.

## 6.3. Effects of perturbing expression of selected *AtCuAO* gene family members by artificial microRNA on growth and development in *Arabidopsis thaliana*

In the presence of the functional redundancy amongst phylogenetically related genes in Arabidopsis (Wang *et al.* 2004), defective genes might be compensated by other family

members (Kafri et al. 2009) which may affect results obtained using single knock-out mutation. Manipulating the expression levels of plant genes using target-specific an artificial microRNA (amiRNA) approach is an effective tool to create transgenic plants silenced in a single or more genes of interest (Sablok et al. 2011). For deeper understanding of the functional role of *AtCuAO* genes and to avoid functional redundancy amongst *AtCuAO* members, two amiRNA lines of *Arabidopsis thaliana* plants transformed with amiRNAs targeting against multiple *AtCuAO* family members were generated.

The phenotype of transgenic lines derived from the two amiRNA constructs deviated from the normal growth and development patterns seen in WT plants (Figure 6-3). A range of significant alterations in plant vegetative and floral development was observed in amiRNA plants grown under optimum controlled conditions, perhaps the most important phenotypic traits were:

- Changes in the timing of flowering and fruiting which subsequently affected other reproductive traits and plant longevity.

- Alterations in the presence of phenolic compounds in the secondary cell walls which in turn affected stem flexibility and phenotype.

The most striking feature of these transgenic plants was the range of phenotypes, sometimes contradictory, and the apparent instability of particular phenotypic traits across generations. For example, flowering time was affected in several lines but transformation with the amiRNAs led to early flowering in plants transformed with the amiRNA CSHL_017399 and late flowering in plants transformed with the amiRNA CSHL_058443. It is possible that this difference is due to different specificities of the two amiRNAs. For example, *AtCuAO7* is one of the amiRNA CSHL_058443 targets while it is not targeted by the amiRNA CSHL_017399 (Figure 6-3). Results displayed in Section 3.3.4 show that expression level of *AtCuAO7* increased suddenly at the floral transition. It is possible that if expression of this gene is down-regulated at this crucial stage in development it reduces the peak of $H_2O_2$ required for flowering as was hypothesized for the *AtCuAO4* mutants. In order to test the hypothesis that *AtCuAO7* might be implicated in flowering process, further investigations in future work using

*AtCuAO7* knock-out mutants or overexpression lines might be helpful in verifying this hypothesis via determination of bolting and flowering times and the related levels of endogenous hydrogen peroxide ($H_2O_2$) in leaves.

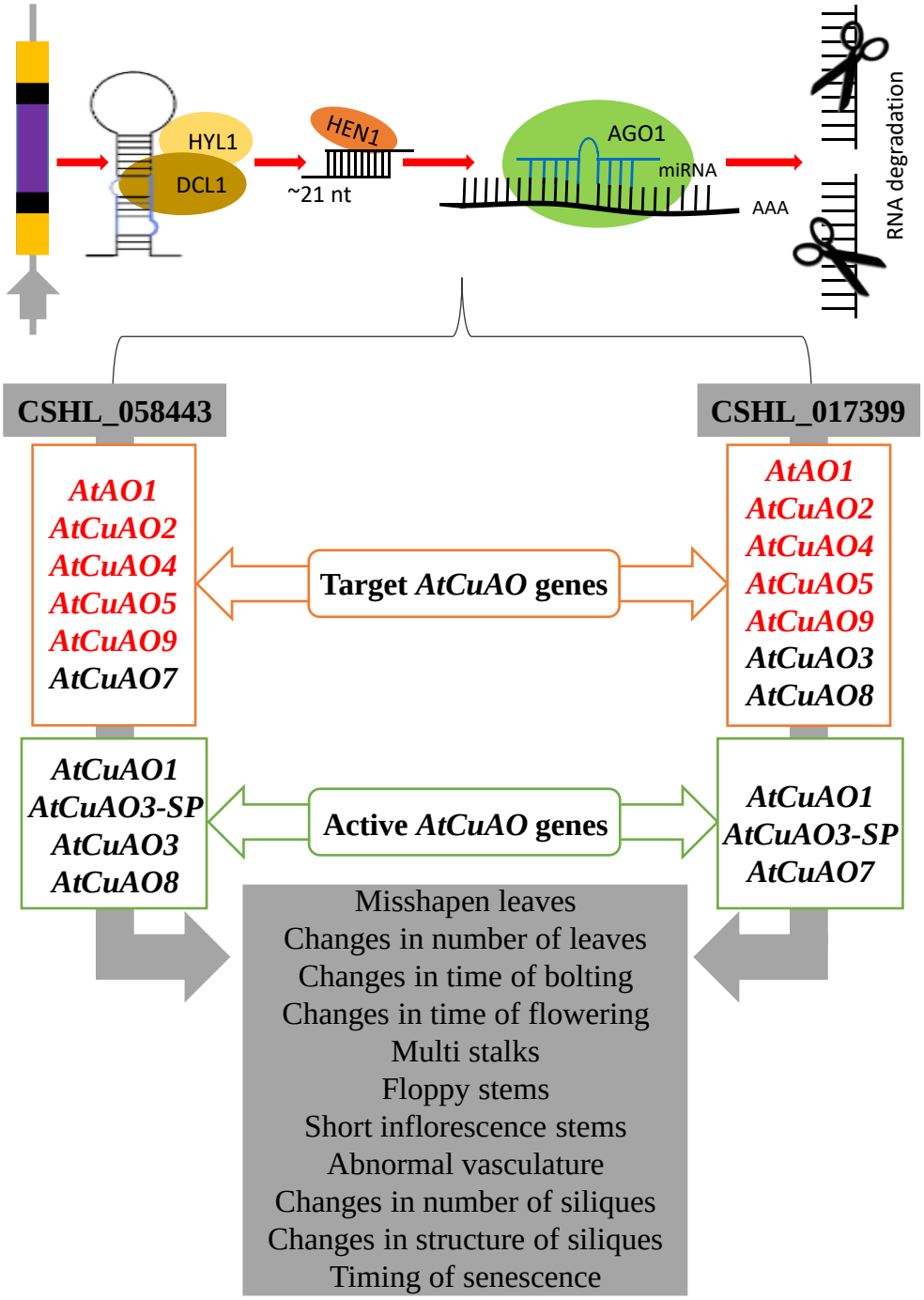



Figure 6-3 A schematic flow chart showing putative manipulated and unaffected *AtCuAO* genes in each amiRNA line and the resulting phenotypic alterations. Genes in red are common targets of both amiRNA constructs.

Another hypothesis to explain the present findings in relation to effects of the amiRNA constructs on flowering time is that defects in the AtCuAO catabolism pathway disturbed PA homeostasis, since catabolising PAs via copper amine oxidases modifies their levels (Rea et al. 2004). Concentrations of polyamines together with a balance in their distribution are critical factors in flowering and reproduction success in various plants (Kushad et al. 1990; Rugini and Mencuccini 1985; Urano et al. 2003). However, conflicting results for the effect of PAs in this regard were reported. For example, overexpression of oat *ADC* (arginine decarboxylase which catalyses Put biosynthesis) in transgenic tobacco plants resulted in accumulation of Put and plants showed an early-flowering phenotype in one line while the rest of transgenic plants which showed higher or lower Put concentrations did not exhibit any changes in flowering from control plants (Masgrau et al. 1997). In contrast, high Put levels in Arabidopsis plants overexpressing *ADC2* delayed flowering time (Alcazar et al. 2005). Recently, it was found that simultaneous silencing of the two *ADC* genes in Arabidopsis by amiRNA:*ADC* reduced PA levels and delayed flowering rate under long day conditions (Sánchez-Rangel et al. 2016). These results highlight the importance of the balance in PA levels in regulating flowering time. Accordingly, alterations observed in the flowering time with both amiRNA constructs is presumably due to perturbing PA homeostasis in response to manipulating one or more *AtCuAO* members. Quantitative analysis of the endogenous PAs in the future work is needed in both amiRNA plants to clarify the link between flowering time and PA levels.

The regulatory network controlling transition to flowering and reproduction in general is complicated and perhaps PA on its own is not sufficient for flowering induction but may be its homeostasis along with other factors are critical for these important processes as was indicated previously (de Cantú and Kandeler 1989). Given the results presented in Section 4.3.8, and in accordance with previously reported results (Alcazar et al. 2005), a hypothesis for effects on flowering could be that the amiRNA affects *AtCuAO* expression which in turn results in Put accumulation and affects gibberellin metabolism. However, this can only be proposed in case of the amiRNA CSHL_058443 which showed late flowering. For the amiRNA CSHL_017399 early flowering refutes this hypothesis. A possible explanation for this effect is that the *FUL* gene which acts redundantly with

*SOC1* to promote flowering (Albani and Coupland 2010) possibly compensated for *SOC1* loss which is positively regulated by gibberellin (Moon et al. 2003). This would need to be tested through an analysis of the expression of these genes, and indeed if this is the case, why was the same effect not seen in the other amiRNA line? This needs further investigation in future work by measuring active GAs and analysing the transcript levels of GA metabolic genes along with the floral integrators for a better understanding of the role played by *AtCuAOs* in regulating this critical process in plant life.

Investigation of the phenotypic characteristics of amiRNA siliques also showed clear alterations in fruit length and number of seeds, which might also be a result of unbalanced PAs. Accumulated evidence demonstrates the critical importance of PA homeostasis in seed production and that perturbation of PA levels may lead to male sterility (Falasca et al. 2010; Zhang et al. 2011), or defects in seed production through affecting pollen germination (Antognoni and Bagni 2008; Biasi *et al.* 2001; Imai et al. 2004b; Song et al. 2002), or in some cases to increased seeds (Masgrau et al. 1997). Metabolites of PA catabolism by CuAOs such as $H_2O_2$ are another possible contributor to alteration in reproduction observed in amiRNA plants, since activity of CuAOs is correlated to $H_2O_2$ production (An et al. 2008; Qu et al. 2014) and PA-derived $H_2O_2$ has a documented role in controlling plant fertility (Wu et al. 2010). In addition, GABA, another PA catabolic product, was suggested as an important factor in guiding pollen tubes (Palanivelu et al. 2003), and their high levels were correlated with formation of short siliques with fewer seeds (Mirabella et al. 2008).

Although the overall organization of vascular bundles and interfascicular fibers was not altered, a remarkable modification in the deposition of phenolic compounds was detected in almost all amiRNA mutant lines. Auto-fluorescence under UV light was more intense in CSHL_058443 plants than in controls especially in the interfascicular regions, indicating an increased deposition of phenolic compounds (McLusky *et al.* 1999), and probably lignification (Oliveira *et al.* 2013). In some cases, plants of this line (CSHL_058443) showed extra-layers of cells, with more intense auto-fluorescence, outside of the usual areas which was detected in the cortex parenchyma and phloem caps. Plants of the other amiRNA line (CSHL_017399) did not show clear differences in the

intensity of auto-fluorescence under UV light, but a clear reduction in the size of vascular bundles was noted. These alterations in stem architecture possibly affected stem flexibility and resulted in the floppy stems seen in the amiRNA CSHL_017399 lines and straight and sometimes wider stems seen in the amiRNA CSHL_058443 lines.

In Arabidopsis, *AtAO1* is expressed during early stages of vascular tissue differentiation in roots and leaves (Møller *et al.* 1998; Moller and McPherson 1998). The contribution of both *AtAO1* and *AtCuAO1* to vascular tissue differentiation was reported (Cona et al. 2006; Paschalidis and Roubelakis-Angelakis 2005a), and the involvement of $H_2O_2$ derived from PA catabolism via both amine oxidases, CuAO and/or PAOs, in plant cell-wall maturation in differentiating xylem was suggested (Moschou et al. 2012). In both amiRNA lines, *AtAO1* is a target gene while *AtCuAO1* is not (Figure 6-3). According to TAIR (https://apps.araport.org/thalemine/portal.do?externalids=AT4G14940.1), the best *Arabidopsis thaliana* protein match to *AtAO1* (At4g14940) is *AtCuAO8* (At1g31690). Hence, along with the activity of *AtCuAO1* in both amiRNAs, it is possibly that defective *AtAO1* in the amiRNA CSHL_058443 was covered by the untargeted gene *AtCuAO8* through functional redundancy, and while *AtCuAO8* is manipulated in the amiRNA CSHL_017399, may be the tight coordination between the two catabolic enzyme machineries, CuAO and PAO, (Planas-Portell et al. 2013), compensated the defect in $H_2O_2$ production.

Confirmation of these suggestions in the future work may reinforce the role of AtAO1 during vasculature differentiation. A first step would be to measure the transcript levels of *AtCuAO* genes which are known to be involved in vascular tissue differentiation, such as *AtAO1* and *AtCuAO2*, along with *AtCuAO8* in the amiRNA lines. In addition, measuring levels of PAs and $H_2O_2$ production might be helpful in explaining these observations as their roles in regulating vascular tissue differentiation are well documented (Clay and Nelson 2005; Cona et al. 2006; de Marco and Roubelakis-Angelakis 1996; Ge et al. 2006; Ghuge 2014; Kakehi et al. 2008; Vera-Sirera et al. 2010). Studying lignification of secondary cell walls using phloroglucinol staining in stems might also be useful to understand in more detail lignin deposition in secondary cell walls in response to simultaneous silencing of several *AtCuAO* members.

Previously, it was suggested that flowering transition and the secondary growth are under shared genetic control (Sibout *et al*. 2008). Loss of function of both flowering time genes, *SOC1* and *FUL*, in Arabidopsis showed late flowering with an extensive secondary growth (Melzer et al. 2008). This suggests another possible explanation for the highly intense auto-florescence observed in stem sections of the amiRNA CSHL_058443 which might be related to their late flowering.

Overall, transformation with the amiRNA constructs has a clear effect on almost all aspects of plant growth and development including transition from vegetative to flowering stage, productivity and plant longevity. However, there is no clear explanation for the pleiotropic developmental defects caused by the presumed simultaneous manipulation of multiple *AtCuAO* family members. Since these observations were focusing on phenotype changes, other methods of analysis should be applied to understand AtCuAO functions in these fundamental events since some of the phenotypes observed could contribute to improvement of some agriculturally important traits such as increased seed production or high lignification. In order to get better understanding of the observed phenotypes and to be able to draw a conclusion regarding the effectiveness of these two amiRNAs in altering *AtCuAO* expression, more investigations are required to analyse the expression levels of target genes of both amiRNAs by qRT-PCR at critical stages of plant development. Of particular importance are the two critical stages where leaf-specific *AtCuAOs* peaked but also the expression of the *AtCuAO* gene family during xylogenesis, floral meristem development, pollen germination and early seed development. It is also important to determine PA and $H_2O_2$ concentrations using DAB assay (Augeri et al. 1990) in transformed plants which may shed light on the action of specific polyamines in different plant developmental processes. In addition, since inflorescence stalks of some individuals lost their apical dominance and produced numerous of lateral branches, testing the pith rib meristem region might be useful in understanding this phenomenon. It is also interesting to discover the interplay between *AtCuAOs* and flowering processes and the role *AtCuAOs* play in floral morphogenesis. Due to redundancy within gene family members, may be it worth also trying simultaneous transformation with both amiRNA constructs, or crossing lines carrying the two different constructs which may extend the number of target genes or, alternatively,

using CRISPR/Cas system to silence multiple *AtCuAO* genes (Feng et al. 2013; Li et al. 2013b).

In conclusion, this work represents a contribution to the understanding of the temporal expression of *AtCuAO* genes in relation to changes in development and senescence, and other fundamental processes in the plant's life. Different approaches used in this thesis support the role played by PAs during vegetative and reproductive stages, and the crucial involvement of the catabolic enzymes AtCuAOs in controlling these functions either directly by regulating PA levels or indirectly by influencing other important pathways which have roles in different physiological processes. Hence, in spite of the pivotal roles PAs play in regulating wide range of plant physiological and developmental processes, their catabolism by AtCuAO is crucial during plant growth and development.

The main things to do next is to understand the effect of other *AtCuAOs* on the whole plant growth and development and the functional diversity expected by their expression pattern by phenotyping plant response to mutation of other *AtCuAO* genes mimicking experiment performed in Chapter 4, in addition to, understanding the mechanism underlying amiRNA effects, by analysing the expression level of *AtCuAO* genes as well as the impact of manipulating the expression of *AtCuAOs* in amiRNA lines on PA content and generation of the catabolic metabolites such as $H_2O_2$ as a result of PA degradation.

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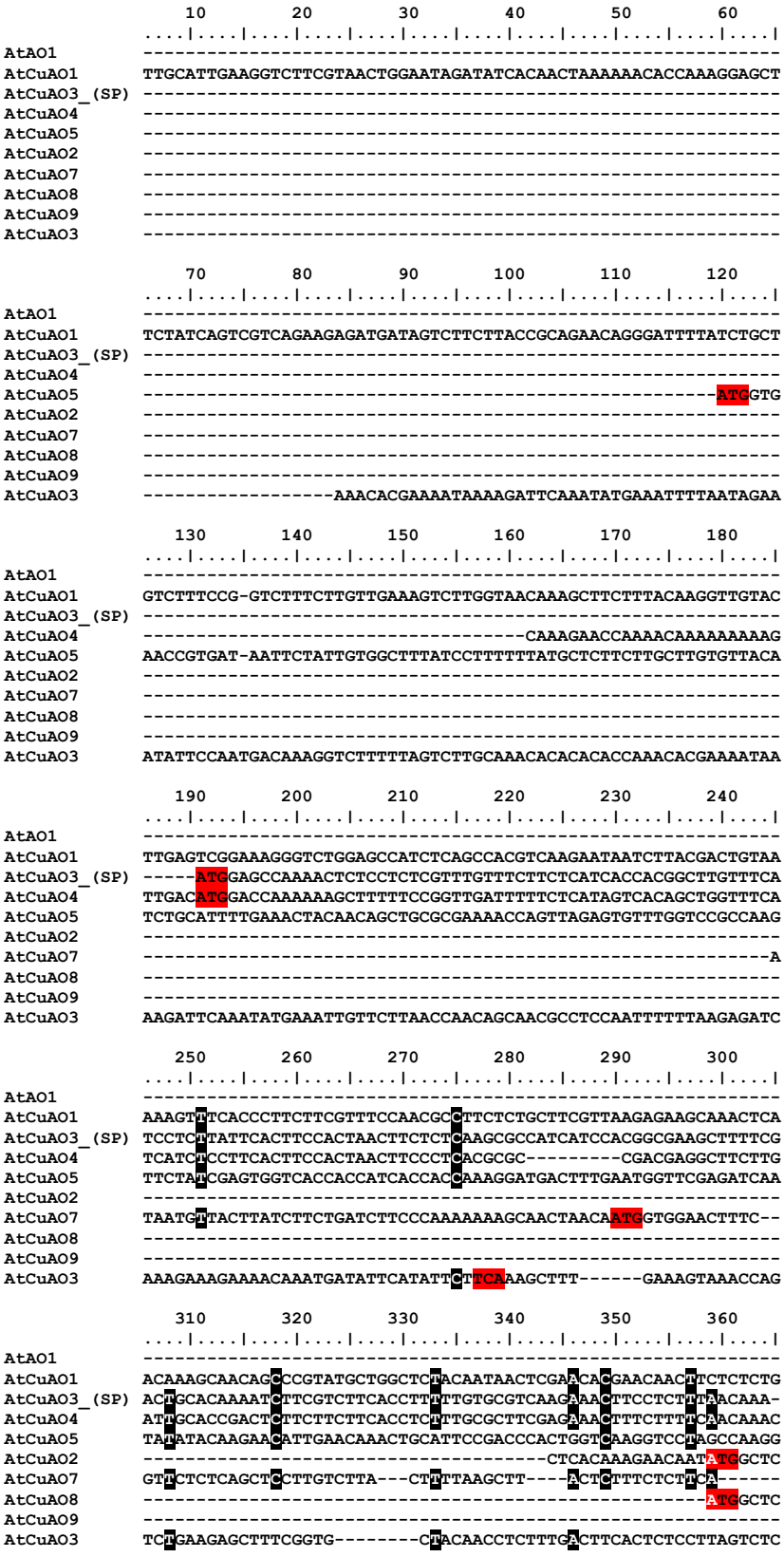
# Appendices

# Appendix A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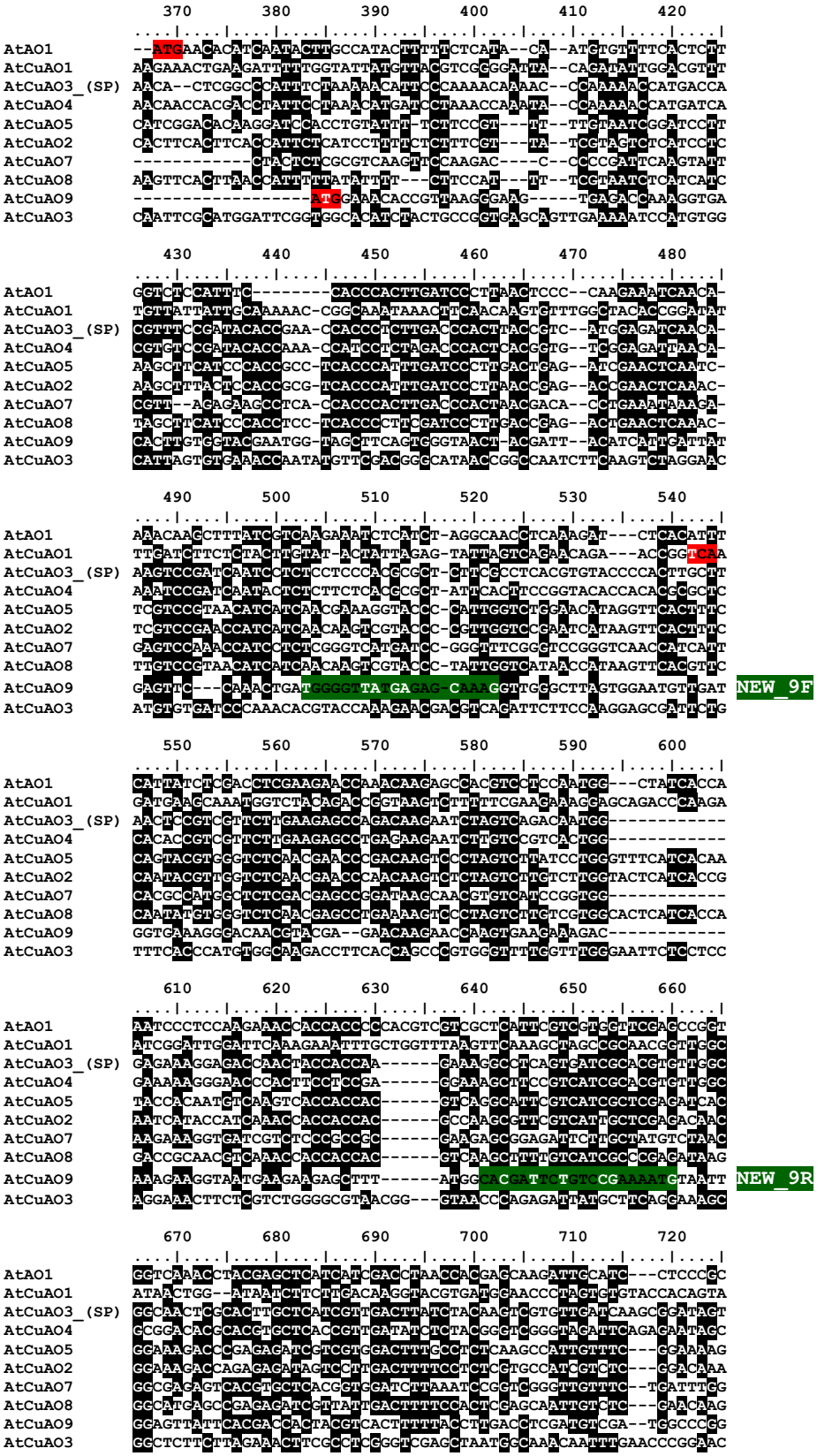
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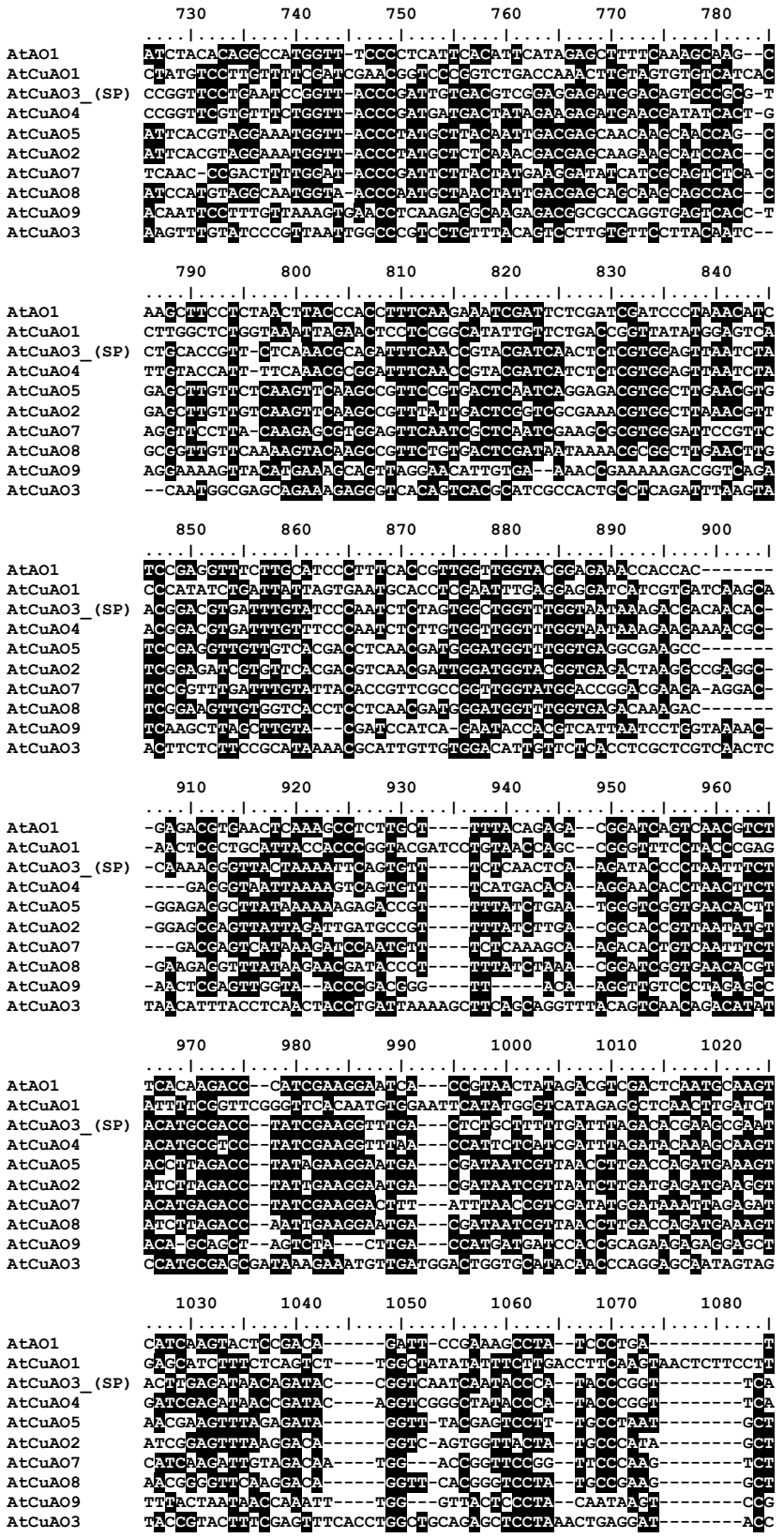
NEW_9F
NEW_9R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AtCuAO4 CCGGTTCGTGTTTCTGGTT-ACCCGATGATGACTATAGAAGAGATCAACGATATCACT-G
AtCuAO5 ATTCACGTAGGAAATGGTT-ACCCTATGCTTACAATTGACGAGCAACAAGCAACCAG--C
AtCuAO2 ATTCACGTAGGAAATGGTT-ACCCTATGCTCTCAAACGACGAGCAAGAAGCATCCAC--C
AtCuAO7 TCAAC-CCGACTTTTGGAT-ACCCGATTCTTACTATGAAGGATATCATCGCAGTCTCA-C
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AtCuAO3 AAGTTTGTATCCCCGTAATTGGCCCCGTCCTCTTTACAGTCCTTGTCTTCCTTACAATC--
790 800 810 820 830 840
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850 860 870 880 890 900
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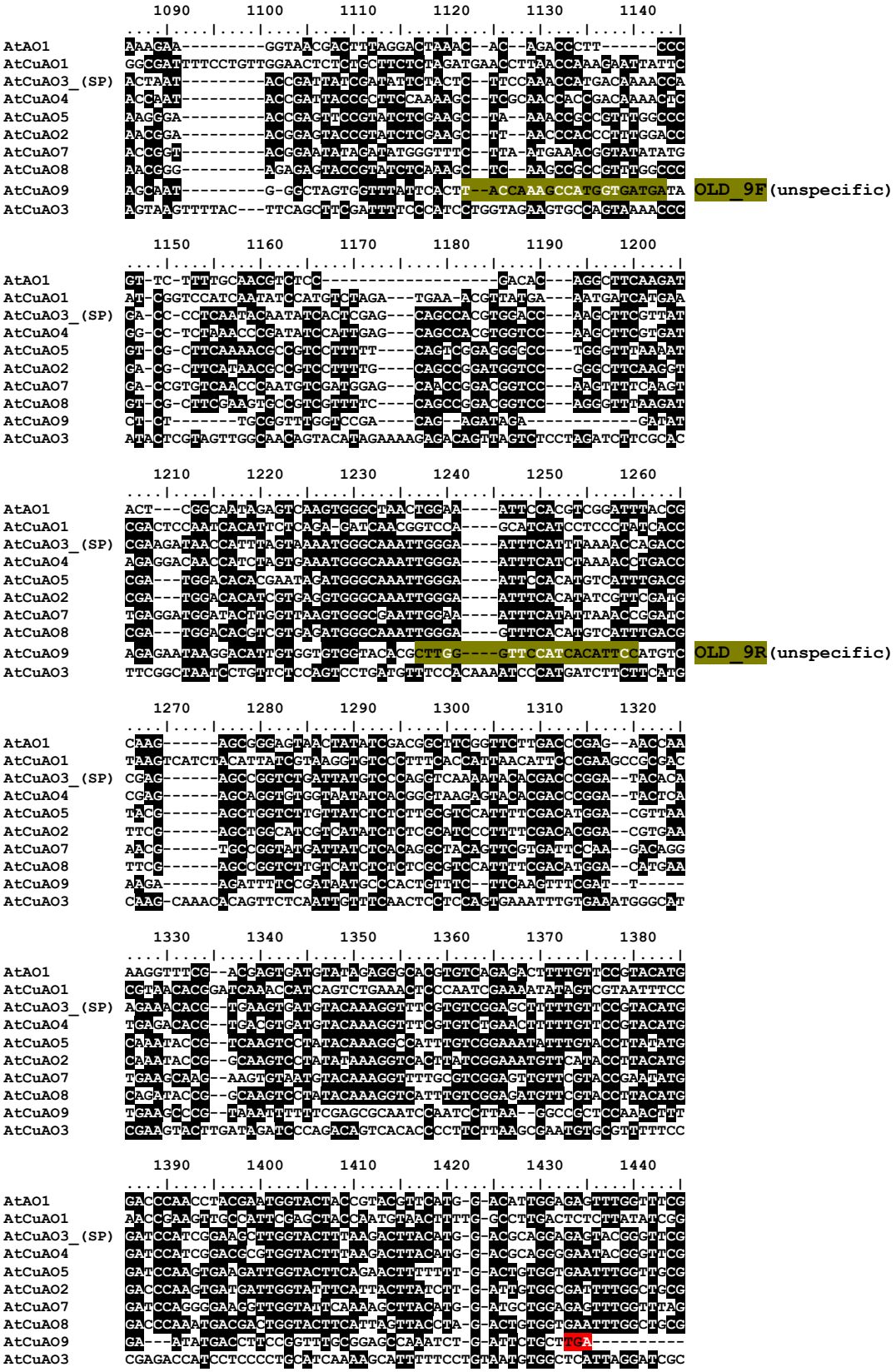
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AtCuAO8            GCCAAACTGCCGTATCCCT-----------------------------TGACCCGT
AtCuAO9            ------------------------------------------------------
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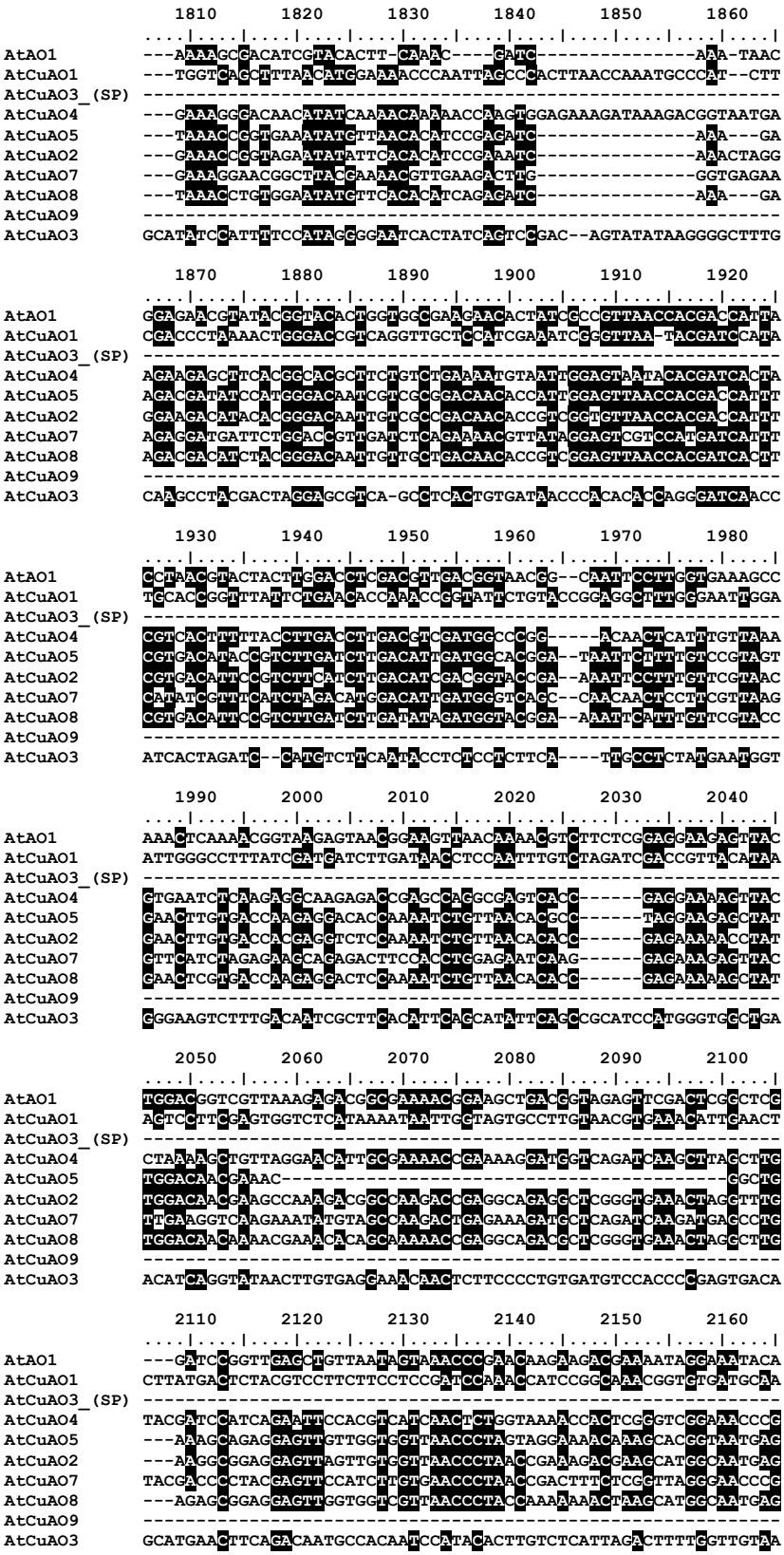
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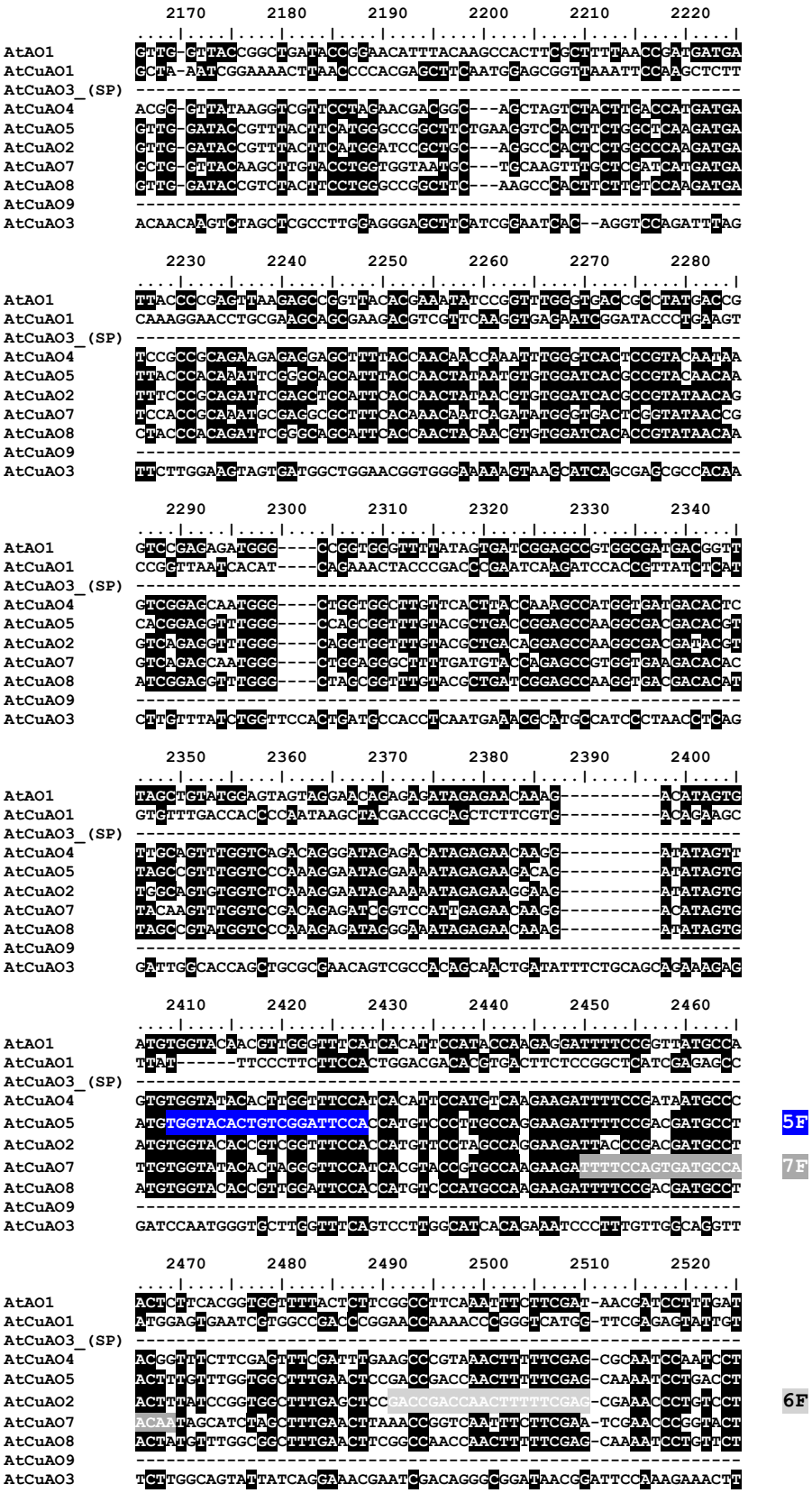
AtCuAO8 ---AGAGCGGAGGAGTTGGTGGTCGTTAACCCTACCAAAAAAACTAAGCATGGCAATGAG
AtCuAO9
AtCuAO3 GCATGAACTTCAGACAATGCCACAATCCATACACTTGTCTCATTAGACTTTTGGTTGTAA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AtCuAO8 GTTG-GATACCGTCTACTTCCTGGGCCGGCTTC---AAGCCCACTTCTTGTCCAAGATGA
AtCuAO9 ----------
AtCuAO3 ACAACAAGTCTAGCTCGCCTTGGAGGGAGCTTCATCGGAATCAC--AGGTCCAGATTTAG
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5F
7F
2470 2480 2490 2500 2510 2520
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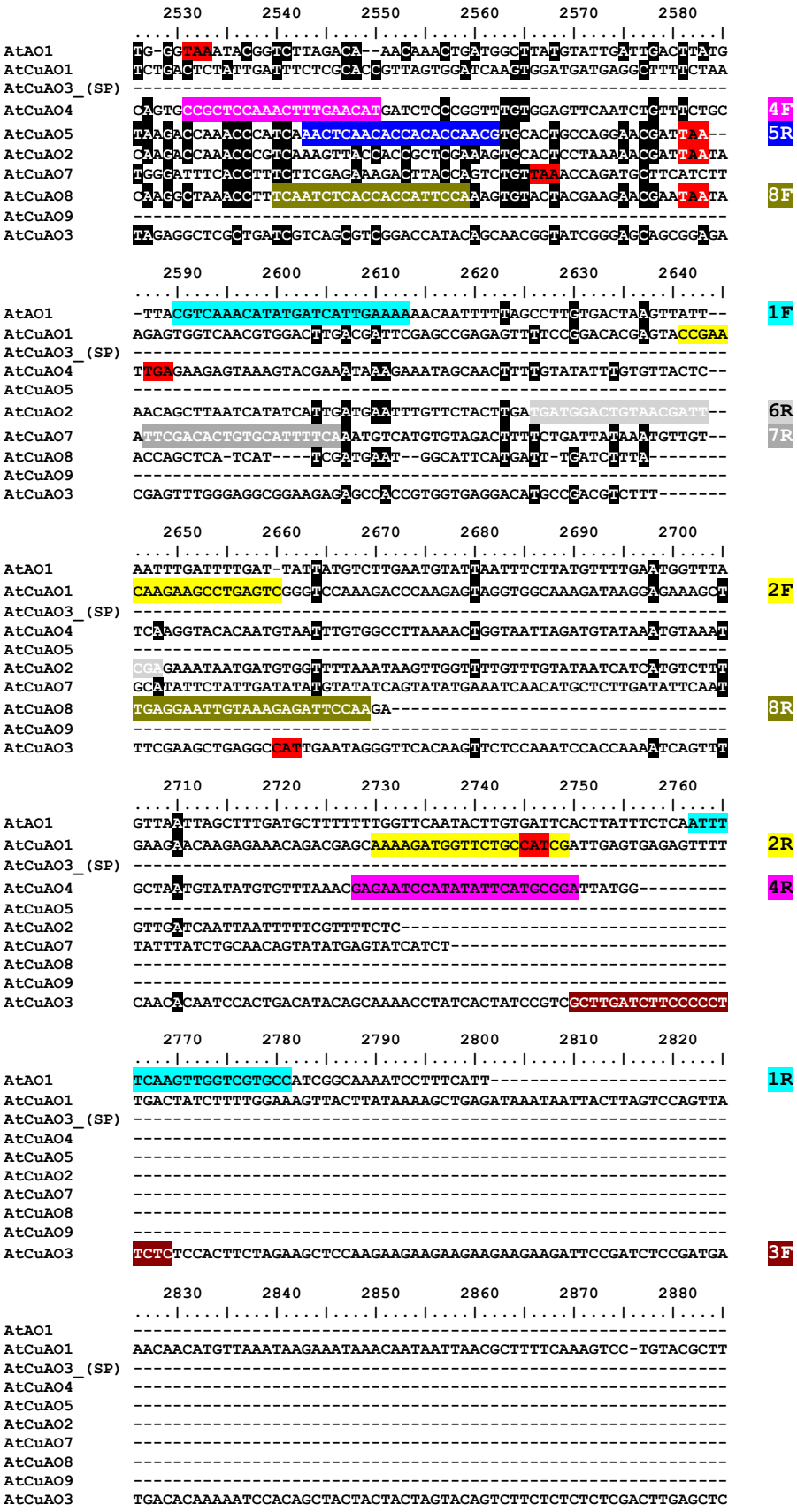
6F


2530 2540 2550 2560 2570 2580
AtAO1
AtCuAO1
AtCuAO3_(SP)
AtCuAO4
AtCuAO5
AtCuAO2
AtCuAO7
AtCuAO8
AtCuAO9
AtCuAO3
4F
5R
8F
2590 2600 2610 2620 2630 2640
1F
6R
7R
2650 2660 2670 2680 2690 2700
2F
8R
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3F
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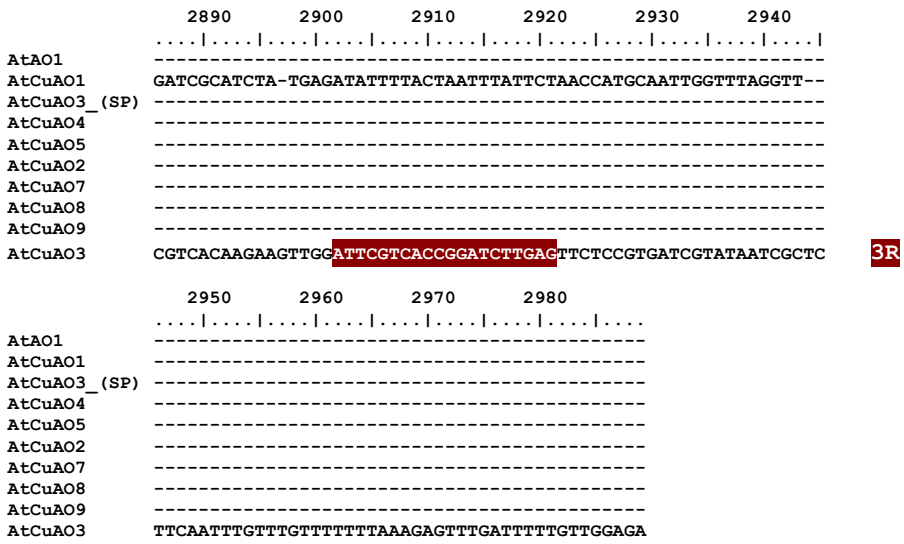
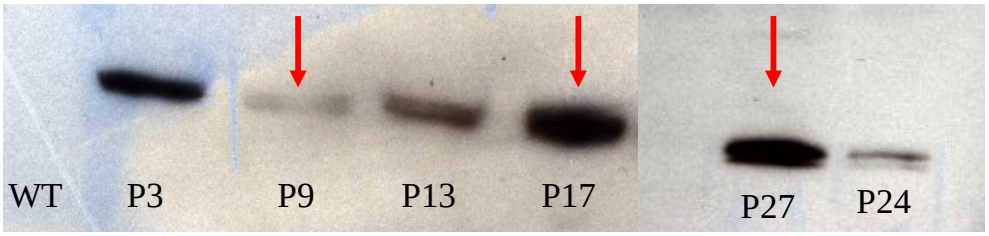


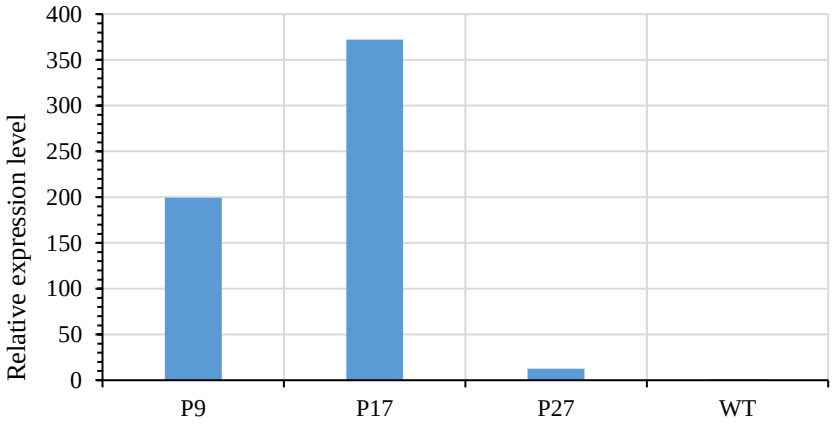

cDNA alignment of *Arabidopsis thaliana CuAO* gene family. Gene codes (TAIR; https://www.arabidopsis.org/): *AtAO1* (At4g14940), *AtCuAO1* (At1g62810), *AtCuAO3-SP* (At4g12270), *AtCuAO4* (At4g12290), *AtCuAO5* (At1g31670), *AtCuAO2* (At1g31710), *AtCuAO7* (At3g43670), *AtCuAO8* (At1g31690), *AtCuAO9* (At4g12280), *AtCuAO3* (At2g42490). Identical nucleotides are indicated by black boxes when they exist in 5 or more genes. The location of each gene primer pair is shown. Start and stop codons are high-lighted in red.

## Appendix B

**(a)**



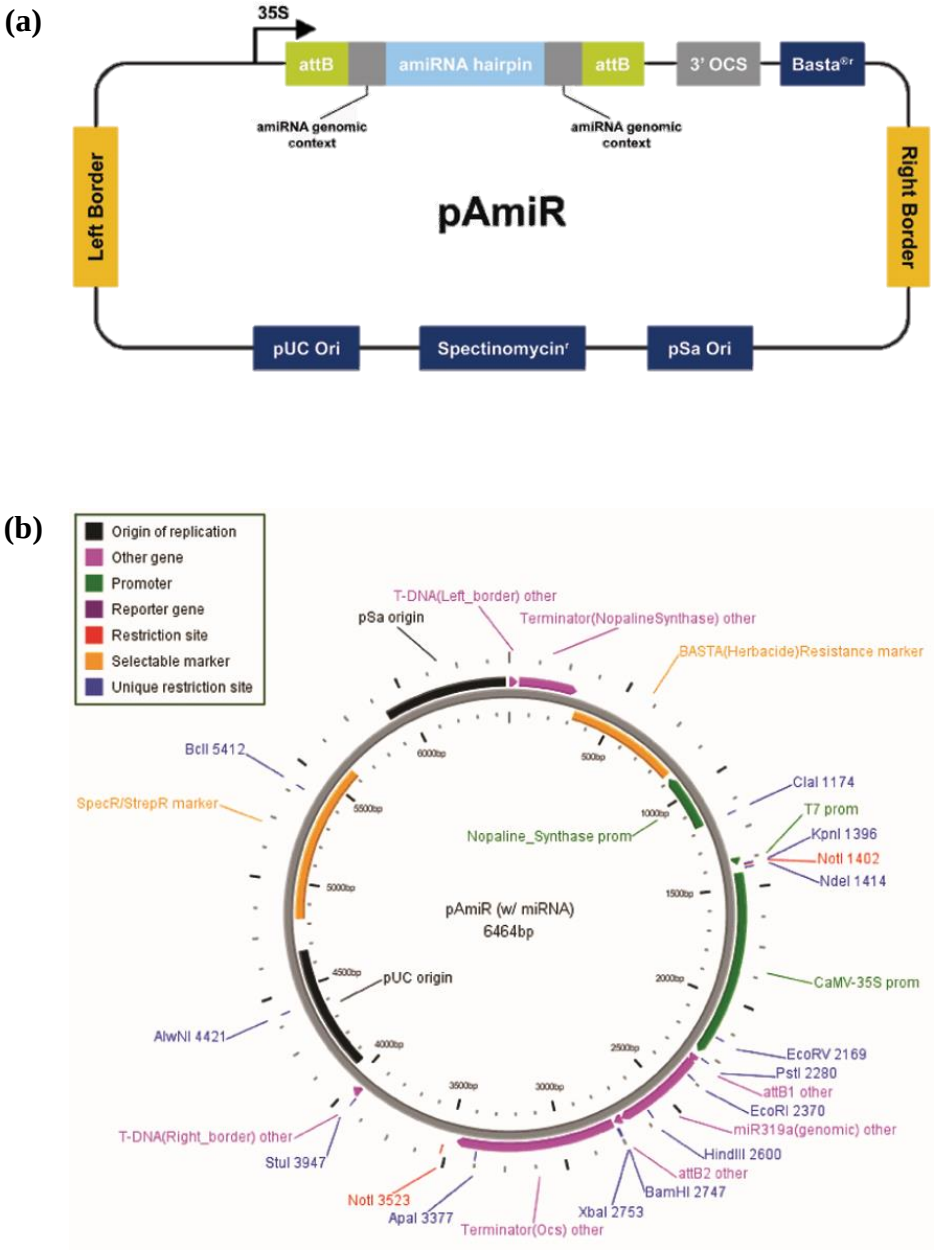

**(b)**




**(c)**

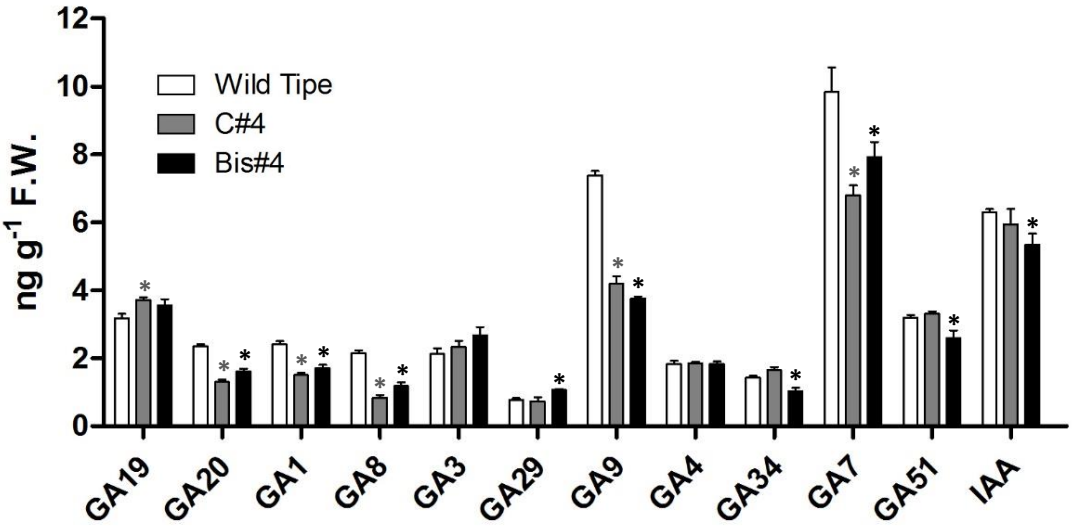

Characterization of *AtCuAO4* over-expressing plants. **(a)** Western blot analysis of crude extract from WT and over-*AtCuAO4* plants using a rabbit anti-His tag antibody (Anti-6X His tag® antibody conjugated to horseradish peroxidase, Abcam), after SDS-PAGE loaded on the basis of total protein content. Red arrows show lines used in the present work. **(b)** Overexpression level of *AtCuAO4* in overexpression lines relative to its level in WT plants assessed by quantitative real-time RT-PCR. Amongst different independent lines analyzed by both methods, line 17 showed the highest level of *AtCuAO4* expression. Presented data are provided by Prof. Alessandra Cona of Roma Tre University where these lines were generated (Ghuge 2014). **(c)** Schematic representation of the construct used for generating *AtCuAO7*::GUS and *AtCuAO8*::GUS lines. Promoter regions upstream of ATG were amplified from *A. thaliana* (Col-0) genomic DNA by PCR using sequence-specific primers and cloned initially into the pDONR221 vector, sequenced by using either a pair of external primers (*M13-for/rev*) or internal primers (*prom-int-AtCuAO for/rev*) to check possible error/ mutation. After confirmation by restriction digestion and colony PCR, promoter regions were then cloned into the pKGWFS7 destination vector upstream of the *GFP-GUS* fusion gene through the GATEWAY recombination system (Ghuge 2014).

# Appendix C




(a) pAmiR™ vector (Left Border for T-DNA boundary; 35S is a promoter for amiRNA expression in plants; AttB are Gateway® cloning sites; 3'ocs is *Octopine synthase* terminator; BASTA® resistance is a plant selection marker; pSa ori is origin of replication for *E.coli* and *Agrobacterium tumefaciens*; Right Border for T-DNA boundary; Spectinomycin resistance is a bacterial selection marker; and pUC ori is origin of replication for *E.coli*. (b) Detailed vector map of pAmiR™ vector (http://www.biocat.com/bc/pdf/Arabidopsis_manual.pdf).

# Appendix D




Quantitative analysis of endogenous gibberellins by GC-MS/MS. Data represent the average of two biological replicates. Asterisks indicate significant differences to WT. (n = 6-7; means ± SE; asterisks indicate significant differences to WT ($P \leq 0.05$) based on T-test where data were normally distributed or Mann-Whitney test where data were not). The analysis has been done by Lorenzo Mariotti group, Department of Biology, University of Pisa, Italy.

# Appendix E

# The effect of senescence related hormones and abiotic stresses on dark-induced senescence of detached leaves and rosettes of WT Arabidopsis plants

## I. Introduction

Leaf senescence is regulated by several internal and external factors such as leaf age, nutrient supply, light intensity and can be hastened via various biotic and abiotic stress factors (Smart 1994). As discussed in the General Introduction (Chapter 1), plant hormones influence the initiation and progression of leaf senescence. Some hormones, such as auxin, cytokinin, and gibberellic acid, have been reported as leaf senescence suppressors (Gan and Amasino 1995; Kim et al. 2011; Yu et al. 2009). In contrast, ethylene, abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) are considered as senescence promoting hormones (Breeze et al. 2011; Cutler et al. 2010; He et al. 2002; Reid and Wu 1992).

Exogenous application of various plant hormones also accelerates senescence (Weaver et al. 1998). ABA, methyl jasmonate (MeJA), and salicylic acid (SA) are well known as senescence-inducing hormones that regulate leaf senescence by mediating the response to environmental signals (Jibran et al. 2013). In fact, MeJA and its precursor JA were first described as senescence promoters in detached oat leaves and were then shown to be a class of plant growth regulators that have a wide range of important roles (Holbrook et al. 1997). Treating either detached or attached leaves of WT Arabidopsis exogenously with JA resulted in an increase in its level in senescing leaves and premature senescence due to the up-regulation of senescence enhanced genes, while no obvious alteration in the senescence phenotype was exhibited in plants defective in JA signalling (He et al. 2002).

ABA is a hormonal promoter of leaf senescence and exogenous treatment of plant with ABA stimulates this phenomenon (Quiles et al. 1995), however, the mechanism by which ABA regulates senescence is still not well understood (Zhang et al. 2012). Weatherwax et al. (1996) indicated that ABA concentration increases upon dark treatment in

Arabidopsis. Under stress conditions and during senescence ABA titres are dramatically increased in many plant species including Arabidopsis which suggested a central role of ABA in plant response to both processes (Buchanan-Wollaston et al. 2005; Guo and Gan 2005; Zhao et al. 2010). It has long been known that treatment of detached leaves with ABA induces several SAGs and reduces chlorophyll content (Weaver et al. 1998; Yang et al. 2002). Expression profiles of Arabidopsis senescent leaves indicate that some ABA biosynthesis and signalling genes are up-regulated during developmental leaf senescence which led to the suggestion that ABA may be part of a mechanism connecting both pathways (leaf senescence and ABA biosynthesis) and thus ABA is an important regulator of plant senescence (Buchanan-Wollaston et al. 2005).

The phenolic compound SA plays a key role in numerous plant responses to stress both biotic and abiotic, while during developmental senescence the SA-signalling pathway can control the change in gene expression (Morris et al. 2000). It has been reported that many *SAGs* are dependent on the SA-signalling pathway as revealed by the transcriptome analysis in senescing leaves of wild type Arabidopsis and SA-deficient mutants. Plants defective in SA signalling showed retardation in developmental senescence while the progression of plant senescence in the dark was normal (Buchanan-Wollaston et al. 2005). These findings suggest that the expression of one or more of senescence associated SA-dependent genes during senescence has an important function in developmental senescence while in dark-induced senescence the SA pathway is not required (Buchanan-Wollaston et al. 2005; van der Graaff et al. 2006).

On the other hand, a large number of developmental and environmental factors can stimulate leaf senescence (Lee et al. 2011) including environmental stresses such as detachment, drought and darkness (Munné-Bosch and Alegre 2004; Oh et al. 1996; Weaver et al. 1998). *SAGs*, chlorophyll loss, and yellowing in detached leaves are all induced by incubation in darkness (Weaver and Amasino 2001). Previous studies investigated the possibility of PA involvement in regulating dark induced senescence. In this regard, Legocka and Zajchert (1999) reported that incubation of barley leaf discs in the dark led to a massive accumulation of the polyamine Put, but this was associated with chlorophyll decline, increase in RNase activity and a rapid senescence. These reactions

to darkness were reversible by exogenous Spd treatment which prevented the degradation of thylakoid membranes during senescence by enhancing their stabilization through the direct interaction with them (Legocka and Zajchert 1999). The concentration of exogenous PA applied also appears to influence the result. Spm at low concentrations (1-10 mM) was more active than other PAs in preventing chlorophyll degradation in detached leaves of oat in darkness (Kaur-Sawhney and Galston 1979).

The role of PAs in leaf senescence retardation might be through blocking the conversion of SAM to ACC (aminocyclopropane carboxylic acid) and of ACC to ethylene, a promoter of leaf senescence (Kaur-Sawhney et al. 2003), or by inducing the synthesis of the DNA and mitotic activity as well as inhibiting the rise in proteases, peroxidases, and RNases (Dumbroff 1990). However, quantitation of levels of various PAs during dark induced senescence of detached rice leaves suggested that the endogenous PAs may not play an important role in the control of dark-induced leaf senescence (Chen and Kao 1991). Recently, Sobieszczuk-Nowicka et al. (2015) investigated the involvement of PA metabolism in dark-induced senescence in barley *Hordeum vulgare* L leaves and they reported an up-regulation in the expression of genes implicated in both pathways of PA metabolism, the anabolic and the catabolic, as well as an increase in the activity of enzymes implicated in the two pathways, indicating that the internal PA pool is subjected to regulation during senescence in barley. These results underline the contradictory effects of PAs in different plants.

In this section, the effect of selected stress hormones and abiotic stresses were examined on dark-induced senescence of *Arabidopsis thaliana* (Col-0) detached leaves or rosettes grown under different conditions and on different media in order to: (a) Verify effects of these treatments on the progression of the dark-induced senescence. (b) Define treatments that affect the progression of dark-induced leaf senescence which can then be used to investigate the role of *AtCuAOs* in this process through examining effects of over-expression and mutagenesis of *AtCuAO* genes.

# II. Materials and Methods

## **II**.1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) were used in all experiments in this Section. In general, unless stated otherwise in the main text, seed sterilization, stratification, sowing and growth were as described in Sections 2.1 and 2.2 in the General Materials and Methods (Chapter 1).

## **II**.2. Dark-induction protocol for Arabidopsis leaves and rosettes

Soil grown Arabidopsis plant material (leaves or rosettes with at least nine leaves) were harvested before bolting then rinsed briefly using sterile water. Plant materials were placed on a 9 cm wet filter paper in the lid of a Petri dish ensuring that rosettes or leaves would not interfere with each other or the side of the dish. Dishes were then incubated at 22° C in complete darkness inside a thick walled plastic box for quantification of dark-induced senescence of the plant materials. Growing Arabidopsis plants on soil was the best way of producing large rosettes suitable for studying dark-induced leaf senescence (Figure 1).

## **II**.3. Quantitation of senescence by image analysis

Plants were photographed every day using a web-cam (Microsoft LifeCam) attached to a computer until the rosettes became completely yellow. Photographs were analysed using the colour histogram function in Image J software to obtain the RGB score for a defined rectangle from the centre of leaf number 5. RGB intensities were normalized using a white-background reference point (Figure 1), and average green/ red ratios provided a quantitative measure of leaf yellowing where a ratio of around 0.8 indicates the initiation of senescence. Data were further analysed using an R script programme to produce values which could be then analysed statistically by SPSS.

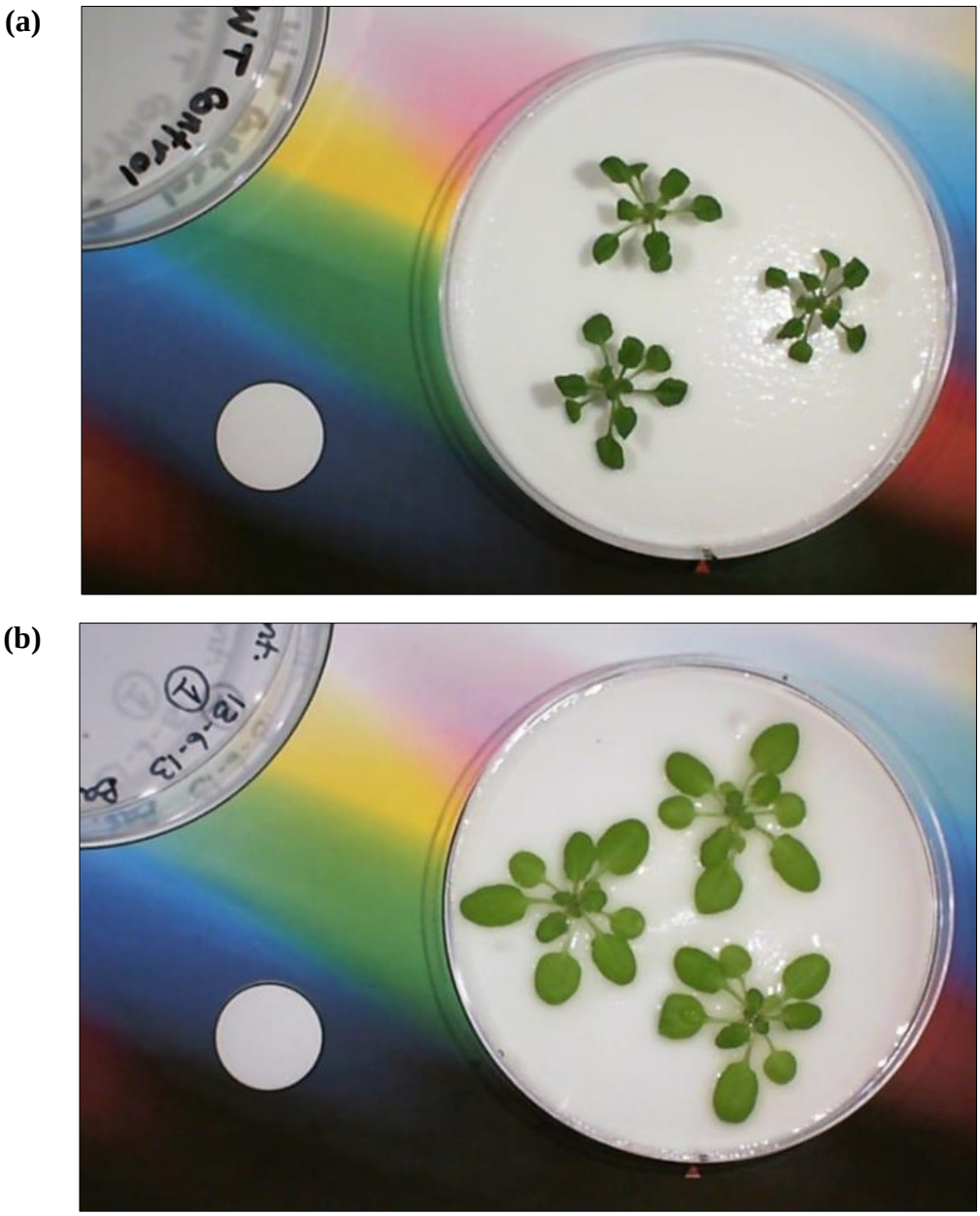



Figure 1. Comparison between plants grown on different media. (a); Rosettes of plants grown on MS medium under short days. (b); Rosettes of plants grown on soil under short days.

## **II**.4. Treatments of Arabidopsis rosettes grown in soil under long days

Rosettes were harvested 32 days after sowing (before bolting) at the base along the level of the soil then rinsed briefly in sterile water. For the hormonal treatment, rosettes (8 rosettes/ treatment; 2 per dish) were placed on a 9 cm filter paper in the lid of a Petri dish soaked with 50 µM MeJA (He et al. 2002), 500 µM Spm (Zheng et al. 2005), or water as a control. The MeJA was first dissolved in ethanol and then diluted in $H_2O$ to a final

concentration of 0.005 % ethanol which was included in the water of controls of MeJA-treated plants. Dishes were then incubated at 22° C under a 16 h photoperiod of illumination at ~68.4 µmol $m^{-2}$ $s^{-1}$ for 72 h. After the incubation period, filter papers were replaced with fresh filter papers saturated with sterile distilled water for assessment of the rosettes' response to dark induced senescence as described above in Section II.2 and II.3.

### **II**.5. Treatment of detached leaves of plants grown in soil under long days

After sowing and growing plants on soil under long day conditions as described in Section II.1, leaf numbers 5 and 6 were detached from 38 d old plants. The leaves were then rinsed briefly using sterile water and placed adaxial side up in Petri dishes containing 100 µM SA (Morris et al. 2000), 50 µM ABA (Fan et al. 1997), MeJA as described in Section II.4, Spm as described in Section II.4, or water as a control (10 leaves/ treatment). Leaves were then incubated as described in Section II.4 for 48 h with SA and ABA, and with Spm and MeJA for 72 h. The hormones SA, ABA, and MeJA were first dissolved in different concentrations of ethanol and then diluted in $H_2O$ to a final concentration of 1 %, 0.5 %, and 0.005 % ethanol respectively, therefore, these concentrations of ethanol were included in all of the treatments, including the water controls. After the hormonal treatments, the response of leaves was quantified as described in Section II.3.

### **II**.6. Treatment of rosettes grown in soil under short-days

Plants were grown in soil for 28 days under short days as described in Section 2.1 in the General Materials and Methods (Chapter 2). Rosettes were then harvested and treated with hormones as described in Section II.5 except for Spm where 350 µM (Mirza and Iqbal 1997) was used rather than 500 µM. Petri dishes containing rosettes were then incubated as described in Section II.4 for 48 h with SA and ABA, 24 h with Spm, and with MeJA for 72 h. After the hormonal treatments, rosettes were treated as described in Section II.2 and II.3 to test the effect of the hormonal treatment on dark-induced leaf senescence. Leaves 5 and 6 were sampled (6 leaves/ time point) at 0 and 6 days of dark treatment for chlorophyll analysis as represented in Section 2.15 in the General Materials and Methods (Chapter 2).

## **II**.7 Induction of senescence by darkness in rosettes grown under short-days on MS medium after pre- and post-harvest treatment with plant growth regulators

Arabidopsis seeds were surface sterilized and stratified as described in Section 2.2 in the General Materials and Methods (Chapter 2). Seeds were then sown onto Petri dishes containing MS medium as described in Section 2.1 in the General Materials and Methods (Chapter 2). For pre-harvest treatment, 500 µM Spm (Zheng et al. 2005) or 50 µM MeJA (He et al. 2002) were added to the MS medium and seedlings were left to grow in short days as described in Section 2.1 in the General Materials and Methods (Chapter 2). Rosettes were harvested 40 d after sowing and hormone treated rosettes placed onto wet filter papers as described in Section II.2 for dark induced senescence (6 rosettes/ treatment; 3 per dish). Alongside these, some rosettes were grown on MS without any hormonal supplements to be used as controls or to be treated after harvest with MeJA or Spm at concentrations similar to those used pre-harvest for 72 h in the dark as described in Section II.4. Following the post-harvest treatment with hormones, filter papers were replaced and rosettes treated as described in Section II.2. In both cases, senescence induced by darkness was quantified as described in Section II.3.

## **II**.8. Treatment of rosettes grown under short-days on MS medium after abiotic stresses

Arabidopsis seeds were sterilized and stratified as described in Section 2.2 in the General Materials and Methods (Chapter 2). Seeds were then sown onto MS medium and seedlings were left to grow under short day conditions as described in Section 2.1 in the General Materials and Methods (Chapter 2). Rosettes were harvested 29 d after sowing and divided into 4 groups (9 rosettes/ treatment; 3 per dish). The first group was placed onto wet filter papers and stored in complete darkness at 21° C as a control, the second was treated for 24 h with cold in the dark at 7° C as a short-term cold treatment, the third was placed on dry filter papers for 24 h in the dark then filter papers were wetted for a dehydration treatment, while the last group was stored on wet filter papers in complete darkness at 7° C throughout the duration of the experiment for long-term cold treatment. For dark-induced senescence assessment, rosettes were treated as described in Section II.2 and II.3.

# III. Results

## III.1. Effect of stress-related hormones on senescence of WT Arabidopsis rosettes grown on soil in long day conditions

Results indicated that treatment of wild type Arabidopsis rosettes grown in soil under long days after harvest with plant hormones such as MeJA and Spm was not effective in modulating the progression of dark-induced leaf senescence in treated rosettes compared with control plants (Figure 2) as no significant differences were seen in the progression of senescence during the experiment.

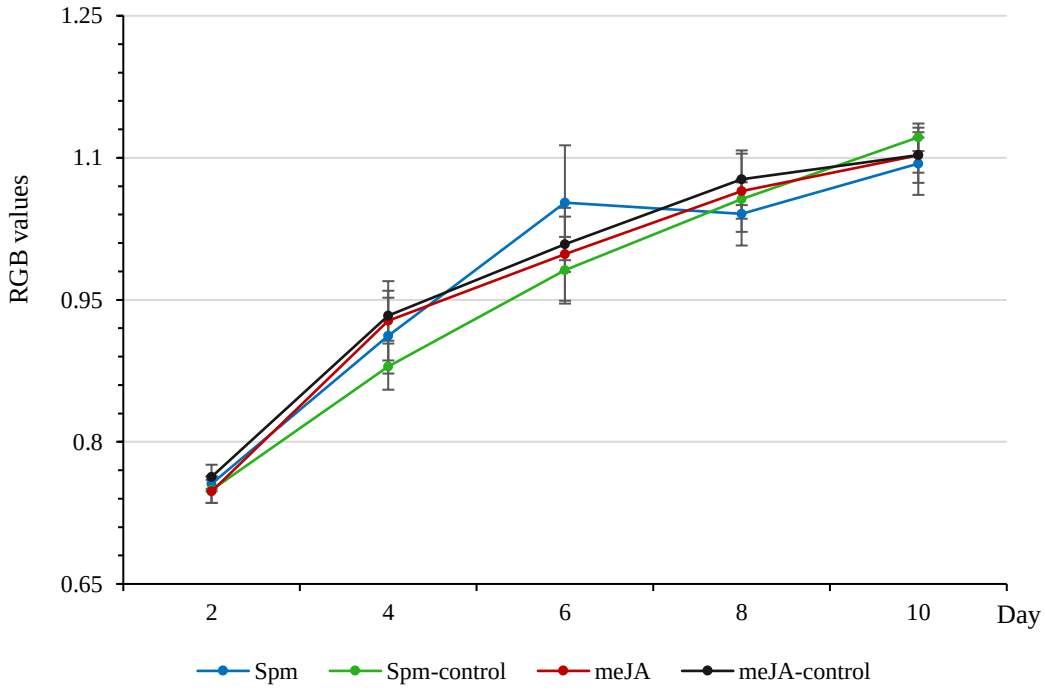



Figure 2. Dark-induced senescence in Arabidopsis rosettes grown in soil under short days after treatment with 500 µM Spm or 50 µM MeJA. Data represent mean values ± SE (n=8).

## III.2. Effect of senescence related hormones on senescence of detached WT Arabidopsis leaves

The response of detached leaves to the senescence hormones ABA, SA, and MeJA as well as the anti-senescence hormone Spm was evaluated in detached leaves (Figure 3). ABA and Spm accelerated senescence significantly as indicated by the higher RGB values. Yellowing following MeJA treatment for 72 h was also slightly higher, but this

induction was not statistically significant relative to the wild type. SA on the other hand had no effect on the progression of dark-induced leaf senescence.

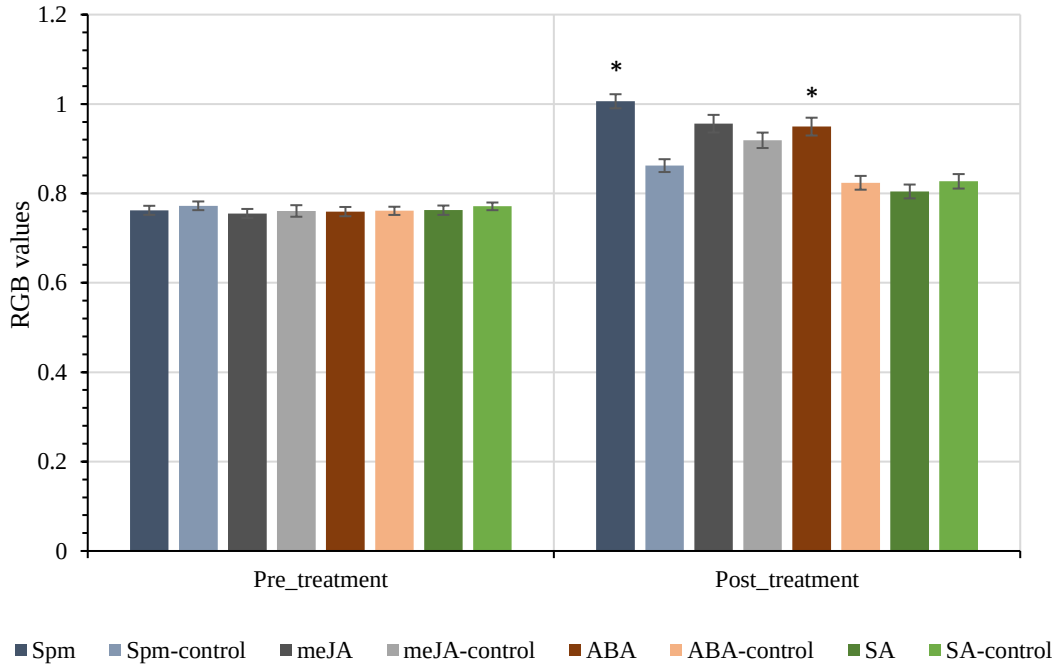



Figure 3. Dark-induced senescence of detached leaves after treatment with senescence-related hormones, 500 µM Spm for 72 h, 50 µM MeJA for 72 h, 50 µM ABA for 48 h, and 100 µM SA for 48 h, at 22° C under a 16 h photoperiod of illumination. Pre-treatment refers to pre-incubation with different hormones. Data represent mean values ± SE (n=10). * $P \leq 0.05$ compared to the respective control.

## III.3. Effect of stress-related plant hormones on dark-induced senescence of WT Arabidopsis rosettes grown in soil under short days

The response of rosettes grown on soil in short days to post-harvest treatments with senescence-promoting hormones, MeJA, ABA and SA, and anti-senescence hormone Spm was evaluated (Figure 4). Exposing rosettes to MeJA accelerated yellowing and this acceleration was significant at several time points (Figure 4.a). On the other hand, ABA and Spm retarded senescence, although this delay was only significant at the sixth and the eighth day respectively of the dark period (Figure 4.b and c). In contrast, SA treatment did not significantly affect the progression of rosette senescence (Figure 4.d).

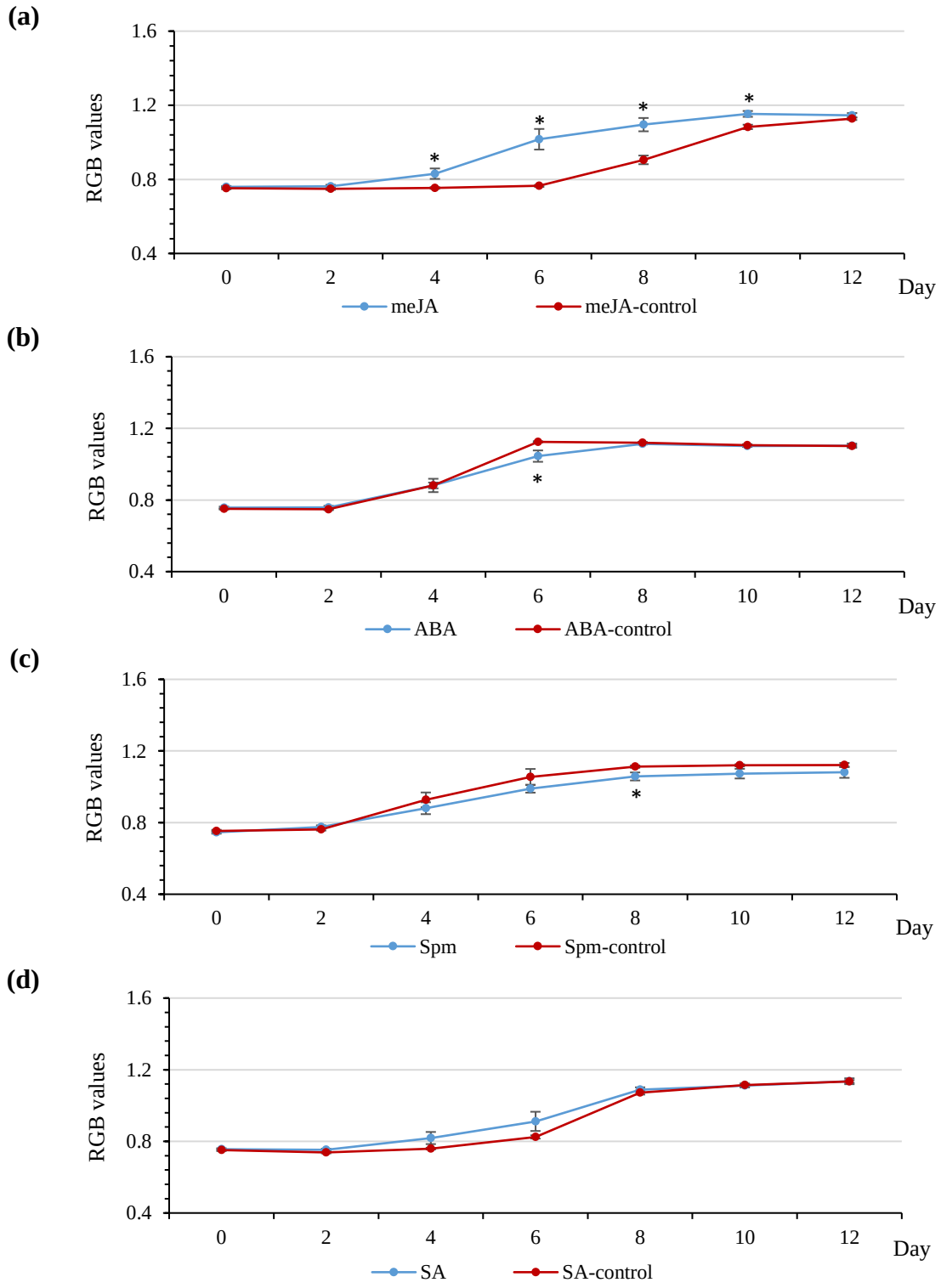



Figure 4. Dark-induced yellowing in rosettes of Arabidopsis upon post-harvest treatment with senescence related hormones. (a) 50 µM MeJA for 72 h, (b) 50 µM ABA for 48 h, (c) 350 µM Spm for 24 h, and (d) 100 µM SA for 48 h. Plants were imaged every 2 days during dark incubation to obtain RGB values until leaves became completely yellow. Data represent mean values ± SE (n=9). * $P \leq 0.05$.

Chlorophyll content as a senescence parameter was determined at the beginning and 6 days after incubation in the dark (Figure 5). At the beginning of dark induction no significant differences in chlorophyll levels were observed between hormone-treated plants and their controls (Figure 5.a). On the sixth day of the dark period, plants treated with ABA and Spm retained higher levels of chlorophyll a, b and the total chlorophyll compared with control plants. In contrast, treatments with either MeJA or SA reduced leaf contents of chlorophyll slightly but this reduction was statistically not significant compared to the control plants (Figure 5.b).

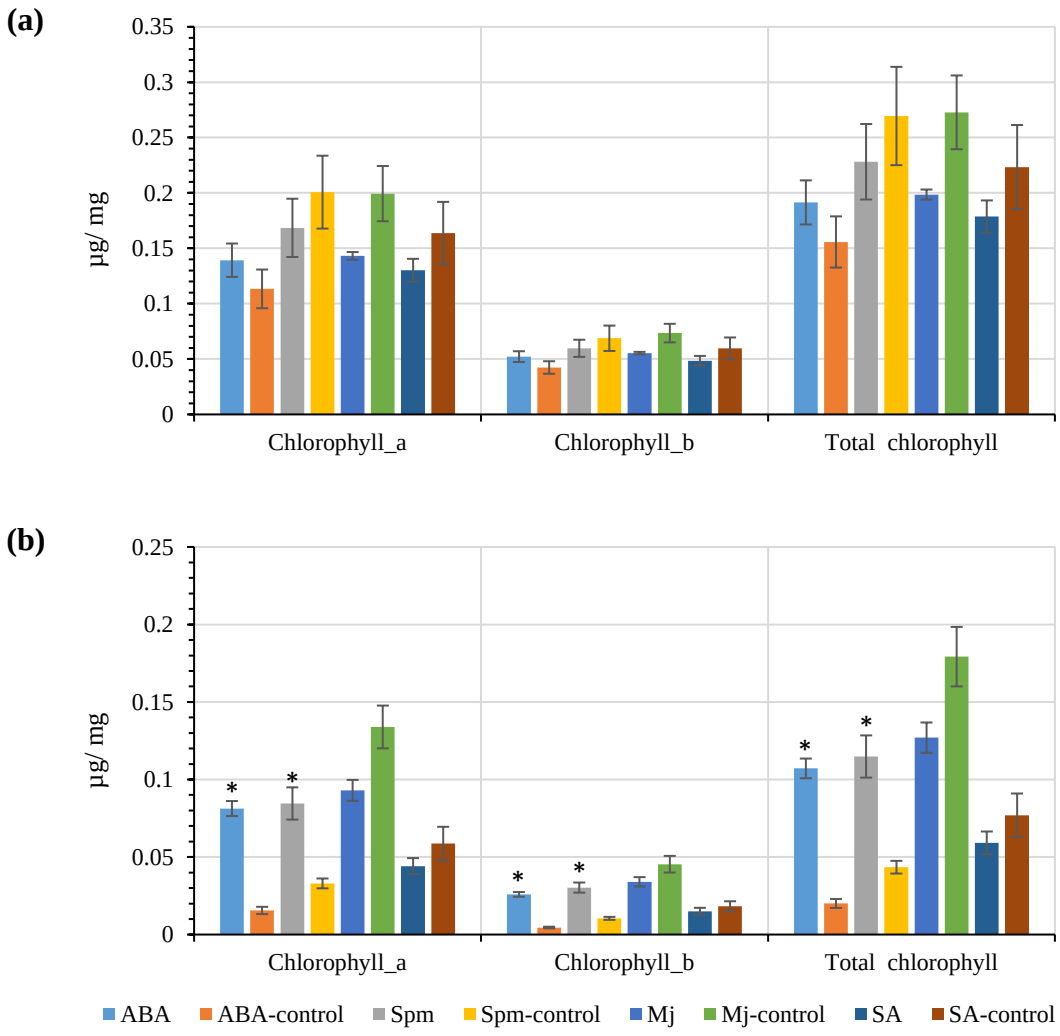



Figure 5. Comparison of chlorophyll content in hormone-treated and untreated plants during dark induced senescence. (a) Chlorophyll levels in leaves at the onset of darkness period. (b) Chlorophyll contents in treated and untreated leaves after 6 days of incubation in the dark. Data represent mean values ± SE (n=6). * $P \leq 0.05$.

## III.4. Effect of pre- and post-harvest treatment with senescence related plant hormones on dark-induced senescence of WT Arabidopsis rosettes grown under short-days on MS medium

Treatment with MeJA during growth produced very small rosettes. They were therefore excluded from the rest of the experiment. Treating rosettes grown in short days with MeJA after harvest accelerated senescence as shown in (Figure 6.a) where it is clearly seen that after day 3 the RGB value increased and at day 5 was significantly higher in the MeJA treated rosettes compared with the control rosettes. Growing seeds on MS medium containing Spm caused a marked retardation in leaf senescence (Figure 6.b) which was obvious early during the dark period (day 1, 3 and 5), however later, the RGB values of Spm-pre-harvest treated rosettes were slightly higher than those of untreated ones (although not significantly). There was possibly a very small delay in yellowing observed as a result of post-harvest treatment with Spm relative to control throughout the dark treatment but this reduction was not significant.

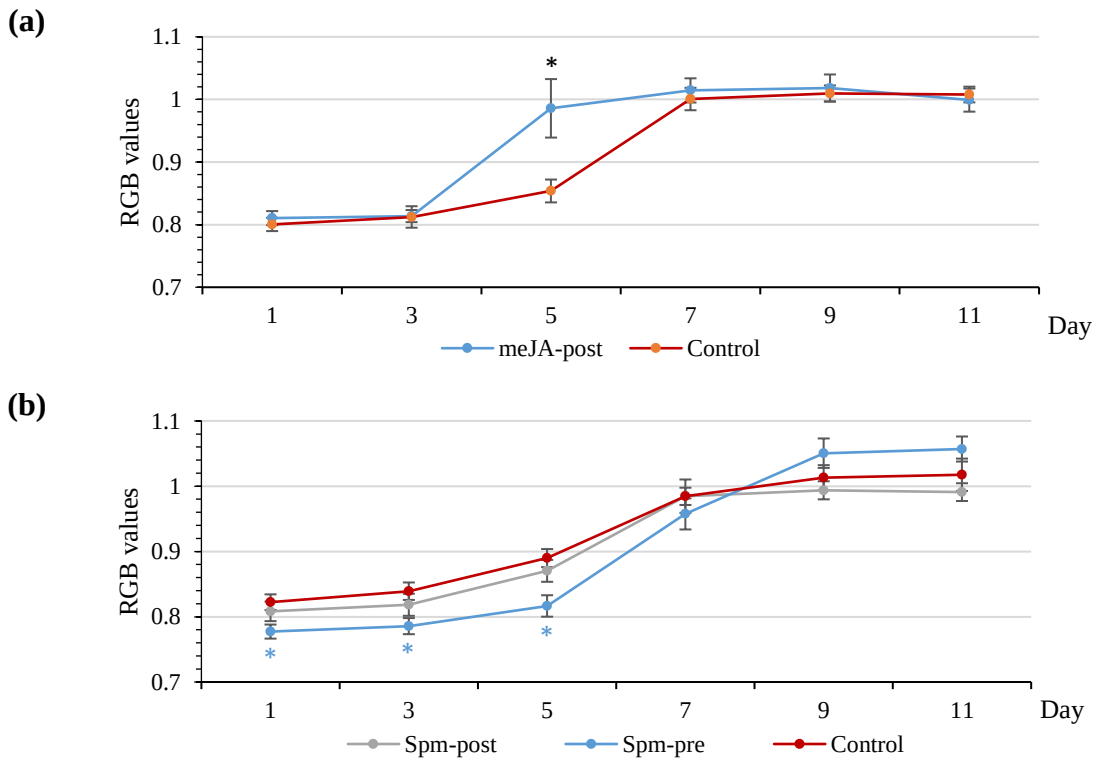



Figure 6. The effects of pre- and post-harvest treatment with senescence related hormones on the progression of dark-induced senescence. Wild type Arabidopsis rosettes grown on MS medium under short-days were treated post-harvest with 50 µM MeJA (a), or pre- and post-harvest with 500 µM Spm (b). Data represent mean values ± SE (n=6). * $P \leq 0.05$.

### III.5. Effects of abiotic stresses on dark-induced senescence of WT Arabidopsis rosettes grown under short-days on MS medium

There was an acceleration in leaf yellowing due to short-term cold and 24 h dehydration treatments compared to the control which was significant only with the drought at 23, 29 and 31 d during the dark period (Figure 7). In contrast, long-term cold treatment abolished the dark-induced senescence in rosettes which remained green until the end of the experiment (41 days).

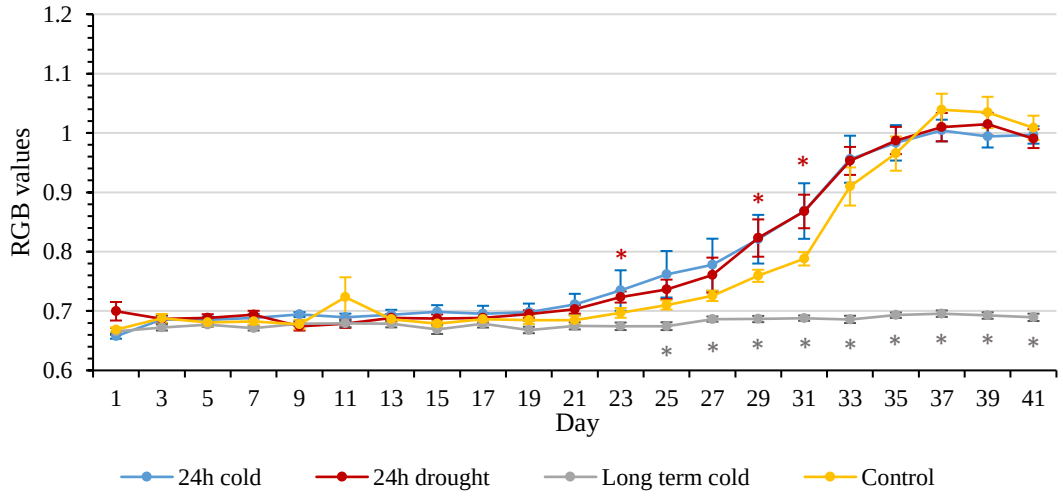



Figure 7. Effects of abiotic stresses (short-term cold, dehydration, and long-term cold) on dark-induced senescence of wild type Arabidopsis rosettes grown on MS medium under short-days. Data represent mean values ± SE (n=9). * $P \leq 0.05$.

## IV. Discussion

The process of senescence can be recognized by a visible leaf yellowing, chlorophyll degradation, sharp decline in photosynthesis capacity, and a decline in total protein and RNA contents (Mishina et al. 2007). In the present work, the effect of selected plant hormones and environmental stresses on dark-induced leaf senescence was examined via changes in leaf colour and in some cases by measuring the variation in chlorophyll, using Arabidopsis plants grown on different media under different growth conditions. In addition, role of *AtCuAO4* in the progression of dark-induced leaf senescence was investigated.

Photoperiod is one of the most important factors which affect the onset of plant flowering. *Arabidopsis thaliana* is a long day plant that flowers rapidly in long days (16 h light, 8 h dark), while short days (8 h light, 16 h dark) postpone flowering in this plant (Martinez-Zapater and Somerville 1990). In many species of annual plants, the reproductive phase is accompanied by the onset of leaf senescence (Hinderhofer and Zentgraf 2001; Ye et al. 2000). In general, results of hormone treatments of plants grown under long day conditions were contradictory and disagreed with results of previous studies where PAs are known to be inhibitors of leaf senescence (Kaur-Sawhney et al. 1982), while MeJA is a promoter of senescence in Arabidopsis attached and detached leaves (He et al. 2002). This could be due to a potential confounding influence of other hormonal pathways induced as a result of the transition to flowering. Growing plants under short day conditions, on the other hand, either on soil or MS media showed more consistent results which is better for understanding the effect of different hormonal treatments on the progression of dark-induced leaf senescence. The medium used to grow plants prior to senescence-induction was also found here to be critical. Growing Arabidopsis plants on soil under short days was more effective in producing rosettes of larger size which were more suitable for studying the progression of dark-induced leaf senescence by measuring the degreening or yellowing using ImageJ analysis.

In general, results of the present experiments indicated that the polyamine Spm has a variable influence on dark-induced Arabidopsis leaf senescence ranging between retardation and stimulation or has no clear effect. A retardation effect would be in agreement with a previous report on the application of Spm to detached oat leaves (e.g. Kaur-Sawhney and Galston 1979). The variation in Spm effects seen here might be attributed to its concentration or to plant growth conditions. Plants grown under short days either in soil or on MS media exhibited delayed dark-induced leaf senescence following treatment with 350 or 500 µM Spm which was characterized by measuring chlorophyll content (Figure 5) and colour change (progression of yellowing) (Figure 4.c and Figure 6.b). Hence, plant growth conditions that affect bolting time and thus flowering is the most probable reason for variations in the effect of Spm treatments in the present experiments.

In all the experiments described here, MeJA hastened leaf senescence and this effect was statistically significant when plants were grown under short day conditions prior to treatment with the MeJA. This effect of MeJA in hastening leaf senescence is consistent with the previous studies (He et al. 2002). On the other hand SA had no clear influence on senescence under any of conditions used here, again in agreement with the lack of an SA regulated pathway in dark-induced senescence (Buchanan-Wollaston et al. 2005; van der Graaff et al. 2006). Investigating the effect of treatments with ABA on leaf senescence revealed a complex pattern that depended on whether plants were grown under long or short days. Leaf senescence was accelerated upon ABA application when treated leaves were from plants grown in long days which is consistent with previous findings (Buchanan-Wollaston et al. 2005; Oh et al. 1996; Sharabi-Schwager et al. 2010), while, treatment of short-day rosette leaves showed late senescence in the dark.

Experiments studying the response of leaf senescence to various environmental stresses during dark incubation of rosettes revealed that dehydration was the only treatment that markedly accelerated senescence. Water stress is a major environmental factor that affects plant growth and development and causes physiological and developmental changes in plants by inducing their genes which in their turn contribute to stress tolerance (Bray 1997; Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 2000; Simpson et al. 2003). Likewise, dehydration treatment of Arabidopsis detached rosettes in dim light induced the expression of about half the SAGs surveyed (Weaver et al. 1998). Exposing rosettes to short term cold (24 h) did appear to accelerate leaf senescence, but the effect was too small to be significant, whereas, long-term cold (throughout the experiment) showed a considerable delaying of leaf senescence during dark incubation. Retardation of leaf senescence by long-term cold is in agreement with several previous studies which confirmed the importance of storage of leafy vegetables at low temperature in reducing chlorophyll loss which contributes to extending the shelf life of produce (Bergquist et al. 2007; Ferrante and Maggiore 2007; Kramchote et al. 2012; Prabhu and Barrett 2009).

# Appendix F

## List of Symbols

| *Symbol* | *Name in full* |
|---|---|
| **cm** | Centimetre |
| **g** | Gram or relative centrifugal force (context specific) |
| **GC-MS/MS** | Gas chromatography/ mass spectrometry |
| **h** | Hour |
| **L** | Litre |
| **M** | Molar |
| **µAU** | Micro-absorption units |
| **mg** | Milligram |
| **µg** | Microgram |
| **min** | Minutes |
| **ml** | Millilitre |
| **µl** | Microliter |
| **µm** | Micrometre |
| **mM** | Millimolar |
| **µM** | Micromolar |
| **mm** | Millimetre |
| **µmol $m^{-2}$ $s^{-1}$** | Micromoles per square meter per second |
| **nmol** | Nanomole |
| **nM** | Nanomolar |
| **pH** | Potential of hydrogen (The molar concentration of hydrogen ions in the solution) |
| **s** | Second |
| **v/ v** | Volume to volume |
| **w/ v** | Weight to volume |
| **° C** | Degree Celsius |
| **$\chi 2$** | Chi-squared |