Transcription Factor Networks Regulating *SAG21*: An Arabidopsis Gene At The Interface Between Stress And Senescence

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By

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ABSTRACT

SAG21/AtLEA5 is an *Arabidopsis thaliana* gene belonging to the late embryogenesis associated (LEA) protein family. Prior research identified tissue specific, spatial and temporal expression of the *SAG21* promoter. A 1685 bp upstream region of *SAG21* contains *cis* elements that act as binding sites for several transcription factor families. The aim here was to investigate the transcription factor network regulating *SAG21* in response to senescence, development and stress responses. To investigate if transcription factors identified in a yeast-1-hybrid screen regulate *SAG21*, expression of *SAG21* was analysed in transcription factor mutants of *WRKY 15, 63* and *67* under abiotic stresses known to elicit *SAG21* expression. *WRKY15* was not essential for induction of the *SAG21* expression. *WRKY63* functioned as a negative regulator of the *SAG21* expression under drought stress. *WRKY67* may act as a positive or negative regulator of *SAG21* under salt stress. SAG21 promoter deletion-GUS reporter constructs were used to understand the role of the *cis*-elements in regulating senescence and wounding responses. *SAG21* showed different expression patterns in floral organs of the deletion constructs. The role of kinetin in regulating senescence and wounding responses was studied in young and older cotyledons and in four-week old wounded leaves. Kinetin inhibited senescence-related and wound-induced *SAG21* expression. Induction of *SAG21* expression by wounding was dependent on the age of wounded rosette. Over-expression of *SAG21* under its own promoter in optimal conditions produced similar primary root length, more lateral roots, and greater lateral root length compared to overexpression of *SAG21* from the 35S constitutive promoter. Under oxidative stress *SAG21* transgenic over expressors lines produced increased primary root length under all concentrations of H_2O_2 whereas the number of lateral roots and length of lateral roots was greater on lower concentrations of H_2O_2.Together, these data suggest effects of specific *SAG21* promoter regions on development and stress responses.

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List of Acronyms and abbreviations

Abbreviation	*Name or term in full*
ABA	Abscisic acid

ABRE	ABA responsive element
ACC	1-Aminocyclopropane-1-carboxylic acid
AD	Activation domain
AHK3	Arabidopsis HIS Kinases
amiRNA	Artificial micro RNA
APX	Ascorbate peroxidase
AP2/ERF	APETALA2/Ethylene responsive transcription factor
ARCs	Age related changes
ARR2	Type-B cytokinin response regulator
AUX	Auxin influx carrier gene
BASTA	Glufosinate ammonium-PESTANAL®
bHLH	Basic helix-loop-helix transcription factor family
BSA	Bovine serum albumin
bZIP	Basic leucine zipper transcription factor family
CAB	Chlorophyll b binding protein
CAT	Catalase enzyme
CAMs	Calmodulin
CaMV	Cauliflower mosaic virus
CBF	C-repeat/dehydration responsive element binding factor
cDNA	Complementary deoxyribonucleic acid

CDPKs	Calcium dependent protein kinase
COI1	Coronatine insensitive
CRE	Cis regulatory element
CRF6	Cytokinin response factor
CRT	C-repeat/cold responsive regulatory element
CUC2	Cup-shaped cotyledon
DAMPs	Damage associated molecular patterns
DAS	Days after sowing
DHAR	Dehydroascorbate reductase
DHE	Dihydroethidium fluorogenic dye
DNA	Deoxyribonucleic acid
dNTP	Deoxy ribonucleotide triphosphate
DOF	One-finger transcription factor family
DPI	Diphenyiodidium
DRE	Drought responsive element
DTT	Dithiothreiol
EDTA	Ethylene diamino triacetic-acid
EMSA	Electrophoretic mobility shift assay
ET	Ethylene
EtBr	Ethidium bromide
ETOH	Ethyl alcohol

GA	Giberellic acid
gDNA	Genomic DNA
GFP	Green-Fluorescent protein
GR	Glutathione Reductase
GRAVY	Grand average Hydropathy plot
GSH	γ-Glutamyl-Cysteinyl-Glycine
GUS	β-Glucuronidase reporter gene
H_2O_2	Hydrogen Peroxide
IPT	Iso pentyl transferase gene
JA	Jasmonic acid
JAZ	Jasmonate ZIM-domain
KCl	Potassium chloride
KD	Knockdown lines
KO	Knockout lines
LB	Luria-Bertani broth medium
LD	Long-Day conditions
LEA	Late Embryogenesis Protein
LTRE	Light regulated responsive element
LUX	Auxin influx carrier gene
MAPK	Mitogen activated protein kinase

MAMPs	Microbial associated molecular patterns
MCP	1-Methylcyclopropene
MDHAR	Mono dehydroascorbate reductase
MEKK1	Mitogen activated protein kinase kinase kinase-1
MKK	Mitogen activated protein kinase kinase
MPK	Mitogen activated protein kinase
MYB	Myeloblastosis family of transcription factors
MYC	Myelocytomatosis
NAA	1-Naphthaleneacetic acid
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NAM	No apical meristem
NaOH	Sodium hydroxide
NOXs	NADPH oxidases
NPA	Naphthylpthalamic acid
NPR1	Non expressor of Pathogenesis related genes
ORF	Open reading frame
OXI1	Serine/threonine-protein kinase gene
PCD	Programmed cell death
PCR	Polymerase chain reaction
PET	Photosynthetic electron transport

RAB	Rav-associated protein
RBOH	Respiratory burst oxidase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-biphosphate carboxylase oxygenase
qRT-PCR	Quantitative reverse transcriptase PCR
SA	Salicylic acid
SAGs	Senescence associated genes
SAM	Shoot apical meristem
SD	Short-day conditions
SDW	Sterile distilled water
SE	Standard error
SMP	Seed maturation protein
SOD	Superoxide dismutase
TAE	Tris acetic acid
T-DNA	Transferred deoxyribonucleic acid
TFs	Transcription factors
TFBs	Transcription factor binding site
WT	Wild type
X-GlucA	5-bromo-4chloro-3-indoyl- ß-D-glucoronic acid.
YEB	Yeast extract broth medium

Y1H	Yeast one hybrid
Y2H	Yeast two hybrid

List of symbols

Symbol	Name in full
bp	Base pair
cm	Centimetre
° C	Degrees Celsius
gm	Grams
g/L	Gram per litre
hr	Hours
Kb	Kilobase
L	Litre
min	Minutes
µl	Microlitre
ml	Millilitre
M	Molar
µM	Micromolar
mM	Millimolar
mg	Milligram

µg	Microgram
µmolm-^{2}sec^{-1}	Micromoles per square metre per second
MgSO4	Magnesium Sulphate
$MgCl_2$	Magnesium Chloride
n	Number of replicates
ng	Nanogram
nM	Nanomolar
rpm	Revolutions per minute
sec	Seconds
v/v	Volume to volume
w/v	Weight to volume

Chapter1: General Introduction

Environmental stresses like drought, heat and cold affect crop yield and productivity by changing metabolic homeostasis and modifying the source sink relationship in plants (Albacete et al., 2014; Sade et al., 2018). Both biotic and abiotic stress can induce premature leaf senescence and yellowing, and also reduced photosynthetic activity because of the accumulation of assimilates in source leaves due to decreased consumption in sink tissues leading to photosynthesis feedback inhibition (Albacete et al., 2014).There is substantial overlap in genes that are regulated by stress and during senescence(Sade et al.,2018b ;Sade et al., 2017) and the signalling networks that regulate them. Genes which are upregulated in senescing leaves are termed as senescence associated genes (SAGs) (Lim et al., 2003) although many of them are also upregulated by stress. *SAG21*, the focus of this thesis is up-regulated by many different stresses and in early senescence (Mowla et al., 2006; Salleh et al., 2012; Weaver et al.,1998)

1.1 Senescence

Leaf Senescence is a developmentally programmed degeneration process, which constitutes the final stage of leaf development (Fischer, 2012; Woo et al., 2013). Generally, senescence refers to the process which leads to the death of a cell, an organ or a whole plant (Lim et al., 2003). Regulation of the senescence process involves the integration of the environmental and developmental signals. During plant development age-related changes like the end of the cell division process, leaf expansion, and accumulation of ROS takes place (Jibran et al., 2013). Leaf senescence depends on age related changes (ARCs), and young leaves are insensitive to signals that induce senescence. As the leaf matures, due to the age -related changes, it is able to respond to senescence inducing signals. This is supported by a study in Arabidopsis showing that senescence cannot be induced until a certain leaf developmental age is reached (Grbic & Bleecker, 1995). In older leaves as the age-related changes occur, senescence is induced despite the environmental signals (Jibran et al., 2013).

Transcription factors like NACs, WRKYs, AP2/EREBP and bZIP play an important role in the regulation of the age induced senescence in Arabidopsis (Breeze et al., 2011).

Understanding of senescence has been achieved by studying various mutants, and senescence associated gene expression, mainly in the model plant *Arabidopsis thaliana* which has revealed the role of regulatory factors and networks involved. Generally, plants show two forms of senescence- replicative (mitotic) and post mitotic senescence. Replicative senescence refers to of the loss of a cell's ability to divide upon aging. Post mitotic senescence is a degenerative process which occurs after maturation and leads to cell death (Weaver et al.,1998).Theoretically, senescence is usually divided into three phases-1) an initiation or signalling phase 2) an execution phase-where nutrient remobilization occurs and 3) a final or terminal phase- during which cell organization and breakdown takes place leading to cell death (Lim et al., 2003).

A number of complex processes occur during senescence including protein degradation, lipid peroxidation, chlorophyll degradation, mobilization of nitrogen and nutrients. However, this is not a passive process, and during senescence cells of the leaf display an orderly changes in cell structure, metabolism and gene expression. The key change in cell structure is the breakdown of the chloroplast organelle which contains 70% of leaf protein. This occurs via changes in grana structure and content and the formation of lipid droplets called plastoglobuli. During this process, there is a loss of photosynthetic proteins such as RUBISCO, and CAB (chlorophyll binding protein) (Hörtensteiner & Feller, 2002; Lim et al., 2007). However, mitochondria and the nucleus, that are required for energy and gene expression respectively stay intact until the last stage of senescence. Cellular biochemical changes are then followed or preceded by reduced anabolism and a reduction in the total cellular content of ribosomes causing a reduction in protein synthesis and RNA levels (Lim et al., 2007) In Arabidopsis and tobacco, during the final stage of senescence, symptoms of programmed cell death (PCD) like vacuolar collapse and chromatin condensation are seen in naturally senescing leaves (Lim et al., 2007)

Leaf senescence is accompanied by global changes in gene expression. Using differential screening and subtractive hybridization of cDNA libraries, studies have found that expression of some genes is up regulated by senescence and other genes are down regulated. For example, expression of genes related with the photosynthetic activity are down-regulated (Breeze et al., 2011) while genes involved in protein degradation, nucleic acid breakdown, lipid remobilization and N2 remobilization are upregulated. The timing of leaf senescence is influenced by multiple internal and environmental signals, including both biotic and abiotic stresses. Abiotic stresses include nutrient deficiency, shading, and exposure to ozone, while biotic stresses inducing senescence are mainly caused by pathogen infection. These environmental factors are mediated by internal signals including several phytohormones (Figure 1.1).

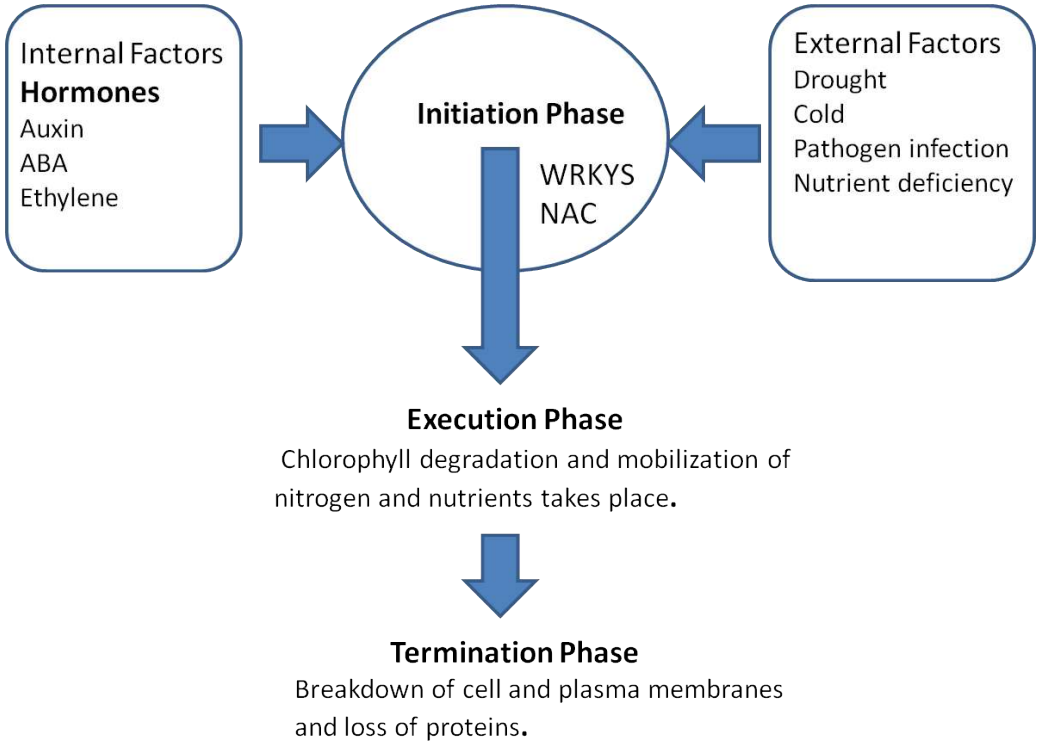


Figure 1.1: A model showing the different phases of leaf senescence and the action of internal and external factors leading to cell death. Adapted from (Lim et al.,2007).

Cytokinin and ethylene are the most studied hormones in senescence regulation, however, other hormones such as salicylic acid (SA) and jasmonic acid (JA) also play a role in promoting senescence (Fischer, 2012). Cytokinin is the most effective

senescence retarding growth regulator. Exogenous application of cytokinin delays or slows down the senescence in Arabidopsis and other plant species. *SAG12*, an *Arabidopsis* gene, encodes a cysteine protease and is expressed specifically in senescent tissues (Noh & Amasino, 1999). Expression of *SAG12* is particularly controlled by the developmental senescence pathways and its transcript levels are down regulated by the treatment with Cytokinin, auxin and sugars. *SAG12* is generally used as a marker to study the senescence regulation in plants (Noh & Amasino, 1999). Gan & Amasino (1995) showed that senescence of tobacco leaves in plants expressing the iso pentenyl transferase gene (IPT) under the control of a senescence associated promoter (*SAG12*) is strongly delayed. IPT encodes a key enzyme in cytokinin biosynthesis, and the *SAG12* promoter activates IPT expression in late senescence causing an increase in cytokinin levels and reduction in senescence in the leaf (Fischer, 2012).

Ethylene accelerates leaf senescence, and ethylene insensitive (*ein2-1*) and ethylene resistant (*etr1-1*) Arabidopsis mutants which lack perception of ethylene and its signal transduction show delay in the senescence process in leaves and expression of SAGs (Grbic & Bleecker, 1995). However, although ethylene signalling speeds up the senescence process it is age-dependent. Thus, only older leaves respond to treatment with exogenous ethylene; young ethylene-treated leaves do not senesce (Jing 2005; Weaver et al., 1998). EIN3, a key TF, plays a major role in the ethylene signalling pathway and functions downstream of EIN2. Transcript levels of *EIN3* show an increase during leaf development and senescence acting as a SAG. The role of EIN3 in senescence was studied in double mutants *ein3 eil1* that showed a delay in senescence; leaves remained green following a dark treatment, in naturally senescing leaves and also when treated with the ethylene precursor ACC when compared with WT (Li et al., 2013). In contrast, overexpression of *EIN3* showed an early senescence phenotype. In young leaves, *EIN3* expression is at a minimum and ethylene sensitivity is low whereas in older leaves ethylene sensitivity increases because of an increase in EIN3 transcription (Li et al., 2013). Thus, *EIN3* provides support for the observation that ethylene promotes senescence in an age dependent manner. Further evidence for this effect comes from cotton plants (*Gossypium hirsutum L*.) treated with the ethylene

inhibiting compound 1-MCP (Cothren et al., 2015).1- MCP delayed senescence caused by developmental age and also reduced soluble sugars and water potential during heat and drought stress.

Exogenous application of ABA and JA also promoted senescence in both attached and detached leaves and induced the expression of several SAGs whereas auxin and gibberellic acid show a negative effect on senescence (Zimmermann & Zentgraf, 2005). In *Arabidopsis thaliana* the YUCCA family of flavin monooxygenase proteins catalyse the rate limiting step in auxin biosynthesis. Kim et al (2011) reported that Arabidopsis plants that overexpress *YUCCA6*, showed a delayed senescence phenotype in detached rosette leaves. Even the accumulation of *SAG12* was delayed in mutant and transgenics when compared with WT which suggests auxin delays the senescence process either directly or indirectly by controlling the expression of SAGs (Kim et al., 2011). In the perennial plant *Paris polyphylla* application of GA3 reduced senescence of the shoots, degradation of proteins and increased endogenous GAs. The role of ABA in senescence was studied through the function of an ABA-inducible gene, *RPK1* (Receptor protein kinase; (Lee et al., 2011). Loss of function mutants, *rpk1-3* and *rpk1-4* showed less senescence-associated symptoms when compared to WT. Even age- dependent cell death in the mutants was reduced which was consistent with a lower level of expression of *SAG12*.The role of mutants in ABA-induced senescence was also studied as RPK1 also acts as a central component in the ABA signalling pathway. Mutants showed again a reduction in ABA-induced cell death and leaves retained 60 % more chlorophyll than WT. Thus, these results suggest that *RPK1* mediates ABA-induced leaf senescence.

1.2 Stress signal transduction

Since plants are sessile they encounter various biotic and abiotic stresses. These stresses may occur at any phase of plant development affecting their growth and productivity. Many genes are expressed in response to these stresses, and function in stress tolerance and response (dos Reis et al., 2012) Stress inducible gene products are divided into two groups – the first group (e.g. Late Embryogenesis Protein (LEA)

proteins) directly protects against environmental stresses and another group (e.g. protein kinases and transcription factors) regulates gene expression and signal transduction in stress responses (dos Reis et al., 2012). Transcriptional regulation of genes is controlled by transcription factor binding sites (TFBS) and transcription factors (TF). Transcription factors are major regulatory proteins that modify the expression of genes by binding to their promoter region (Singh & Laxmi, 2015). TFBS are DNA elements that are usually located in the regions directly upstream of protein coding sequences (Aarts & Fiers, 2003). Transcription factors (TFs) play a major role in transducing perception of stress signals to changes in expression of stress-responsive genes. They also act as molecular switches in different stress related genes by interacting with *cis*-regulatory elements in promoter regions (Banerjee & Roychoudhury, 2015; Liu et al., 2014). In plants, 7% of the genome encodes putative TFs which belong to large gene families such as WRKY, bZIP, AP2/ERPB, MYB, and NAC. The Arabidopsis genome includes 1500 TF genes and studies from gene expression analysis in Arabidopsis leaves have identified 827 genes (Breeze et al., 2011; Fischer, 2012) whose transcript levels show 3-fold upregulation during leaf senescence.

1.3 WRKY transcription factors

WRKY transcription factors belong to the WRKY-GCM1 superfamily of zinc finger transcription factors which have evolved from mutator like (Mule) transposases (Chen et al., 2012).WRKY TFs are believed to have originated in eukaryotes and they are one of the largest family of transcriptional regulators present in plants. The first member of the WRKY superfamily was identified in sweet potato (SPF1), (Ishiguro & Nakamura, 1994; Jiang et al., 2017). About 74 WRKY genes in Arabidopsis, more than 100 genes in rice, 197 genes in soybean and 120 genes in cotton have been identified (Banerjee & Roychoudhury, 2015; Jiang et al., 2017). WRKY TFs comprise a highly conserved 60 amino acids long DNA binding domain or WRKY domain, which contains a novel zinc finger like motif at the C-terminus and a conserved seven amino acid sequence motif WRKYGQK at the N-terminal end (Banerjee & Roychoudhury, 2015). Both the motifs

are important for the TF binding to consensus *cis*-elements in the promoters of responsive genes called W-BOX (TTGACT/C) (Chen et al., 2012).

On the basis of the number of WRKY domains and the type of zinc finger motif, WRKY proteins are classified into three groups. Group 1 WRKY TFs have 2 distinct WRKY domains and group 2 and 3 have a single domain. Group 1 and 2 have zinc finger motif C2H2(C-X4-5-C- X-22-23-H-X1-H) where X can be any amino acid and group 3 contains a C2-HC(C-X7-C-X23-H-X- C) zinc finger motif (Jiang et al., 2017). Based on phylogenetic analysis, of their WRKY domain, WRKY TFs are classified further into five groups (Jiang et al., 2017; Y. Zhang & Wang, 2005) - group I (I-N terminal & I-C-terminal), group IIa+group IIb, group IIc, group IId+group IIe and group III. WRKY TFs play a role in both biotic stress responses and also in developmental processes such as during embryogenesis, seed coat development and development of trichomes as well as, leaf senescence. They are also involved in regulation of physiological processes such as biosynthetic pathways, and hormone signalling (Jiang et al., 2017). WRKY TFs form the second largest family of TFs in the senescence transcriptome (Guo, et al., 2004; Rushton et al., 2010). The first sign of their role in senescence was identified from a study on *WRKY6* (Robatzek & Somssich, 2001). *WRKY6* was used as a probe on RNA blots to study gene expression at different stages of leaf development and *WRKY6* showed strong expression in senescent leaves. Expression of *WRKY6* was also increased by wounding and by treatment with SA, JA or ethylene, and can act as a positive or negative regulator. *WRKY6* negatively regulates the activity of its own promoter and functions as a positive regulator of senescence and pathogen defence gene expression (such as expression of PR1) through the involvement of NPR1 (an upstream regulator of PR1; (Balazadeh et al., 2008). Another WRKY TF which has been well studied in its role in the regulation of senescence is *WRKY53* which is involved in cross talk between biotic and abiotic stress responses. A pull-down assay of genomic DNA fragments using recombinant WRKY53 protein revealed many SAGs and TFs as direct or primary targets of WRKY53 (Zentgraf et al., 2010). Transient expression of *WRKY53* promoter :: GUS constructs showed that it binds to its own promoter and regulates its own expression. Using the *WRKY53* promoter as a bait in a yeast -1-hybrid

screen, three proteins which bind to the promoter were identified (Miao et al.,2008). One of these proteins belongs to the GATA4 transcription factor family and over expression of it increases *WRKY53* expression. Furthermore, GATA4 mRNA levels increased with H_2O_2 treatment as is the case for *WRKY53*. Another DNA binding protein identified was MEEKK1, which has a DNA binding motif in the WRKY53 promoter. Promoter :: GUS analysis showed that this promoter region acts as a switch for *WRKY53* expression from leaf age dependent senescence to plant age dependent senescence during bolting (Miao et al., 2007). MEKK1 interacts directly with the WRKY53 protein and phosphorylates the transcription factor by an alternative pathway in a kinase signalling cascade without interacting with MAPKKs and MAPKs (Figure 1.2). Another DNA binding protein identified contains a transcriptional activation domain (AD) and a kinase domain. The AD domain can phosphorylate itself and phosphorylation enhances binding of it to the *WRKY53* promoter Over expression and knockout of this gene caused a change in transcriptional levels of *WRKY53* demonstrating AD as a positive regulator of *WRKY53* expression. Zentgraf et al (2010) also reported that AD protein interacts with MEEK1 but is not phosphorylated by MEKK1 which was confirmed by bimolecular fluorescence complementation and also indicates that AD and WRKY53 may compete for MEKK1 interaction (Bakshi & Oelmüller, 2014; Zentgraf et al., 2010). In contrast, *WRKY54* and *WRKY70* play a negative role in senescence (Besseau et al., 2012). Leaves of single mutants of *WRKY54* and *WRKY70* showed a weak senescence phenotype, whereas, *wrky54 wrky70* double mutants showed an obvious senescence phenotype (Woo et al., 2013; Zentgraf et al., 2010) indicating the role of these two mutants as negative regulators.

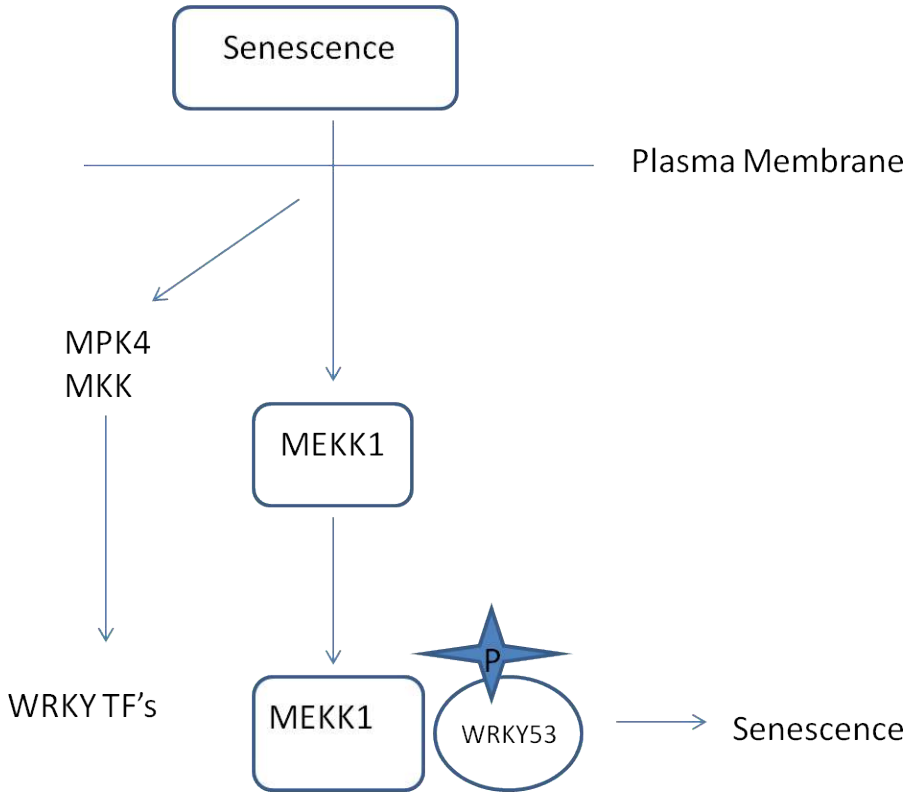


Figure 1.2: MAPK signalling process through MEKK1 in Arabidopsis MEKK1 interacts directly with WRKY53 phosphorylating the transcription factor, and taking an alternative pathway instead of interacting with MAPKs. MEKK1 (Mitogen-activated protein kinase kinase kinase 1); MPK (Mitogen-activated protein kinase); MKK (Mitogen-activated protein kinase kinase). Adapted from Zentgraff et al (2010); Rushton et al (2010).

AtWRKY15 (At2g23320), a ROS inducible TF, regulates plant growth and salt or osmotic stress responses. AtWRKY15 was classified under group IId of the WRKY TF family. WRKY15 protein is primarily located in the nucleus (Inzé et al., 2012). Studies of its spatial and developmental expression pattern using promoter reporter fusion studies showed that *WRKY15* is mainly expressed in young vascular and growing tissues. Transgenic plants overexpressing *WRKY15* showed an increased leaf area, plant biomass and endoreduplication index. As no T-DNA insertion mutants for *AtWRKY15*, were available, transgenic plants were produced that contain artificial microRNA constructs (amiR) constructs. These transgenic plants, which showed a 20% decrease in *WRKY15* levels., had a smaller average cell area than WT plants (Vanderauwera et al., 2012). Plants with elevated *WRKY15* levels ($WRKY15^{OE}$) were used to study the effect of oxidative and salt stress. $WRKY15^{OE}$ plants were more sensitive to oxidative stress while WRKY15-amiR plants were similar to WT (Vanderauwera et al., 2012).

With salt stress, WRKY15^{OE} were affected by chlorosis whereas amiR plants showed a reduced rosette area compared to WT. WRKY15 also plays a crucial role in disease resistance and cotton plant development (Yu et al., 2012). Overexpression of *GhWRKY15* showed increased resistance to viral and fungal infections compared with WT and also increased expression of pathogen related genes. When these transgenic plants were exposed to abiotic stresses, such as cold, wounding, and drought, they also showed higher accumulation of *GhWRKY15* transcripts. *WRKY63* belongs to the 3rd sub-group of the WRKY transcription factor family. The role of WRKY63 in abiotic stress was shown using T-DNA insertion mutant (*abo3*) created by insertion into the WRKY63 transcription factor (Ren et al., 2010). In plant responses to ABA and drought stress AtWRKY63 plays an important role: *abo3* mutant plants exhibit reduced ABA response and were less drought-tolerant than WT plants, and the *abo3* mutation also lowered the expression of stress responsive genes in response to ABA.

1.4 NAC transcription factors

Transcriptomic studies have demonstrated that many genes from the NAC transcription factor family play a role in senescence (Podzimska-Sroka et al., 2015). NAC TFs form one of the main families of plant specific transcription factors and are involved in a number of different processes including in the shoot apical meristem (SAM), cell division, flower development, secondary wall formation, leaf senescence, biotic and abiotic stress responses (Shao et al., 2015). Approximately 35 Arabidopsis NAC genes show upregulation during senescence and the whole NAC TF family comprises about 117 genes identified in Arabidopsis, 152 in soybean and 151 in rice, and (Puranik et al., 2012). NAC proteins get their names from three discrete proteins with an individual domain (NAC domain) from petunia NAM (no apical meristem), Arabidopsis ATAF1/2 and CUC2 (cup shaped cotyledon (Shao et al., 2015). Generally, NAC proteins contain a conserved N-terminal domain of 150 amino acids and contain five conserved regions and a variable C-terminal regulatory transcriptional region (Puranik et al., 2012). Arabidopsis thaliana NAC019 NAC domain structure, determined

by x- ray crystallography, shows that the NAC domain lacks a helix turn helix motif but includes a TF fold enclosed by helical elements containing a twisted β sheet (Nakashima et al., 2012). Some of the NAC genes which function in leaf senescence include in *Arabidopsis thaliana* include: *AtNAP (ANAC029*), *Oresara1 (ORE1; ANAC092), Oresarasister1 (ORS1; ANAC059), Jungbrunnen1 (JUB1; ANAC042)*, and *Arabidopsis thaliana* Activating factor1 *(AtAF1; ANAC002*; (Podzimska-Sroka et al., 2015). Overexpression of *AtNAP (ANAC029*), and *ORE1 (ANAC092)* resulted in early senescence whereas T-DNA insertion knockout of the *AtNAP (ANAC029)* gene delayed senescence indicating that they act as positive regulators of senescence (Guo & Gan, 2006). *JUB1 (ANAC042) and VNI2 (ANAC083)* act as negative regulators of senescence (Wu et al., 2012; Yang et al., 2011). Overexpression of JUB1 *(ANAC042)* strongly reduces senescence while early senescence is seen in *jub-1-1* mutant and miRNA lines. *JUB1* affects the gene regulatory network which involves *DREB2A,* an important TF which reacts to abiotic stresses (Sakuma et al., 2006). Heat shock TF gene *HSFA3* is a down stream target of *DREB2A* during heat stress, as is *RD29A,* a gene responsive to desiccation. *JUB1* is also a heat stress responsive gene (Shahnejat-Bushehri et al., 2012) and a transcriptional loop is formed with these three positive regulators: *JUB1-DREB2A-HsfA3.HsfA3* is involved with two additional TFs: *HsfA1e* and *HsfA2* (Wu et al., 2012). *HsfA1e* activates *HsfA2* which activates *HsfA3* and then *HsfA2* and *HsfA3* control the expression of H_2O_2 scavenging enzymes causing a decrease in intracellular H_2O_2 levels.

1.5 Reactive oxygen species

ROS, partially reduced forms of molecular oxygen are produced as unwanted by-products of aerobic metabolism (Mittler et al., 2011; Raja et al., 2017). Different forms of ROS include hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$). Major organelles involved in ROS production include chloroplasts, peroxisomes, and mitochondria due to their high metabolic activity and rapid rates of electron flow (Mittler, 2017). During evolution plants have evolved to acclimatize or accustom themselves to the harmful effects of the ROS and also use ROS as signalling molecules

(Noctor et al., 2018). However, imbalance between ROS production and scavenging induces oxidative damage to the proteins, lipids, DNA and RNA and ultimately leads to cell death (Nath et al., 2016). To defend themselves, plants also produce ROS detoxifying proteins which reduce the effects of the oxidative damage caused to the plant (Gilroy et al., 2016). Every cellular compartment in plants contains its own ROS homeostasis control and ROS signalling is modified based on the developmental stage, cell type and stress level. During abiotic stress conditions, production of ROS in cells is perceived by different sensors which generate stress related signals causing an adaptation or acclimation response. These stress specific signals are then processed by redox reactions that change protein function and structure and control the binding of transcription factors to DNA thus regulating transcription (Dietz, 2016).

1.5.1 Reactive oxygen species formation

Reactive oxygen species (ROS) formation in plants takes place in different steps or reactions in which O_2 undergoes a reduction process leading to the formation of Superoxide, Hydrogen peroxide and Hydroxyl radical (Noctor et al., 2018). The ground state molecular form of oxygen is present in a triplet state ($^3O_2$) and has an unpaired electrons with parallel spins, however, on the absorption of sufficient energy these spin restrictions are removed leading to the formation of singlet oxygen ($^1O_2$) (Tripathy & Oelmüller, 2012). Chlorophyll which is the light absorbing pigment in plants is located in the light harvesting complex and photosynthetic reaction centres. The excited form of chlorophyll is usually long lived and also allows the conversion of the energy to electrochemical potential by charge separation, whereas insufficient energy transfer leads to the generation of the triplet form of chlorophyll (Sharma et al., 2012; Tripathy & Oelmüller, 2012). Singlet oxygen is formed in the photosystem (PSII) reaction centre by the photodynamic activation of ground state oxygen which reacts with triplet chlorophyll (Das & Roychoudhury, 2014; Noctor et al., 2018).

$$\text{Chl} \xrightarrow{\text{Light}} {}^3\text{Chl}$$

$$^3\text{Chl} + {}^3O_2 \longrightarrow \text{Chl} + {}^1O_2$$

Although it also has a short life of 3 µs, (Hatz et al., 2007) it causes severe damage to molecules including lipids, nucleic acids, pigments and proteins and it functions as a major ROS responsible for causing cell death. Superoxide radical ($O_2^{\bullet-}$), anothertype of ROS is generated by the monovalent reduction of O_2 (Sharma et al., 2012). $O_2^{\bullet-}$ has a short half -life of 2-4 µs and does not cause much damage by itself, but it undergoes conversion or transformation to produce the more reactive and toxic $^1O_2$ and $OH^{\bullet}$ which are responsible for membrane lipid peroxidation (Halliwell, 2006). H_2O_2 is formed when the superoxide radical ($O_2^{\bullet-}$) undergoes a univalent reduction and protonation (Figure 1.3). It forms non-enzymatically by becoming dismutated to H_2O_2 or by a reaction catalysed by superoxide dismutase (Das & Roychoudhury, 2014; Noctor et al., 2018). It functions as a signalling molecule at low concentrations for processes like photosynthesis and senescence but damages the cell at higher concentrations (Das & Roychoudhury, 2014; Peng et al., 2005).

$$2\ O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$2\ O_2^{\bullet-} + 2H^{+\cdot} \xrightarrow{SOD} H_2O_2 + O_2$$

Hydroxyl radical ($OH^{\bullet}$) is the most toxic ROS, and is generated by the reaction between H_2O_2 and $O_2^{\bullet-}$ which is catalyzed by metals like Fe^{+2} or Fe^{+3} through the Haber Weiss or Fenton reaction (Figure 1.3) (Das & Roychoudhury, 2014; Gill & Tuteja, 2010).

$$H_2O_2 + O_2^{\bullet-} \longrightarrow OH^- + O_2 + OH^{\bullet}$$

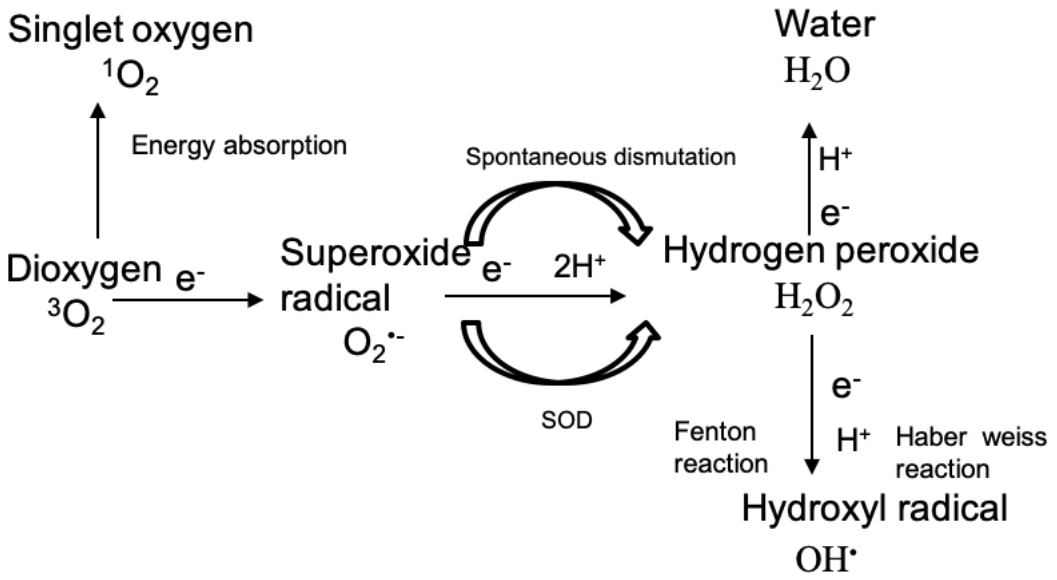


Figure 1.3: Generation of the different forms of Reactive oxygen species in the plants.(SOD-superoxide dismutase). Adapted from Pallavi Sharma et al (2012)., Gill & Tuteja (2010)., Das et al (2014).

1.5.2 ROS Scavenging and antioxidants

To protect against these reactive oxygen intermediates plant cell organelles like chloroplasts and mitochondria employ antioxidant defence machinery. Generally, the components of antioxidant defence are divided into enzymatic and non-enzymatic antioxidants (Gill & Tuteja, 2010). Enzymatic antioxidants include catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR). Non enzymatic antioxidants are divided into water soluble antioxidants like GSH (γ-glutamyl-cysteinyl-glycine), and ascorbic acid, and lipid soluble, that include antioxidants like carotenoids, and tocopherol (Gill & Tuteja, 2010).

1.5.3 ROS Perception and Signal transduction

To show a response, plants perceive external stimuli and transmit a signal to the nucleus of the plant cell. Generally, signal perception is at the plasmalemma which

then activates the internal signalling components (Jalmi & Sinha, 2015). The most important change which occurs immediately upon perception is a change in the redox state of the plant. This occurs because of the production of ROS in the chloroplast and the mitochondria (Mittler et al., 2004), but also by cell wall NADPH oxidases. The accumulation or level of ROS determines if it serves as a protective or destructive molecule. This is in turn controlled by the ROS production and turnover (Mittler et al., 2004). The important signalling cascade which works in transmitting external stress responsive stimuli is the mitogen activated protein cascade (MAPK). The MAPK cascade is highly conserved and contains three different components MAPKKKs, MAPKKs, and MAPKs. The Arabidopsis genome contains 20 MAPK, 10 MAPKK, and 60 MAPKKK encoding genes (Ichimura et al, 2002). Abiotic stresses like salt, drought, cold and wounding activate MAPKKK, MEKK1 (Pitzschke et al., 2009). When the MAPKKKs are activated, a phosphorylation induced signal is activated that causes the phosphorylation and activation of downstream MAPKs (Figure 1.4). Two types of MAPK cascade operate in defence against environmental stress and pathogen attack. The MEKK1-MKK4/5-MPK3/6 cascade is activates ROS in pathogen defence response (Asai et al., 2002). Another cascade which works down stream of ROS to protect against biotic as well as abiotic stresses is the MAPK cascade (MEKK1-MKK2-MPK4/6) (Pitzschke et al., 2009). Although MAPK cascades are activated by ROS, they are known to function in the regulation of ROS production by a feedback mechanism. Findings reported from a study on maize showed that ABA activates MAPK cascade that works downstream of ROS, regulating the activity of RBOH for the production of ROS (Lin et al., 2009). Another cascade which is activated by ROS includes the OXI1-MPK6 cascade which also plays a role in regulation of ROS production (Asai et al., 2008). These findings indicate that ROS and MAPKs are interconnected with each other. Downstream components associated with ROS signalling involve Ca^{+2} and Ca^{+2} binding proteins like calmodulin, G proteins. A serine /threonine protein kinase (Oxidative signal inducible; OXI1) also plays an important role in ROS detection by the activation of MAPKs (MAPK 3 and 6) through Ca^{+2}. The expression of OXI1 is induced by H_2O_2 and is also required for plant immunity against *Pseudomonas syringae* in Arabidopsis (Tripathy & Oelmüller, 2012).

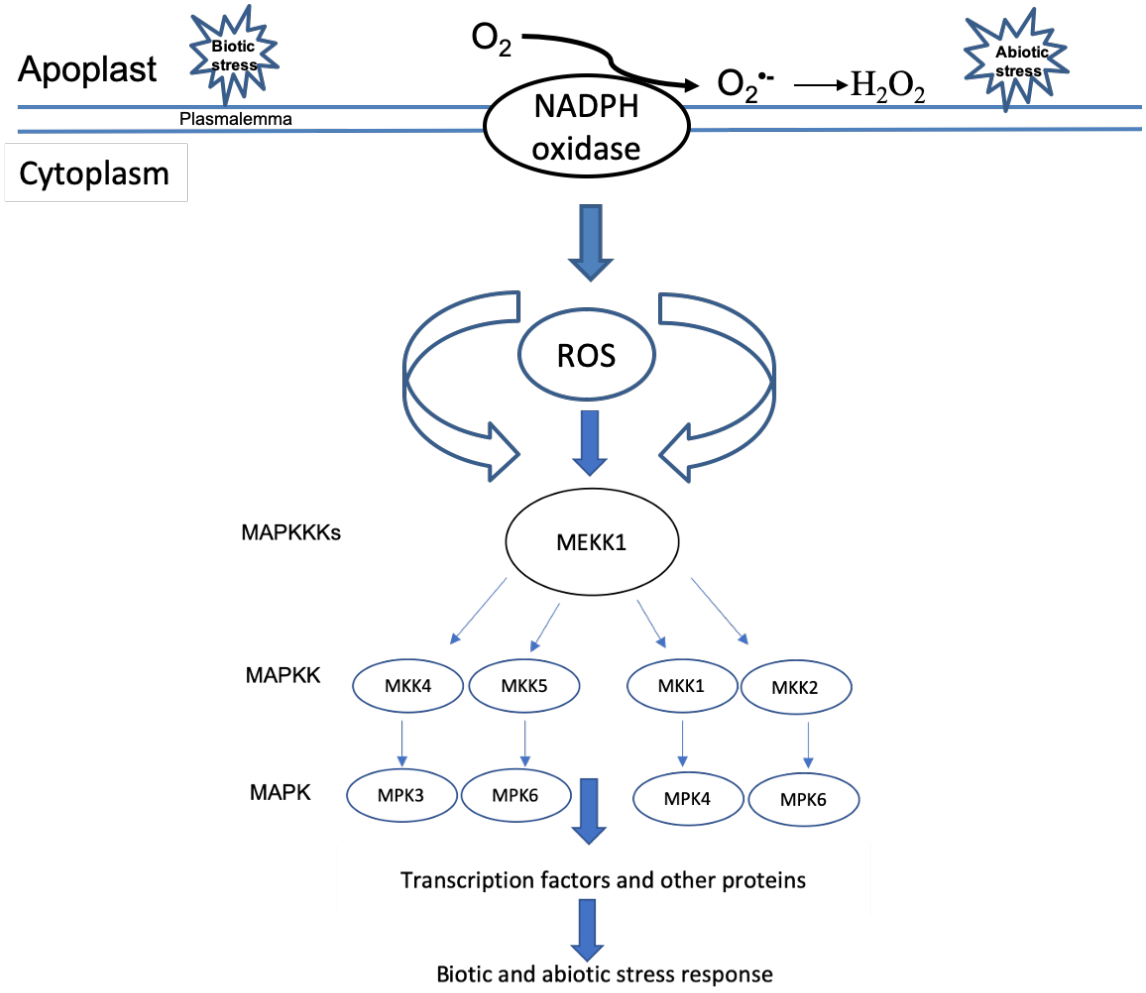


Figure 1.4: MAPK cascade activation by ROS in biotic and abiotic stresses. MEKK1 is a common MAPKKK (Mitogen Activated protein kinase kinase kinase) cascade activated by ROS leading to the activation of the downstream components of MAPK cascade in Arabidopsis in defense against the biotic and abiotic stresses. (MAPKK-Mitogen Activated protein kinase kinase; MAPK- Mitogen Activated protein kinase). Adapted from Jalmi and Sinha (2015).

1.5.4 Reactive oxygen species in abiotic stress responses

Membrane bound NADPH oxidases are enzymes which catalyse the production of superoxide radicals and as they are functionally homologous with those in mammals, plant NADPH oxidases have been named respiratory burst oxidase homolog (Rboh) enzymes (Sewelam et al., 2016). Rboh enzymes usually work together with other signalling components such as Ca^{+2} and protein phosphorylation, which produce ROS. Rboh enzymes function in diverse biological process in cells, making them an important component of the ROS signalling network. In Arabidopsis ten genes encoding NADPH oxidases have been reported. In relation to the involvement of Rboh

in abiotic stress it has been demonstrated that the *RbohD* gene was involved in ROS induced activation of a rapid systemic signal by various abiotic stresses including wounding, light, heat and salinity (Miller et al., 2009). ROS are also found to be involved in the regulation of Na^+/K^+ homeostasis and H_2O_2 has also been reported to decrease the Na^+/K^+ ratio and increases salt resistance in *Populus euphratica* calluses under saline stress (Zhang et al., 2007). ROS generated by *AtrbohD* and *AtrbohF* function as signalling molecules and control NA^+/K^+ homeostasis by improving tolerance to salt in Arabidopsis (Ma et al. 2012). The double mutants *atrbohD1/F1* and *atrbohD2/F2* produced less ROS and were much more sensitive to salt treatment.

1.5.5 Reactive oxygen species in rapid systemic signalling

ROS were also shown to be involved in rapid systemic signalling in plants in response to abiotic stress by a process coupled to calcium signalling and electric waves (Gilroy et al., 2016; Miller et al., 2009). An auto propagating wave of ROS production is mediated by RBOHD in each cell that can reach a rate of up to 8.4 cm/min, and was found to be linked with calcium and electric signals. When the abiotic stress affects local or neighbouring cells this ROS wave causes the production of a flux of calcium into the cytosol. This activates RBOHs, which induce a cascade of events that activates calcium dependent protein kinases, which phosphorylate and activate RBOHs (Figure1.5). These activated RBOHs generate ROS in the apoplast which are then detected by the neighbouring cells causing a calcium flux which in turn activates their own RBOHs. This activation of a ROS calcium flux connected with calcium- activation of RBOHs is generated automatically from a cell to a neighbouring cell and activates systemic responses to the abiotic stress (Choudhury et al., 2017; Miller et al., 2009).

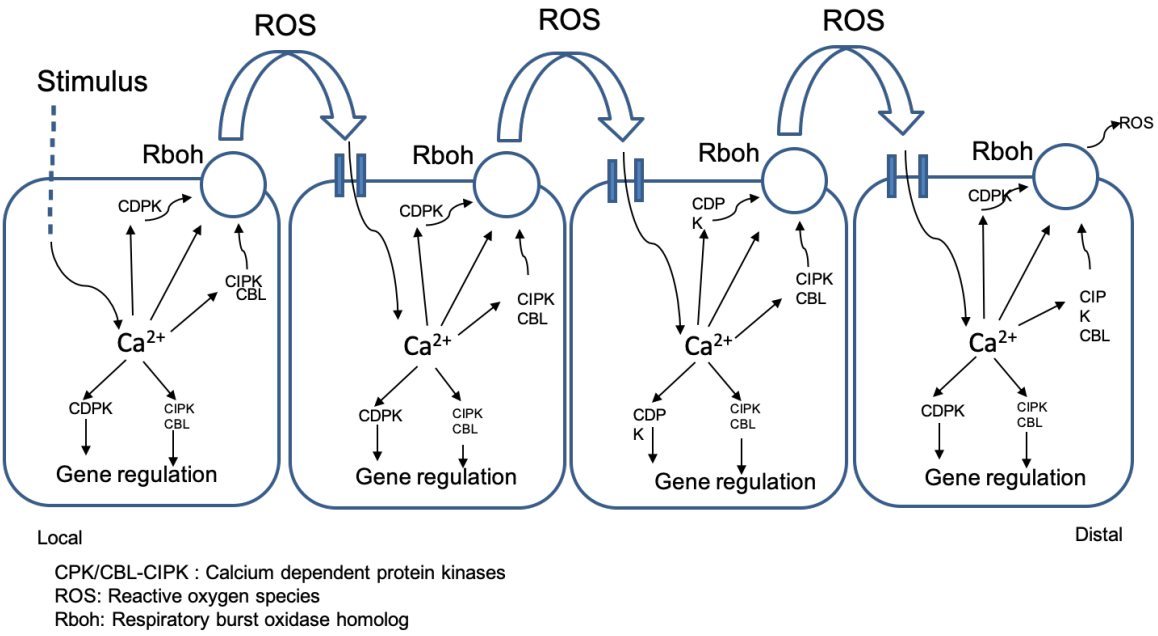


Figure 1.5: A model showing the Calcium and ROS autopropagation wave leading to the activation of the rapid systemic signaling response in plants. Adapted from Steinhorst and Kudla (2013); Choudhary et al (2017).

1.5.6 Reactive oxygen species in wound responses

Mechanical wounding produces an increase in cytosolic Ca^{+2} levels and Ca^{+2} oscillations that function as a for stimulus response mediator for several calcium binding proteins such as CAM-like proteins, calcineurin b-like proteins, calmodulins (CaMs) and calcium dependent protein kinases (CDPKs). ROS function as important secondary messengers in wounding or defense responses which are associated with calcium signalling. RbohD plays a major role in the generation and signalling of ROS (Takahashi et al., 2011) as discussed above. Although previous studies reported (Orozco-Cárdenas et al., 2001; Sagi et al., 2004) that ROS function as downstream secondary messengers during the wound response (Miller et al., 2009) it was not known whether ROS accumulation is needed for rapid systemic signalling and how it links with the Ca^{+2} signaling. Miller et al (2009) showed that RbohD is needed in these process and superoxide generated by the RbohD functions as a cell to cell communication mediator in plants over long distances. Plants produce superoxide and H_2O_2 during the early stage of the wound response, (Suzuki & Mittler, 2012). Wounding processes activate the expression of cytosolic ROS detoxifying enzymes such as ascorbate peroxidase2 (APX2). In Arabidopsis when DPI (diphenyliodonium),

an inhibitor of NADPH oxidase was added, to fully expanded wounded leaves after 6 hr it prevented the induction of *APX2* expression by the inhibition of photosynthetic electron transport (PET) indicating that *APX2* expression in wounded leaves needs NADPH dependent H_2O_2 and PET. (Chang et al., 2004).

Mitogen activated protein kinases are also an essential mediator which participate in the plant wounding response (Nakagami et al., 2006). In the wound signalling pathways the Arabidopsis mitogen activated protein kinase MPK8 connects protein phosphorylation, Ca^{+2} and ROS (Takahashi et al., 2011). MPK8 is activated by mechanical wounding, and requires calcium dependent direct binding of calmodulins (CaMs). MPK8 is also phosphorylated and activated by MKK3 in the cascade and the complete activation of MPK8 requires both MKK3 and CaMs in plants. MPK8 negatively regulates the ROS accumulation by controlling the expression of RbohD gene which suggests the existence of two major mechanisms, Ca^{+2}/CaMs and phosphorylation of the MAP kinase which join at MPK8 to maintain ROS homeostasis (Takahashi et al., 2011). Thus ROS, Ca^{+2} and protein phosphorylation all play an important role in the wound response in plants (Suzuki & Mittler, 2012).

1.5.7 Reactive oxygen species in plant growth and development

Growth of roots depends on the balance between cell proliferation and expansion at the root tip. In the elongation zone H_2O_2 accumulates, whereas superoxide is found to accumulate, in the meristematic zone (Dunand et al., 2007). When this balance is altered or changed the size of the root meristem is affected. *UPB1* a bHLH transcription factor controls the ROS homeostasis by inhibiting the action of class III peroxidases in the elongation zone (Tsukagoshi et al., 2010). The mutant *upb1-1* shows a longer meristem, and the level of H_2O_2 was reduced in the elongation zone. However, superoxide levels increased in the meristematic zone which indicates that ROS homeostasis plays a major role in cell transition from the zone of division to elongation and differentiation. ROS also function as signalling molecules during lateral root formation. When roots of Arabidopsis are exposed to ROS, they show an increase in

lateral root number and lateral primordia (Orman-Ligeza et al., 2016). Lateral root initiation and emergence require *AUX1* and *LAX3* genes which encode the family of auxin influx carriers, (Lavenus et al., 2013). Mutation of both these genes results in a rootless mutant phenotype but surprisingly when double mutants *aux1 lax3* were treated with H_2O_2 this caused the appearance of lateral roots (Orman-Ligeza et al., 2016). ROS were also found to be deposited in the apoplast of these cells overlying the developing lateral roots. Spatial expression analysis of auxin inducible *RBOH* genes overlaps with H_2O_2 localization in the peripheral cells, indicating lateral root production could be promoted by the RBOH mediated ROS generation by the cell wall remodelling of peripheral cells. (Orman-Ligeza et al., 2016). Therefore, lateral root production is controlled by auxin-ROS signalling mediated by the RBOH genes with ROS functioning downstream of auxin.

NADPH oxidases, AtrbohD and AtrbohF negatively regulate lateral root development by altering peroxidase activity and promoting the local superoxide generation in the mature area of primary root independently of auxin (Li et al., 2014). Double mutants of *AtrbohD* and *F* showed an increase in peroxidase activity compared to the wild type in the mature root zone and lateral root density was also increased, which supports that ROS modulation is required for the lateral root emergence. Double mutants were not responsive to exogenous application of auxin, NAA and NPA with respect to lateral root formation. Treatment of roots of WT and double mutants with auxin transport inhibitor NPA caused the inhibition of lateral root development in both, and co-treatment with auxin and NPA increased the number of lateral root primordia of both mutants and WT. The authors conclude that the regulation of lateral root formation by AtrbohD and AtrbohF does not depend upon the accumulation of the auxin in the root, and that ROS regulation of lateral root emergence takes place independently of auxin (Li et al., 2014; Tsukagoshi, 2016).

Arabidopsis *mpk6* mutants produce more lateral roots after treatment with H_2O_2 (Wang et al., 2010), which again supports the function of ROS in lateral root development. *atmpk6* seedlings exhibit an increase in root cell elongation when treated with H_2O_2 and ABA. Addition of a calcium ionophore to roots containing H_2O_2

showed enhanced root cell elongation and growth which supports the model where Ca^{+2} is required for the H_2O_2 induced elongation of root cells in Atmpk6 (Han et al.,2015). Roots and root hairs are both developed by a cell expansion process and root hair elongation involves absorption of minerals and water from soil. Ca^{+2} influx from the extra cellular store is required for cell elongation in roots (Cramer & Jones, 1996). *rhd2* mutants which are impaired in Ca^{+2} uptake show much less cell expansion and have short root hairs and stunted roots (Foreman et al., 2003). RHD2 is an NADPH oxidase involved in the formation of ROS and levels of ROS in *rhd2* mutants have been found to be reduced which indicates that *RHD2* controls development by generating ROS which regulates cell expansion by the activation of Ca^{+2} channels (Foreman et al., 2003).

1.5.8 ROS and Transcription factors

Transcription factors are major regulatory proteins which modulate the expression of specific sets of genes by binding to their promoter region (Singh & Laxmi, 2015). They play an important role in the downstream signalling cascades and control the expression of stress responsive genes to provide tolerance against environmental stress in the plants (You & Chan, 2015). The response of 1500 Arabidopsis TFs to ROS was analysed (Gadjev et al., 2006) and it was found that different ROS repress or induce the expression of 500 annotated TFs in Arabidopsis (Sewelam et al., 2016). Regulation of TF activity mediated by ROS signalling is controlled at different levels. Even though expression of many TF genes is upregulated by ROS, the role of the TFs in oxidative stress is not well established. A study of *erf6* mutants, an ethylene responsive transcription factor, under oxidative stress demonstrated that RbohD and calcium signalling are essential for *ERF6* ROS responsive expression (Sewelam et al., 2013). Zinc finger proteins are generally considered as major players in the regulation of ROS defence genes in Arabidopsis. In *AtAPX1* knockout plants where an ascorbate peroxidase gene is down-regulated, and hence ROS increases, expression of three zinc finger TFs: *ZAT7, ZAT10* and *ZAT12* is upregulated by the oxidative stress (Miller et al., 2008). Members of WRKY, NAC, and AP2/ERF families were also shown to be involved

in ROS homeostasis in crop plants (You & Chan, 2015) as well as in Arabidopsis, as discussed in a previous section. *ZFP179* belongs to the zinc finger transcription factor protein family. Transgenic rice plants overexpressing ZFP179 showed an increase in salt and enhanced oxidative stress tolerance. ZFP179 increases the ROS scavenging activity to reduce the ROS accumulation caused by the salt stress (S. J. Sun et al., 2010). SERF1 (salt responsive ERF1), ERF transcription factor shows an induction in rice roots upon the treatment with H_2O_2 and salt and overexpression of it increases oxidative tolerance. It was also reported that the SERF1 strengthens the ROS activated MAPK cascade signal generated during the salt stress and translates it into a salt induced response, causing an increase in salt tolerance (Schmidt et al., 2013). Stress responsive gene, *GmWRKY27* overexpression showed an increase in salt and drought tolerance by reducing the ROS levels in transgenic soybean hairy roots. GmWRKY27 interacts with GmMYB174 and inhibits the transcription of *GmNAC29*. The inhibition of the expression of GmNAC29 results in increasing the expression of GmSPOD1 which causes the reduction in intracellular ROS levels and thereby increasing stress tolerance (Wang et al., 2015). SNAC3 overexpression in rice showed increased tolerance to drought and to oxidative stress while SNAC3 suppression showed sensitivity to these stresses. SNAC3 functions as a positive regulator under heat stress as over expressors exhibited lower levels of H_2O_2 indicating stress tolerance is modulated by the ROS homeostasis by controlling the expression of ROS associated enzymes (Fang et al., 2015).

1.6 Late Embryogenesis proteins (LEA) proteins

Environmental stresses like drought, excessive heat, freezing and increases in salinity lead to the loss of intracellular water of plants, a process known as dehydration (Kovacs et al., 2008). Some plants, animals and microorganisms are able to dry out completely and however remain viable, a process called anhydrobiosis(Tunnacliffe et al., 2010). Desiccation tolerant organs like seed and pollen are able to withstand the dehydration process for a long period of time. In anhydrous organs during the dry conditions bioglasses are formed by the cell solutes such as sugars which provide protection against molecular denaturation and also in aggregate formation (Buitink &

Leprince, 2004). This bioglass formation also fills spaces in the tissues during dehydration process increasing cytoplasmic viscosity which stops chemical reactions that need molecular diffusion (Buitink & Leprince, 2004). Important seed proteins which are involved in bioglass formation are LEA (late embryogenesis proteins) (Buitink & Leprince, 2004; Tunnacliffe et al., 2010). LEA proteins were originally discovered during late embryogenesis in cotton seeds (Dure III & Chlan, 1981) but later also found in the seeds and vegetative organs of other plants under stress conditions (Hundertmark & Hincha, 2008a). LEA protein production is associated with cellular dehydration tolerance to drying, freezing, and saline conditions (Hundertmark & Hincha, 2008b). The hydrophilin family of proteins are a group of highly hydrophilic intrinsically unstructured proteins which are characterized or defined by their high glycine content(> 6 %) and a high hydrophilicty index(> 1.0) which differentiates them from the other LEA proteins as well as the proteins from the different taxons (Garay-Arroyo et al., 2000; Olvera-Carrillo et al., 2011).The most striking feature of the LEA proteins is a biased composition of amino acid which results in high hydrophilicity similar to hydrophilins. Indeed, there is considerable overlap between proteins classified or graded as hydrophilins and LEA proteins. As the most discriminating feature of hydrophilins is their high glycine content and hence not all the LEA proteins are classified as hyrophilins and alternatively, non-LEA proteins are also members of this group (Hincha & Thalhammer, 2012). LEA proteins are not only present during water deficit conditions caused by environmental conditions but also found to be associated with water limitation conditions produced during the development of seeds and pollen grains and a few stages of shoot and root development. LEA proteins are also found to be associated with vascular tissues and in meristematic regions (Battaglia & Covarrubias, 2013; Colmenero-Flores et al., 1999). LEA proteins are ubiquitous in higher plants and are also found in other organisms including bacteria (such as *Deinococcus radiodurans*), algae, bryophytes, pteridophytes (ferns) seedless vascular plants (Selaginella) and also in yeast, nematodes and rotifers (Amara et al., 2014; Hundertmark & Hincha, 2008b; Kahl, 2015). The protective role of LEA proteins has been demonstrated by over-expression of genes encoding LEA proteins in transgenic plants that increases their stress tolerance. For example, expression of the barley gene *HVA1* in rice and wheat (Xu et al., 1996) conferred or contributed to

drought tolerance in plants. Although not much information is known about the participation of the LEA genes in biotic stress it is known that the overexpression of the group 2 LEA from Arabidopsis affects the expression of genes involved in plant defence response like PR proteins (Hanin et al., 2011).

1.6.1 LEA characteristics

LEA proteins are small (10 to 30 kDa) and are highly hydrophilic. They are predicted to be present as largely unstructured proteins in the hydrated state and hence form a part of the large set of natively unfolded proteins which constitute a significant proportion of proteomes (Tompa, 2002; Tompa et al., 2005; Uversky et al., 2000). Many of the unfolded proteins on binding with partner molecules or other molecules generally become structured but such molecules were not characterized as those involved in desiccation tolerance (Tunnacliffe et al, 2010). Contrastingly, LEA proteins also form a defined or alpha-helical structure during the dehydration process caused by freezing or drying (Tunnacliffe et al., 2010). Because of the absence of the secondary structure, LEA protein family members are part of the large class of proteins known as intrinsically disordered, unstructured or natively unfolded proteins (Uversky et al., 2000). The hydrophilic nature of LEA proteins is calculated based on the hydropathy scale or plot in which each amino acid is given a value reflecting its hydrophilicity and hydrophobicity (Kyte & Doolittle, 1982). This is characterized by a GRAVY (grand average hydropathy value) score; the value of 7 to 21 residues of amino acid is used to measure the hydrophobic nature of side chains along the protein length. In the case of LEA proteins, the whole sequence falls into the hydrophilic space on a hydropathy scale whereas for example a globular protein like BSA shows a distribution between the hydrophilic and hydrophobic spaces. This hydrophilic feature of LEA proteins is explains the absence of the secondary structure in a hydrated state and also makes them remain soluble at high temperatures (Tunnacliffe & Wise, 2007a). Most of the unstructured proteins play an important role in cells; although they lack three-dimensional structure, they contain flexible structural elements including polyproline helices which make them bind with other interacting molecules like DNA, RNA or other proteins (Hundertmark & Hincha, 2008b).

1.6.2 LEA classification

Dure and Bray classified (1981) LEA proteins into 3 groups on the basis of similar sequence motifs in LEA proteins from various plant species. LEA protein group 1 is identified by a 20 amino acid hydrophilic motif, group 2 proteins have at least two or three well defined motifs named Y(DEYGNP), S(S_n) and K(EKKGIMDKIKEKLPG). Group 3 consists of several copies of an 11 amino acid (TAQAAKEKAXE) motif (Tunnacliffe & Wise, 2007b). A different naming pattern was carried out by Dure which labelled the groups based on the cotton prototype: D-19 for group 1, D-11 for group 2, D-7 for group 3. Although most of the LEA proteins are present under these 3 main groups, a few other minor groups are also included: D-113 for group 4, D-29 for group 5 and D-34 for group 6. Wise et al, (2003) later again defined the classification based on the POPP analysis (peptide profile) which lead to the characterization of LEA proteins superfamilies with one or more super families making up each of the main groups (Tunnacliffe & Wise, 2007b). LEA proteins are further classified based on the Pfam nomenclature (Bateman, 2004). Each Pfam domain is characterized by multiple sequence alignments which correspond to the specific protein domains. The most recent classification using the physiochemical properties of LEA proteins was carried out by Jaspard et al. (2010) which has led to the classification of 710 LEA proteins into 12 non-overlapping classes with definite or distinct properties (Hunault & Jaspard, 2010). Although the Arabidopsis LEA proteins are highly diverse, they are grouped into eight families in the Pfam database (Finn et al., 2009) according to the primary sequences-Dehydrin, LEA_1, LEA_2, LEA_3 LEA_4, LEA_5, LEA_6, and SMP (seed maturation protein) (Table 1.1). Group 3 is considered as the major LEA group characterized in plants whereas Group 4 ,5, and 6 have fewer members. Most typical LEA proteins studied belong to Group 3, 2 and 1 (Leprice, 2010). *SAG21/AtLea5* belongs to Group 6 of the Bies-Ethève et al. (2008) Classification and LEA_3 family (PF03242) according to the classification developed by Pfam (Table 1.1).

1.6.3 Expression profiles of LEA proteins

Genome-wide analysis of the LEA proteins lead to the recognition of 51 LEA protein-encoding genes in *Arabidopsis* which were categorised into distinct groups. Expression analysis was performed on these individual LEA genes from different organs including leaves in response to different abiotic stress and hormone (ABA and GA) treatments (Hundertmark & Hincha, 2008b). Twenty two of the LEA genes showed high levels of expression in the non-seed organs and transcript levels for most of the LEA genes were high in seedlings. Most (82%) of genes encoding LEA proteins contain ABRE and 69% contain LTRE *cis*-acting regulatory elements in their promoter regions. ABRE responsive *cis*-elements function in ABA signalling during seed development and also under abiotic stress whereas LTRE binds to CBF/DREB1 transcription factors which function in drought and cold tolerance (Hundertmark & Hincha, 2008a).

1.6.3.1 Group 1 LEA (Pfam00477-LEA_5) D-19,132

Group 1 proteins (*Gossypium hirsutum* D19, *Triticum aestivum Em*) are characterized by an internal twenty amino acid conserved motif, and also a high proportion of charged amino acid residues. Proteins similar to group 1 have been identified in *Bacillus subtilis*. In plants, group 1 LEA proteins accumulate in dry seeds and also in organs which go through dehydration such as pollen grains (Ulrich et al., 1990) and many of this group of genes are responsive to ABA.

1.6.3.2 Group 2 LEA (Pfam00257-Dehydrins) D-11

Group 2 LEA proteins also known as dehydrins were found to accumulate during late embryogenesis and as the dehydrin expression is up-regulated by ABA they are also called RAB (Ras-associated binding proteins) proteins. They are ubiquitous in the plant kingdom and also found to be present in non-vascular plants like the moss *Physcomitrella*. patens, and seedless vascular plants such as the lycopod *Selaginella lepidphylla* (Battaglia et al., 2008; Hanin et al., 2011). A peculiar feature of group 2

LEA proteins is that they contain a conserved lys rich 15 residue motif named the K-segment and also contain an additional motif called a Y-segment present in one to 35 copies at the N-terminus of the protein. Dehydrins also possesses a stretch of Ser residues called the S-segment which undergoes phosphorylation (Battaglia et al., 2008; Hanin et al., 2011). Many of the group 2 LEA proteins accumulate in the cytoplasm although some of them were found to be localized in the nucleus (Battaglia et al., 2008).Stress inducible dehydrin encoding genes show the presence of ABRE, CRT, myelocytomatosis (MYC) and MYB *cis*-regulatory elements in their promoter regions (Hanin et al., 2011). Many studies have shown the importance of dehydrin gene expression on plant stress tolerance. Over-expression of multiple Arabidopsis dehydrin genes *(RAB18, COR47, LTI30, ERD10)* increase freezing resistance and survival under low-temperature conditions (Battaglia et al., 2008; Puhakainen et al., 2004). Ecotopic expression of wheat dehydrin *DHN-5* in Arabidopsis plants showed increased tolerance to water deprivation and high salinity (Brini et al., 2007). Houde et al, (2004) reported that the expression of the *WCOR410* gene from wheat caused an increase in frost tolerance in strawberry. However, over-expression of RAB18 did not confer freezing and drought tolerance in Arabidopsis (Lång & Palva, 1992), while co-expression of *RAB18* with *DHN(Cor47)* in Arabidopsis showed an improved freezing tolerance but not drought tolerance (Puhakainen et al., 2004). When dehydrins *LTI29* and *LTI30* were co-expressed in Arabidopsis they also conferred better freezing tolerance than lines over-expressing a single *DHN* gene (Hanin et al., 2011).

1.6.3.3 Group 3 LEA (Pfam PF02987,Pfam:LEA_4) D-7/D- 29

Group 3 proteins are defined by an 11 amino acid repeat motif (Dure, 1993). Differences in the molecular mass of the proteins in this group are due to the number of repetitions of this 11 mer motif. The variability of this 11 mer motif divides the classification of Group 3 LEA proteins into two subgroups-3A characterized by the cotton D-7 LEA protein and 3B by the cotton-29 LEA protein (Amara et al., 2014; Battaglia et al., 2008). The first subgroup is highly conserved while 3B is more heterogeneous. Group 3 LEA proteins are distributed extensively in the plant kingdom with their transcripts being identified in nonvascular plants and in seedless vascular

plants. Proteins similar to group3 LEA proteins are also found to accumulate in non-plant organisms in response to dehydration (Battaglia et al., 2008). Evidence to support the tolerance of water stress in invertebrates is from studies in the nematode *Caenorhabditis elegans* in which the dauer larvae show increased dessication resistance when compared to adult worms, which are susceptible to dessication. The increased desiccation resistance in dauer larvae is connected with the expression of *Ce- LEA-1*, a gene that encodes an LEA like protein. Furthermore, when *Ce- LEA-1* expression is silenced, the dauer larvae show an increase in mortality on dehydratin (Gal et al.,2004; Tunnacliffe & Wise, 2007a). Gene *Aav- LEA-1*, which encodes a group 3 LEA protein in the anhydrobiotic nematode *Aphelenchus avenae* , shows upregulation by dessication and osmotic shift but not by oxidative, heat or cold stresses (Browne et al., 2004; Tunnacliffe & Wise, 2007b). Group 3 proteins were also found to be present in desiccation-tolerant bdelloid rotifers (Tunnacliffe & Wise, 2007b) and the crustacean *A. franciscana* also accumulates group 3 LEA proteins in the dessicated state (Amara et al., 2014).

In plants, the expression of group 3 LEA proteins appears to be regulated by ABA at particular developmental stages or under stress conditions. The role for the increase ingroup 3 LEA proteins and or transcripts in protection from stress caused by cold, freezing, or salinity is supported by the analysis of protein expression in different plant species. Wheat roots, where LEA 3 proteins are absent, could not resume growth and died when dehydrated in comparison to the shoot and the scutellar tissues where there were high levels of LEA proteins (Battaglia et al., 2008). Another well-studied example of this LEA group includes transgenic rice plants in which the barley gene *HVA1* is regulated by the rice constitutive promoter, *ACTIN1*. This causes high-level accumulation of *HVA1* protein in leaves and roots and results in tolerance to high salt and water deficit (Battaglia et al., 2008; D. Xu et al., 1996). The effects of the same gene when constitutively expressed in wheat, bentgrass and mulberry also showed similar results(Fu et al., 2007; Lal et al., 2008; Sivamani et al., 2000). In vitro assays on Group 3 LEA proteins from Arabidopsis and pea were found to protect enzymes such as malate dehydrogenase and citrate synthase, against partial dehydration. (Battaglia et al., 2008).

1.6.3.4 Group 4 LEA (Pfam03790) D-113

Group 4 LEA proteins are widely spread across the plant kingdom in both vascular and non-vascular plants (Battaglia, et al., 2008). LEA proteins of this group are characterized by a conserved N-terminal region which is of 80 residues in length and is responsible for the formation of amphipathic-alpha helices, and a less conserved variable C-terminal region. Due to the presence of the variable C-terminal region, this group is further classified into 2 subgroups, 4A and 4B (Cuevas-Velazquez et al., 2017). Sub group 4A contains small proteins which are of 80-124 residues long and group 4B consists of longer representatives of 108-180 residues long (Amara et al., 2014; Battaglia et al., 2008). Group 4 LEA proteins were initially discovered to be accumulated in the dry embryos and one among them is cotton D-113 protein (Battaglia et al., 2008; Roberts et al., 1993). Group 4 LEA transcripts were also found to be accumulated in leaves during water deficit conditions in tomato plants (Battaglia et al., 2008; Cohen et al., 1991). Unlike the other LEA proteins, Group 4 LEA proteins also prevent the inactivation of the lactate dehydrogenase enzyme during dehydration or water deficit conditions (Cuevas-Velazquez et al., 2017; Cuevas-Velazquez et al.,2016).

1.6.3.5 Group LEA_5 (Pfam3242-LEA_3)

Members of this group differ from the groups of LEA proteins as they contain a very high fraction of hydrophobic residues and also an 11 mer amino acid repeat similar to group 3 LEA proteins. After boiling these proteins are not soluble, indicating that they form a globular structure (Battaglia et al., 2008). Their transcripts generally accumulate during the late phase of seed development and also during abiotic stress conditions like salinity, drought, cold, wounding and UV light (Battaglia et al., 2008; Kiyosue et al., 1992). In Arabidopsis, there are four members of this LEA group, including *SAG21*, the focus of this thesis. The other three members encode proteins which show a drought response, AtDi21 (At4g15910), At1g02820, and At3g53770.

These encoded proteins are either targeted to mitochondria or plastids (Hundertmark & Hincha, 2008a).

1.6.3.6 Group 6 SMPO (Pfam 4927) D-34

Unlike the typical LEA proteins, LEA proteins belonging to this group contain a very high proportion of hydrophobic residues. The first proteins discovered in this group are D-34, D-73, D-95 from cotton (Amara et al., 2014; Baker et al., 1988; Battaglia et al., 2008). Although not much is known about this group of proteins their transcripts were found to accumulate during the late stage of the development of the seed and also in response to abiotic stress conditions like drought, salinity, cold and wounding (Battaglia et al., 2008).

Table 1.1-LEA proteins classification based on the Pfam nomenclature and their main characteristics features

Pfam Classification	Dure *et al* 1989	Tunnacliffe and Wise 2007	Battalgia *et al* 2008	Hundermark and Hincha 2008	LEAPbd 2010	Characteristics
PF03760	D113	Group 4	Group 4A,4B	LEA_1	Classes 10	Contain a conserved N-terminal and variable C-terminal region Prevent the inactivation of the enzymes
PF03168	D95	Group Lea14	Group5C	LEA_2	Classes 7 and 8	Hydrophilic Characterized by their small size and highly conserved. PvLEA18 first protein discovered in bean
PF03242	D73	LEA_5	Group5B	LEA_3	Classes 9	Contain hydrophobic residues and also 11-mer motif. These proteins are insoluble forming a globular structure.

PF02987	D7, D29	Group3	Group 3A,3B	LEA_4	Classes 6	Contain an 11 amino acid motif and Protects the enzymes against partial dehydration
PF00477	D19, D132	Group1	Group1	LEA_5	Classes 5	Characterized by a conserved twenty amino acid motif. Accumulate in dry seeds.
PF00257	D11,	Group2	Group 2	Dehydrin	Classes 1 to 4	Ubiquitous in the plant kingdom Contain different motifs named as K, Y and S segments. Over-expression of them increases freezing tolerance
PF04927(SMP)	D34,73,95	Group6	Group 5 A	SMPO	Classes 11	Contain Highly Hydrophobic residues. Not much characterized

1.6.4 LEA functions

1.6.4.1 Protein stabilization

Although the exact molecular function of LEA proteins is unclear they are found to function as membrane protectants. Many proteins, including enzymes like lactate dehydrogenase, and citrate synthase form insoluble aggregates when dried or frozen but in the presence of LEA proteins aggregation of these enzymes is reduced. Group2 LEA proteins ERD10 and ERD14 usually accumulate in response to abiotic stress and these proteins protect cells against dehydration (Kovacs et al., 2008). LEA proteins have also been demonstrated to prevent heat-induced aggregation of various substrates like alcohol dehydrogenase, citrate synthase, and lysozyme. (Kovacs et al., 2008). Their role of protecting proteins against aggregation has also been demonstrated in living cells (Chakrabortee et al., 2007). The group 3 LEA protein of

the desiccant tolerant nematode *Aphelenchus avenae* prevents in vitro and in vivo aggregation of other proteins (Chakrabortee et al., 2007). When the nematode LEA protein is co-expressed in a human cell line it reduces the disposition of polyglutamine and polyalanine expansion proteins to form aggregates in vivo (Chakrabortee et al., 2007). This anti-aggregation activity of LEA proteins might be due to their hydrophilic unstructured nature. LEA proteins display some similar properties to those of holding chaperones which stabilize passively protein species in a partially unfolded state, preventing aggregation during stress. This process requires ATP, but although LEA proteins resemble holding chaperones they function without ATP hydrolysis (Amara et al., 2014)

1.6.4.2 Membrane protection

To ensure the survival of cells during dessication or freezing, membrane protection is needed. As LEA proteins have no transmembrane segment it is not possible to integrate them into membranes as intrinsic proteins. However, some of them form amphipathic alpha-helices during the drying process which makes them interact with membranes peripherally. This was demonstrated in both plants and rotifers (Hincha & Thalhammer, 2012a). Plant specific dehydrins which contain a 15 mer Lys-rich sequence are proposed to be associated with membrane binding (Close 1996); the maize dehydrin DHN1 was found to bind to liposomes which contain anionic phospholipids, resulting in increased helicity leading to membrane stabilization. Arabidopsis dehydrins ERD10, ERD14, also bind to anionic phospholipids through peripheral electrostatic interactions without modifying the fluidity of the membrane (Kovacs et al., 2008).

1.6.4.3 Ion sequestration and antioxidant capacity

One of the important effects of cell dehydration is an increase in the concentration of intracellular components including ions, that damages the macromolecular structure and function. LEA proteins, as they contain charged amino acids, might function to sequester ions. A protein from the dehydrin family in celery was found to bind to Ca^{+2}

when phosphorylated and also three group 2 proteins(ERD10, COR47, ERD14,) were also found to demonstrate phosphorylation-dependent Ca^{+2} binding (Alsheikh et al., 2003).Group2 LEA proteins were also found to bind to other metal ions through the His residues which are overrepresented in the group 2 LEAs (Amara et al., 2014). This feature may be corelated with their antioxidant properties. This was shown for the citrus protein CuCOr19 which protects against peroxidation of liposomes and also reduces cold-induced electrolyte leakage in tobacco seedlings (Tunnacliffe & Wise, 2007b) as well as exhibiting scavenging activity for hydroxyl and peroxyl radicals. Thus LEA proteins may reduce oxidative stress in dehydrated cells by sequestering metal which generates ROS or by scavenging ROS (Amara et al., 2014).

Other functions-of LEA proteins might be as hydration buffers by slowing down the rate of water loss during freezing or osmotic stress (Amara et al., 2014). They may also act as nuclear proteins that unwind or repair DNA, regulate transcription and also associate with chromatin or the cytoskeleton (Wise & Tunnacliffe, 2004).

1.6.5 Localization of LEA proteins

LEA proteins have been localized to the cytosol, chloroplasts, mitochondria, endoplasmic reticulum, peroxisomes, and chloroplasts. Candat et al. (2014) studied the localization of LEA proteins encoded by 51 genes in *Arabidopsis thaliana*. LEA-GFP fusions of LEA 37 (*SAG21*), 48, and 41 showed a typical pattern of localization to the mitochondrial matrix shown by the perfect co-localization between GFP signal and mitochondrial marker. LEA 42 and 48 were found to be targeted dually to the mitochondria and plastids. Six of the LEA families (LEA_1, LEA_2, LEA_5, LEA_6, Dehydrin, and SMP) show a cytosolic localization with all of their members whereas three out of the four members of LEA_3 family displayed mitochondrial and the last one showed a cytosolic localization (Candat et al., 2014). LEA_ 4 which is the largest LEA family and has 18 members showed multiple localizations (cytosol, plastid, mitochondria, and endoplasmic reticulum).

Three of the four LEA_3 proteins (37, 38 (*SAG21*), and 41) of the LEA3 family were targeted to mitochondria whereas LEA2 showed cytosolic localization. LEA2 and

LEA38 (*SAG21*) were found to be paralogous proteins (Avelange-Macherel et al., 2018; Hundertmark & Hincha, 2008b). Although they are targeted to different locations and share a very similar sequence, the difference in location is thought to be due to the deletion of six amino acids in the LEA2 region just before the putative cleavage site of LEA38 (Candat et al., 2014). As each cellular compartment shows the presence of LEA protein it is clear that LEA proteins play a wide role in stress tolerance during stress conditions like freezing, cold and oxidative stress.

1.7 *AtLEA5/SAG21*

Senescence associated genes are generally defined as those genes which show an increase in their mRNA levels during age mediated or natural senescence (Weaver, et al.,, 1998). The Arabidopsis gene, *AtLEA5/SAG21*(At4g02380) belongs to the LEA_3 protein family. *SAG21* shows a very strong amino acid sequence similarity to the other members of this LEA family in Arabidopsis. The other three genes of the Arabidopsis LEA_3 family, AtDi21(At4g15910), At3g53770, and At1g02820 encode drought responsive proteins which are either targeted to the chloroplast or mitochondria (Hundertmark & Hincha, 2008). These three genes show different expression patterns in floral organs (Winter et al., 2007). *SAG21* was first identified as a senescence associated gene whose expression levels increased just before the senescence of leaf, followed by a decline in expression. *SAG21* shows a strong induction in both dark treated attached leaves and light treated detached leaves and it also showed an unusual response to ethylene treatment where the younger leaves showed a strong response compared to the older ones and this was in contrast to the other SAGs which show stronger induction in the older leaves (Weaver et al., 1998). *SAG21* transcript levels were found to be upregulated by ozone-induced leaf senescence (Miller et al.,1999) and also by hormone treatments like ethylene (Weaver et al., 1998) and jasmonate (Jung et al., 2007). *SAG21* is also a ROS-inducible gene, and is thought to play a role in oxidative stress protection. It shows upregulation by oxidants like H_2O_2, menadione, paraquat and superoxide which indicates that it functions in ROS mediated signalling processes. An investigation into the role of *OXI1* (H_2O_2

inducible serine/threonine protein kinase) in ROS mediated induction showed that *SAG21* induction takes place independently of *OXI1* (Mowla et al., 2006). *SAG21* transcripts were also detected at very low levels in stem, root and cauline leaves but found to be more abundant in flowers which might be due to its high expression in pollen and anthers. Expression of *AtLEA5* in leaves also showed a diurnal pattern of regulation where expression was abundant in the dark and decreased quickly upon exposure to light (Mowla et al., 2006).

Confocal analysis of plants expressing a SAG21-YFP fusion showed a mitochondrial location (Salleh et al., 2012). The regulation of *SAG21* by various abiotic stresses was explored by the analysis of the transgenic plants. Two homozygous transgenic promoter reporter constructs *SAG21*(1685)::GUS and *SAG21*(325)::GUS were generated using respectively 1685 bp and 325 bp of the SAG21 promoter region upstream of the ATG start codon. Expression of *SAG21(1685)*:: GUS and SAG21*(325)*:: GUS was induced by several abiotic stresses including cold, drought, salt and H_2O_2 (Salleh, 2011). Expression of *SAG21* was observed both in the elongation and maturation zones of the root, but expression was found to be absent in lateral root tips and primordia. In transgenic lines of *SAG21(1685)*::GUS, strong upregulation was seen by cold stress followed by drought, salt and H_2O_2 (Salleh, 2011). Spatial and temporal expression patterns of the promoter were investigated by Salleh (2011) to understand the tissue specific expression of *SAG21*. Analysis of the upstream 1685 bp promoter region of *SAG21* showed several regulatory elements upstream of the transcriptional start point. Histochemical GUS staining of transgenic lines carrying these constructs revealed that *SAG21* is mainly expressed in the cotyledons, roots and pollen and its expression is repressed by the light treatment in the roots. No GUS activity was detected in the younger leaves of promoter GUS rosettes but an occasional expression was seen in early senescing leaves confined to the junction between green and yellow tissue. In flowers of *SAG21* (1685):: GUS lines expression was confined to male reproductive organs and pollen. However, pollen specific expression was completely absent in lines expressing the shorter construct. Promoter lines exposed to a light/dark cycle demonstrated that the promoter activity was high in the dark and decreased upon the exposure to light (Salleh, 2011).

Constitutive overexpression of *SAG21* did not confer much tolerance to drought stress and the photosynthetic assimilation of drought treated plants was greatly reduced (Mowla et al., 2006). However an upregulation of *SAG21* gene expression was seen with dehydration stress. However, overexpression of *SAG21* did confer increased tolerance to H_2O_2. When exposed to oxidative stress, the transgenic plants were able to produce greater root and shoot growth than WT at proportionate levels of H_2O_2, indicating the tolerance conferred by expression of *SAG21* to the oxidative stress (Mowla et al., 2006) The effect of overexpression of *SAG21* on overall plant growth and development was investigated by using *SAG21* over-expressor and antisense lines. Antisense plants produced a dwarf stature and early senescence phenotype in the leaves, while the over expressors were taller and had a delayed senescence phenotype. Bolting time was also different where antisense lines displayed an early bolting whereas the over-expressors showed a delay in time for bolting (Salleh, 2011). Below ground phenotype was also affected where the over expressors produced longer primary roots and more lateral roots compared to antisense lines which produced less lateral roots and lateral root primordia. The proportion of longer root hairs showed an increase in over-expressors lines whereas in antisense lines there were no longer root hairs than 300 μm. However, the total number of root hairs was similar in both lines(Salleh, 2011). The effect of *SAG21* perturbation towards pathogens was studied using over-expressor and antisense lines to understand the role of the *SAG21* in defence response. Inoculation of *SAG21* (1685):: GUS constructs with fungal a nectotroph showed an induction in the expression of *SAG2*1. The effect of the bacterial pathogen was also studied on the antisense lines and over-expressors lines and the bacterial numbers were reduced significantly in over expressor lines whereas antisense lines showed an increase in bacterial number(Salleh, 2011).

1.8 Experimental Aims and Objectives

SAG21 protein has been localized to the mitochondria and is an atypical Type III LEA protein (Mohd Salleh, 2011; Weaver et al., 1998). Its expression is upregulated by

dehydration, oxidants and a range of other stresses such as ozone, cold, pathogen infection as well as plant growth regulators like ethylene and jasmonate (Jung et al., 2007). Numerous W- Boxes, MYC motifs, light regulating elements like the GATA box and GT1, root specific and pollen specific motifs (POLLEN1LELAT52, ROOTMOTIFAPOX1) have been identified in the upstream 1685 bp region as well as a 325 bp promoter fragment of the *SAG21* promoter (Mohd Salleh, 2011). Recently yeast-1-hybrid screens have identified a number of stress-related and developmentally- related transcription factors that bind to the *SAG21* promoter (Rogers lab. unpublished results).

This work aimed to study the transcription factor networks regulating the expression of *SAG21* in response to development, senescence, and stress responses. As the prior research had identified the tissue specific, spatial and temporal expression of the SAG21 promoter this work had three main objectives:

(1) Verify whether selected transcription factors previously identified to bind to the *SAG21* promoter are necessary for its expression under stressed and non-stressed conditions.

Four transcription factors (WRKY15, 63, 67 and NAC042) were studied using real time PCR in mutants (*wrky15*, *wrky 63, wrky 67, wrky 042)* and WT lines to assess the effect of the TFs on *SAG2*1 expression (Chapter 3).

(2) Understand the role of the transcriptional regulatory *cis*-elements identified in the upstream region of the SAG21 promoter region by deletion analysis of the promoter region.

Promoter-GUS reporter fusion constructs were used to understand the role of the *cis* elements. Transgenic lines were analysed for GUS expression in different tissues to establish the role of promoter fragments under stress and during development as well as the role of cytokinin in regulating senescence and wounding responses (Chapter 4).

(3) Investigate whether the overexpression of *SAG21* under its own promoter has any effect on development and stress response.

This was investigated by studying the effect of overexpression of the SAG21 coding region under its own promoter in optimal conditions and under oxidative stress

conditions and then comparing its effect to the overexpression of SAG21 from 35S constitutive promoter (Chapter 5).

Chapter 2 General Materials and Methods

This chapter describes the general methods which were employed throughout the study. Methods used for specific experiments are described in their individual chapters. All the chemicals used for the experimentation were purchased from Sigma (UK) and Melford (UK) unless otherwise stated.

2.1 Plasmids

All the plasmids were in the *E.coli* strain DH5 α and *Agrobacterium tumefaciens* strain GV3101. Promoter GUS constructs were selected on the plates with the antibiotic kanamycin at a concentration of 50 µg/ml and transgenic lines were selected on soil with BASTA at a concentration of 120 µg/ml.

Table 2-1 Complete List of Plasmids used in this work

Constructs	Plasmid	Selection	Chapter	origin
Insert (GUS-GFP-NOS) isolated from plasmid pKGWFS7	pKGWFS7-GUS-GFP-NOS	Kanamycin	4	This work
Backbone construct	pGREENII0229-GUS-GFP	Kanamycin	4	This work
SAG21 Promoter Cloning Template	pGEM-T Easy SAG21::1685 promoter	Ampicillin	4	Salleh, 2011
Promoter Constructs	pGREENII0229::SAG21 (1685) promoter::GUS-GFP	Kanamycin	4	This work
	pGREENII0229::SAG21 (1439) promoter::GUS-GFP	Kanamycin	4	This work
	pGREENII0229::SAG21 (1225) promoter::GUS-GFP	Kanamycin	4	This work

	pGREENII0229::SAG21 (965)promoter::GUS-GFP	Kanamycin	4	This work
	pGREENII0229::SAG21 (737) promoter::GUS-GFP	Kanamycin	4	This work
	pGREENII0229::SAG21 (489) promoter::GUS-GFP	Kanamycin	4	This work

2.2 Arabidopsis Seed stocks

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were used for the study in all the experiments in this thesis.

Table 2-2 Seed stocks of *Arabidopsis thaliana* used in this work

Description	Seed Stock	Selection	Chapter	Origin
Promoter GUS Constructs	*pSAG21*(1685)::GUS-GFP	Basta, HM* Line: T2-JJ-3	4	This work
	pSAG21(1439)::GUS-GFP	Basta, HM* Line: T2-M-1	4	This work
	pSAG21(1225)::GUS-GFP	Basta, HM* Line: T2-G-5	4	This work
	pSAG21(965)::GUS-GFP	Basta, HM* Line: T2-F-5	4	This work
	pSAG21(737)::GUS-GFP	Basta, HM* Line: T2-J-2	4	This work
	pSAG21(489)::GUS-GFP	Basta, HM* Line: T2-O-5	4	This work
SAG21-OEX	SAG21P::SAG21 2B	HM*	5	This work
	SAG21P::SAG21 2C	HM*	5	This work

Control	WT	*Arabidopsis thaliana* Ecotype Columbia (Col-0).	3,4,5	This work

(HM*: Homozygous OEX: over expressors)

2.3 Primer Stocks

All the primers used in this work were desalted (DST, grade 0.025) and were purchased from Sigma-Genosys (UK).

Table 2-3 All primer sequences used in this work for PCR

Experiment	Temperature (Tm °C)	Primer set name	Oligonucleotide sequence (5'-3')	Product size in base pairs
Amplification of insert (GUS-GFP-NOS)	57 °C	DCF1R1	DCF1-ATATGGATCCATATGGTGAGCAAGGGCGAGG DCR1-AATTGAGCTCAGGTCACTGGATTTTGGTTTTAGG	2700bp
SAG21 promoter reporter Constructs	55 °C	SAG21F1R (489) promoter ::GUS-GFP	F1-TAGAATTCTGAAAGTTTGCATTTTTCAAATAAATCATTG R-ATGGATCCTTTTCGAAGTAAGTGGTTTCTTG	489bp
	55 °C	SAG21F2R (737) promoter ::GUS-GFP	F2-TAGAATTCACAGATTAAAAAAATATATTAAAACTAATCAT R-ATGGATCCTTTTCGAAGTAAGTGGTTTCTTG	737bp

	65 °C	SAG21 F3R (965) promoter ::GUS-GFP	F3-TAGAATTCCACTTTGCCTACTTTACACACG R-ATGGATCCTTTTCGAAGTAAGTGGTTTCTTG	965bp
	65 °C	SAG21F4R (1225) promoter ::GUS-GFP	F4-TAGAATTCTCTCGTGATAGTATGAAGCTGG R-ATGGATCCTTTTCGAAGTAAGTGGTTTCTTG	1225bp
	65 °C	SAG21 F5R (1439) promoter ::GUS-GFP	F5-TAGAATTCGAATTGGTTATACGATTATCTATCTAGTTAGC R-ATGGATCCTTTTCGAAGTAAGTGGTTTCTTG	1439bp
	67 °C	SAG21 F6R (1685) promoter::GUS-GFP	F6-TAGAATTCTCCAAAACATTGTGAAAAATTGG R-ATGGATCCTTTTCGAAGTAAGTGGTTTCTTG	1700bp
Colony screening	55 °C	SEQ GNF-F GUS R 389	F-GCATCAAGGTGAACTTCAAGATC R-TACGTACACTTTTCCCGGC	650bp
	53 °C	CP-FR	F-CTCATCTCTTCAAACCATTTTCGAAAGC R-GTCGCCCTCGAACTTCACCTCGG	500bp

Real time PCR (housekeeping gene)	60 °C	ACTIN 2	F - TGTGCCAATCTACGAGGG R-TTTCCCGCTCTGCTGTTGT	120bp
Real time PCR of *SAG21*	60 °C	SAG21FR	F-TGCTTGTTGTTCAAGAGAGCTG R-GGAAGAAGTGGAGCTGTTGC	137bp
Sequencing primers used for the insert verification		M13 FR	F-TGTAAAACGACGGCCAGT R-CAGGAAACAGCTATGACC	

2.4 Nucleic acid Quantification.

RNA and DNA samples were quantified on a Nanodrop uv-Spectrophotometer (Thermo Fisher Scientific ND-1000, UK) by measuring absorbance of 1µl of sample at 260 nm. The purity of the sample was assessed by the 260/280 ratio.

2.5 Agarose gel electrophoresis

Generally 1-1.5 % agarose gels were prepared for separation of PCR products or nucleic acids. Agarose (Bioline, UK, 1-1.5 g) was dissolved in 50 ml 1 X TAE Buffer (4.84 g/L Tris-HCl, 1.142 ml/L glacial acetic acid, 2 ml/L 0.5M EDTA) and boiled in a microwave for 1-2 minutes until the agarose was completely dissolved. The agarose was cooled down to 60 °C under tap water and 2 µl of Ethidium bromide (10 mg/ml) or Safeview (NBS Biologicals) was added to the 50 ml of agar and mixed gently. The agarose was then poured into a gel tray, a comb was inserted, and it was allowed to solidify at room temperature for 15-20 mins. Then the comb was removed, the gel was transferred to a gel tank, and covered with 1 X TAE buffer. For RNA the gel comb, tray and tank were soaked in 0.1 N NaOH for 20-30 mins before pouring the gel to remove any contaminating RNAse which might degrade the RNA. Prior to use the comb, tray and tank were rinsed with sterile distilled water.

RNA or DNA samples were mixed with 6X Loading dye buffer (10 mM Tris-HCl, 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol, 50 mM EDTA) before loading on to the wells for running the gel. A ladder (1Kb^{+} DNA ladder, Invitrogen) was run alongside the samples for comparison of product size. Generally, gels were run in 1 X TAE Buffer at a voltage of 60-100 V depending upon the sample. Gels were then visualized under uv-illumination using a GelDoc®-It 310 imaging system (UVP).

2.6 PCR Reactions

PCR reactions were set up in a 20 µl volume in a GeneAmp® 3700 Applied Biosystems thermocycler or a Techne Flexigene (Techne, UK), or Veriti® 96 well thermal cycler (Applied Biosystems) PCR machine. Reactions contained 0.5 µL of dNTPs (10 mM), 5X Green Go Taq Flexi buffer (Promega), 1.5 µl $MgCl_2$ (25 mM), of forward and reverse primers (10 µM) (Table 2.3), and 0.125 µl Go Taq polymerase (Promega 5U/µl). Cycling was carried out for 35 cycles of: 94.0 °C - 3.00 min, 94.0 °C - 30-60 sec, primer dependent temperature - 30-60 sec, 72.0 °C -30 sec-2 min dependent on fragment size, 72.0 °C - 7-10 mins, 4.0 °C -hold. Products were then checked on a 1% agarose gel. In order to obtain equal concentrations of template cDNA for real time PCR, the samples were normalized to 100 ng/µl and further diluted to 10 ng/µl and PCR was carried out on the 10 ng/µl stock with primers to check uniformity of products.

2.7 Seed Sterilization

Approximately 50-100 *Arabidopsis thaliana* seeds were transferred to 1.5 ml Eppendorf tubes and surface sterilised by treating with 0.5-1 ml of 10% sodium hypochlorite. Tubes were mixed by inverting for 1 minute and then allowing the seeds to settle to bottom of the tube, at room temperature for 1-2 mins .Then the hypochlorite solution was removed and seeds were washed with 1 ml of ETOH mix (ETOH: sterile water: sodium hypochlorite (7:2:1) ratio) and again the tube was inverted for a few times. The ETOH mix was then removed and the seeds were washed two or three times with 1 ml sterile distilled water to ensure that the bleach was removed completely. The seeds were then stratified in sterile water at 4 °C for 24 hrs.

2.8 Plant growth conditions.

For growth in soil, stratified seeds were sown onto a 3:1 ratio of sterilized multipurpose compost (John Innes no 3 UK) and sand (v/v) in 5-9 cm pots. When grown under sterile conditions, seeds were sown onto autoclaved 1 X Murashige and Skoog medium (MS; 4.708g/L) basal salt (Duchefa Biochemie, Melford) supplemented with 1% Difco™ Agar (Becton, Dickinson and Company) and 1-3% (w/v) Sucrose (Thermo Fisher Scientific UK) adjusted to pH 5.5-5.7. Stratified seeds were sown onto 100 mm Petri dishes individually with the help of a 1 ml pipette by slowly releasing seeds onto the surface of the media plates. After drying plates in an laminar air flow for a few minutes Petri plates were sealed with 3M™ Micropore™ Medical Tape (Thermofisher UK) and moved to growth chambers for growing under controlled conditions.

2.9 Statistical Analysis

All the statistical work in the entire study was carried out by using the R Studio (version 1.2.5019) programming. The normality of the data was tested using a suitable parametric test on a normally distributed data and non-parametric test was applied on the non-normal data. Significance of the differences was determined if the P value < 0.05 with all the statistical test used.

Chapter 3 Analysis of effect of WRKYs and NAC TF on *SAG21* expression

3.1 Introduction

Plants, when exposed to abiotic and biotic environmental stresses have evolved mechanisms to combat these stresses. At a cellular level these include closure of stomata, and inhibition of vegetative growth, and at a molecular level induction of stress responsive genes and stress tolerance genes (Chen et al., 2012). Transcriptional regulation of stress induced genes plays an important role in the development of stress tolerance and is dependent on the spatial and temporal function of transcription factors (Banerjee & Roychoudhury, 2015). These TFs are components of the process of signal transduction. They interact with the *cis*-elements in stress inducible genes thus regulating their expression and thus providing tolerance against multiple stresses (Banerjee & Roychoudhury, 2015). Some of the important stress-related *cis*-regulatory elements (CRE) include the ABRE (abscisic acid responsive element), the DRE (dehydration responsive element), and W-boxes (Zou et al., 2011).

SAG21 is developmentally regulated, and also up regulated by biotic and abiotic stresses (Salleh et al., 2012), forming an interesting gene to study the interface between stress and senescence. To understand the transcriptional regulatory network and *cis*-elements involved in regulating *SAG21* gene expression in response to stress, the Y1H (yeast 1 hybrid technique; (Castrillo et al., 2011; Hickman et al., 2013) was applied to the analysis of the *SAG21* promoter (Rogers lab. unpublished results). A library of TFs was fused to the GAL4 activation domain within a plasmid carrying a tryptophan synthesis gene and transformed into yeast (strain AH109). SAG21 promoter fragments were cloned into another vector which contains leucine and histidine synthesis genes and transformed into yeast strain Y187). Mating between the two yeast strains enables interaction between promoter and TF. Yeast growth on media deficient in leucine and tryptophan demonstrates successful mating whereas growth on medium deficient in leucine, tryptophan and histidine indicates an interaction between a TF and the promoter fragment, resulting in activation of the histidine synthesis gene.

Y1H analysis was carried out using seven overlapping fragments from the promoter of *SAG21* covering 1685bp upstream from the ATG site to identify the TFs which bind to

the promoter (Figure 3.1 a).

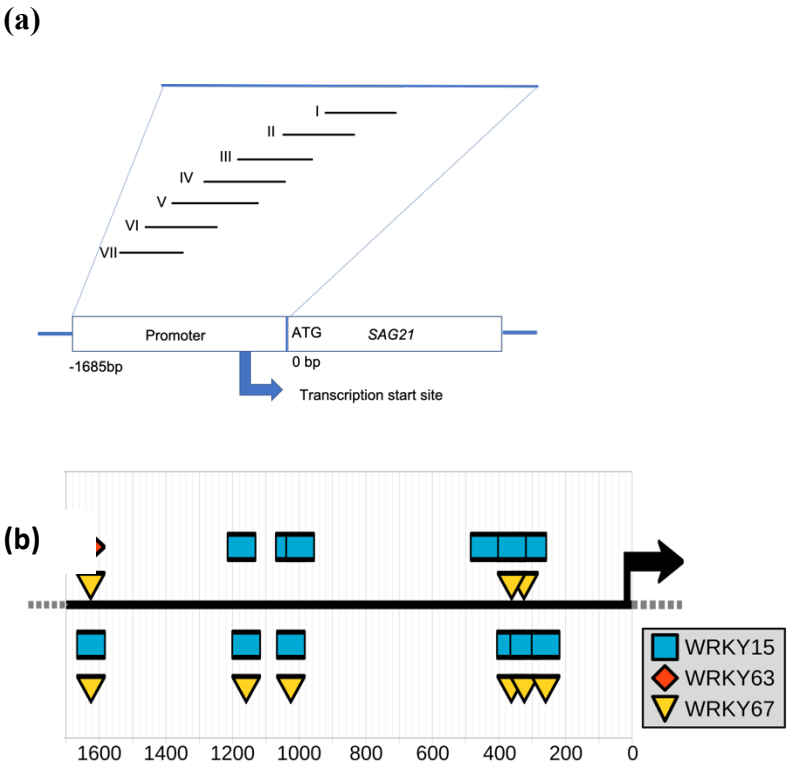


Figure 3.1: (**a**) SAG21 promoter region split into seven overlapping fragments upstream from the ATG for screening by Y1H. (**b**) WRKY transcription factors (WRKY15,63,67) showing the positions of binding on the 1685 bp SAG21 promoter region.

The seven overlapping promoter fragments were screened against a library of 75 *WRKY* TFs (Hickman et al., 2013). Bioinformatic analysis (Salleh, 2011) indicated that the promoter region of *SAG21* contains four W-boxes which are the binding sites for WRKY TFs. Two of these W-boxes were identified close to the ATG and the other two were identified upstream at position -1020 and -1620. Out of the 75 WRKY TFs screened, 13 were found to interact with four of the seven fragments, where one of the fragments contained two of the predicted W-boxes. These 13 WRKY TFs play a role in regulating different process like ABA signalling, salt stress response and pathogen defence. Of the 13 WRKY TFs that bind with the *SAG21* promoter, three WRKY TFs– WRKY15, WRKY63 and WRKY67 were chosen for further study. *WRKY15* was selected as it responds to ROS (Vanderauwera et al., 2012) which are thought to be important in the activation of *SAG21*. *WRKY 63* and *WRKY67* were selected as they are responsive to two other stresses known to elicit *SAG21* expression: salinity and drought

(Ren et al., 2010; Vanderauwera et al., 2012).

The seven overlapping fragments of the *SAG21* promoter region were also screened against a library of NAC TFs using Y1H to identify any TFs which could bind to the promoter region. Sequence analysis of promoter region revealed a CGTGA, *cis*-element which is a recognition site for NAC TFs (Wu et al., 2012) and was located between 1125 and 1436 bp upstream of the ATG. Of the 96 NAC TFs screened, four of them (NAC013, NAC042, NAC038/39, NAC071) showed a positive interaction. NAC042 was selected for further study as it is regulated by ROS (Wu et al., 2012). Real time PCR was used to investigate further the role of the selected *WRKY* and *NAC* TFs in regulating *SAG21* expression. Expression was determined with and without stress in knock out or knock down mutants of two *WRKY* TFs: *WRKY63* and *WRKY67*, and in an artificial micro RNA line of *WRKY15* in which expression of *WRKY15* had been down-regulated (Vanderauwera et al., 2012). In addition the effect of mutation and overexpression of *NAC042* on *SAG21* expression was also analysed by RT-PCR using a KD line and a line in which *NAC042* overexpression was driven by the 35S promoter (Wu et al., 2012).

3.2 Methods

3.2.1 Plant material and Seed sterilization

An artificial micro RNA (amiRNA) line for *Arabidopsis thaliana WRKY15* (AT2G23320) was obtained from the lab of Prof Van Breusegem (Vanderauwera et al., 2012) T-DNA insertion lines for *WRKY63* (At1g66600; SALK_068280C), and *WRKY67* (AT1G66550; SALK_027849) were obtained from the Nottingham Arabidopsis Stock Centre. T-DNA insertion line of *NAC042* (AT2G43000; SALK_036474) was obtained from the lab of Prof Mueller-Roeber and Prof Balazadeh (Wu et al., 2012). Mutant lines used here were grown horizontally on plates in a Sanyo Fitotron growth chamber under long day (LD) conditions with 16h light and 8h dark conditions at 21 °C with light intensity of 70-90 $\mu molm^{-2}s^{-1}$.

Approximately 50 seeds were transferred to a 1.5 ml Eppendorf tube and surface sterilized according to procedure described in Section 2.7. The seeds were then stratified

in a Petri dish with sterile distilled water at 4 °C for 24 hrs. The surface sterilized seeds were then sown onto 1% MS agar medium containing 1% sucrose, 1% agar (Difco™ Agar), and 1 x basal salt medium (Duchefa Biochemie) in Petri dishes and seedlings were grown vertically in a controlled growth chamber at 21°C, under 16hr light /8hr dark (90-100 µmol m^{-2} s^{-1}) for 14 days

3.2.2 Abiotic stress treatments

Seedlings were pre-treated with 12 hr light (90-100 µmol $m^{-2}s^{-2}$) before stress treatment to repress expression of the *SAG21* gene which is light regulated (Mowla et al., 2006; Salleh et al., 2012). For drought stress, *wrky*63 seedlings were removed from Petri plates and placed on Whatmann filter paper for 30 mins and exposed to air flow in a laminar chamber (Microflow, Hampshire, United Kingdom), and for the control treatment, Petri plates were left in the growth chamber for 30 mins (Kilian et al., 2007). Hydrogen peroxide (H_2O_2) stress treatment was performed on *wrky*15, *nac042*, $NAC042^{OE}$ and WT seedlings. Seedlings were submerged in 1 X MS liquid medium with 10 mM H_2O_2 for 6 hrs and control seedlings were left on plates (Wu et al., 2012). For salt treatment, *wrky*67 seedlings were grown on MS Petri plates to which 100 mM NaCl was added and they were grown for 14 days (Vanderauwera et al., 2012). Whole seedlings from three biological replicates of the control and stress treatments were harvested and frozen in liquid nitrogen and stored at -80 °C.

3.2.3 RNA isolation

RNA isolation was carried out according to the QIAGEN RNeasy Plant Mini Kit. Before use mortars and pestles were soaked in 0.1 N NaOH for at least 1-2 hr to make them free from RNases and then rinsed with sterile distilled water. They were then wrapped with aluminium foil and autoclaved at 120 °C for 20 min. After drying they were stored at -20 °C. Approximately 100-150 mg of plant material was ground into a fine powder with liquid nitrogen in a mortar and pestle, was collected in 1.5 ml Eppendorf tubes, and then frozen at -80°C. On the day of RNA isolation, 450 µl of Buffer RLT was added to tissue along with β-mercapto ethanol (Sigma Aldrich) and vortexed vigorously. Then the lysate was transferred to a QIA-shredder spin column (lilac) placed in a collection tube and was centrifuged at 13,000 rpm in a microcentrifuge (miniplus Eppendorf) for 2min. The

supernatant was transferred to a new microcentrifuge tube without disturbing the cell debris and 0.5 volume of ethanol (96-100 %) were added to the supernatant and mixed immediately by pipetting up and down. The entire sample was transferred to an RNA easy spin column and was centrifuged for 30 sec at 13,000 rpm in a microcentrifuge and flow through was discarded. The column was then washed with 700 µl of RW1 buffer and centrifuged as above at 10,000 rpm for 30 secs and flow through was discarded. Next, 500 µl of RPE buffer was added and centrifuged for 2 mins at 10,000 rpm. Generally, a long centrifugation time was used at this step to make ensure the column was completely dried since there should not be any presence of ethanol for further steps. The column was then placed in a new 1.5 ml centrifuge tube and 30-50 µl of RNase free water were added to the membrane and incubated for 1 min at room temperature. The column was then centrifuged as above at 10,000 rpm for 60 sec to elute the RNA. Eluted RNA was stored at -80 °C until further use.

3.2.4 Dnase treatment of RNA

Samples were treated with DNase to remove genomic DNA contamination. Reactions were set up in a 20 µl volume using 2 µg RNA per sample. For each reaction 2 µl of RQ1Dnase 10x Buffer (400 mM Tris-HCl (pH 8.0), 100 mM MgSO4 and 10 mM CaCl2), 2 µl of RQ1Dnase (1 U/µl Promega), were added to the RNA in the PCR tubes and the volume was made up to 20 µl with sterile distilled water. Samples were then incubated at 37°C for 30 mins. The DNase was inactivated by adding 2 µl of stop solution (20 mM EGTA (pH 8.0) and the whole mix was incubated at 65 °C for 10 mins. To confirm whether DNase treatment was successful 1 µl sample of RNA was used for PCR with reference gene primers (*ACTIN2*).

3.2.5 cDNA synthesis

First strand cDNA was synthesized from RNA samples using M-MLV RNase H Reverse Transcriptase (Promega). To 19 µl of DNase treated RNA, 1 µl of oligo dt (Promega) was added which anneals to the poly A tail of mRNAs. The mix was incubated at 70 °C for 10 min. Samples were then placed on ice for 10 min. Then 6 µl of 5x first strand buffer (250 mM Tris-HCl (pH 8.3 at 25 °C), 375 mM KCl, 15 mM MgCl2, 50 mM DTT), 2 µl of 0.1 M DTT (Dithiothreitol), 1 µl of 10 mM dNTPs were added and the

mix incubated at 42 °C for 2 min. The reaction was started by adding 1 µl of M-MLV RNase H Reverse Transcriptase (Promega 200 U/µl) and the mix was incubated at 42°C for 50 min and then the reaction was terminated at 70 °C for 15 min. The concentration of DNA was then checked on a Nanodrop uv spectrophotometer (Thermo Fisher Scientific ND-1000) and quality was confirmed by PCR with the reference gene (*ACTIN2)*.

PCR reactions were set up in a 20 µl volume in a Gene Amp 3700 Applied Biosystems thermocycler. Reactions contained 0.5 µL of dNTPs (10 mM), 5X Green Go Taq Flexi buffer (Promega), 1.5 µl MgCl2 (25 mM), 1 µl of forward and reverse *ACTIN2* primers (Table 2-3), and 0.125 µl Go taq polymerase (Promega 5U/µl). Cycling was carried out for 35 cycles of: 94.0 °C - 3.00 min, 94.0 °C - 0.30 sec, 60.0 °C - 0.30 sec, 72.0 °C - 0.30 sec, 72.0 °C – 10 mins, 4.0 °C -hold. Products were then checked on a 1% agarose gel. In order to obtain equal concentrations of template cDNA for real time PCR, the samples were normalized to 100 ng/µl and further diluted to 10 ng/µl and PCR was carried out on the 10 ng/µl stock with *ACTIN2* primers to check uniformity of products.

3.2.6 Quantitative Real time PCR

Real Time PCR was conducted using a Light Cycler 96 (Roche) machine. A 20 µl reaction was set up using 6 µl of cDNA (60 ng) as a template, 0.4 µl of forward and reverse primers (10µM),10 µl of 2x qPCRBIOSyGreen Mix Lo-ROX (PCR Biosystems), 3.2 µl of sterile distilled water. PCR thermal profiling conditions were as follows: 95.0 °C -120 sec, 95.0 °C - 0.30 sec, 60 °C - 0.30 sec, 72.0 °C -0.30 sec for 35 cycles followed by melting curve analysis from 60.0 to 98.0 degrees to check for primer specificity and primer dimers. Gene expression analysis was carried out using the relative comparative method (Livak &Schmittgen 2001) using the $2^{-\Delta\Delta ct}$ method.

3.3 Results

3.3.1 RNA Extraction

RNA was extracted from whole seedlings of *wrky15, nac042 KO* and *NAC042^{OE}, wrky67, wrky63* and WT lines. For each line, seedlings were either stressed or non-stressed. For stress treatments, *nac042 KO, NAC042^{OE},* and *wrky15* were treated with

H_2O_2 while *wrky67* was salt stressed and *wrky63* was drought stressed. Isolation of RNA was successful showing a clear banding pattern of 28s and 18 s rRNA on agarose gels (Figure 3.2).

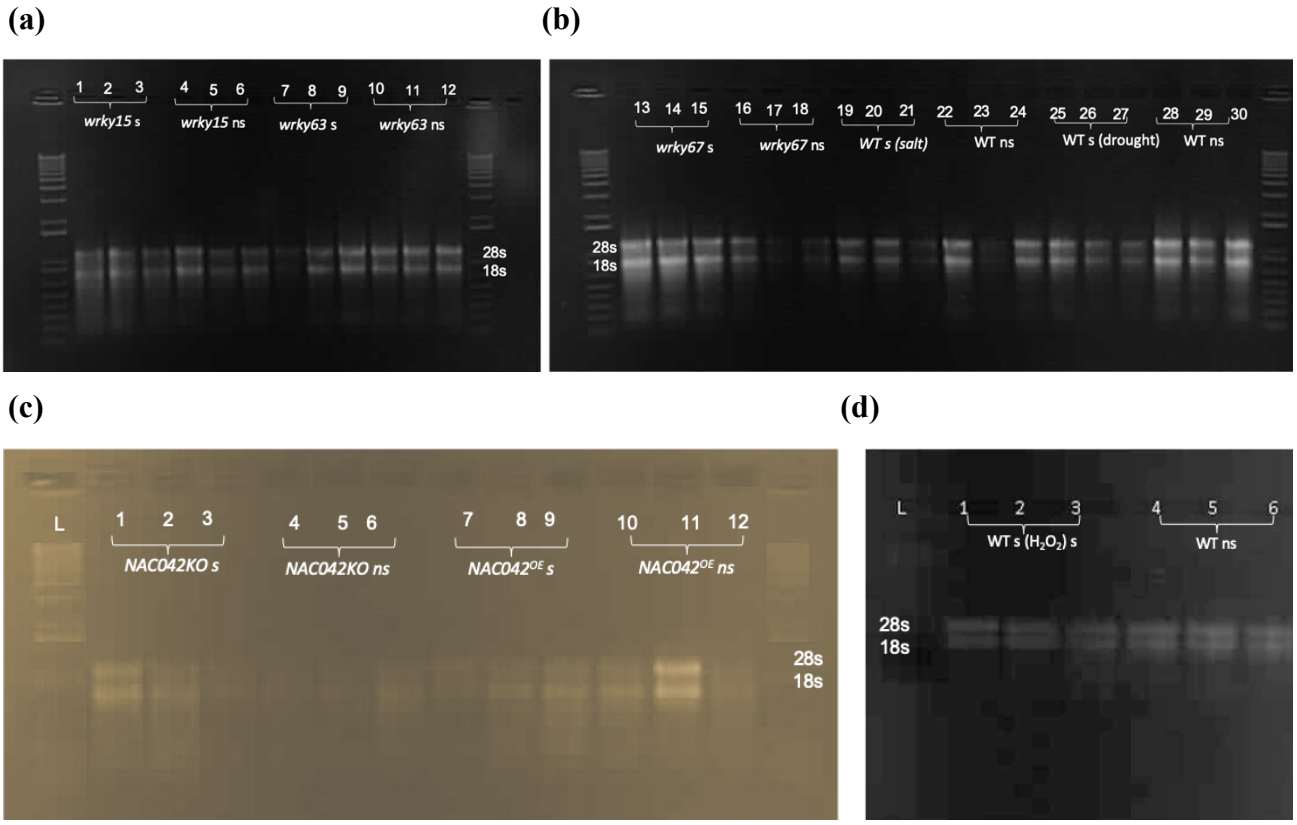


Figure 3.2 : Agarose gels showing integrity and concentration of RNA extracted from stressed (s) and non stressed (ns) seedlings**(a)** RNA isolation from *wrky15* and *wrky63* seedlings. Lanes1-3: *wrky15* H_2O_2 stressed samples. Lanes 4-6: *wrky15* non stressed samples. Lanes 7-9: *wrky63* drought stressed. Lanes 10-12: *wrky63* non stressed. **(b)** RNA isolation from *wrky67* and WT samples. Lanes 13-15: *wrky67* salt stressed. Lanes 16-18: *wrky67* non-stressed. Lanes 19-21: WT salt stressed. Lanes 22-24: WT non stressed. Lanes-25-27: WT drought stressed. Lanes 28-30: WT non stressed. **(c)** RNA isolation from *nac042KO and* $NAC042^{OE}$ seedlings. Lanes1-3: *NAC042KO* H_2O_2 stressed. Lanes 4-6: *NAC042KO* non stressed. Lanes7-9: *NAC042OE* H_2O_2 stressed. Lanes 10-12: *NAC042O*E non stressed **(d)** RNA isolation from WT H_2O_2 stressed and non stressed seedlings. Lanes1-3: WT H_2O_2 stressed. Lanes4-6: WT non stressed. Equal loading in each lane (2µg) based on Nanodrop spectrophotometer.

RNA samples were then subjected to DNAse treatment to remove residual genomic DNA. To test whether the DNAse treatment was successful PCR was carried out with housekeeping gene *ACTIN2* primers. PCR products were checked on an agarose gel and were completely absent indicating that all residual genomic DNA had been removed. (Appendix 3, Figure A3.2). Following DNAse, treatment cDNA was synthesized and checked by PCR with the reference gene (*ACTIN2*) primers. Based on the above PCR, and assessment of concentration using a Nanodrop spectrophotometer, cDNA was

diluted accordingly to 10 ng/µl and again PCR was carried out with reference gene primers. Analysis by agarose gel electrophoresis of the PCR products indicated an equal amount of cDNA concentration across all the samples which were then used for real time PCR (Appendix 3, Figure A3.2).

3.3.2 Expression of *SAG21* in Transcription factor mutants and *NAC042* ^{OE} with and without abiotic stress treatments by real time PCR

To determine whether mutation of TFs that bind to the *SAG21* promoter had any effect on expression of *SAG21,* real time PCR on WT, knock down mutants (KD) of *WRKY15, WRKY63, WRKY67* and *NAC042,* as well as *NAC042*^{OE} (over-expressor) was carried out to study gene expression with and without abiotic stress treatments. The expression of *SAG21* was compared in stressed and unstressed WT and stressed and unstressed mutant seedlings.

Oxidative stress induced a significant upregulation of *SAG21* expression in both WT and *wrky15* amiRNA mutant seedlings ($P < 0.05$). The extent of the upregulation was similar, although the actual expression in stressed mutant seedlings was slightly (but not significantly) lower than in WT. Without oxidative stress, there was a slight downregulation of *SAG21* in the *wrky15* mutant seedlings compared to WT (Figure 3.3), although again this was not statistically significant.

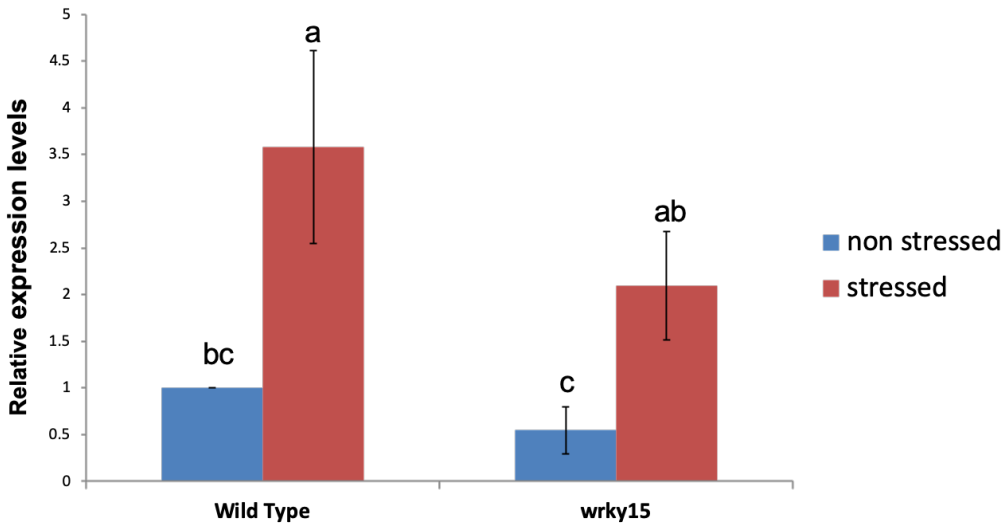


Figure 3.3: Effect of Hydrogen peroxide (H_2O_2) stress treatment on *SAG21* expression levels in WT and *wrky15* amiRNA stressed and non-stressed seedlings as measured by real time PCR(n=3; error bars ± S.E.; different letters indicate significantly different values, Kruskal Wallis followed by Dunn's test, $P < 0.05$).

Under drought (ambient dehydration) stress both WT and *wrky63* mutant seedlings showed a slight upregulation of *SAG21* when compared to non-stressed seedlings, but the effect was not statistically significant. However, in non-stressed *wrky63* mutant seedlings, *SAG21* was expressed at a significantly higher level ($P < 0.05$) than in WT non-stressed seedlings. When stressed, *SAG21* also appeared to be more highly expressed in the *wrky63* mutant seedlings compared to WT but the difference was not statistically significant (Figure 3.4).

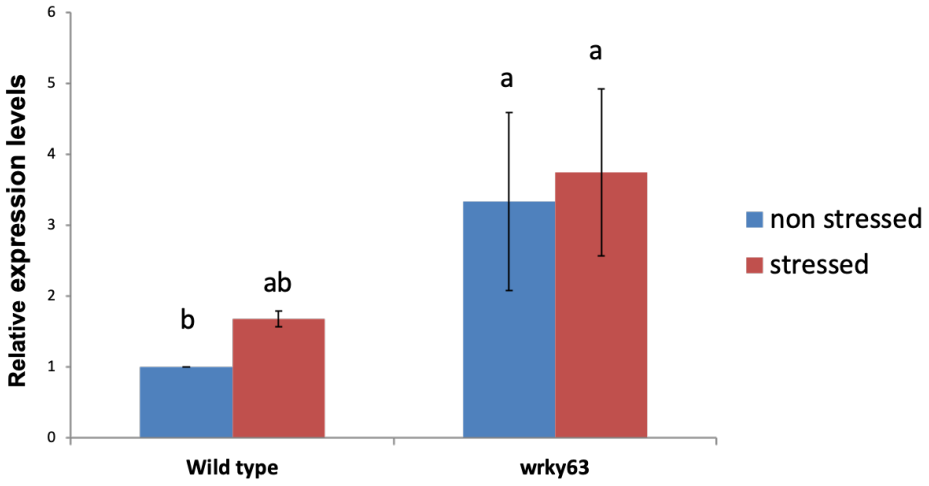


Figure 3.4: Comparison of *SAG21* expression levels as determined by real time PCR in WT and *wrky63* stressed and non -stressed seedlings (n=3; error bars ± S. E.; different letters indicate significantly different values, Kruskal Wallis followed by Dunn's test, $P < 0.05$).

Analysis of *SAG21* expression with NaCl stress treatment revealed that WT grown on media containing salt showed a significant downregulation ($P < 0.05$) when compared with non stressed WT. In contrast, in the *wrky67* mutant, there was a significant upregulation ($P < 0.05$) of *SAG21* (Figure 3.5) with salt stress compared with unstressed seedlings. Furthermore, under non-stressed conditions, *SAG21* was expressed at a lower level in *wrky67* mutants compared to WT ($P < 0.05$), while under stressed conditions expression of *SAG21* was greater in the mutant compared to WT ($P < 0.05$).

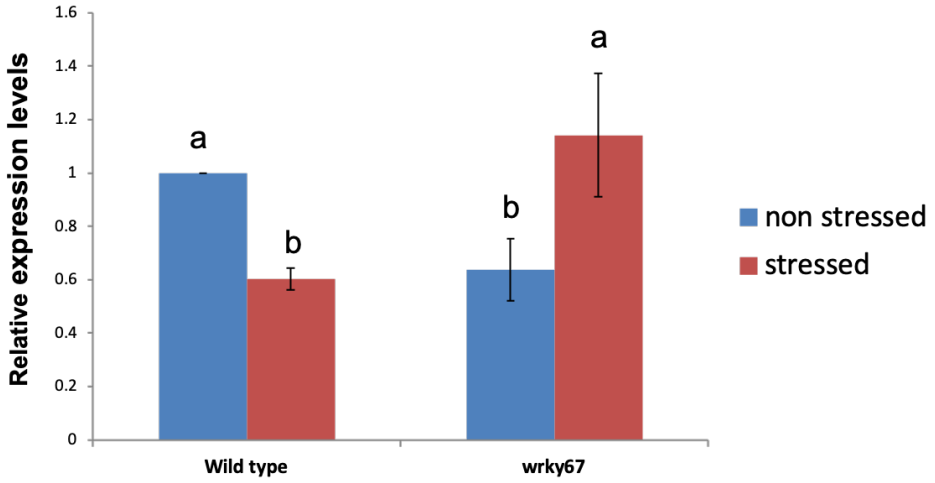


Figure 3.5: Comparison of *SAG21* expression levels as determined by real time PCR in WT and *wrky67* stressed and non-stressed seedlings (n=3; error bars ± S. E.; different letters indicate significantly different values, Kruskal Wallis followed by Dunn's test, $P < 0.05$).

Real Time PCR analysis of the *nac042* knockout and $NACO42^{OE}$ with respect to oxidative stress showed an upregulation of *SAG21* in seedlings of all three genotypes although the increase was only significant ($P < 0.05$) in the KO line (Figure 3.6). Without stress, there appeared to be a slight decrease in *SAG21* expression in the over-expressor line, although it was not statistically significant.

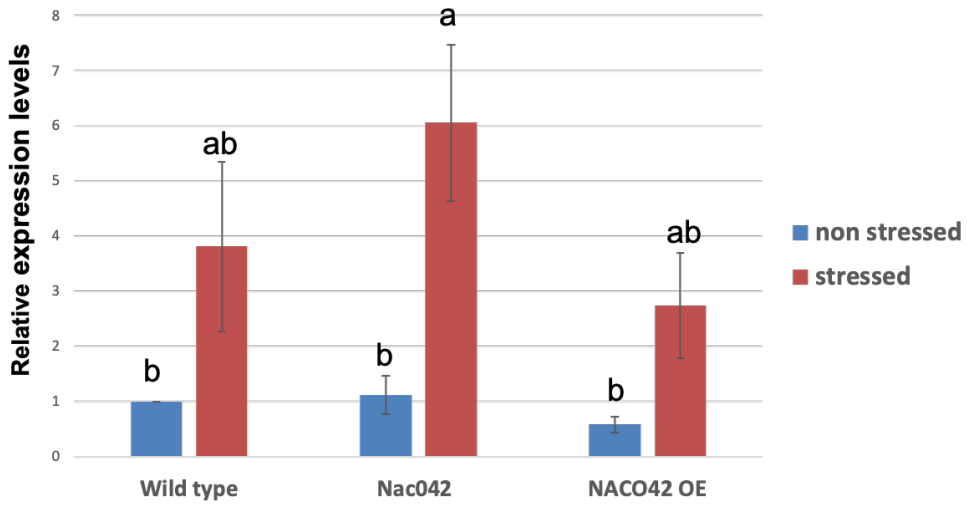


Figure 3.6: Comparison of *SAG21* expression levels as determined by real time PCR in WT, *nac042* (knockout) and *NAC042*^{OE} (over-expressor) stressed and non-stressed seedlings with oxidative stress treatment(n=3; error bars ± S. E.; different letters indicate significantly different values, Kruskal Wallis followed by Dunn's test, $P < 0.05$).

3.4 Discussion

When plants are exposed to stress as a whole organism they generally first recognize the external signal at the membrane level via receptor like kinases or histidine kinases which later then activate signalling molecules like ROS and Ca^{2+} (Tuteja, 2007) and MAP kinases that activate transcription factors, switching on the expression of protective genes. In this study the role of *WRKY* and *NAC* transcription factor mutants in regulating *SAG21* expression under abiotic stress was investigated.

WRKY 15 is not essential for the induction of *SAG21* expression with oxidative stress

Real time PCR results showed an up regulation of *SAG21* in wild type and *wrky15* seedlings when they were challenged with oxidative stress. There was a very slight down regulation of *SAG21* in *wrky15* mutant seedlings compared to wild type both when stressed and without stress, however, it was not statistically significant. This result is consistent with the literature which showed that *SAG21* is induced by oxidative stress (Mowla et al., 2006; Salleh et al., 2012). Here seedlings in these study were exposed to 6 hr of stress treatment whereas previously it was carried out for 30 mins (Salleh et al.,

2012). Although, exposure to stress treatment is different in each study *SAG21* shows an induction with oxidative stress which suggests that even a short term stress treatment is enough for the gene to be activated by stress which also suggests a role for *SAG21* in the ROS-signalling process (Salleh et al., 2012). However, the lack of change in *SAG21* expression in *wrky15* mutants indicates that *WRKY15* is not essential for *SAG21* expression under non-stressed conditions, or for its induction by ROS. This further indicates that other TFs must be involved in the ROS induction of *SAG21* either on their own or acting as a complex.

WRKY63 acts as negative regulator of *SAG21* expression with response to drought stress

With the drought treatment imposed in this study (ambient dehydration for 30 min) *SAG21* expression was slightly up regulated in wild type and mutant *wrky63* seedlings but not significantly. This is in contrast with previous data (Mowla et al., 2006; Salleh et al., 2012) who showed a significant up-regulation of *SAG21* in response to drought. Although the method used in this study is similar to that used by Salleh et al. (2012) it was different to that used by Mowla et al. (2006) where plants were dehydrated by withholding water. In Salleh et al. (2012) the change in *SAG21* expression was monitored through the use of GUS assays in the nine day old seedlings. So the lack of induction by drought seen in this study may be due to the age of the seedlings (here seedlings were 14 days old) or assay method. However, expression of *SAG21* was significantly higher in mutant seedlings under non drought conditions. Hence this suggests that *wrky63* may function as a negative regulator of *SAG21* under non-stressed conditions. This is partly consistent with the findings of Ren et al., (2010) who report that *WRKY63* is a negative regulator of ABA signalling but a positive regulator of drought signalling.

WRKY67 acts as a positive or negative regulator of *SAG21* depending on the stress condition

Salt treatment of WT and *wrky67* mutants revealed a significant up regulation of *SAG21* in the mutant and down regulation in wild type. Furthermore, under non stressed conditions *SAG21* was significantly down regulated in the mutant compared to wild type. The down regulation of *SAG21* under non-stressed conditions does not agree with Salleh et al., (2012) where up regulation of *SAG21* was seen with salt stress. However,

the method of stress treatment used in this study was according to Vanderauwera et al. (2012) where seedlings were grown for a long period (2 weeks) on MS medium containing salt. This was quite different to Salleh et al., (2012) where the seedlings were exposed to short term stress treatment for one hour. Generally, exposure of a plant to long term salt stress causes ion imbalance and osmotic stress (Golldack etal., Shavrukov, 2013). Two phases of gene expression are seen relating to a salt shock: in the first hour of treatment and then salt stress to prolonged or step up treatments. Hence in future work this experiment should be repeated to assess whether *SAG21* responds to both types of salt treatment. Under the conditions used here the up-regulation of *SAG21* expression under prolonged salt stress in the *wrky67* mutant suggests that WRKY67 may be acting as a negative regulator of *SAG21* However, in non-stressed conditions the real time PCR results indicate that *wrky67* functions as positive regulator of *SAG21.*

***NAC042* had no effect of *SAG21* expression in response to oxidative stress**

Real time PCR analysis of oxidative stress treatment on the *nac042* knockout and NACO42^{OE} compared to WT revealed an up regulation of *SAG21* in WT and both mutant and over- expressor lines. However, varied levels of NAC042 had no significant effect on *SAG21* expression levels either with or without the stress treatment. The up-regulation of *SAG21* expression with oxidative stress agrees with the literature and with the results above in the experiment comparing *SAG21* expression in WT and *wrky15* mutants showing that *SAG21* is induced by H_2O_2 (Mowla et al., 2006; Salleh et al., 2012). The lack of change in *SAG21* expression in response to changes in *NAC042* levels indicates that this NAC TF is not required for *SAG21* expression either under stressed or non-stressed conditions. *SAG21* expression in these lines is consistent with the transcriptomic data for the *NAC042* mutant and over-expressor lines: *SAG21* was down-regulated in *NAC042* over-expressors and slightly upregulated in the *NAC042* knock-down line (Prof Salma Baladazeh, personal communication).

Overall regulation of *SAG21* by transcription factors

Thus, evidence from real time PCR in this chapter indicates that two WRKY TFs may be affecting *SAG21* expression in different ways. Oxidative stress induced a significant upregulation of *SAG21* expression in both WT and *wrky15* amiRNA mutant seedlings but because of the lack of change in expression in mutant seedlings it can be concluded that *WRKY15* is not essential for *SAG21* expression under non-stressed conditions. *SAG21* shows up regulation with drought stress in wild type and mutant *wrky63* seedlings

but not significantly. As the expression of *SAG21* was significantly higher in mutant seedlings under non drought conditions therefore it suggests that *wrky63* may function as a negative regulator of *SAG21* under non-stressed conditions. With regard to salt stress *SAG21* functions as positive or negative regulator but the effect depends on the stress condition. Up-regulation of *SAG21* expression with salt stress in the *wrky67* mutant compared to WT suggests that WRKY67 may be acting as a negative regulator of *SAG21* under salt stress. With respect to oxidative stress treatment *SAG21* showed upregulation in *nac042* knockout and $NACO42^{OE}$ compared to WT but due to the varied levels of expression it is concluded that it has no effect with oxidative stress.

Many WRKYs are found to be involved in the drought and salinity response. In Arabidopsis *AtWRKY25* and *AtWRKY33* transcripts were found to be increased by the drought, ABA and salt stress (Li et al., 2011). It was also shown that they are phosphorylated by the MAPKs and thus are involved in heat stress response (Mao et al., 2011). Abiotic stress results in the production of reactive oxygen species, and H_2O_2 functions as a signalling molecule providing tolerance to various stress conditions (Miller et al., 2008). In Arabidopsis expression of several WRKYs like *AtWRKY75*, *AtWRKY53*, and *AtWRKY6* were found to be induced by oxidative stress (Davletova et al., 2005). *AtWRKY53* also functions in drought response and its expression is also induced by oxidative stress (Miao et al.2004; Shen et al., 2012). MAPKs also function in the regulation of the downstream signals during the ABA mediated stress response. Drought stress causes the phosphorylation and activation of MPK3 which phosphorylates the Ser residue of OsWRKY30 and activates the WRKY protein which leads to the initiation of transcription by binding to the W-box of the gene leading to the drought tolerance (Shen et al., 2012). Hence more work is needed to establish if other WRKY TFs or other TF family members are regulating *SAG21* expression under stress, and how upstream signalling feeds into this network.

Chapter 4 Functional Analysis of *cis*-Regulatory elements in *SAG21* gene

4.1 Introduction

Knowledge of gene promoters is required to understand the global regulation of gene expression in plants. Promoters are usually classified into three different classes: constitutive promoters show constant levels of gene expression in all tissues, spatiotemporal promoters confine their expression to certain cells, tissues or organs or developmental stages (Hernandez et al., 2014). Inducible promoters are usually responsive to endogenous, external physical and chemical stimuli. Inducible promoters regulate the expression of stress-related genes activated by biotic and abiotic stresses.

Plants being sessile encounter numerous biotic and abiotic stresses. These stresses may occur at any stage of plant development affecting their growth and productivity. Many genes are expressed in response to these stresses, and function in stress tolerance and response (dos Reis et al., 2012). Plants have evolved mechanisms to tolerate the stress by the activation of inactive transcription factors or by the translation of premade mRNAs. In general transcriptional regulation is controlled by transcription factors and transcription factor binding sites (TFBS) (Aarts & Fiers, 2003). Transcription factors are regulatory proteins that modify the expression of genes by binding to their promoter (Singh & Laxmi, 2015). Transcription factors (TFs) play a major role in the conversion of stress signal perception to stress-responsive gene expression and they also act as molecular switches in different stress-related genes by interacting with *cis*-elements present in the promoter region (Banerjee & Roychoudhury, 2015; Liu, et al., 2014). TFBS are the DNA elements that are usually located upstream of the coding sequences. Binding of transcription factors to TFBS is controlled by activator proteins which promote conformational changes and activate the transcription whereas the suppressor proteins compete with transcription factors for binding to TFBS. This whole process involves a series of signalling cascade events determined by tissue, development, and environment (Aarts & Fiers, 2003). A single TF can change the expression of many

genes and a collection of genes under the regulation of the same protein is called a REGULON. Plants activate these regulons under stress conditions and optimize or enhance or advance or develop plant growth (Singh & Laxmi, 2015).

Several approaches have been applied to understand these expression regulatory networks and one of them is studying the interaction of transcription factors with DNA *cis*-elements present in the promoter region. The classical way to study the promoter elements is to fuse a promoter to a reporter gene, such as GUS or GFP and make a deletion series of the promoter. Promoter reporter fusions are then introduced into plant cells and the regions of the promoter important for the regulation of transcription can be identified (Aarts & Fiers, 2003). A very well-known example is the study of the CaMV 35S promoter (Naru et al., 1960). Analysis was performed on many different plant promoters and a whole array of *cis*-elements have been identified and made available in public databases such as PLACE and Plant CARE (Aarts & Fiers, 2003).

In order to develop multiple stress-tolerant crops, it is essential to identify multiple stress-responsive promoters that can be used for the regulation of expression of transgenes that can mitigate the effects of the stresses. As plants share transcriptional machineries and regulatory elements Arabidopsis has been widely used as a model plant for studying multiple stress-responsive promoters (Liu et al., 2014). Promoters of stress-inducible genes contain many stress-responsive cis-elements which allows their specific stress expression. They contain elements such as ABRE (abscisic acid-responsive element), LTRE (low-temperature responsive element), MYC and DRE /CRT elements (Dehydration responsive element) which are the likely targets for regulating stress-inducible expression of transgenes in transgenic plants (Maruyama et al., 2012; Kazuko Yamaguchi-Shinozaki & Shinozaki, 2005; Bhuria et al., 2016). DRE/CRT elements with the core sequence, GCCGAC play a crucial role in regulating gene expression in ABA-independent regulatory systems and were found to be present in the promoter regions of many inducible genes in Arabidopsis such as *rd29A*, *kin1* and *cor15a* (Hou et al., 2012; Msanne et al.,2011; Yamaguchi-Shinozaki & Shinozaki, 1994).

Leaf senescence is a developmentally programmed degeneration process, which constitutes the final stage of leaf development (Fischer, 2012; Woo et al., 2013). The

timing of leaf senescence is influenced by multiple internal and environmental signals, including both biotic and abiotic stresses. These environmental factors are mediated by internal signals including several phytohormones. Transcriptional regulation of leaf senescence is mediated by many transcription factors among them WRKYs, NACs, MYB, C_2H_2 zinc finger and bZIP are mostly studied (Balazadeh et al., 2008). AtNAP/ANAC029 functions as major regulator of senescence in Arabidopsis. overexpression of it causes precocious senescence and knockouts shows a delayed senescence (Guo & Gan, 2006). Another major transcription factor which functions in regulating leaf senescence in Arabidopsis is ORESARA1 (ORE1, ANACO92) which shows induction during senescence (Balazadeh et al.,2010).

The tissue-specific, developmental and stress-induced expression patterns detected for *SAG21* are presumed to be controlled by the various regulatory elements present in the promoter region. Analysis of the 1685 bp region of *SAG21* included several regulatory elements upstream of the transcriptional start point. A group of two W-box elements were identified in the region between -360 and -316 and another two W-boxes were present as single motifs upstream at positions -1023 and -1623. Light regulating elements GATA-BOX and GT1CONSENSUS (Lam & Chua, 1989) were identified all over the promoter region. A CIRCADIANLELHC (Piechulla et al., 1998), POLLEN1LELAT52 (Bate & Twell, 1998), MYCCONSENSUS and root-specific elements, ROOTMOTIFTAPOX1 (Elmayan & Tepfer, 1995) were also identified. Liu et al (2016) reported that based on microarray data from rice flag leaves they have identified that W-box and G-box, binding sites for bZIP, bHLH and NACs play a major role in the rice flag leaf senescence process (Liu et al., 2016).

Cotyledon senescence like leaf senescence also exhibits a genetically programmed sequence of events that eventually leads to cell death (Peterman & Siedow, 1985). The cotyledon plays a major role in providing nutrients for seedling formation (Du et al., 2014; Peterman & Siedow, 1985). During the cotyledon senescence a number of complex and metabolic changes take place like DNA, RNA protein and chlorophyll levels drop along with enzyme activities (Peterman & Siedow, 1985). Arabidopsis leaves begin to yellow or senesce after 21 days (Lim et al.,2007). Cotyledons also follow a similar developmental process and enter the senescence or programmed cell death stage after 21 days (Du et al., 2014). Cotyledons of *Arabidopsis thaliana* unlike the other plants

are epigeal and they enlarge markedly and develop into leaf like photosynthetic structures (Tsukaya et al.,1994). Stoynova et al (2004) reported that cotyledon development or growth in light-grown WT seedlings takes place solely by the cell expansion process and also show that cells do not show any division with dark treatment (Stoynova- Bakalova et al., 2004).

Cytokinin is one of the most studied hormones in senescence regulation, (Fischer, 2012) and is the most effective senescence retarding growth regulator. Exogenous application of cytokinin delays senescence in Arabidopsis and other plants. Gan & Amasino (1995) showed that senescence of tobacco leaves is strongly delayed in plants expressing the isopentyl transferse gene (IPT) under the control of a senescence associated promoter (*SAG12*). IPT encodes a key enzyme in cytokinin biosynthesis, and the *SAG12* promoter activates IPT expression in late senescence causing an increase in cytokinin levels and reduction in senescence in the leaf (Fischer, 2012). Similar to leaf senescence, cytokinin restricts or prevents chlorophyll disruption in cotyledon senescence, whereas ethylene promotes initiation of the cotyledon senescence process (Ananieva et al., 2008).

SAG21 transcripts were detected at very low levels in stem, root and cauline leaves but found to be more abundant in flowers which might be due to its high expression in pollen and anthers (Mowla et al., 2006). *SAG21* was absent in rosette leaves and dry mature seeds with the samples harvested in light. Expression of *AtLEA5(SAG21)* in leaves showed a diurnal pattern of regulation where expression was abundant in dark and decreased quickly upon exposure to light (Mowla et al., 2006). Weaver et al. (1998) reported earlier that the *SAG21* showed very high expression just before senescence followed by a reduction in detached leaves (Weaver et al.,1998). Miller et al. (1999) also reported that *SAG21* transcript levels were found to be upregulated in ozone-induced leaf senescence (Miller et al., 1999)

To determine the spatial and temporal expression patterns of the promoter and to investigate the tissue-specific expression of *SAG21*, two homozygous transgenic promoter-reporter constructs, *SAG21(1685)*::GUS (Salleh et al., 2012) containing 1685 bp upstream of the translational start site and *SAG21(325)*::GUS (Salleh, 2011) containing 325 bp upstream of the translational start site, were generated. Histochemical GUS staining on these constructs revealed that *SAG21* is mainly expressed in the

cotyledons, roots, and pollen, and is light repressed. Salleh et al. (2012) reported that no GUS activity was detected in the younger leaves of promoter GUS rosettes, which was in agreement with the results reported by Mowla et al. (2006) but noticed an occasional expression in early senescing leaves confined to the junction between green and yellow tissue. In flowers *SAG21(1685)*::GUS expression was confined to male reproductive organs. Upon anthesis a very strong expression in pollen was seen which was high in mature pollen. The pollen specific expression was totally absent in lines of the shorter *SAG21(325)*:: GUS promoter construct, but the expression was seen in sepals, and petals and with a marked expression in anthers and filaments. The *SAG21(325)*:: GUS promoter construct also showed a strong expression in the lower part of the stigma post-anthesis (Salleh, 2011).

Promoter lines exposed to light/dark cycles demonstrated that the promoter activity was high in the dark and decreased upon exposure to light. In addition expression of *SAG21(1685)*:: GUS and SAG21*(325)*:: GUS was induced by several abiotic stresses including cold, drought, salt and H_2O_2 .Expression of *SAG21* was observed both in the elongation and maturation zones of the root but expression was found to be absent in lateral root tips and primordia. With respect to *SAG21(1685)*::GUS, strong upregulation was seen by cold stress followed by drought, salt and H_2O_2 (Salleh et al., 2012).These expression patterns were also supported by quantitative GUS assays. With respect to the shorter construct SAG21*(325)*::GUS expression was observed only in the stele region of the roots but expression was absent in the epidermis and the cortex. With drought stress a strong upregulation was detected in roots whereas with the salt and cold stress a mild expression of *SAG21* was observed in roots oxidative stress showed a complete absence of GUS expression in roots which was confirmed by the quantitative GUS analysis on roots which showed that the drought stress showed strong upregulation followed by salt and cold stress compared to the controls (Salleh, 2011).

When plants are exposed to biotic stress conditions like herbivory, bacterial, fungal infections and damage by pests and abiotic stress like salinity, drought and mechanical injury or wounding have developed a defence mechanism to tolerate these stresses and also to prevent infection (Prasad & Balukova, 2020; Savatin et al., 2014). Plants activate defence responses by the perception of damage associated molecular patterns (DAMPs) or microbe associated molecular patterns (MAMPs). Defence responses activated by the wounding process are similar to those switched on by the DAMPs and MAMPs

demonstrating that pathogen and injury are controlled by the plants in a similar way (Savatin et al., 2014). Plants have developed many pathways of defence to combat wounding, one of them is through the constitutive structures like cuticle and trichomes which prevent the entry of the pathogen (Savatin et al., 2014). Local response activation to repair the damage also occurs through the stress responsive genes, accumulation of phytoalexins, lectins and also by an oxidative burst (Prasad & Balukova, 2020; Reymond et al., 2000; Savatin et al., 2014). ROS is one of the major defence mechanisms found to be associated with biotic and abiotic stresses activating both local and systemic responses (Prasad & Balukova, 2020). The wounding response is also mediated by hormones like jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) (Pena-Cortes et al., 1995; Savatin et al., 2014). In Arabidopsis the wounded or damaged leaves synthesize JA and its active form, JA-Ile (jasmonoyl-isoleucine) near the wound site and the concentration of JA was found to increase within 30 sec of wounding (Farmer et al., 2014). JA-I1e then activates the MYC transcription factors through binding to JAZ and COI1 and later the JAZ proteins are degraded releasing the transcription factors which activate the defence gene expression (Ruan et al., 2019; Sheard et al., 2010). In Arabidopsis rosette leaves are connected through the vasculature. Wounding leaf no 8 was found to increase the accumulation of hormone JA in leaf no 13 as they share a common vasculature (Dengler and Kang, 2001; Savatin et al., 2014). *Cis*-elements responsible for wound induced expression were identified in the promoters of the wound induced genes. The promoter of *FAR6* shows the presence of a WUN motif (TCATTAA/CA/GAA) called a wound responsive *cis*-element (Stanford et al., 1989). T/G box (AACGTG), binding sites for the bHLH proteins which function in JA induction are also identified (Boter et al., 2004). Apart from these *cis*-elements W-boxes (TTGAC) are present which bind to the WRKY transcription factors (Rushton & Somssich, 1998). The *SAG21* 1685 upstream region from ATG contains cis elements like W-box, TGA binding sites which play a role in wound response in leaves (Salleh, 2011). Transgenic lines expressing the *SAG21(1685)*:: GUS construct with wounding showed expression of GUS around the wound site whereas with the shorter construct SAG21*(325)*:: GUS wound expression was detected at the wound site and also across the vascular tissues (Salleh et al., 2012; Salleh, 2011).

Recently yeast-1-hybrid screens were performed to identify transcription factors binding to the *SAG21* promoter region (Rogers lab. unpublished results). The promoter was divided into seven fragments that cover different *cis*-elements and identified several

stress-related and developmentally-related transcription factors that bind to the *SAG21* promoter. Here a complementary approach was taken to assess the effects on expression of the regions covered by the promoter fragments

The main hypothesis of this chapter is that by making deletion promoter-reporter constructs it is possible to map the function of transcriptional regulatory *cis*-elements identified in the upstream region of the *SAG21* promoter, relating to expression during development, senescence and responses stress. Another objective of this study was aimed at understanding the role of the kinetin in regulating senescence and wounding responses of the *SAG21* deletion promoter constructs.

4.2 Methods

4.2.1 DNA Manipulation

4.2.1.1 Digestion of DNA with restriction Enzymes

Restriction digestion enzymes and buffers were purchased from Promega. The 10 X reaction buffers supplied with enzymes which had 100 % activity were selected for restriction digestion. Restriction digestion of the vector was set up in a 50 µl total volume with 10 µl of vector DNA, (4 µg) 1 µl of restriction enzymes, (Promega 10U/µl) 5 µl of buffer (Promega) and the final volume was made up with sterile distilled water. The reaction mix was incubated for 3 hrs at 37 °C.

The insert restriction digestion was set up using 27 µl of insert DNA, 1 µl of restriction enzymes, 5 µl of 10X Multicore buffer, and the final volume was made to 50 µl with sterile distilled water. The reaction mix was incubated at 37 °C for 3hrs.

4.2.1.2 PCR amplification with high fidelity Taq polymerase

PCR reactions were set up in a 25 µl volume in a GeneAmp® 3700 Applied Biosystems thermocycler or a Techne Flexigene (Techne, UK), or Veriti® 96 well thermal cycler (Applied Biosystems) PCR machine. Reactions contained 0.5 µL of dNTPs (10 mM), 5

µL of 5X Q5 Reaction buffer (NEB), 5 µL of 5X Q5 high GC Enhancer(optional), 1.25 µl of forward and reverse primers (10 µM) (Table 2-3), and 0.25 µl Hot start Q5 High fidelity Taq polymerase (NEB,M0491). General Cycling conditions for PCR: 98.0 °C – 30sec, 25-35 cycles of 98.0 ºC - 5-10sec, 50-72.0 °C -10-30 sec, 72.0 °C -20-30 sec dependent on fragment size, 72.0 °C -2 mins, 4.0 ºC -hold. Products were then checked on a 1% agarose gel.

4.2.1.3 Purification of DNA fragments

Gel extraction was carried out on a 1% agarose gel by excising the band of interest with a scalpel and then the entire gel piece was transferred into an Eppendorf tube and then it was processed for isolation of DNA with a QIA quick gel extraction kit (QIAGEN.)

4.2.1.4 Ligation Reactions

Ligation reactions were set up in a 10 µl volume with 25-50 ng vector and 50 ng insert DNA using 1.0 µl T4 DNA ligase (Promega 3 U/µl) and 1.5 µl 10x Ligase buffer (10mM Tris-HCl (pH 7.4 at 25 °C), 50mM KCl, 1mM DTT, 0.1mM EDTA and 50% glycerol) and the final volume was made up to a total volume of 15 µl with sterile distilled water. The reaction mix was incubated at 4 º C overnight. Control reactions were set up without insert DNA to check for plasmid re-ligation and without insert and ligase to check whether the vector had undergone complete digestion.

4.2.1.5 DNA Sequencing

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

4.2.2 Transformation of constructs into bacterial cells

4.2.2.1 Transformation into E. coli

E.coli DH5α Competent cells (50 µl) were removed from -80 °C and thawed on ice for a few minutes. Then 2 µl of ligation mix was mixed with the competent cells and incubated on ice for 20 mins. The mix was then heat shocked at 42°C for 45 sec and transferred to ice immediately for 2 min. LB medium (Bacto yeast extract 5 g/L, Tryptone 10 g/L, NaCl 10 g/L pH5-5.5) 950 µl was added to the mix and incubated at 37°C, 200 rpm for 1 hr in a Gallenkamp cooled orbital incubator. The transformation mix was then plated on LB agar plates (Tryptone, Bacto yeast extract, Nacl, and Difco™ LB Agar) with appropriate antibiotic. The plates were then incubated overnight at 37 °C. Transformed colonies were screened by colony PCR using specific primers.

4.2.2.2 Transformation into Agrobacterium tumefaciens (GV3101)

pGreenII0229 can replicate in *Agrobacterium tumefaciens* only if another plasmid, pSoup is co-resident in the same strain. pSoup provides replication functions for the pGreen plasmid (Hellens *et al*., 2000). GV3101 strain *Agrobacterium* competent cells were thawed on ice for 2 min and approximately 1 µg of pGreenII0229 plasmid DNA and 0.5 µg of pSoup plasmid DNA were then added to the competent cells. The competent cells were then frozen in liquid nitrogen and thawed at 37 °C. Then 1 ml of LB liquid medium was added to the cells and incubated for 4 hrs at 28-30 °C at 100 rpm in a Gallenkamp orbital shaker incubator. Cells were then centrifuged at 8000 rpm for 1 min at room temperature using an Eppendorf miniplus microcentrifuge. The supernatant was then removed and the pellet was resuspended in 100 µl of liquid LB and then spread on LB solid plates containing kanamycin (50 µg/ml) and gentamycin (25 µg/ml) which were then incubated for two days at 30 °C. For each construct, different ratios of plasmid DNA were used. Following growth on the solid medium around 3-4 colonies were picked from the plates and inoculated in 5 ml liquid LB medium containing kanamycin (50 µg/ml), rifampicin (50 µg/ml), gentamycin (25 µg/ml) and grown for 24 hr at 28-30 °C in a Gallenkamp orbital shaker incubator. From these liquid cultures, 1 µl was then

used as a template for PCR using CP-FR (Table 2.3) primers. Colonies which showed a positive result with colony PCR were then prepared as glycerol stocks and stored at -80 °C. For each clone around 2-3 glycerol stocks were prepared.

4.2.2.3 Colony Screening by PCR

Around 5-10 colonies were selected from each transformation plate using a pipette tip and streaked onto LB agar with appropriate antibiotics. The plate was incubated overnight at 37 °C to create a master plate. Colonies were picked from the master plate and inoculated into 5-10 ml LB liquid medium with suitable antibiotics and incubated at 37 °C (*E. coli*) and 30 °C (*A. tumefaciens*) 200 rpm overnight. Overnight liquid cultures (1 µl) were used as a template for PCR.

4.2.2.4 Glycerol stock preparation and plasmid purification

Five ml of LB liquid medium with suitable antibiotic was inoculated with a bacterial colony and used for plasmid purification using QIA prep spin Miniprep kit (QIAGEN). The inoculated culture was harvested by centrifugation at 8000 rpm for 3 minutes at room temperature. Pelleted bacterial cells were resuspended in 250 µl Buffer P1 and transferred to a micro centrifuge tube. Buffer P2 (250 µl) was added and mixed thoroughly by inverting the tube 4-6 times then 350 µl Buffer N3 were added and mixed immediately and thoroughly by inverting the tube 4-6 times. Tubes were centrifuged for 10 min at 13,000 rpm in a table-top micro centrifuge. The supernatant was applied to a spin column and centrifuged for 30-60 sec. The flow-through was discarded and the spin column washed by adding 0.75 ml Buffer PE and centrifuging for 30-60 sec. The flow-through was discarded, and the spin column was centrifuged at full speed for an additional 1 min to remove residual wash buffer. The column was placed in a clean 1.5 ml micro centrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water were added to the centre of each spin column, let stand for 1 min, and centrifuged for 1 min. For making glycerol stocks 1 ml of overnight inoculated culture was centrifuged at 13,000 rpm in a microcentrifuge at room temperature. The supernatant was then removed and the pellet was resuspended in 500 µl of sterile 50 % glycerol and stored at - 80 °C.

4.2.3 Assembly of *SAG21* promoter deletion constructs

The Gibson cloning method was attempted (Appendix 4.1) but was not successful so an alternative approach using classical directional cloning was employed. In the directional cloning method both the vector and insert are digested with the two different restriction enzymes to create non complementary sticky ends allowing the insert to be ligated into a vector in a specific orientation and preventing recircularization of the vector. A plant transformation vector which contained both GUS and GFP reporters in a suitable poly-linker was not available therefore the first step was to construct a suitable vector, into which the fragments of the *SAG21* promoter were then inserted. To obtain a greater quantity of the eGFP-GUS-NOS cassette, the cassette was first amplified by PCR from pKGWFS7 (Appendix 4.2 c) then sub cloned into the pZErO-2 vector (Figure 4.1a). The insert was then isolated by restriction digestion from the pZErO-2 and ligated into the plant transformation vector, pGreenII0229 (Appendix 4.3 a) using with BamHI and SacI restriction enzymes (Figure 4.1b, c).

4.2.3.1 Construction of a backbone pGreenII0229 plasmid vector – cloning the eGFP-GUS-NOS cassette into pZErO2

pZErO2 vector was digested with EcoRV (Thermofisher scientific ,UK) to create blunt ends (Section 4.2.1.1). Digested product was checked on a 0.8% agarose gel (Section 2.9) and it was gel extracted using a QIA quick gel extraction kit (QIAGEN) (Section 4.2.1.3). The concentration of eluted DNA was determined using a Nanodrop UV spectrophotometer (Section 2.8)

The insert (GUS-GFP-NOS) was amplified from the pKGWFS7 plasmid by PCR using DCF1R1primers (Table 2-3) and Q5, high fidelity DNA polymerase (NEB) (Section 4.2.1.2). The amplified PCR product was checked on a 1% agarose gel (Section 2.9), purified using a QIA quick PCR purification kit (Section 4.2.1.3) and isolated DNA was then quantified using a Nanodrop UV spectrophotometer (Section 2.8). Ligations (Section 4.2.1.4) were set up with 25 ng vector (3 µl) and 50ng insert DNA (1.5 µl). Ligations were incubated and transformed into competent cells as described (Section 4.2.1.4 and 4.2.2.1).

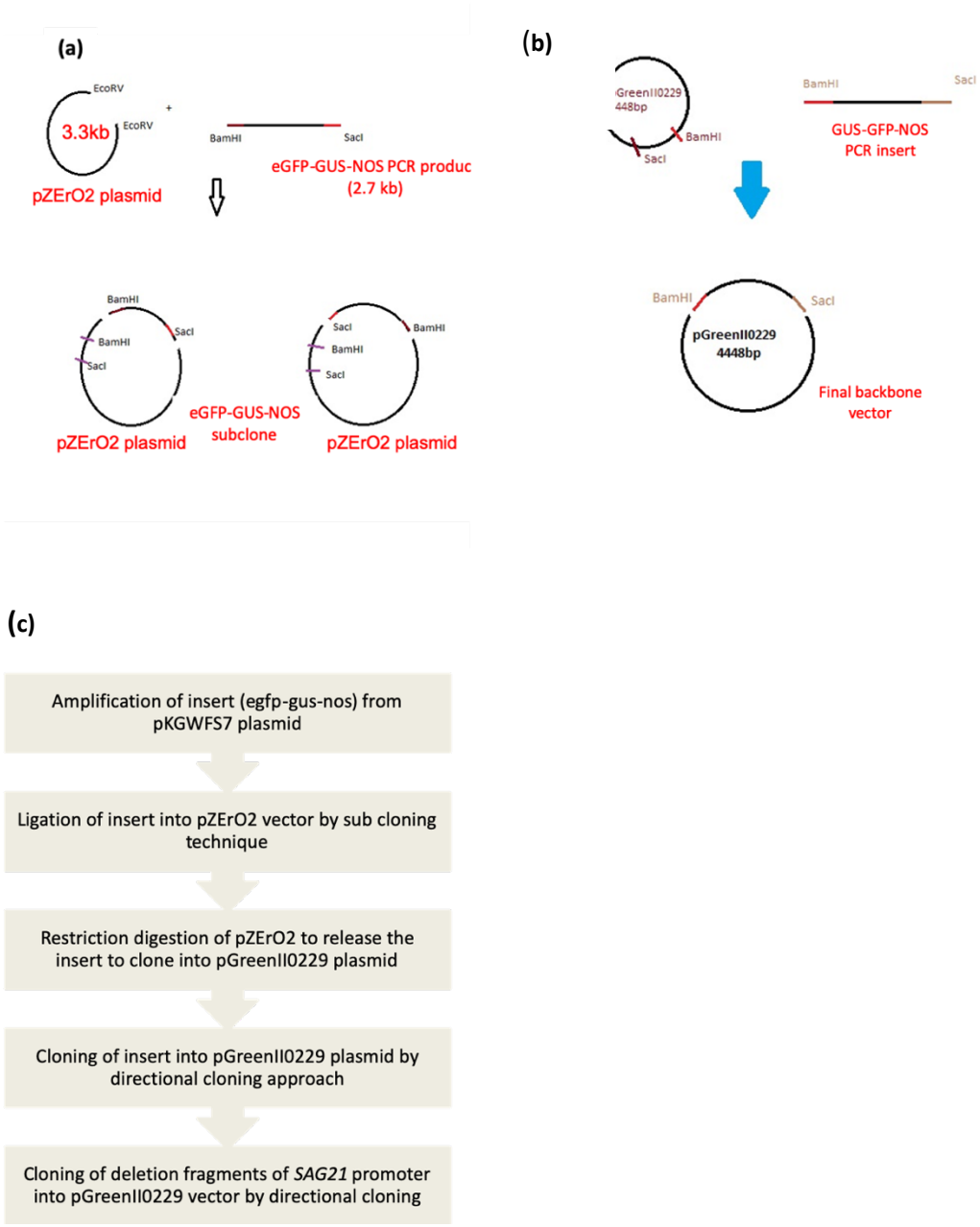


Figure 4.1: Sub cloning GUS-GFP-NOS insert into pZErO-2 vector **(a)** Sub cloning GUS-GFP-NOS insert into pZErO-2 vector, **(b)** Restriction digestion of GUS-GFP-NOS insert from the pZErO-2 and ligation into the plant transformation vector, pGreenII0229, **(c)** Flow chart showing the whole cloning process of the insert into pGreenII0229 vector and deletion promoter fragments of SAG21.

4.2.3.2 Construction of a backbone pGreenII0229 plasmid vector – directional cloning of the GUS-eGFP-NOS cassette into pGreenII0229

pGreenII0229 (4 µg) DNA was digested with restriction enzymes BamHI and SacI (Promega 10U/µl) with 10X multicore buffer (Promega) (Section 4.2.1.1). Digested product was checked on a 0.8 % agarose gel (Section 2.9), and it was gel extracted with

a QIAGEN Qia quick gel extraction kit (Section 4.2.1.3). The concentration of eluted DNA was determined using a Nanodrop UV spectrophotometer (Section 2.8). The insert was isolated from pZErO2 by digesting with restriction enzymes BamHI and SacI and gel extraction was carried out on a 1% agarose gel. The eluted DNA was then used to set up a ligation reaction. Ligation was set up in 1:1 ratio with 50 ng vector (5 µl) and 50 ng insert DNA (5 µl) (Section 4.2.1.4) with the control reactions. The mix was then incubated and transformed into DH5α competent cells (Section 4.2.2.1).

4.2.3.3 Inserting SAG21 promoter deletion fragments into the pGreenII0229 vector containing the eGFP- GUS-NOS cassette

Deletion constructs of the *SAG21* (AT4G02380) promoter were made starting at the 5'end upstream from ATG site to make promoter reporter cassettes. These were carried out by designing a common reverse primer with a different forward primer for each fragment. Restriction sites for EcoRI and BamHI enzymes were introduced in forward and reverse primers. These primer pair combinations were used to amplify six different length deletion promoter fragments from the *SAG21* promoter sequence using Q5 high fidelity hot start DNA polymerase (NEB). These were later cloned into a pGreenII0229 vector by following a directional cloning approach where both vector and insert were digested with the same restriction enzymes creating six expression constructs with GUS-eGFP fusions. pGreenII0229 plasmid (Hellens et al., 2000) confers resistance to kanamycin in bacteria and BASTA in plants. (Figure 4.2).

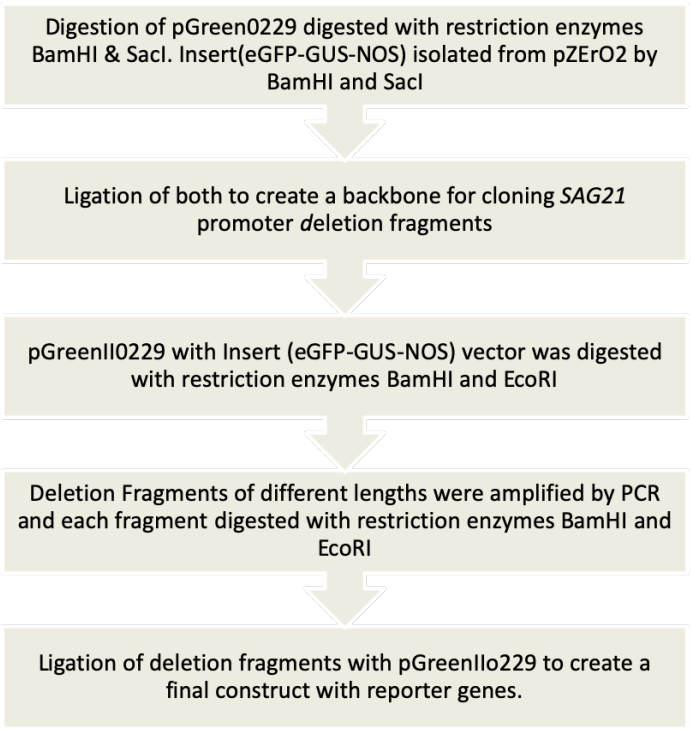


Figure 4.2**:** Flow diagram showing the digestion of vector and cloning of the *SAG21* promoter deletion fragments to create a final construct.

pGreenII0229 with insert (GUS-GFP-NOS) vector was digested with restriction enzymes BamHI and EcoRI (Promega) to create sticky ends for the directional cloning approach. The digestion was set up in 10 x multicore buffer (Promega). After incubation, the pGreenII0229 was gel extracted and purified using a Qiagen Qiaquick gel extraction kit. The concentration of eluted DNA was measured using a Nanodrop uv spectrophotometer as described above.

Plasmid DNA was isolated from a pGEM-T Easy SAG21:: 1685 promoter clone (Salleh, et al., 2011) (Section 4.2.2.4) and was used as a template for the amplification of *SAG21* promoter fragments. Deletion Fragments of different lengths (489, 737, 965, 1225, 1439, and 1700 bp) were amplified from the *SAG21* promoter region using F1R, F2R, F3R, F4R and F5R primer pairs (Table 2.3) according to the PCR conditions listed in Table 4.1 using Q5 high fidelity hot start DNA polymerase (NEB). Amplification of the PCR product was then confirmed on a 1 % agarose gel. The PCR product was then digested with restriction enzymes BamHI and EcoRI (Promega) in 10 x multicore buffer creating sticky ends. Following incubation, the PCR product was purified using a Qiagen QiaQuick PCR purification kit. The concentration of purified DNA was then checked

using a Nanodrop uv spectrophotometer and was then used for ligation of vector and fragment DNA using a ratio of 1:3. The ligation reactions were then incubated overnight at room temperature and transformed into *Ecoli* DH5α competent cells as described (Section 4.2.2.1). After transformation, colonies were then screened using colony PCR (Section 4.2.2.3) and glycerol stocks (Section 4.2.2.4) for positive clones were made and stored at -80°C.

Table 4.1 PCR conditions used for amplification of SAG21 promoter fragments with Q5 hot start DNA polymerase

pSAG21(489) *::*GUS-GFP	*pSAG21*(737) *::*GUS-GFP	*pSAG21*(965) *::*GUS-GFP	*pSAG21*(1225) *::*GUS-GFP	*pSAG21*(1439) *::*GUS-GFP	*pSAG21*(1700)*::* GUS-GFP
94°C-2 min	94°C-3 min	94°C-3 min	94°C-3 min	94°C-3 min	94°C-3 min
94°C-0.30sec	94°C-0.60sec	94°C-0.45sec	94°C-0.30 sec	94°C-0.30 sec	94°C-0.30sec
55°C-0.30sec	55°C-0.60sec	65°C-0.45sec	65°C-0.30 sec	65°C-0.30sec	67°C-0.30sec
72°C-1.5min for 30 cycles	72°C-0.60sec for 35 cycles	72°C-1 min for 35 cycles	72°C-1.30 min for 35 cycles	72°C-min for 35cycles	72°C-2min for 35 cycles
72°C-7 min	72°C-7 min	72°C-7 min	72°C-7 min	72°C-7 min	72°C-7 min

4°C-hold	4°C-hold	4°C-hold	4°C-hold	4°C-hold	4°C-hold

4.2.4 Transformation into *Arabidopsis thaliana*

4.2.4.1 Floral dipping

Four week old *Arabidopsis thaliana* (Col-0) WT plants were grown in glass house conditions at the green house facility in Tal-y-bont, Cardiff University at around 21 °C. Six pots of plants each containing 5-6 plants per pot were used for dipping of each construct transformed into *Agrobacterium tumefaciens* GV3101 strain (Section 4.2.2.2). The primary inflorescences were removed to produce more secondary inflorescences with more pre opened flowers. Approximately 15 days (i.e. 7 weeks after germination) after removing primary stems, plants were ready for dipping. *Agrobacterium* clones were streaked onto LB agar plates with antibiotic kanamycin (50 µg/ml) and were incubated at 30 °C for 2 days to obtain a single colony. The single colony was then picked and resuspended in 10 µl of sterile distilled water. Half of the volume was then plated onto solid YEB (beef extract 5 g/L ,yeast extract 1 g/L, peptone 5g/L, sucrose 5g/L, $MgSO_4$ 300 mg/L and agar 20 g/L) plates with Kanamycin(50 µg/ml) and incubated at 30 °C to form a complete lawn of bacteria for 2 days. Bacteria were then collected from the plates by gently scraping the plates with the help of a spatula and bacteria were resuspended in 30 ml YEB liquid medium. The optical density of the culture was then measured at 600 nm to obtain approximately OD 2.0. On the day of dipping, a solution containing 5 % sucrose and Silwett 77 (0.03 % [v/v], surfactant, Lehle Seeds) was prepared fresh, and for each transformation, 150 ml of sucrose solution containing Silwett was transferred to a plastic container and the *Agrobacterium* was then added to the mix. The inflorescences of plants were dipped into the solution for approximately 60 sec by gently agitating them until a coating of liquid was formed on flowers. Plants were then placed in a plastic tray sideways covered with a black bag to maintain humidity and left overnight. Subsequently, the plants were grown under optimal growth conditions and watering was stopped after the seed set. Plant material

was then dried for 3 weeks and T0 seeds were harvested (Figure 4.3).

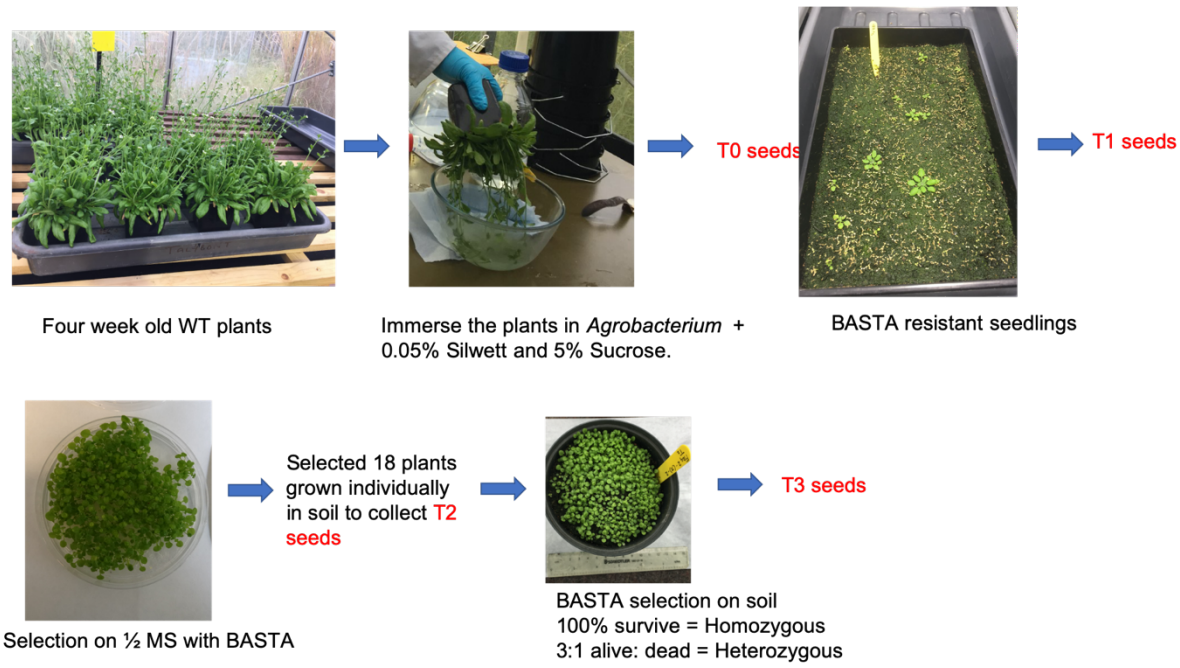


Figure 4.3 :Transformation of Arabidopsis WT plants by floral dipping method showing the various steps involved for the generation of homozygous T2 plants

4.2.4.2 Screening of transgenic seeds on BASTA

T0 seeds were screened on soil for the identification of BASTA resistant plants in glass house conditions at the green house facility in Tal-y-bont, Cardiff University. For the identification of positive plants, a control experiment using *Arabidopsis thaliana* (Col-0) WT and BASTA resistant seeds (Landsberg *erecta*) were also grown in trays alongside the transgenics. T0 Seeds were distributed evenly onto the soil by making a mixture of seeds and fine sand at the ratio of 0.5:10 g/g and were then sprinkled on the soil with help of a sieve. Trays were then covered with lids and incubated at 4 °C for 72 hours for stratification of seeds and were later then moved into glass house with a temperature of approximately 21 °C under long day conditions. When the seeds germinated the lids were removed to prevent excess humidity. For selection of transgenic seedlings, commercial herbicide BASTA (13.52 % w/w, Glufosinate-ammonium, Kaspar, Aventis CropScience, UK Limited) was sprayed onto plants at 120 µg/ ml three times, (once a week for 3 weeks) starting when seedlings were at the cotyledon stage. After spraying, approximately after 3 weeks of germination, the positive T1 plants were identified from the non-transgenics by the presence of green leaves, while WT were white and they failed to develop beyond cotyledons and first

primary leaves. Each positive T1 plant was then picked with a spatula, taking care not to disturb the root system and then moved into single pots and grown individually for 3 weeks until maturity to produce T1 seeds. Plant material was then dried and sieved; seeds were collected and stored in Eppendorf tubes. Seeds of Independent T1 lines were then sown on ½ MS agar (Murashige and Skoog salt, bacto agar, pH 5.5) supplemented with BASTA at a concentration of 5 µg/ml and grown for 15 days on Petri dishes in controlled conditions. From each Petri dish of independent T1 lines, 18 plants which survived the selection on BASTA were then selected to the soil to grow individually until maturity to collect T2 seeds.

For each deletion promoter construct, 10 plants of 5-10 independent lines were screened on soil for selecting homozygotes. Approximately 100 T2 seeds of each promoter line were then sown on soil in a 5 cm pot and grown until the cotyledon stage and sprayed with BASTA. The number of seedlings that germinated in each pot were counted before spraying with BASTA. For each pot two sprays of BASTA were given and the gap between the first and second spray was kept a week apart. The seedlings which survived the selection were counted after the first and second spray. Control WT and BASTA positive plants were also included along with the transgenic lines.

4.2.5 Promoter GUS analysis

4.2.5.1 Histochemical GUS assays

GUS staining solution was prepared using the following: 0.5 M Sodium phosphate pH-7, 200 mM potassium ferricyanide, 200 mM potassium ferrocyanide, 20 mg/ml chloramphenicol, 0.01 % TritonX-100, 5 mg/ml X-Gluc A Salt (5-bromo-4-chloro-3-indolyl-P-D-glucuronide cyclohexylammonium, Melford) and the final volume was made up with distilled water. Tissues or samples were immersed in the staining solution and then subjected to vaccum infiltration for 2-3 minutes and incubated at 37 °C overnight or 1-6 hr depending on the development of the staining. Tissues were then destained in 70 % ethanol until the solution became colourless and the process was repeated 1-2 times. Then tissues were stored at 4 °C for microscopic imaging.

4.2.5.2 Microscopy and imaging

Samples were visualized using a Nikon Dissecting microscope and imaging was carried out with a GXCAM Hi Chrome-SMII camera. Flowers are imaged at 3.5X objective under dissecting microscope.

4.2.6 Plant Treatments

4.2.6.1 Kinetin treatment of cotyledons

Promoter deletion GUS-GFP construct seeds were stratified, sown on 1X MS media plates with 1 % sucrose (Section 2.13) and grown horizontally for 14 days and 24 days under long day conditions of 16 h light and 8 h dark in a Pervical growth chamber. A 10 mM stock solution of kinetin (K0753-Sigma ,UK) was prepared by dissolving powder in 1 ml of 1 M NaoH and the final volume of the solution was made to 10 ml with distilled water and filter sterilized using a 0.20 µm filter (Minisart®,Sartorius stedim). A 1 mM stock solution of kinetin was prepared from the 10 mM stock and then added to a final volume of autoclaved MS liquid solution. Two 24 well cell culture plates (Grenier Bio-one) were taken and each well was filled with 3-5 ml of kinetin solution and the other plate was filled only with MS liquid solution as a control for the experiment. Pairs of cotyledons were detached from the plant using scissors and were then placed in the 24 well cell culture plate wells. The culture plates were then sealed with micropore tape and incubated for 18 hr in continuous light at 21°C. After incubation, the kinetin solution was removed and cotyledons were washed with sterile distilled water and GUS staining was carried out as described in Section 4.3.5.1.

4.2.6.2 Wounding and Kinetin Treatment

Promoter deletion GUS-GFP lines were sown on soil (see Section 2.13) and were grown for 4-5 days under normal growth conditions until seeds germinated. Seedlings were then thinned out with forceps gently by placing a single plant in each plug tray and then the trays were moved into the growth chamber for growing under short day conditions of 8 h light and 16 h dark, watering every 2-3 days. After 4 weeks of growth, plants were then given a continuous light treatment of 18 hr. Leaves were then numbered according to Falmer et al., (2013). Leaves 4, 5 and 6 were wounded on the plant using forceps creating

an imprint on the surface of the leaf. The leaves were then detached from the rosette and placed in kinetin solution in a Petri plate and also in water as a control for the experiment. Petri plates were then moved to continuous light for 18 hr at 21°C and then GUS stained according to the protocol described in section 4.2.5.1.

4.2.6.3 Wounding and Age of rosette

Promoter deletion GFP-GUS-seeds were sterilized and sown as described in Section 2.12 and Section 2.13. The seedlings were then grown horizontally on 1 x MS with 3 % sucrose under long day conditions of 16 hr light and 8 hr dark for the complete duration of the experiment. Wounding was performed on the plants at weekly intervals aged, 22 days, 29 days and 36 days after sowing. One Petri dish containing seedlings from each line was opened every week, the remaining dishes were left undisturbed in the growth chamber until used. Every week, three plants were wounded from each of the lines and unwounded plants were selected as controls for the experiment. Leaves were numbered and leaf 5 was selected for wounding, by crushing with forceps. After 15 mins of wounding on plant, the entire rosettes (both wounded and unwounded) were carefully removed from the agar by cutting at the base of the stem just above the root, and placed in 90 % acetone. After 20 mins of acetone treatment, the rosettes were washed with 50 mM sodium phosphate buffer and also in sterile distilled water once and GUS stained by the protocol mentioned in Section 4.2.5.1

4.3 Results-

4.3.1 Construction of a backbone pGreenII0229 plasmid vector

Sub cloning of the GUS-GFP-NOS cassette into the pZErO-2 vector was successful. Positive clones were selected by colony PCR using primers to the vector and GUS (Figure 4.4a), which resulted in the expected product size of 650 bp and then positive clones were further confirmed by restriction digestion with Bam HI and SacI showing fragment sizes comparable to the vector (3.3 kb) and insert (2.7 kb) (Figure 4.4b).

Sequencing using M13 Forward and Reverse primers (See section 4.2.1.5 and Table 2-3) from the vector backbone verified the sequence of the insertion to be correct (Appendix 4.3).

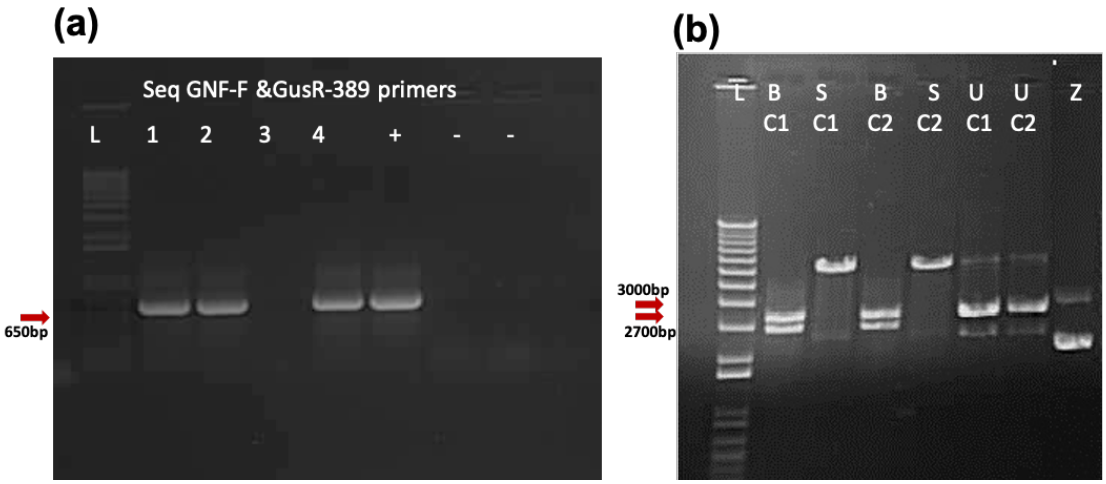


Figure 4.4: Selection of sub-clones of the GUS-GFP-NOS cassette **.(a)** colony PCR products with Seq GNF-F&GUS-R 389 primers**.** Lanes 1-4: colonies from ligation reaction; (+) pKGWFS7 plasmid DNA as positive control; (-) colonies from negative ligation reaction and water as a negative control for PCR **(b)** Restriction digestion of plasmid from two selected positive clones (C1 and C2) with BamHI & SacI enzymes. Lanes 1-4: digestion of the two clones with BamHI (B) and SacI (S); U: undigested plasmid DNA from the two clones. Z: uncut pZErO-2 Plasmid DNA; L: 1Kb plus DNA ladder.

Digestion of pZErO-2 with BamHI and SacI restriction enzymes resulted in the isolation of insert DNA of 2.7 kb (Figure 4-5a).Vector pGreenII0229 was also digested with the same enzymes to clone insert (2.7kb) into it by the directional cloning approach (Figure 4-5b).Two positive clones (SN02 and SN06) were obtained from ligation of the GUS-GFP-NOS cassette insert into the plant transformation vector, pGreenII0229 and transformation into *E. coli.* PCR using SeqGNF-F and GUS-Reverse primers confirmed that SN06 contained the correct construct and it was then used a backbone for cloning the deletion fragments of the *SAG21* promoter.

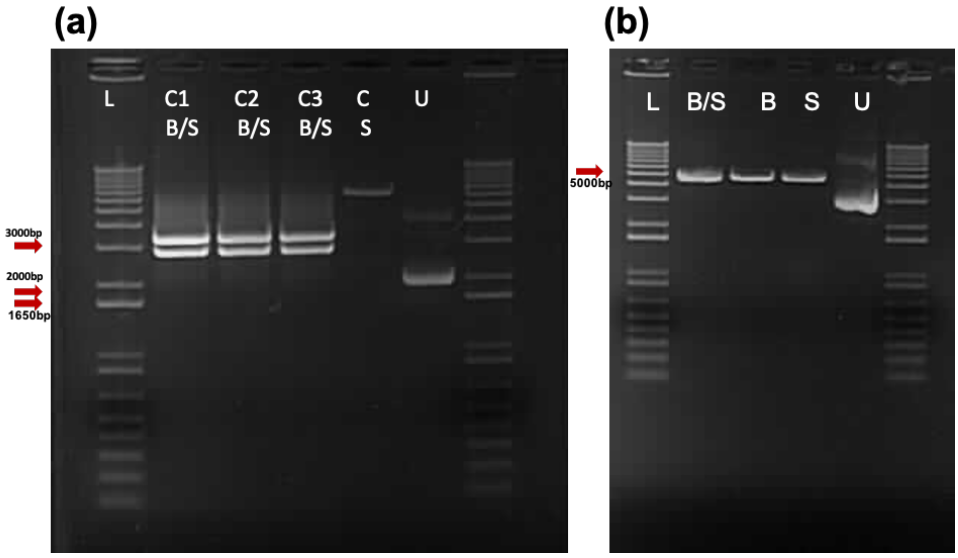


Figure 4-5**:(a)** Restriction digestion of pZErO-2 clones containing the GUS-GFP-NOS cassette with BamHI and Sac I restriction enzymes. Lane: 1-3 pZErO-2 clones (**C1 C2 C3)** digested with BamHI and SacI; C: control SacI restriction digestion; U: Uncut pZero2 Plasmid DNA.**(b)** Restriction digestion of vector pGreenII0229 plasmid DNA with BamHI and SacI restriction enzymes**.** B/S: Digestion with BamHI and SacI. B: control BamHI restriction digestion; S: control SacI restriction digestion; U: uncut pGreenII0229 Plasmid DNA; L: 1Kb plus DNA ladder.

4.3.2 Cloning the SAG21 promoter fragments into the pGreenII0229-eGFP-GUS-NOS vector

SAG21 promoter sequence was previously analysed and Yeast-1 hybrid carried out to study the function of *cis*- elements (see Introduction). Primers were designed to amplify fragments from the promoter that matched the fragments used in the Yeast-1-hybrid analysis. These were used to create the deletion constructs and contained various cis-elements (Figure 4.6a). *SAG21* promoter constructs of different lengths were amplified from a pGEM-T Easy *SAG21*:: 1685 promoter clone (using primers listed in Table 2-3) and showed expected bands of different sizes on an agarose gel (Figure 4.6). Each fragment was further digested with Bam HI and EcoRI enzymes and ligation was carried out with the backbone vector. Following ligation of each fragment with the vector plasmid, and transformation into *E. coli,* around 8-10 colonies were screened by colony PCR with CP-FR primers showing a 500 bp PCR product (Appendix 4 A4.4). CP-FR primers were designed from the promoter region of *SAG21* and GFP region of the insert.

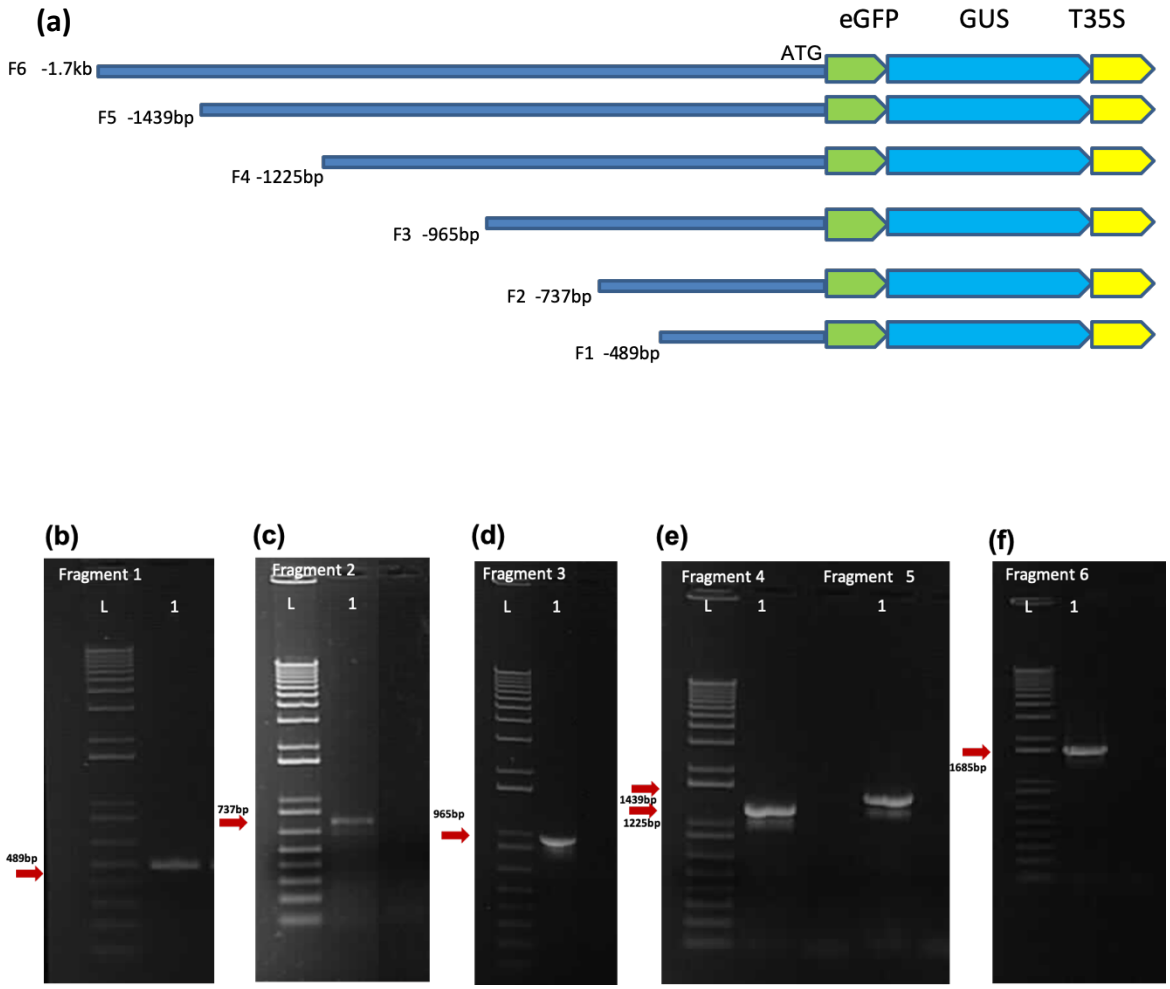


Figure 4.6: Amplification of PCR fragments from pGEM- T Easy SAG21::1685 promoter **(a)** Deletion analysis of *SAG21* promoter fragments which are fused to reporter genes. L: 1Kb Plus DNA ladder**. (b**) fragment 1 (F1-489bp) **(c)** fragment 2 (F2-737bp) **(d)** fragment 3 (F3-965bp) **(e)** fragment 4 (F4-1225bp **(f)** fragment 5 (F5-1439bp) **(g)** fragment 6 (F6-1700bp).

Use of these primers in colony PCR produced a 500 bp product showing that cloning of fragments was successful. Plasmid DNA was isolated from 2-3 clones for each fragment and restriction digestion was carried out with BamHI and SacI enzymes releasing the desired fragment to confirm they carried the correct fragment size (Figure. 4.6). Clones of each fragment were sequenced with M13 reverse primer from the vector and CP-F primer from the GUS region of the insert to confirm that no errors had been introduced into the sequences during PCR.

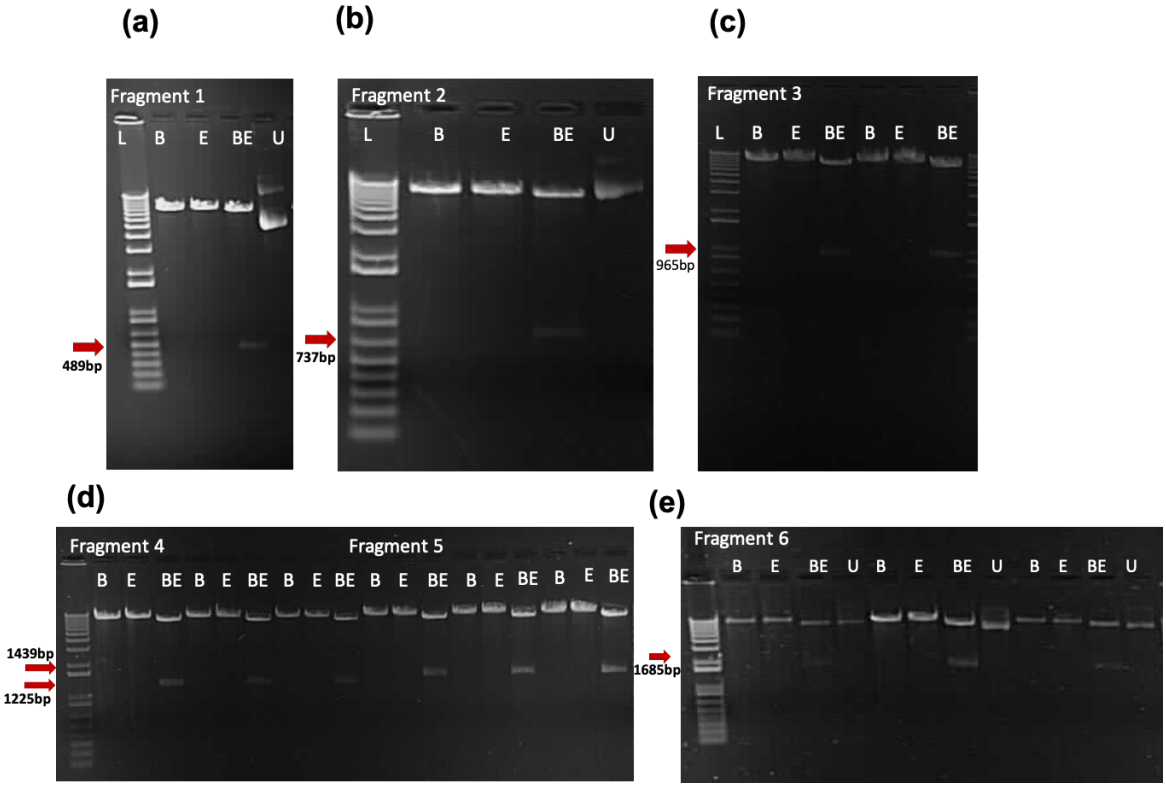


Figure 4.7 :Restriction digestion on three selected positive clones with BamHI & EcoRI restriction enzymes releasing the respective fragments.**(a) (b) (c) (d) (e)** Restriction digestion on three selected positive clones with BamHI & EcoRI restriction enzymes releasing the respective fragments. Lanes B: digestion with BamHI. Lane E: digestion with EcoRI. Lane BE digestion with BamHI & EcoRI. LaneU: undigested plasmid DNA

Plasmid DNA was isolated from positive clones of each fragment used for transformation into competent *Agrobacterium tumefaciens* (see Methods Section 4.2.2.2). As pGreenII0229 cannot replicate without pSoup both pSoup and the pGreenII0229 plasmids were used for transformation. After transformation into *Agrobacterium* colonies were selected by colony PCR using CP-FR primers (Appendix 4, Figure A4.5). Colonies which showed the desired band of 500bp were confirmed as positive *Agrobacterium* clones which were then used for floral dipping to create transgenic lines with respect to each fragment.

4.3.3 Transformation of *SAG21* promoter fragment – GUS GFP reporter constructs into Arabidopsis

After floral dipping, T0 seeds obtained were then screened for BASTA resistant plants (Figure. 4.8). Positive plants were grown individually in pots to collect T1 seeds. Between 11 and 34 positive plants were recovered for each construct (Table 4.3).

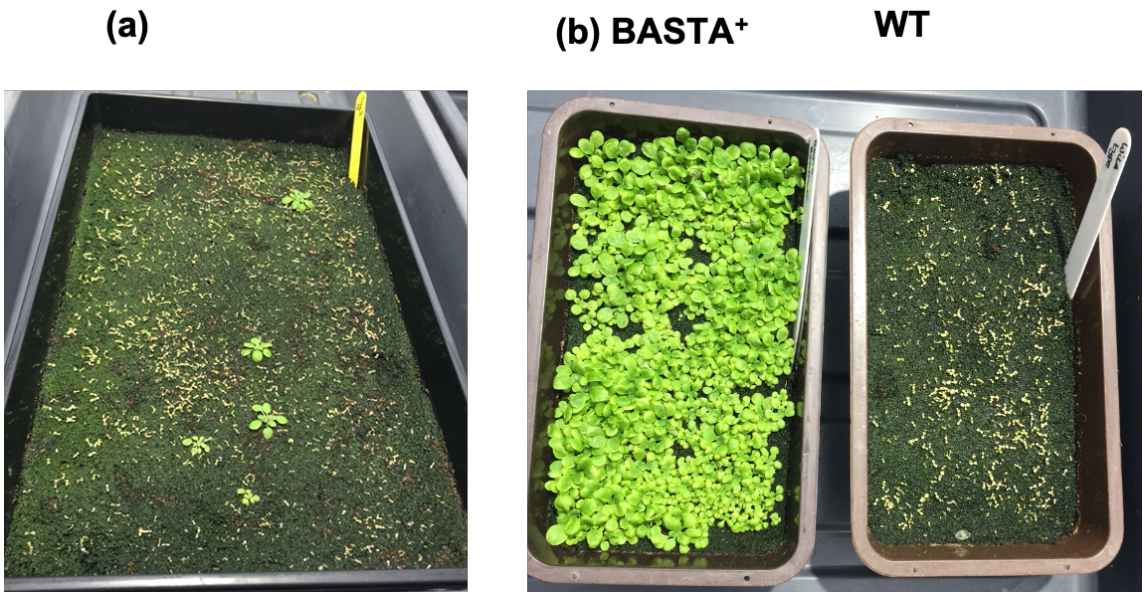


Figure 4.8 :BASTA selection of T0 transformed seeds on soil **(a)** Four week old Transgenic plants recovered by the screening of T0 seed on soil after spraying with herbicide BASTA at a concentration of 120 µg/ml; **(b)** BASTA resistant plants and WT seeds were also included to serve as a controls for the experiment.

T1 seeds obtained from each independent line of the constructs were then screened on plates with BASTA to check the segregation of the transgene (Figure 4.9). Lines which followed a segregation of 3:1 ratio on BASTA were selected, and 18 positive plants from each independent T1 line after selection were then taken to soil and grown individually to obtain the T2 seeds.

Table 4.3- Transgenic plants recovered for each construct after BASTA selection.

Promoter deletion reporter constructs	No of Transgenic plants recovered after transformation	Independent T1 Positive lines obtained after selection on BASTA.	No of final T2 lines used for selection of homozygotes
pSAG21(1700)::GUS-GFP	11	5	5
pSAG21(1439)::GUS-GFP	18	5	5
pSAG21(1225)::GUS-GFP	14	9	5
pSAG21(965)::GUS-GFP	16	9	9
pSAG21(737)::GUS-GFP	34	18	10
pSAG21(489)::GUS-GFP	28	10	10

Ten independent plants from each T2 line were again screened on soil with BASTA for the final selection of homozygotes (Figure 4.10). Lines which showed 100 % seedling survival on soil were considered as homozygotes and lines which still showed segregation were considered as heterozygotes.

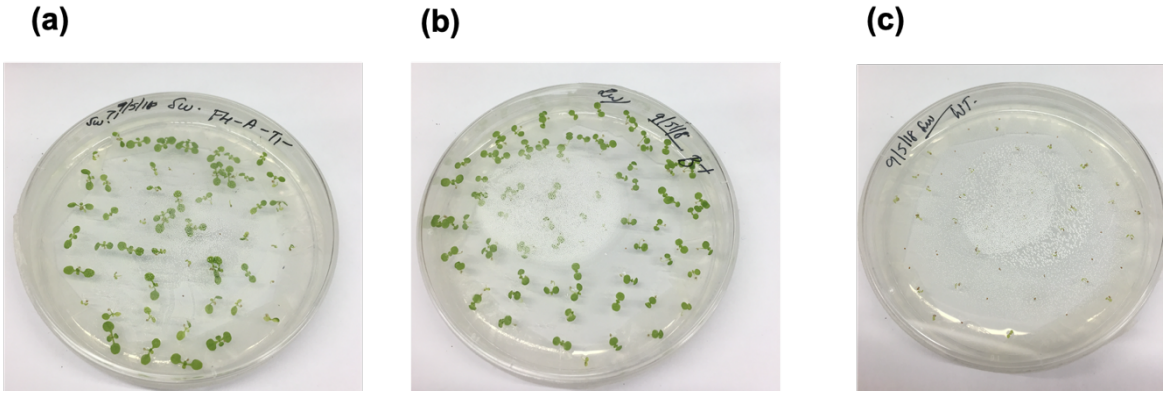


Figure 4.9 : Segregation of T1 seeds on MS plates with BASTA selection at a concentration of 5 ug/ml **(a)** Seedlings showing the segregation of transgene; **(b)** BASTA resistant plants used as a positive control; **(c)** WT as a negative control.

As the germination rate on the soil was variable for the lines with selection on BASTA, final lines were selected by taking into consideration the seed germination percentage. For each promoter deletion construct at least two homozygous lines were obtained which were used for the study.

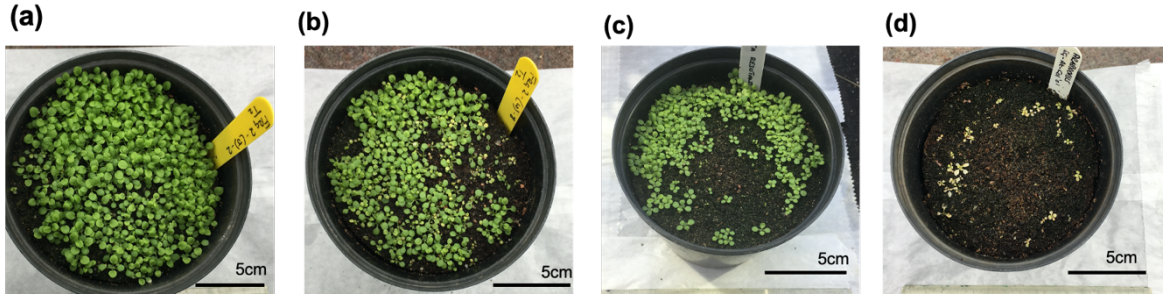


Figure 4.10 :Soil selection of T2 seeds on BASTA for identification of Homozygotes **(a)** Homozygote line showing 100% percent survival on soil after spray with BASTA**(b)** Heterozygote line showing still the presence of WT.(**c)** BASTA resistant seeds used as positive control. **(d)** WT as negative control.

4.3.4 *SAG21* showed different expression patterns in floral organs of promoter deletion construct lines

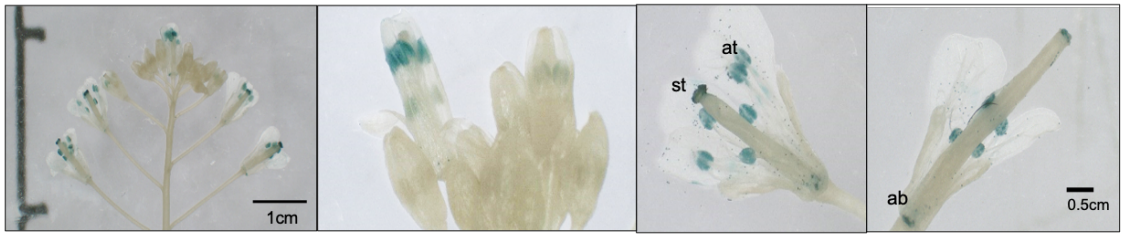
SAG21 promoter deletion constructs showed a varied floral expression pattern (Figure. 4.11 and summarized in Table 4.4). Flowers containing the p*SAG21*(1700)::GUS-GFP construct showed expression only in stigma and anthers but expression was absent in sepals, petals and filaments (Fig. 4.9a). Deletion of promoter region from 1700 bp to 1439 bp showed an increase in expression in petals and filaments when compared to *pSAG21*(1700)*::* GUS-GFP (Figure. 4.11b). Deletion promoter construct p*SAG21*(1225)::GUS-GFP showed expression of *SAG21* in stigma, anthers, petals, sepals and filaments (Figure. 4.9c). In contrast, p*SAG21*(965)::GUS-GFP construct

expression was weaker or absent from the stigma (Fig. 4.11d). Flowers of p*SAG21*(737)::GUS-GFP constructs showed clear expression in the stigma as well as sepals, petals and anthers while p*SAG21*(489)::GUS-GFP showed expression only in stigma and anthers (Fig. 4.11e,f).

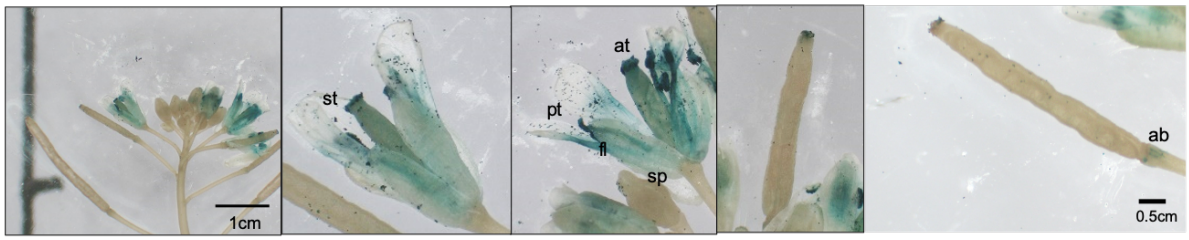
Table 4.4-Expression of *SAG21* promoter constructs in Different floral parts of a flower

Promoter constructs	Sepals	Petals	Stigma	Anther	Filament
p*SAG21*(1700)::GUS-GFP	×	×	✓	✓	×
p*SAG21*(1439)::GUS-GFP	×	✓	✓	✓	✓
p*SAG21*(1225)::GUS-GFP	✓	✓	✓	✓	✓
p*SAG21*(965)::GUS-GFP	✓	✓	(✓)	✓	✓
p*SAG21*(737)::GUS-GFP	✓	✓	✓	✓	×
p*SAG21*(489)::GUS-GFP	×	×	✓	✓	×

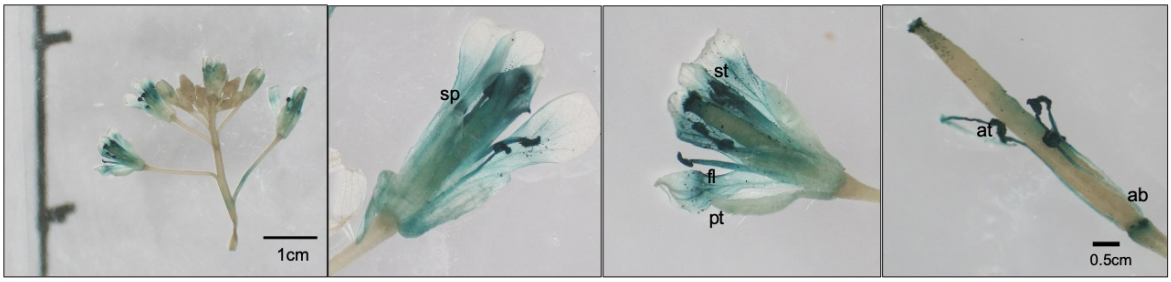
(a) *pSAG21*(1700)*::*GUS-GFP



(b) *pSAG21*(1439)*::*GUS-GFP

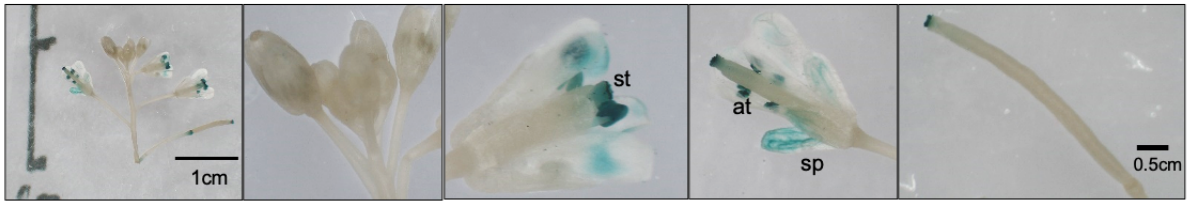


(c) *pSAG21*(1225)*::*GUS-GFP



(d) *pSAG21*(965)::GUS-GFP

(e) *pSAG21*(737)::GUS-GFP



(f) *pSAG21*(489)::GUS-GFP

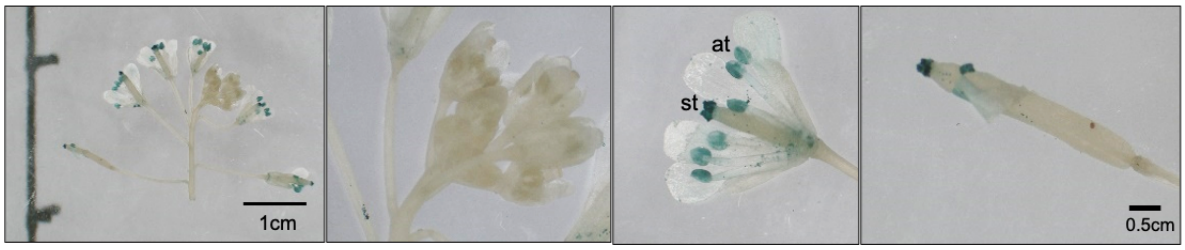


Figure 4.11: Histochemical GUS staining*: SAG21* GUS expression patterns in different floral organs of promoter deletion GUS-GFP constructs. **(a)** p*SAG21*(1700)::GUS-GFP; **(b)** p*SAG21*(1439)::GUS-GFP*;* ***(c)*** p*SAG21*(1225)::GUS-GFP; **(d)** p*SAG21*(965)::GUS-GFP; **(e)** p*SAG21*(737)::GUS-GFP ; **(f)** p*SAG21*(489)::GUS-GFP. Scale bar= 1 cm and 0.5 cm. sepal(sp), petals(pt), anthers(at), filament(fl), stigma(st), abscission zone(ab).

4.3.5 Kinetin inhibits *SAG21* expression in cotyledons but effect is dependent on cotyledon age

To investigate the expression of *SAG21* in cotyledons and the effect of cytokinin on its expression, young seedlings of homozygous transgenic Arabidopsis promoter deletion GUS-GFP lines of *SAG21* generated were used for treatment with kinetin. Cotyledons were completely green before the start of the treatment with Kinetin. Two ages of cotyledon were used, 14 and 24 day old, to assess the effects of cotyledon ageing.

In 14 d old cotyledons, there was a differential expression pattern depending on the SAG21 promoter fragment used: p*SAG21*(1700)::GUS -GFP cotyledons were stained blue at the wound site and also the expression of *SAG21* was spread lightly across the surface of the cotyledons. However, with the promoter deletion construct p*SAG21*(737)::GUS-GFP of 737 bp, cotyledons were stained blue only at the wound site with less expression on the surface of the cotyledons (Figure 4.12 b). With respect to promoter deletion construct p*SAG21*(489)::GUS-GFP the control cotyledons showed a very high expression at the wound site and the expression was spread more intensely across the surface of cotyledons when compared to the constructs *pSAG21*(1700)::GUS-GFP and p*SAG21*(737)::GUS -GFP (Figure 4.12c).When treated with kinetin, 14 day old cotyledons from all the construct lines turned completely colourless. (Figure 4.12).

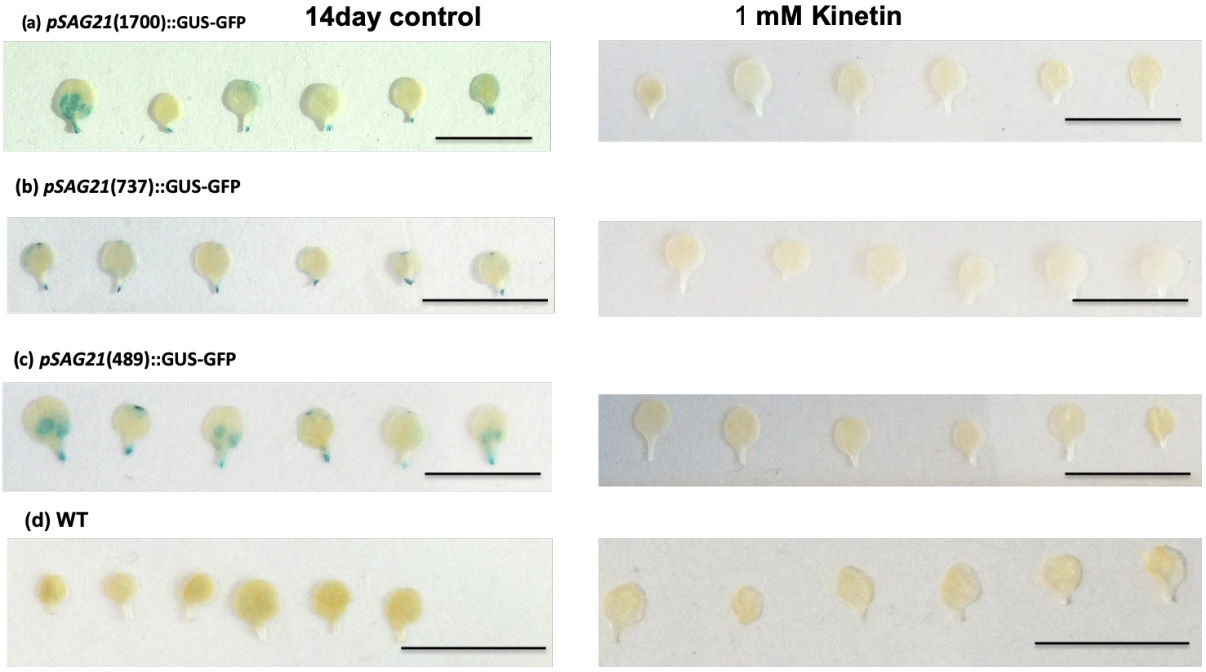


Figure 4.12 :GUS staining of 14 day old cotyledons of promoter deletion constructs of *SAG21* exposed to 1mM Kinetin and control MS liquid**.(a)***pSAG21*(1700)::GUS-GFP**(b)**p*SAG21*(737)::GUS-GFP.**(c)** p*SAG21*(489)::GUS –GFP.(d) WT. Scale bar=1cm

A similar pattern of expression of the SAG21 promoter fragments was seen in 24 d old cotyledons: cotyledons of p*SAG21*(1700)::GUS-GFP showed expression at the wound site and staining was spread in patches across the surface of the cotyledons (Figure 4.13a). Promoter deletion construct p*SAG21*(737)::GUS-GFP and p*SAG21*(489)::GUS-GFP showed similar expression of *SAG21* again at the wound site and also across the surface of the cotyledons. When 24 d old cotyledons of p*SAG21*(1700)::GUS-GFP were

treated with kinetin expression of *SAG21* was only seen at the wound site and in a localized spot at the tip of the cotyledon in all construct lines, but the patchy expression across the cotyledon was abolished.

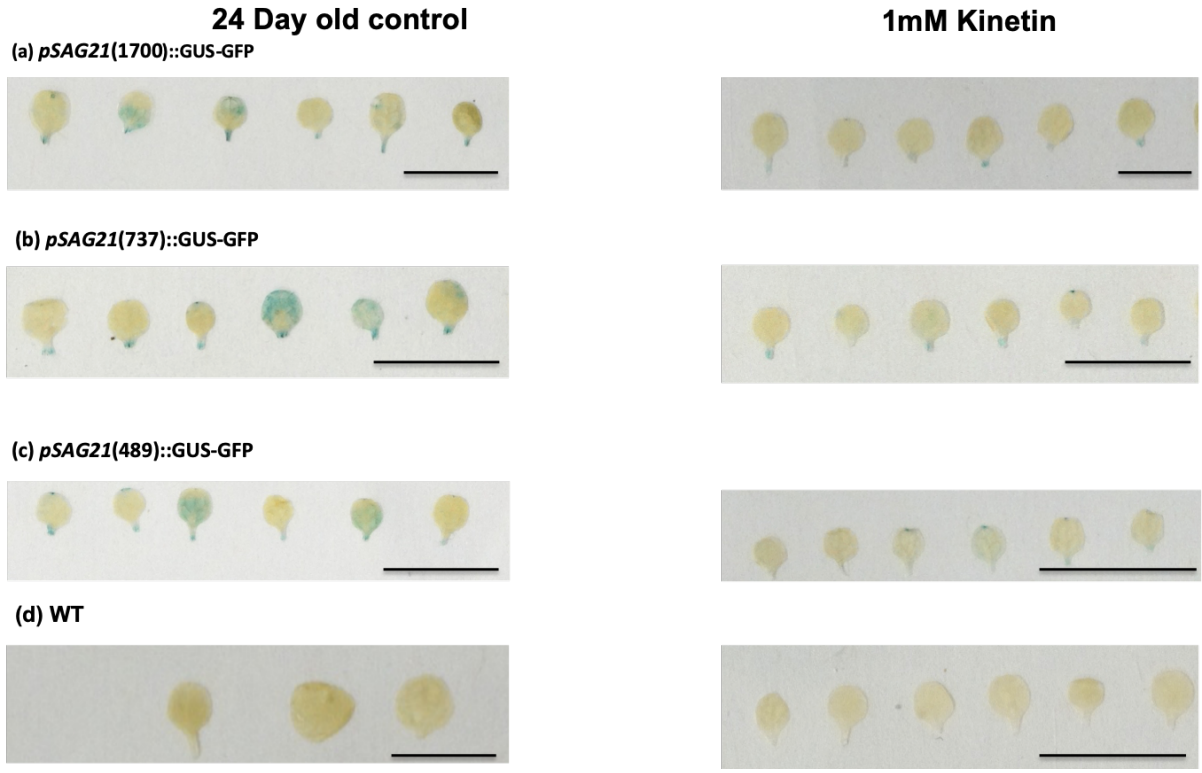


Figure 4.13 : GUS staining of 24 day old cotyledons of promoter deletion constructs of SAG21 exposed to 1mM Kinetin and control was MS Liquid.**(a)** *pSAG21(1700)::GUS.*(**b)** p*SAG21(737)::GUS* **.(c)** p*SAG21(489)::GUS* .(**d)** WT. Scale bar =1 cm.

4.3.6 Effect of kinetin on wound-induced expression of *SAG21* in mature leaves

As kinetin was completely abolishing the expression likely related to senescence but not the wound related expression of *SAG21* in 24 day old cotyledons, it was interesting to study the effect of wounding on leaves of four week old promoter deletion construct lines. In addition the effect of kinetin on the GUS expression was studied. After GUS staining the wounded leaves of all the promoter deletion GUS construct lines treated with 1 mM kinetin showed no response to wounding whereas the control wounded leaves of the constructs showed a very clear response to wounding and stained blue showing clearly the imprint pattern of the wounding. Promoter deletion constructs p*SAG21*(1700)::GUS-GFP, p*SAG21*(1225)::GUS-GFP and p*SAG21*(489)::GUS-GFP showed a weaker expression of *SAG21* with wounding while constructs

p*SAG21*(1439)::GUS-GFP and p*SAG21*(737)::GUS-GFP displayed stronger expression of *SAG21* in response to wounding (Figure 4.14 a-e). This observation showed that the kinetin completely inhibits the *SAG21* wounding response in mature leaves

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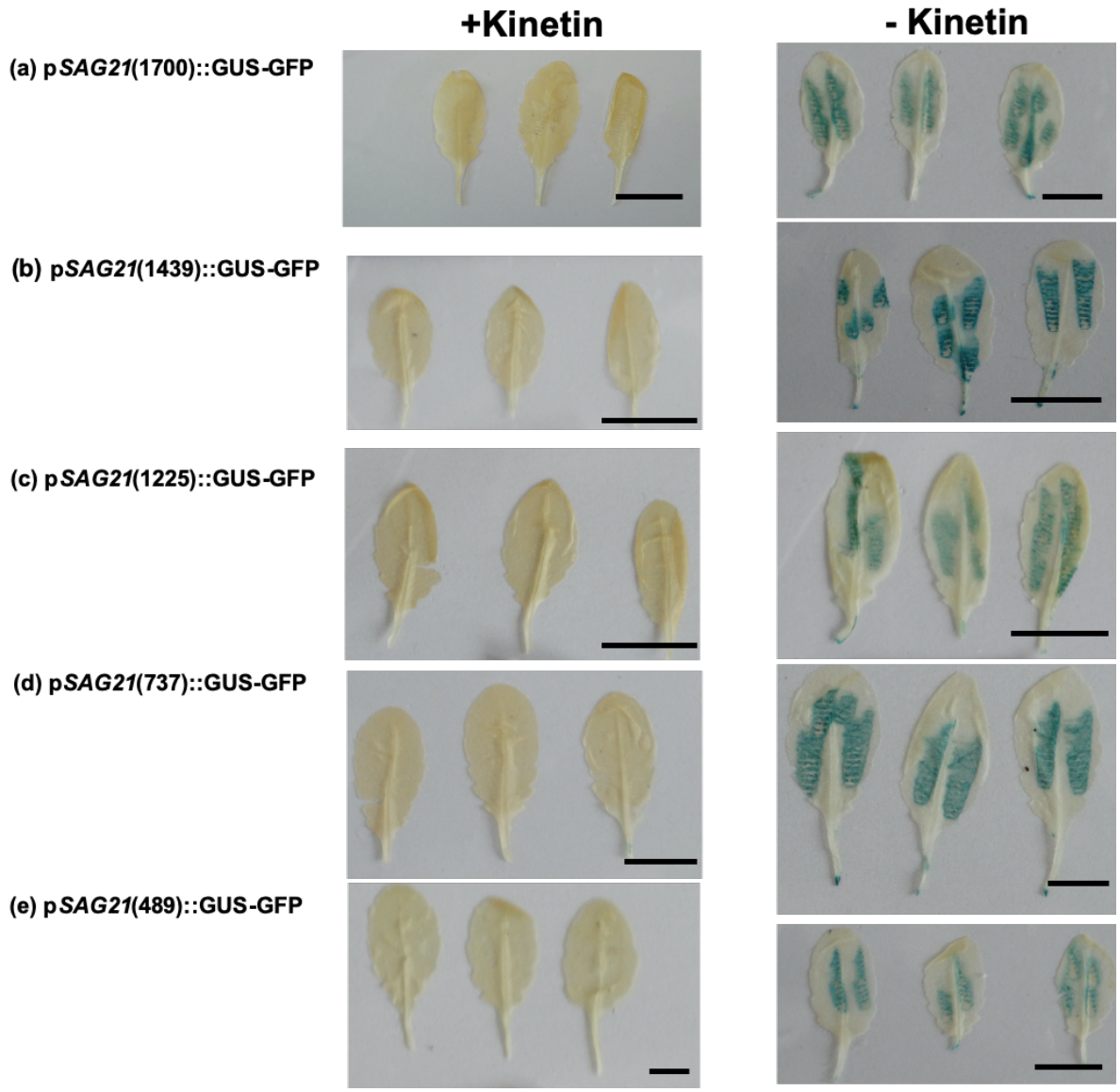


Figure 4.14 :GUS staining of four week old wounded leaves of promoter deletion constructs of *SAG21* treated with 1mM Kinetin for 18 hr in continuous light and water as control.**(a)** *pSAG21*(1700)::*GUS.***(b)**p*SAG21*(1439)*::GUS.***(c)**p*SAG21*(1225)*::GUS.***(d)**p*SAG21*(737)::*GUS.*(**e**)p*SAG 21*(489)*::GUS.*Scale bar=1cm

4.3.7 Age of rosette is important for induction of *SAG21* by wounding

To understand how the length of deletion promoter constructs affects the response to wounding with age, rosettes of different ages were wounded and stained with GUS.

Comparison of the results at different weeks with respect to age of rosette in p*SAG21*(1700)::GUS-GFP construct lines revealed that in 22 day old rosettes the expression of *SAG21* was very weak on the wounded leaf showing no visible wounding pattern. In contrast, in 29 day old rosettes *SAG21* expression in response to wounding increased and GUS staining was very intense on the wounded leaves. However, in 36 day old rosettes, the induction of *SAG21* expression by wounding decreased and the expression was very similar to 22 day old rosettes. Control (unwounded) rosettes showed expression of *SAG21* in the cotyledons and sporadically in rosette leaves (Figure 4.15). Wounded rosettes at 29 days also showed *SAG21* expression in cotyledons while at 22 and 36 days there was much less expression. These results clearly demonstrate that age affects the response to wounding.

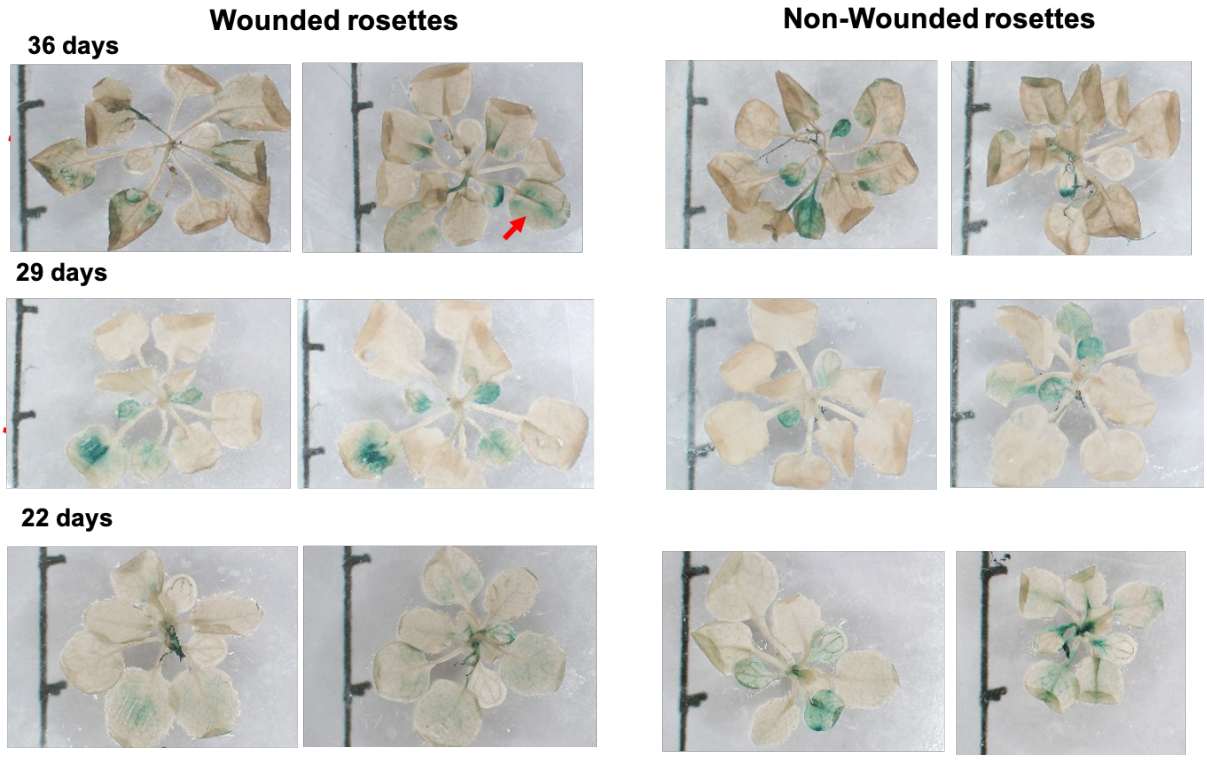


Figure 4.15: Wounding response on p*SAG21(1700)::GUS* rosettes by crushing the leaf five with forceps at four weekly intervals of 36 ,29, and 22 days. Then the entire rosette was stained by a GUS assay. Wounded leaves are indicated by red arrows. Scale bar=1 cm

When lines expressing the p*SAG21*(737)::GUS-GFP construct were treated in the same way, there was apparently no sign of expression of *SAG21* on the wounded leaves at 22 and 29 days (Figure 4.16). In cotyledons as well *SAG21* expression was absent or very weak both at 22 and 29 days in both wounded and control rosettes. However, in the rosettes at 36 days, expression of *SAG21* on the wounded leaves was evident from GUS staining and was also visible in the cotyledons. In control non-wounded rosettes, *SAG21* expression was not detected in the rosettes in either the 22or 29 day old plants. These observations demonstrate that p*SAG21*(737)::GUS-GFP construct lines showed a response to wounding only at 36 days.

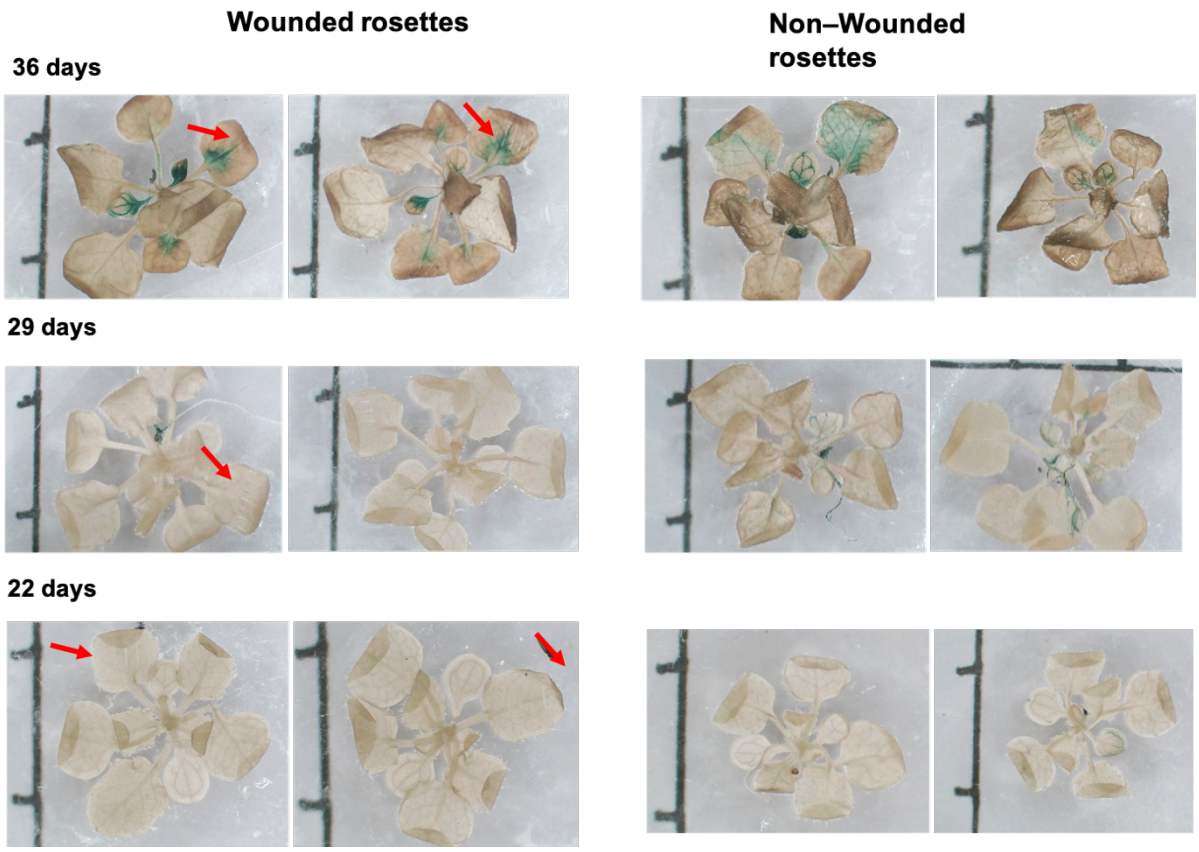


Figure:4.16: Wounding response on p*SAG21(737)::GUS* rosettes ;Wounding was carried out by crushing leaf five with forceps at weekly intervals over a four week period of 36, 29, 22 days. The entire rosette was then stained by a GUS assay. Wounded leaves are indicated by red arrows. Scale bar=1 cm

pSAG21(489)::GUS-GFP rosettes demonstrated no clear response to wounding leaf 5 at different week intervals with respect to the age of the rosette. However, cotyledons exhibited a high expression of *SAG21* in 36 day old wounded and control non-wounded rosettes, and some expression at 22 and 29 days. Expression in the primary veins of the rosette leaves was also observed in both wounded and control rosettes (Figure 4.17). These observations show that *pSAG21*(489)::GUS-GFP shows no effect with regard to wounding, but there is an effect of age on expression in the cotyledons.

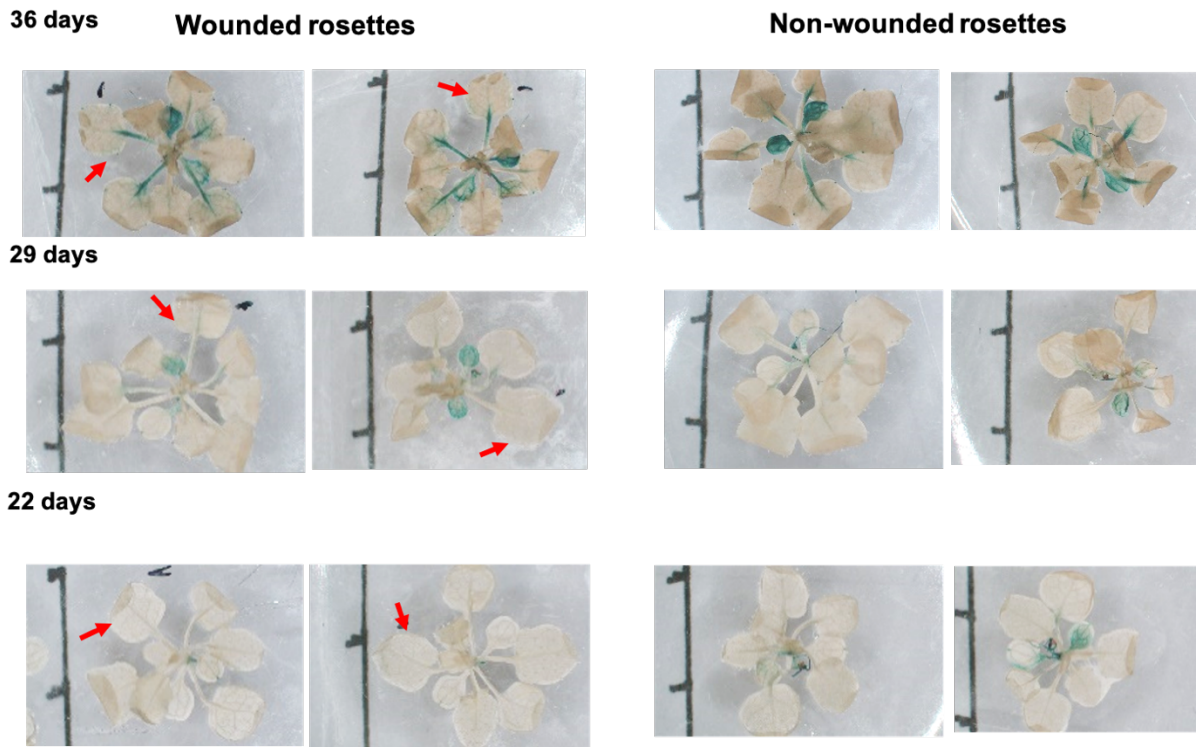


Figure 4.17 : Wounding response on *pSAG21(489)::GUS* rosettes; leaf 5 was wounded by crushing the leaf with forceps at four week intervals of 36 ,29, and 22 days and the entire rosette was stained by a GUS assay. Wounded leaves are indicated by red arrows. Scale bar=1cm

4.4 Discussion

To analyze the function of the *cis*- elements in the *SAG21* promoter, six promoter deletion constructs were successfully assembled using directional cloning. The very first approach in making the deletion constructs was to create a backbone of plasmid with a GUS-GFP insert into which *SAG21* deletion fragments were to be cloned. An alternative strategy to the directional cloning would have been to use Gibson assembly, a new and rapid method that joins multiple DNA fragments in a single isothermal reaction. The assembly Master Mix contains three different enzymes that perform the reaction in a single buffer. Insert DNA with 15-20 base pairs overlap are ligated with a linearized vector in a single reaction in the presence of the enzymes (Gibson et al., 2009). This was indeed attempted, and ligation and transformation of vector and insert DNA by Gibson cloning produced many hundreds of colonies on reaction plates. However, colonies when screened by PCR showed that ligation was not successful. The reason for the unsuccessful transformation of the insert is perhaps that the size of insert (GUS-GFP) DNA which is of 2.7 kb might be difficult to clone into a plasmid of size 4.4 kb, and also control plates with vector-only also produced many colonies on the plates indicating the presence of some undigested vector DNA. Several modifications

to the Gibson protocol were attempted which included trying different molar ratios of vector and insert DNA. The primers were also redesigned for amplification of the insert and digested the vector with a single restriction enzyme instead of two restriction enzymes according to the protocol. However, it was still not possible to obtain positive colonies through this approach. Hence directional cloning was adopted and was successful.

The directional cloning approach used here in making the promoter deletion constructs ensured that the promoter region and ATG start codon were ligated without the extra additional poly-linker bases inserted in the constructs generated by Salleh et al. (2012). These additional extra poly-linker bases might have affected the promoter activity and therefore directly affect the expression patterns of the promoter when fused to the reporter genes. Promoter GUS fusion constructs generated by Salleh et al. (2012) also employed only GUS as a reporter gene, whereas the deletion constructs generated in this work with the pGreenII0229 plasmid contain both GUS and GFP as reporter genes. GUS as a reporter gene has an advantage because of its broad substrate versatility (Oliver, 2013) but GFP can also be evaluated or studied using non-destructive methods and does not diffuse from its place of expression (Kavita & Burma, 2008). GFP is therefore considered as a better choice for the analysis of tissue-specific promoter activity. So the pgreenII0229 plasmid generated here, which contains both reporter genes can be used for future research for both live imaging and for GUS staining of plant tissues.

Binary vectors are usually the vectors of choice in *Agrobacterium* plant transformation because of their size and copy number in *E.coli* which provides increased efficiency, and pGreen can replicate in *Agrobacterium* only if another plasmid pSoup is co-resident in the same strain of plasmid (Hellens et al., 2000). However, in this work with the deletion promoter constructs, the number of the transformants obtained after floral dipping was very low which might be due to the efficiency of the plasmid or might be due to the method of transformation by floral dipping. The success rate of obtaining enough transformants depends on several factors during the process of floral dipping: the very first factor is the developmental stage of the plant with the maximum number of unopened buds, but also presence of sugar and surfactant (Clough & Bent, 1998). In this study with the floral dipping experiment, I found that the transformation efficiency also depends upon the season because the number of transformants obtained during

summer was usually high when compared to the other times of the year. Ultimately sufficient numbers of transformants were obtained through multiple dipping events.

Developmental pattern of GUS expression

Overall, the pattern of expression of the longest construct (*pSAG21*(1700)*::* GUS-GFP) was similar to that seen previously (Salleh et al., 2012; Salleh, 2011) However, expression in seedlings was more variable than that seen previously, perhaps reflecting the difference in construct or subtle differences in growth of the seedlings. Due to lack of time, the analysis therefore focussed on expression during reproduction and in response to wounding and cytokinins.

***SAG21* showed different expression patterns in floral organs of promoter deletion construct lines**

Floral expression of the promoter deletion constructs was compared to the two promoter GUS constructs generated by Salleh et al. (2011). The *pSAG21*(1700)*::* GUS-GFP deletion construct showed expression in anthers and pollen and high expression in pollen upon anthesis, which was a very similar pattern of expression to the 1685:: GUS construct of Salleh et al. (2011). However, there was some expression in the stigma seen with the *pSAG21*(1700)*::* GUS-GFP construct which was not seen with the construct generated by Salleh (2011). This might be due to the insertion of polylinker in the Salleh (2011) construct repressing expression in the stigma. The 325:: GUS construct showed expression in sepals, petals, and filaments and also near the style but expression was not seen in the stigma. In contrast, deletion construct *pSAG21*(489)*::* GUS-GFP showed very strong expression in stigma and also expression in anthers but no expression in sepals, petals, and filament which is a quite different pattern of expression when compared to 325:: GUS construct (Salleh, 2011).This may be due to the extra 164 bp of promoter in the *pSAG21*(489)*::* GUS-GFP construct, directing expression to the stigma but down-regulating expression to sepals, petals and filaments. This region contains 95 putative cis elements from 17 different TF families (Appendix Table A4.3.1). These include several TF families known to be involved in regulating pollen development such as bHLH (DYT1;Feng et al., 2012), bZIP (bZIP18;Gibalová et al., 2017), and MYB-related (MYB80; Phan et al., 2011).

Deletion of the promoter region from -1700 to -1439 caused an increase in expression in the *pSAG21*(1439):: GUS-GFP construct lines making an expression gain in petals and filaments when compared to *pSAG21*(1700)*::* GUS-GFP construct. This suggests *cis*-elements present in this promoter region spanning from -1700 to -1439 function as repressors of expression. Further deletion from -1439 to -1225 did not change the pattern of expression suggesting that this promoter region does not contain major elements for expression in these tissues. However, deletion from -1225 to -965 apparently reduced expression in sepals, petals and filaments indicating cis- elements as activators of gene expression in this region. However, quantitative GUS assays would be required to confirm this trend. The deletion from -965 to -737 and -737 to -490 caused further loss in expression in sepals, petals, and filaments indicating the further *cis*-elements in these regions as activators of *SAG21* expression. Expression in stigma and anthers was relatively unaffected by the deletions of the promoter across the floral organs, although stigma expression was almost abolished in the *pSAG21*(965)*::* GUS-GFP construct but then restored in the *pSAG21*(737)::GUS-GFP suggesting the presence of negative and positive regulators.

Many other plant promoters that direct strong pollen expression have been studied. For example, *ACA7,* encodes for a plasma membrane Ca^{+2} P-type ATPase in Arabidopsis. Its expression is restricted to pollen, and its protein product was essential for proper pollen development. GUS expression constructs of this gene have identified the presence of a cis-regulatory element in the promoter upstream from the start codon which activates gene expression in the pollen but represses gene expression in the sporophyte. This 88 bp cis-regulatory element sequence shows the presence of a motif GAATATTCCT recognized by *GARP* and *KANADI1* family transcription factors. This regulatory function of *cis*- elements might be by the interaction with other elements which may be closely located to the transcription start site (Hoffmann et al., 2017). Similarly with respect to the *SAG21* the gain and loss in expression in sepals, petals and filaments might be controlled by a similar kind of motif which interacts with other elements and regulated by the transcription factors involved in sepal and petal expression.

Bioinformatic analysis of the region between 1439 and 1700 lost in the deletion from *pSAG21*(1700):: GUS-GFP to *pSAG21*(1439):: GUS-GFP revealed that this promoter region contains potential binding sites for 24 different TF families (Appendix A4.3.2)

including several binding sites for the Dof transcription factor family which might function as activators or repressors of expression. Dof transcription factors contain a conserved DNA binding domain that binds to the *cis*-regulatory elements containing a recognition core sequence T/AAAAG named as the Dof core element. Dof transcription factors contain a variable C terminal domain which functions in transcriptional activation or repression of gene expression by interacting with other regulatory proteins (Peng et al., 2017). In this region of the *SAG21* promoter there are also several cis elements able to bind zinc finger protein TFs. The C_2H_2-zinc finger transcription factor family are involved in regulation of cell division and proliferation in floral organ development and also play a role in the process of flower partition into four distinct whorls (Lyu & Cao, 2018). Hence these elements may also be involved in the change in expression between these two deletion constructs.

Compared to *SAG21*, the other three genes of the LEA_3 family (At4g15910, At3g53770, and At1g02820) show different expression patterns in floral organs (Winter et al., 2007). At4g15910 is mainly expressed in the shoot apex and developing seeds, with low or no expression in floral organs and pollen, At3g53770 shows generally weak expression but is expressed in pollen as well as in sepals and pistils while At1g02820 is strongly expressed in developing seeds, sepals and mature anthers, but not in pollen before germination. Mowla et al. (2006) reported that *SAG21/AtLEA5* transcripts were more abundant in the flowers but found that *SAG21* was absent in female tissues or reproductive organs and thought that expression in flowers might be due to the high expression in pollen and anthers. Hence results here show that *SAG21* expression in flowers is more complex than previously thought and contrasts with the expression of the other members of the LEA_3 family.

pSAG21(1700)::GUS-GFP and *pSAG21*(1225)::GUS-GFP constructs show strong expression near the abscission zone. However this expression appears to be lost in the *pSAG21*(965)::GUS-GFP, *pSAG21*(737)::GUS-GFP and *pSAG21*(489)::GUS-GFP constructs. After pollination of the flower abscission occurs, during which the unwanted floral organs are shed from the main plant body. One of the members of the Dof transcription factor family *DOF4.7* was found to be expressed in the abscission zone. This protein binds to the typical Dof *cis*-elements in the promoter of an abscission related PG gene, PGAZAT. The promoter of PGAZAT contains AAAG elements which

function as binding sites for AtDOF4.7 (Wei et al., 2010). This promoter region of *SAG21* contains several Dof *cis*-elements one of which has the sequence tctTAAAGt. This element may be responsible for abscission expression in the longer constructs which are possibly regulated by Dof transcription factor family members.

Kinetin inhibits *SAG21* expression in cotyledons but the effect is dependent on cotyledon age

Cotyledons are short lived organs that senesce along with the progress of the seedling development and usually die after the appearance of the differentiated leaves (Mishev, et al., 2005). Cotyledons serve as specific reserve organs whose major function is to ensure the development of the growing seedling until the differentiation of the photosynthetically efficient leaves (Mishev et al., 2005). Although, cotyledon senescence is not much different from leaf senescence, organ-specific differences between cotyledons and leaves with regard to their photosynthetic activity in natural senescence has been reported (Mishev et al., 2005). To study the *SAG21* expression in cotyledons and to understand how kinetin affects its expression, cotyledons from promoter deletion constructs of *SAG21* of two ages 14 and 24 days old were used.

pSAG21(1700):: GUS -GFP cotyledons showed staining at the cut site and also across the surface of the cotyledons. The *pSAG21*(737):: GUS-GFP construct cotyledons also showed expression at the wound site with less spread across the surface however, *pSAG21*(489):: GUS-GFP construct cotyledons showed very intense expression at the wound site and also across the surface of the cotyledon. Given the age of the cotyledons it seems likely that they were entering senescence, although further work using expression of early and late senescence markers (Breeze et al., 2011), and measurement of the chlorophyll content and yellowing of the cotyledons, which are the main definitive signs of the natural senescence process (Mishev et al., 2005). should be carried out to verify the senescence status. Treatment with kinetin completely inhibited the expression both on the surface of the cotyledon and on the cut edge suggesting that it was repressing both *SAG21* expression activated by senescence and wounding. In 24-day old cotyledons *pSAG21*(1700):: GUS-GFP showed a similar expression to 14 day old cotyledons, whereas the reduction in GUS expression across the surface of the *pSAG21*(737):: GUS-GFP cotyledons was not seen in 24 day old cotyledons. This

suggests that promoter elements between 737 and 1700 bp upstream of the ATG are affecting senescence-related expression of *SAG21* in cotyledons. Further work would be useful examining the expression in cotyledons of the intermediate constructs *pSAG21*(1225):: GUS-GFP and *pSAG21*(965):: GUS-GFP to identify more closely the region in which these elements are contained. There was also a difference in the effect of kinetin: after 24 days kinetin no longer abolished the wound-related *SAG21* expression. These results indicate that the kinetin was completely inhibiting the senescence-related expression after 24 days in these constructs but not the wound-related expression of *SAG21.*

Given the results from these two time-points a time course analysis or measurement of senescence parameters and GUS expression for the different construct lines in the cotyledons from 3-4 days after germination to senescence stage would be interesting to know how far the cotyledons reach in the senescence process by 24 days in Arabidopsis. This would give a clearer indication to determine the correct age of cotyledons for treatment with the kinetin to explore natural senescence.

Although few studies have tried to understand the link between ROS metabolism and cotyledon senescence, a study in mung bean has found that O^{2-} is associated with cotyledon development and H_2O_2 with storage mobilization followed by senescence (Pal & Kar, 2019). It might be interesting to understand the localization of ROS in intact and detached cotyledons to understand the role of ROS in senescence in the cotyledons and whether localisation of ROS is linked to the GUS expression of the promoter deletion constructs.

Gilbert et al. (1979) showed that the application of benzyladenine to young bean seedlings delayed senescence of cotyledons and the explanation of this effect was due to the disruption of the source-sink nutrient relationship which happens during early seedling development. This was shown by study of parameters like cotyledon weight, delay or lag in the breakdown of the starch and proteins, and delay in the translocation of hydrolyzed food reserves from storage cells and the delay in onset of membrane leakiness which usually facilitates translocation of the hydrolysis products (Gilbert et al., 1980). So probably a similar disruption in source-sink nutrient relationship is taking place when cotyledons are treated with kinetin in this study, with *SAG21* being switched off.

The senescence delaying process is well known but more recently the mechanism behind this phenomenon in Arabidopsis has been identified as cytokinin specific signalling components: the cytokinin receptor (AHK3), the type-B response regulator (ARR2) and cytokinin response factor (CRF6) through which senescence is regulated (Zwack & Rashotte, 2013). All these components work through a two-component signalling pathway and CRF6 was found to act as a side branch of the two component cytokinin signalling pathway. In Arabidopsis perception of cytokinin by AHK leads to the activation by phosphorylation of type B response regulator ARR2 and induces the expression of CRF6. ARR2 and CRF6 proteins also may interact in the process of the regulation of downstream genes, and cell wall invertase is one such gene that has been found to be needed for senescence inhibition in tobacco and tomato (Zwack & Rashotte, 2013) The downstream mechanism involved in the cytokinin delay of leaf senescence involves the regulation of sink/source relations as discussed above, and the influence of cytokinin upon it is exerted by the regulation of the cell wall invertase activity (Roitsch & Ehneß, 2000). Studies on tobacco proSAG12::ipt lines found a link between cytokinin induced cell wall invertase and delayed leaf senescence where long-lived leaves of the plants had high levels of cell wall invertase activity (Godt & Roitsch, 1997). It is therefore possible that the repression of *SAG21* expression in cotyledons may be mediated by the AHK3-ARR2-CRF6 signalling pathway. Indeed recently CRF6 (an *AP2/ERF* transcription factor) was found to be activated by oxidative stress, and to act as a repressor on downstream genes (Zwack et al., 2016). Although the *SAG21* promoter did not appear to have any *AP2/ERF* transcription factor binding cis elements, it would still be interesting to discover whether CRF6 may regulate *SAG21* expression directly or indirectly.

Kinetin effect on wound-induced expression of *SAG21* in mature leaves.

Mechanical injury activates a similar kind of defence response as induced by herbivores and insects (Reymond et al.,2000; Savatin et al., 2014). Signal transduction events that are usually triggered by wounding include electric signals, ion fluxes, ROS/Ca^{+2} signalling, MAPKs, and hormones. JA usually accumulates in wounded plants and activates the expression of many defence genes (Savatin et al., 2014). Promoter deletion constructs *pSAG21*(1700):: GUS-GFP, *pSAG21*(1225):: GUS-GFP and *pSAG21*(489):: GUS-GFP showed a weaker expression of *SAG21* with wounding while constructs *pSAG21*(1439):: GUS-GFP and *pSAG21*(737):: GUS-GFP displayed stronger

expression of *SAG21* in response to wounding. This suggests that *cis*-elements across the SAG21 promoter are both activating and repressing expression in response to wounding in adult leaves.

MAPKs are known to function during the early stage of wound signalling (Cell et al., 1988). In Arabidopsis wounding activates MPK4 and MPK6 (Ichimura et al., 2000). More recently, Cozen et al. (2019) have identified that two independent MAPK cascades or modules are activated by wounding in Arabidopsis. The first cascade has MKK4/5-MPK3/6 and the other cascade is regulated by the MKK3-cladeC MAPKs. The MKK3-MPK2 cascade is activated by JA and wound-induced transcription of clade III MAP3Ks like MAP3K18, 17, 19, and 20. MPK3 and MPK6 were found not to be activated by JA and the wound-induced activation of MPK3/6 was not reduced in the JA sensing deficient *coi-34* mutant indicating that the rapid MKK4/5-MPK3/6 cascade is independent of JA signalling. In contrast the MKK3-MPK2 cascade depends on JA signalling (Sözen et al.,2017). *SAG21* promoter deletion constructs show a rapid wound response immediately after the wounding, within fifteen minutes, so it might be that *SAG21* expression is controlled by the pathway of rapid MKK4/5-MPK3/6 MAPK cascade, functioning downstream in the cascade.

In this study with promoter deletion constructs, it was found that kinetin completely abolished the wounding expression activated by the *SAG21* in mature leaves. An explanation behind this inhibition of wounding by kinetin may be that *SAG21* might function downstream of the AHK3-ARR2-CRF6 APK pathway discussed above. Treatment of wounded leaves with kinetin completely downregulates the MAPK pathway, thus downregulating the expression of *SAG21* in wounded leaves. It would be interesting to understand how *SAG21* is regulated in mutants of this pathway.

The *SAG21* promoter region contains several MYC/MYB recognition sites, TGA binding sites and W-boxes which function in response to wounding, pathogen defence, and stress. Four W- boxes are present in the promoter region of *SAG21* with two of them situated very close to each other serving as excellent binding sites for *WRKY* TFS (Salleh, 2011). The promoters of other genes which show wound-induced response also contain mainly W-Box and T/G box cis-element AACGTG. For example the promoter region of the *FAR6* gene in Arabidopsis showed the presence of a WUN motif

(TCATTAA/CA/GAA) which functions as a wound responsive element and also contains W-BOX (TTGAC) known to bind the WRKY family of transcription factors (Gupta et al., 2012). The promoter region of *At4CL1* and *At4CL2* genes contains the/G box cis-element AACGTG which is the binding site for JAMYC2 and JAMYC10 bHLH transcription factors which direct wound-induced expression in tomato and Arabidopsis (Soltani et al., 2006). No G boxes were identified in the *SAG21* promoter region, however, so other TFs are more likely to be involved.

JA plays a major role in rapid and systemic wound responses in plants (Farmer et al., 1992). Arabidopsis leaves when damaged synthesize substantial amounts of JA and concentration of JA increases in crushed tissue within 30s after wounding. Generally crushed wounds are more effective in exciting jasmonate synthesis than the clean wounds produced by insects and chewing or scissors (Farmer et al., 2014). The 1685 bp SAG21 promoter-GUS construct responded to JA (Salleh et al., 2012) hence the response seen here with wounding on the deletion construct lines may also relate to SAG21 responsiveness to JA. BHLH transcription factor, MYC2 is a major regulatory protein which interacts with the JAZ protein family leading to the activation of the downstream genes. The *SAG21* promoter contains cis-elements or binding sites for the MYC/MYB transcription factors and bHLH transcription factors, hence it is possible that the wound-induced expression in leaves of the deletion constructs might also proceed through the JA pathway via MYC transcription factors leading to the activation of expression of *SAG21.* WRKY and NACs are also found to be involved in JA signalling. AtWRKY33 and WRKY8 were also upregulated by wounding, ROS and abiotic stress like *SAG21* and therefore they might also function as possible regulators of *SAG21* via the W boxes (Chen et al., 2010; Zheng et al., 2006)

Age of rosette is important for induction of *SAG21* by wounding

AtWRKY53 positively regulates developmental senescence and mRNA expression was seen in the old leaves of 6-week old rosettes and also expressed in all leaves at the 7-week stage and decreased by the 8-week old stage (Zentgraf, 2001). This suggests that this gene plays a role in senescence, independent of the leaf development age. It was demonstrated that WRKY53 regulates SAG expression and functions in an upstream position in the WRKY signalling cascade (Zentgraf et al. 2010). MEKK1 interacts directly with the WRKY53 promoter and binds to the MYC related motif (Miao et al.,

2007). *SAG21* is upregulated in the *mekk1* mutant as well as in mutants of MKK1/2 and MPK4 indicating *SAG21* might function downstream of WRKY TFs with WRKY53 as a possible regulator in the MEKK1-MKK1/2-MPK4 cascade (Pitzschke et al., 2009).

To understand whether the wounding response of promoter deletion construct lines is affected by age, rosettes of different ages were wounded. At 22 days, the *pSAG21*(1700):: GUS-GFP construct showed very weak expression in wounded leaves, but at 29 days old rosette expression in wounded leaves was increased, indicating that there might be some regulatory elements which function as activators of *SAG21* expression at the wound site that are only activated in older rosettes. However, the expression was decreased in the 36 day old wounded leaves, suggesting a further age-related effect. Bioinformatic analysis in this study shows that the promoter region *pSAG21*(1700):: GUS-GFP contains binding sites for WRKY and NAC TFs which may function in leaf age-dependent wound expression regulating the expression of *SAG21*. The expression of cotyledons here also was dependent on the age showing high expression at 29 days but very little expression at 22 or 36 days. This fits with the expression pattern of SAG21 as an "early senescence" expressed gene (Weaver et al., 1998).

pSAG21(737):: GUS-GFP showed no expression of *SAG21* at 22 days and 29 days but showed expression in 36 day old rosettes, which indicates some regulatory elements functioning as activators of *SAG21* expression during the early stages of development both in response to wounding, and in cotyledons, located between 738-1700 bp. Analysis of the 1225 and 965 bp promoter fragment constructs may be useful to narrow down the region(s) responsible. The *pSAG21*(489):: GUS-GFP construct did not respond to wounding either at 22, 29 or 36 days indicating that this region of promoter is not sufficient for wound regulated expression of *SAG21* in mature rosette leaves. As this experiment was carried out in *in vitro* conditions growing in Petri plates, an experiment on soil might be interesting as the conditions may be less stressful, and hence responses may differ.

Thus overall, analysis of GUS expression from deletion constructs of the SAG21 promoter indicated several regions in which *cis*-elements may be directing expression in response to leaf and cotyledon age, wounding and treatment with cytokinin. Further work is required to identify these elements with more precision.

Chapter 5 Analysis of *SAG21* over expressors under optimal and oxidative stress conditions

5.1 Introduction

Plants, when exposed to abiotic stress conditions, have an acquired adaptive response to improve their growth and survival. Even though developments have been made in identifying the genes, proteins, and metabolites associated with stress adaptation the understanding of most of these elements is still unclear (Dang et al.,2014). Stress-responsive genes identified in plant species include those that encode LEA proteins (Dang et al., 2014; Hincha & Thalhammer, 2012b). The functional role of LEA proteins in plant stress tolerance has been investigated by transgenic approaches. Many investigations of the LEA proteins have used heterologous gene expression where a gene is expressed in a different plant species, although in some cases, genes from similar species have also been constitutively overexpressed (Dang et al., 2014; Hincha & Thalhammer, 2012).

LEA proteins are a heterogeneous group of proteins originally discovered in cotton seeds (Dure III & Chlan, 1981) and they accumulate in the seeds of other plants and vegetative organs during abiotic stresses such as cold, salinity, drought (Amara et al., 2014). The remarkable feature of LEA proteins is that they are hydrophilic and intrinsically unstructured but however, they acquire α-helical structure upon desiccation or drying or in extreme temperature (Battaglia & Covarrubias, 2013; Hundertmark & Hincha, 2008). Based on the appearance of different sequence motifs, plant LEA proteins were classified into different groups. The model plant *Arabidopsis thaliana* contains 51 LEA proteins that were divided into nine different groups based on amino acid sequence analysis. PfaM_LEA4 is the largest group among them as it is present even in non-plant organisms (Hincha & Thalhammer, 2012) and the other group which is extensively studied is the dehydrins. Dehydrins belong to group 2 LEA proteins and pfaM_LEA4 belongs to group 3A LEA proteins (Battaglia et al.,2008).

Some LEA proteins function as molecular chaperones or shields by protecting the cellular proteins from aggregation during the stress conditions. The anti-aggregation function was demonstrated for Group 3 LEA proteins (that include *SAG21*) in living cells (Amara et al., 2014; Tunnacliffe & Wise, 2007b). Membrane protection is necessary for the survival of the cell during freezing or dehydration. LEA proteins have no transmembrane segments but they also play a role in membrane protection by integrating within the membrane as intrinsic proteins. They also form amphipathic alpha-helices during freezing which enables them to connect or interact externally with membranes (Hincha & Thalhammer, 2012a). Overexpression of genes encoding LEA proteins increases the stress tolerance of transgenic plants. For example, expression of the barley gene *HVA1* in rice and wheat (Xu et al., 1996) conferred or contributed to drought tolerance in plants.

To exhibit responses plants generally recognize the external signals or stimuli and then transmit the signal to the nucleus of the plant cell. This occurs with the help of cell wall receptors which interact with other signalling components and cause a change in the redox state of the plant. This is mediated by the production of reactive oxygen species (Jalmi & Sinha, 2015). Increased levels of ROS cause oxidative damage to the nucleic acids, lipids, and proteins and this situation leads to oxidative stress. At a low concentration, they function as a crucial component of the signalling pathway and provide tolerance against the oxidative damage (Singh et al., 2019). The major sources of ROS during abiotic stress include ROS generated as a result of disruption in metabolic activity and ROS produced for the function of the abiotic stress response signal transduction (Choudhury et al.,2017; Miller et al., 2010). ROS are usually formed as by-products of aerobic metabolism. Chloroplasts are one of the major sites for the production of metabolic ROS in a plant cell (Choudhury et al., 2017). Plants deal with excess ROS production by producing enzymatic and non-enzymatic antioxidants which work in a coordinated manner (Cruz De Carvalho, 2008). Metabolic ROS controls the redox status by changing the metabolic fluxes in a cell and therefore affecting the metabolic reactions to protect against the effect of the stress. Signalling ROS is mediated by calcium signals or phosphorylation-derived activation of NADPH oxidase present at the plasma membrane (Choudhury et al., 2017; Gilroy et al., 2014). Signalling ROS also modifies the redox state of regulatory proteins producing an acclimation response to reduce the effect of the stress on metabolism. Although the two processes occur in different sub-compartments in a cell, they can affect each other and H_2O_2 can move in between compartments. (Choudhury et al., 2017). Another important signalling

cascade that works in transmitting the external signal is the MAPK cascade. The entire cascade of components is activated by phosphorylation and also activates other proteins causing or leading to the exhibition of stress mediated responses.

As mentioned above, many of the LEA proteins function in stress tolerance, and among LEA proteins at least one of them: *SAG21/LEA5* plays a role in oxidative stress tolerance (Mowla et al., 2006b). *SAG21* is a ROS-inducible gene, and is thought to play a role in oxidative stress protection. As evident from microarray data and various reports it is also strongly upregulated by other stresses (Weaver et al.,1998; Mowla et al., 2006; eFP Browser: Winter et al., 2007; Salleh et al., 2012). The regulation of *SAG21* by various abiotic stresses was shown by the analysis of transgenic *SAG21* promoter GUS plants (Salleh, 2011; Salleh et al., 2012). Light repression of *SAG21* and its expression in non-photosynthetic tissues like flowers and roots suggests it might function as an antioxidant in mitochondria. However, its upregulation in response to oxidants and low reactivity to H_2O_2 in plants overexpressing *SAG21* indicate a function in ROS mediated signalling (Mowla et al., 2006; Salleh, 2011; Salleh et al., 2012).

Constitutive promoters show constant levels of gene expression in most tissues under different conditions. CaMV35S promoter is a well-known constitutive promoter used for the development of transgenic plants (Hernandez-Garcia & Finer, 2014). It has been most widely used in the transformation of dicots and is very efficient when compared to monocots. Genes, when expressed under the constitutive promoters, are active in most of the cells throughout development but the expression levels depend usually on cell type (Porto et al., 2014). Antisense lines and transgenic *Arabidopsis* lines overexpressing S*AG21* under the transcriptional control of the CaMV35S promoter were generated by Mowla et al (2006) and analysed by Salleh et al. (2012). Antisense plants were smaller with few flowering stalks and showed early senescence phenotype in the leaves while the over-expressors showed a taller and delayed senescence phenotype with more flowering stalks. Bolting time was also different, where antisense lines displayed an early bolting whereas the over-expressors showed a delay in time for bolting (Salleh, 2011). Below ground phenotype was also affected where the primary root length was similar to WT in the over-expressors but shorter in antisense lines. However, there was an increase in lateral roots in relation to primary root length in over-expressors and a decrease in antisense lines (Salleh et al., 2012). Antisense lines also exhibited very short root hairs compared to WT but the

opposite phenotype was shown by the over-expressors (Salleh et al., 2012). Root and shoot biomass, and rosette fresh weight under control conditions were increased in over-expressors but when exposed to oxidative stress the transgenic plants were able to produce higher root and shoot growth than WT at proportionate levels of H_2O_2, indicating the tolerance conferred by expression of *SAG21* to the oxidative stress (Mowla et al., 2006a).

Since the CaMV35S promoter shows expression in almost all the organs of the plant, all through development, transgenic Arabidopsis plants have been created in which *SAG21* is overexpressed under its own promoter. In these transgenic lines it is expected that the additional copy of *SAG21* is switched on only at the location where the endogenous gene was expressed and might produce a different phenotype to plants in which *SAG21* expression is driven by the CaMV35S promoter. These over-expressor lines were generated before the start of my Ph D in the Rogers' lab and these transgenic seeds were further used for my work (Figure 5.1).

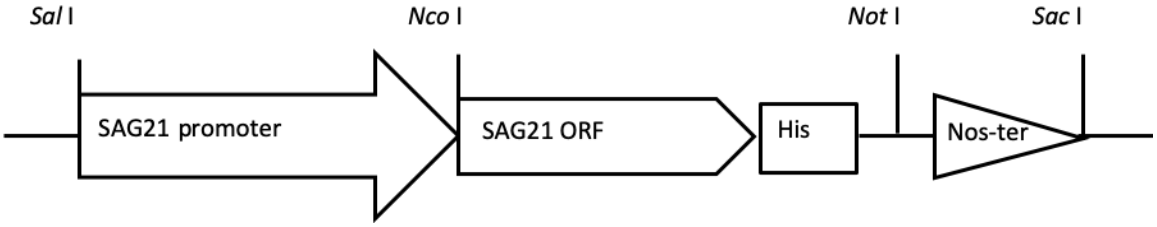


Figure 5.1 : Illustration of pSAG21::SAG21-OEX overexpression construct under the control of its own promoter.

This chapter aims to understand whether the overexpression of *SAG21* under its own promoter has any effect on development and stress response. This was investigated by studying the effect of overexpression of the *SAG21* promoter under its own promoter in optimal conditions and under oxidative and drought stress conditions and then comparing its effect to the overexpression of *SAG21* from 35S constitutive promoter.

5.2 Materials and Methods

5.2.1 Plant material

Arabidopsis thaliana lines included Wild type (Col-0), *SAG21* over-expressors driven by the constitutive 35S promoter (Mowla et al., 2006) and *SAG21* over-expressors driven by *SAG21*'s own promoter (Rogers Lab, unpublished; Table 2.2). Three lines all derived originally from OE2-5 (Mowla et al., 2006), but which had been multiplied independently for several generations were used for this work called here: 35s2-5 (line 1), oex 3 (line 2) and oex 1a (line 3). Likewise three lines of WT that had been multiplied independently were also used as controls.

Several lines were selected from the screening of the primary transformants for SAG21p::SAG21ORF (prior to the start of this project) and among them *SAG21* ORF H2B, H2C were selected for further work. Seeds of T2 generation from both lines were sown on 1 X MS and were grown until seedlings germinated and then transferred on to 1 X MS selection plates with kanamycin 50 µg/ml. The seedlings were grown for 12-14 days on the antibiotic selection to check the survival of seedlings on the selection media. Seedlings which survived the antibiotic selection from each plate were selected and transferred to sterilized autoclaved soil for growing until eight weeks for seed collection. Seeds that were collected after harvest were marked as T3 generation. T3 seed of 13 independent lines of *SAG21* ORF H2B and ORF H2C were selected further on kanamycin plates by sowing 100 seed individually on to the plates to check the homozygosity of the independent lines. For each independent line homozygotes which showed a hundred percent survival on kanamycin were obtained which were then used for study in the experiments (Table 2.2.).

5.2.2 Growth of over-expressors of SAG21 under optimal conditions

SAG21 over-expressor lines driven by its own promoter, SAG21p::SAG21ORF-H2B, SAG21p::SAG21ORF-H2C, over-expressor lines of *SAG21* from the 35S promoter, 35S:SAG21ORF (35s2-5, OEX-3,OEX-1a) along with WT were used for studying root architecture under optimal conditions. Before sowing seeds onto the media the seeds were sterilized according to the protocol described in Section 2.13. After sterilization, the seeds were then sown on ½ MS media prepared with 1 % sucrose poured onto 120 mm square Petri dishes. Ten to twelve seeds of each genotype were sown 1-2 cm apart in a row on the top of the Petri dish with the help of a pipette tip. Plates were then dried in a laminar hood

for a few minutes and sealed with micropore tape and moved into a fridge at 4 °C for stratification for 24 hr. Petri dishes were then moved to a growth chamber (Percival growth chamber) for growing vertically under 16h light and 8h dark long-day conditions for 13-14 days. After 14 days of growth, the Petri dishes were then scanned with an EPSON PERFECTION V500 PHOTO scanner and primary root length, number, and length of laterals were measured using Image J software.

5.2.3 Growth of over-expressors of SAG21 under oxidative stress conditions

Over expressor lines SAG21p::SAG21ORF-H2B, SAG21p::SAG21ORF-H2C, 35S:SAG21ORF (35s2-5, OEX-3, OEX-1a) and WT were used to study the effect of different concentrations of H_2O_2 on primary root length and lateral roots. Seeds were sterilized following the method described in Section 2.12 and 5-6 seeds were sown in a row 1-2 cm apart on a 120 mm Petri dishes with ½ MS media supplemented with 1 % sucrose. Petri dishes were then sealed with micropore tape and moved to a fridge at 4 °C for stratification for 24hr. After 24 hr the Petri dishes were then moved to Percival growth chambers and grown vertically under long-day conditions of 16 hr light and 8hr dark for 6 days. After 6 days of growth the seedlings were then transferred aseptically in the laminar hood with the help of a spatula to Petri dishes prepared with different concentrations of H_2O_2 (1mM, 2mM, 3mM) and were then sealed with micropore tape and replaced back into the growth chamber and grown vertically for another 5 days on H_2O_2. After 5 days of growth on H_2O_2, the Petri dishes were then scanned with an EPSON PERFECTION V500 PHOTO scanner and length of primary root, lateral root length and number were measured using Image J software.

5.3 Results

Seeds of SAG21p::SAG21-H2B, and SAG21p::SAG21-H2C were selfed and seeds of 13 lines of the T3 generation were then screened individually on kanamycin selection plates to obtain homozygous lines which showed 100% survival of seedlings on the selection plates. For each over expressor line at least two to three homozygous lines were obtained which were used for the study (Figure 5.2).

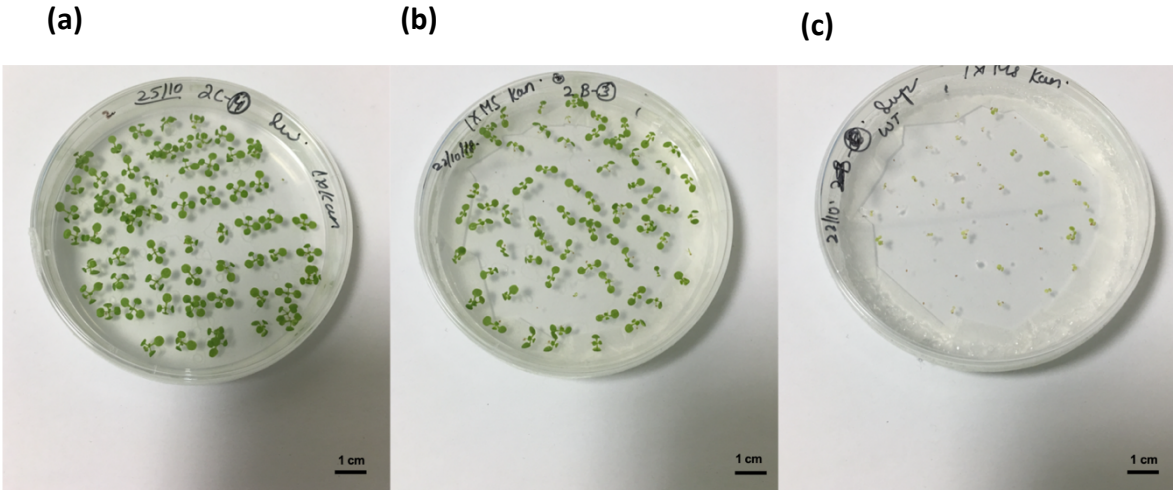


Figure 5.2 : Selection of T2 seeds with antibiotic Kanamycin **(a)(b)** Homozygous transgenic lines obtained from *SAG21* over expressors (SAG21p::SAG21-H2B, and SAG21p::SAG21-H2C) showing 100% survival on kanamycin selection plates. **(c)** WT used as a negative control. Scale bar=1cm.

SAG21 when expressed under the control of *CamV35S* promoter, produced longer primary roots than WT and the total number of lateral roots was also increased (Salleh, 2011), so it was interesting to understand the effect of over-expression of *SAG21* under its own promoter when compared to the 35S promoter. *SAG21* over-expressor lines driven by its own promoter, (SAG21p::SAG21-H2B, SAG21p::SAG21-H2C), three over-expressor lines of *SAG21* driven by the 35S promoter, 35S:SAG21 (35s2-5, oex3, oex1a) along with three WT lines were grown vertically on agar for studying root architecture under optimal (Figure 5.3) and oxidative stress conditions.

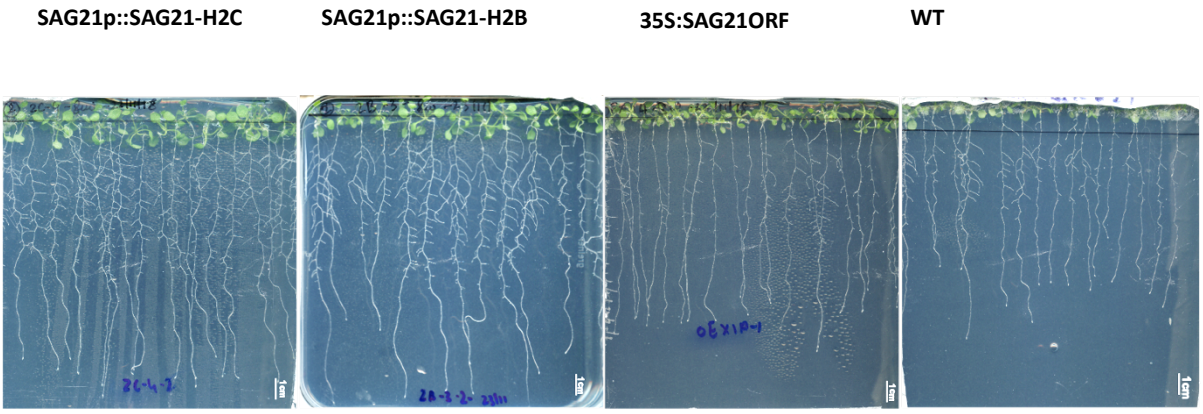


Figure 5.3 :Root architecture of Transgenic SAG21 over-expressor lines under its own promoter compared to CaMV35S promoter and WT when grown vertically on MS agar plates for 14 days under optimal conditions. scale bar=1cm

5.3.1 Effect of SAG21 over-expression under its own or the 35S promoter on primary roots, lateral roots and root hairs when grown under optimal conditions

When root architecture was measured on 14 day old seedlings under optimal conditions, it was found that over-expressor lines of *SAG21* under its own promoter showed a similar average primary root length when compared with expression of *SAG21* under the 35S promoter. There was no significant difference between primary root length of SAG21p::SAG21ORF-H2B, SAG21p::SAG21ORF-H2C, 35S::SAG21(35s2-5-line 1) or 35S::SAG21 (oex-1a- line 3). However, 35S::SAG21 (oex-3- line 2) primary roots were significantly shorter than SAG21p::SAG21ORF-H2B. All *SAG21* over-expressors were significantly longer ($P < 0.05$) in average primary root length when compared with the three WT lines (Figure 5.4).

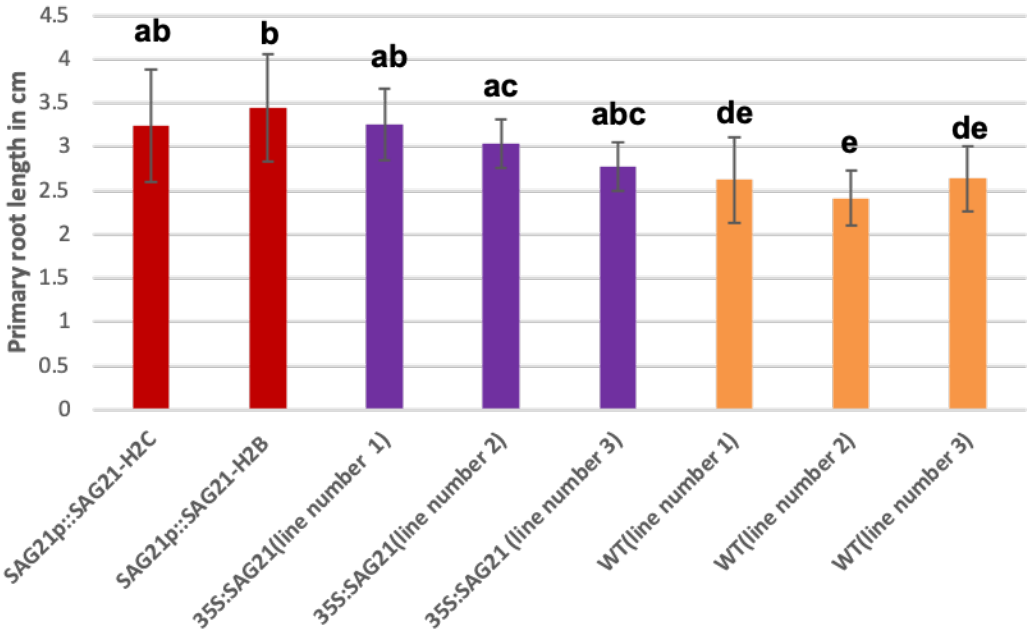


Figure 5.4 : Mean primary root length measurement of transgenic SAG21 over-expressor lines SAG21p::SAG21-H2B, SAG21p::SAG21-H2C (SAG21 driven by its own promoter) compared to three 35S::SAG21 lines after 14 days of growth on agar under optimal conditions. (n=35; error bars ± S.E.; Different letters indicate significantly different values, two-way ANOVA,, $P < 0.05$).

The mean number of lateral roots of both *SAG21* transgenic lines SAG21p::SAG21-H2B and SAG21p::SAG2-H2C was greater than WT, while there was no significant difference in the number of lateral roots between the 35S:SAG21 lines and WT (Figure 5.5).Indeed, SAG21p::SAG21-H2C and SAG21p::SAG21-H2B produced more lateral roots compared to both 35S:SAG21 and wild type (two way ANOVA; $P < 0.05$).

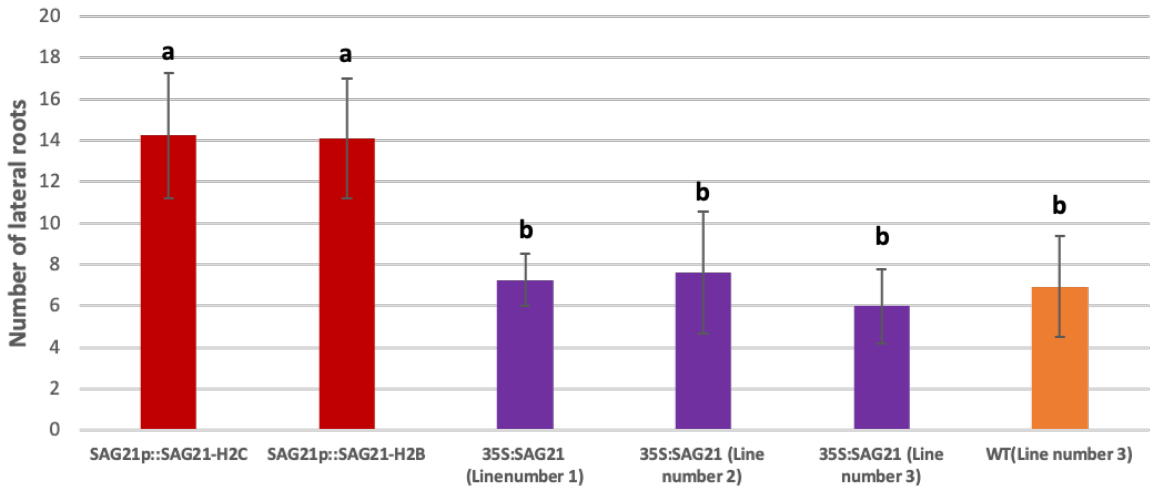


Figure 5.5: Average number of lateral roots measurement on SAG21 over-expressor lines under its own promoter when compared to 35S promoter and WT after 14 days of growth under optimal conditions; (n=20; error bars ± S.E.; different letters indicate significantly different values, two-way ANOVA, $P < 0.05$).

SAG21p::SAG21-H2B, and SAG21p::SAG21-H2C lines showed greater average lateral root length when compared to 35S:SAG21ORF lines and wild type under optimal conditions (two way ANOVA, $P < 0.05$, Figure 5.6).

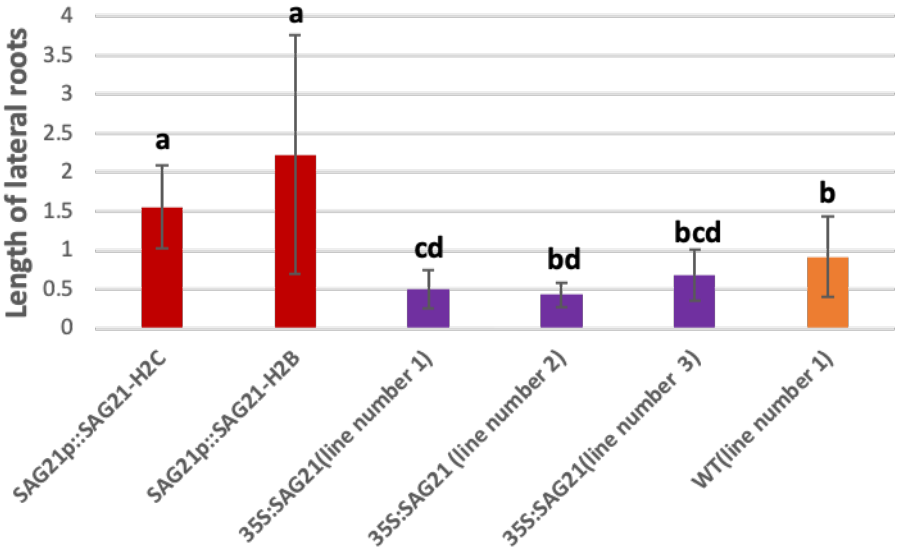


Figure 5.6 : Mean length of lateral roots of SAG21 over-expressor lines and WT after 14 days of growth under optimal conditions. (n=20; error bars ± S.E.; different letters indicate significantly different values, $P < 0.05$ based on two-way ANOVA).

Mean root hair length of 35S::SAG21 (line 1) seedlings was significantly greater than that of WT and SAG21p::SAG21-H2C seedlings. However, there was no consistent difference in root hair length between the over-expressors of *SAG21* driven by the *SAG21* or the 35S promoters. (Figure 5.7 a). The distribution of root hair lengths across the different lines was

also not consistently different between SAG21 over-expressors and WT or between the two different types of *SAG21* over-expression. (Figure 5.7 b).

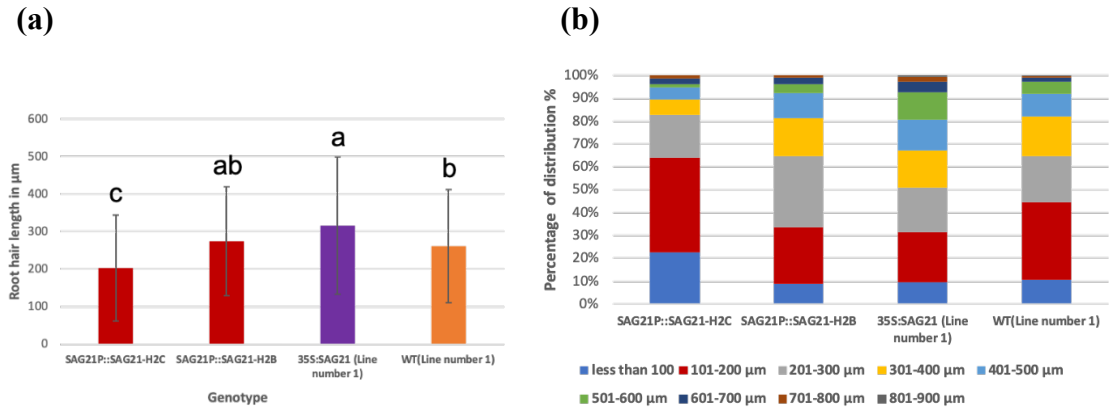


Figure 5.7 :Root hair length of SAG21 over-expressor lines and WT after 8 days of growth under optimal conditions; **(a)** mean root hair length (µm); **(b)** distributions of root hair lengths (expressed as percentage of whole population);(n>350 error bars ± S.E.; different letters in (a) indicate significantly different values based on a one-way ANOVA test)

5.3.2 Effect of over expression of *SAG21* under its own promoter or the 35S promoter on primary root length, lateral roots under oxidative stress conditions

Over expressor lines SAG21p::SAG21-H2C, SAG21p::SAG21-H2B, where *SAG21* expression was driven from its own promoter, a 35S:SAG21 (line 35s2-5; line 1) where *SAG21* expression was driven from a constitutive promoter and WT were used to test the effect of different concentrations of H_2O_2 on root growth. When grown on 1 mM H_2O_2 there was a significant increase in average primary root length compared to the WT, but no significant difference amongst the different over-expressor lines (Figure 5.8). When grown on 2 mM H_2O_2, again over-expressors produced longer primary roots compared to wild type. In addition, SAG21p::SAG21-H2B produced a significantly longer primary root length compared to the 35S::SAG21 line. On the 2 mM H_2O_2 concentration, SAG21p::SAG21ORF-H2B showed an increase in primary root length when compared with growth on 1 mM H_2O_2, however, primary roots of the SAG21p::SAG21-H2C line grew to the same length. When grown on 3 mM H_2O_2, primary root length was greater in all over-expressors compared to wild type but there were no consistent significant differences between lines in which *SAG21* expression was driven from its own or from the 35S promoter (Figure 5.8).

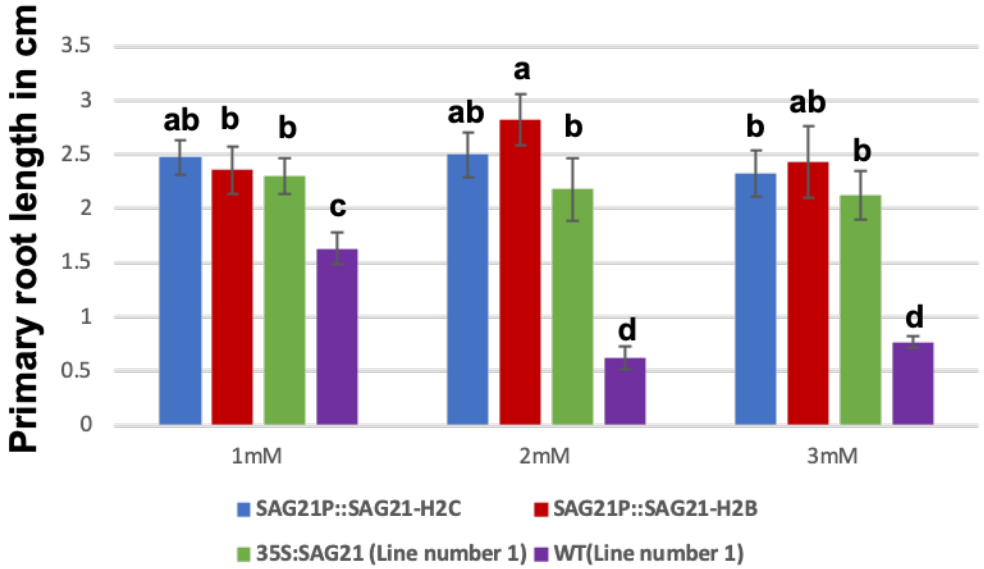


Figure 5.8 : Mean primary root length of SAG21 over-expressors and WT Seedlings were grown for 5 days vertically on 1X MS media and then transferred to plates containing H_2O_2 and grown for a further 5 days under oxidative stress conditions. (n=6; error bars ± S.E.; different letters indicate significantly different values, $P < 0.05$ based on a two-way ANOVA).

Mean number of lateral roots produced by the SAG21 over-expressors and WT under different concentrations of H_2O_2 were also measured. When grown on 1 mM H_2O_2, over expressor lines SAG21p::SAG21-H2C, SAG21p::SAG21-H2B seedlings produced more lateral roots when compared to WT. In comparison, the number of lateral roots produced by 35S:SAG21 seedlings was not significantly different to wild type or to seedlings where SAG21 was over-expressed under its own promoter. When the lines were grown on 2 mM or 3 mM H_2O_2 differences amongst lines were not statistically significant (Figure 5.9). Over expressor lines SAG21p::SAG21ORF-H2B, SAG21p::SAG21ORF-H2C, 35S:SAG21ORF and WT all displayed a decrease in number of lateral roots when grown on 3 mM H_2O_2 compared with the 1mM concentration. At 2 mM H_2O_2 the number of lateral roots produced by all the over-expressor lines was reduced compared with growth on 1 mM H_2O_2 whereas the number of lateral roots produced by WT at 1 mM or 2 mM H_2O_2 was not significantly different.

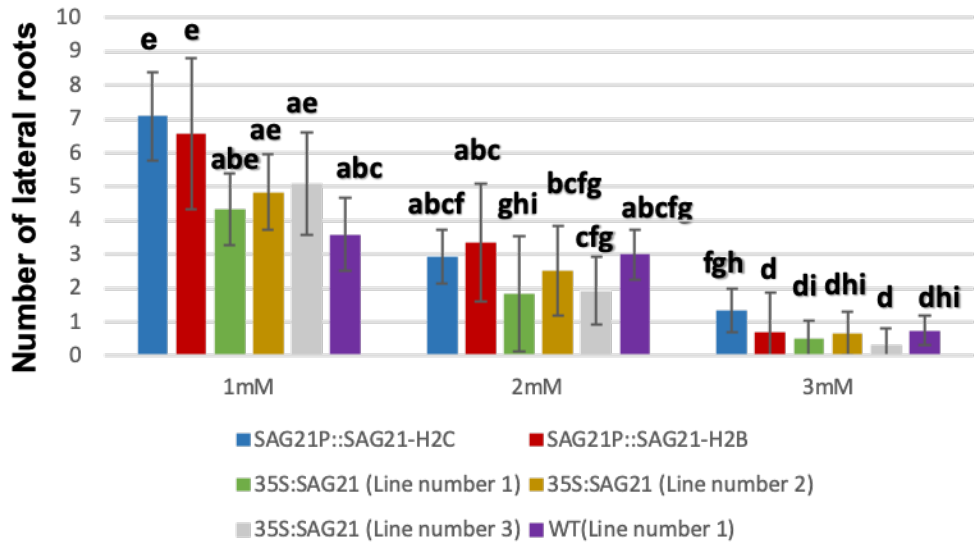


Figure 5.9 : Mean number of lateral roots produced by SAG21 over-expressor lines and WT. Five day old seedlings were transferred to H_2O_2 plates for growing for a further 5 days under oxidative stress conditions. (n=6; error bars ± S.E.; different letters indicate significantly different values $P < 0.05$, based on a two-way ANOVA test).

SAG21p::SAG21ORF-H2C grown on 1mM H_2O_2 produced a mean length of lateral roots which was significantly higher than all the other lines (Figure. 5.10). However this increase in lateral root length was not consistent between the two lines in which SAG21 was expressed from its own promoter. There were no consistent significant differences in the mean length of lateral roots between the 35S::SAG21 lines and WT. When grown at 2 mM H_2O_2 mean lateral root length was dramatically reduced when compared to the 1 mM concentration of H_2O_2 in all the lines. However, there was no statistically significant difference in mean lateral root length when seedlings were grown at either 2 mM or 3 mM H_2O_2 (Figure 5.10).

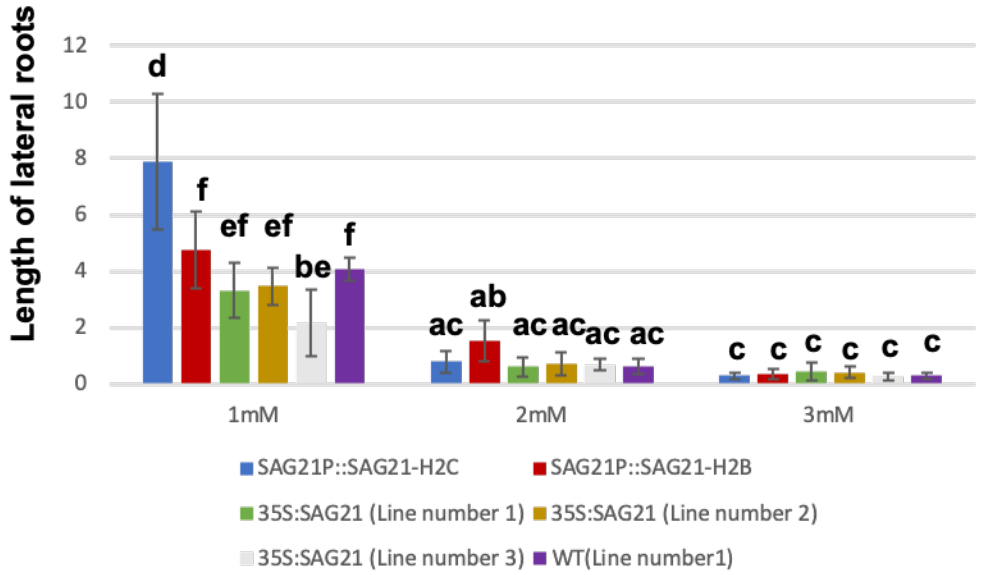


Figure 5.10 : Mean length of lateral roots of SAG21 over-expressor lines and WT after 5 days of growth under oxidative stress conditions. (n=6; error bars ± S.E.; different letters indicate significant difference $P < 0.05$, based on a two-way ANOVA).

5.4 Discussion

Overexpression of *SAG21* under the control of the CaMV35S constitutive promoter resulted in seedlings that produced longer primary roots, and the number of lateral roots and primordia were also reported to be significantly increased when compared to wild type (Salleh, 2011). Based on those findings the data presented in this work investigated the effect of overexpression of *SAG21* under the control of its own promoter compared with overexpression of *SAG21* under the control of the 35S promoter under optimal and oxidative stress conditions.

The findings reported in this chapter clearly demonstrate that the overexpression of *SAG21* has a considerable effect on the root architecture under optimal conditions. Seedlings of *SAG21* over-expressor lines where *SAG21* was expressed under its own promoter, SAG21p::SAG21-H2C, SAG21p::SAG21-H2B produced an increased primary root length when compared to wild type although the difference to 35S:SAG21 lines was not significant. These findings were in agreement with previous findings reported by Salleh (2011) for the 35S:SAG21ORF over-expressors.

In previous findings it was reported that 35S:SAG21ORF seedlings where *SAG21* was over-expressed from a constitutive promoter produced a significant increase in number of lateral roots and lateral root primordia compared with wild type (Salleh, 2011). However, here there was no significant difference in number of lateral roots between 35S:SAG21ORF and wild type seedlings. In contrast, mean number of lateral roots in seedlings where *SAG21* was over-expressed from its own promoter were significantly greater when compared with both 35S:SAG21ORF and WT seedlings.

Length of lateral roots measured in this experiment was significantly greater when *SAG21* was over-expressed from its own promoter compared with *SAG21* over-expressors driven from the 35S promoter, and wild type. Mowla et al (2006) showed that total lateral root length in 35S:SAG21ORF lines was greater than WT, however mean lateral root length was not reported.

In this study, 35S:SAG21ORF seedlings produced longer root hairs when compared to WT which supports the previous findings reported by Salleh et al. (2012). However, *SAG21* over-expressed from its own promoter there was no consistent increase in root hair length compared to WT.

When root architecture is studied on the plates containing MS medium factors such as concentration of the growth medium, temperature, light and aeration might affect the root growth (Dubrovsky & Fordeb, 2012). Full strength MS contains more nitrogen supply which inhibits root growth when compared to ½ MS (Dubrovsky et al., 2009; Dubrovsky & Fordeb, 2012). Another issue might be the contact of surface of roots with sucrose which affects the lateral root formation (Dubrovsky & Fordeb, 2012; MacGregor et al., 2008). Therefore comparisons to other studies may be affected by these factors and may explain different results obtained. However, in this study all the above factors were kept constant while measuring the phenotypes of over-expressor lines. Therefore, comparisons between lines presented here provide interesting insights into the effect of over-expression of *SAG21* constitutively or driven by its own promoter.

Root growth is regulated by the balance between cell proliferation and expansion at the root tip and different ROS moieties maintain this balance and function as signalling molecules along with hormones (Tsukagoshi, 2016). Dunand et al. (2007) showed that H_2O_2 accumulates in the elongation zone of the root whereas the superoxide accumulates in the meristematic zone and promotes root hair growth. The disruption of the balance among these ROS moieties affects the size of the root meristem. *UP BEAT1* (*UPB1*, a bHLH

transcription factor) overexpressing plants showed an increase in the level of the H_2O_2 in the elongation zone and a decrease in superoxide levels and also showed longer meristems (Tsukagoshi et al.,2010). ROS accumulates in root cells even under normal growth conditions (Dunand et al.,2007). Over-expressors of *SAG21* under its own promoter or under the 35S promoter produced longer primary roots under optimal conditions. In the dark, *SAG21* shows expression in the elongation and differentiation zone (Salleh et al., 2012) and the 35S promoter drives expression in most root tissues. Although SAG21 over-expression does not appear to affect overall redox state (Mowla et al., 2006) it might be possible that it affects local ROS levels that in turn affect root development.

In these experiments, expression of *SAG21* from its own promoter had a greater effect in increasing lateral root number and length compared to expression driven by the 35S promoter. This may be either quantitative: because levels of overexpression when *SAG21* over-expression is driven by its own promoter are lower than over-expression from the 35S promoter and at a more optimal level for stimulating root growth. Alternatively, the timing or spatial expression of the *SAG21* may be affecting ROS signalling more specifically to induce root growth. The increase in the number of lateral roots suggests an activation of cell division to create more lateral meristems, which is known to be mediated by auxin and by ROS (Orman-Ligeza et al., 2016). However cell expansion is also affected by ROS for example in root hair growth (Tsukagoshi, 2016). It would be interesting to study cell division and cell elongation, and ROS homeostasis and distribution in *SAG21* over-expressors to determine where the cells in the root tip transition from zone of proliferation to elongation and differentiation. This might provide a better understanding of the involvement of ROS regulation in primary root growth.

An explanation for the increase in root hair length in over-expressors of *SAG21* under the 35S promoter might involve the regulation of ROS responses. ROS participates in the regulation of root hair development (Tsukagoshi, 2016) and is interconnected with calcium and pH in the cell expansion process (Mangano et al., 2016; Singh et al., 2016). The cell takes up Ca^{+2} either from external sources or it may be stored in the cell wall and these calcium levels are controlled and released by the changes in apoplast pH which is regulated mainly by the plasma membrane located activation/deactivation of H^+ pumps, AHA. Low concentration of cytoplasmic Ca^{+2} is maintained by the activity of ACAs which transport back Ca^{2+} to the apoplast and CAX which transports Ca^{2+} to the apoplast and also imports H^+ into the cytoplasm. In the root hair tip increased levels of $_{cyt}Ca^{2+}$ activate the generation of apoplastic ROS by NADPH oxidases (NOXs). NOX proteins are also regulated by the

Ca^{2+} ions, ROP proteins and kinases. In the root hairs ROP generates the production of reactive oxygen species (Mangano et al., 2016). Another mechanism in root cell elongation or hair growth involves auxin, which triggers the NOXS proteins and increases the auxin responsive transcription factors and RSL4 transcription factors. Auxin responsive transcription factors activate RSL4 TFs which target the genes involved in ROS production like RBOH or class III peroxidases, affecting the apoplastic ROS balance leading to cell wall loosening and thickening and thus promoting root hair growth (Mangano et al., 2017). Over-expression of SAG21 may interact with this ROS-auxin signalling to promote hair growth. The lack of effect on root hair growth seen when *SAG21* is over-expressed from its own promoter may again be quantitative: maybe insufficient extra SAG21 is produced to affect root hair growth.

In over-expressors of SAG21 driven by its own promoter, upstream regulators may also play a role. Mutants of *MPK6*, a downstream regulator of MEKK1, produced an increased primary root length, more lateral roots and increased root hair length (López-Bucio et al., 2014) resembling the phenotype seen in *SAG21* over-expressors. MAPK signal transduction modules consist of three different kinases which function in stress and developmental responses (López-Bucio et al., 2014). Although the *SAG21* expression is independent of *OXI1* (Mowla et al., 2006) it might be controlled through the MEKK1-MKK1/2-MPK4/6 cascade which functions in oxidative burst mediated signalling (Pitzschke et al.,2009). Although *MPK6* in this pathway functions as a negative regulator of root development, the phenotypes produced might be due to cell elongation and division processes mediated by ROS which are not yet known. In addition, not much is known about the signalling components operating downstream of this pathway that affect root architecture and development, so it might be that *SAG21* might operate downstream in these pathway by controlling the cell expansion mediated by ROS.

In order to understand whether *SAG21* over-expressor lines under the transcriptional control of the CaMV35S promoter and also under the control of its own promoter were able to provide protection against the oxidative stress, an experiment was carried out to study the effect of different concentrations of H_2O_2 on root growth and development. In this study, with low oxidative stress, *SAG21* over-expression from its own promoter resulted in a similar primary root length when compared to a 35S over-expressor line, and both had a longer primary root length than WT. This supports previous findings reported by Mowla et al. (2006) with respect to 35S:SAG21ORF lines. At 2 mM and 3 mM concentrations of H_2O_2 the primary root length was still increased in over-expressors which is in agreement

with previous findings reported by Mowla et al. (2006), although the difference seen in the experiments reported here was much greater. In the case of Mowla et al. (2006) the seedlings were grown on H_2O_2 for 7 days whereas, here the seedlings were grown on H_2O_2 for 5 days and measurements were then recorded. The difference in timing may explain the difference in the primary root length with respect to higher concentrations.

SAG21 over-expression under its own promoter produced an increase in number of lateral roots with 1 mM H_2O_2 compared to WT, whereas over-expression of *SAG21* from the 35S promoter did not. The number of lateral roots under H_2O_2 stress was not reported by Mowla et al. (2006). Differences in phenotype resulting from over-expression of *SAG21* from the 35S promoter compared to its own promoter may be due to the up-regulation of the SAG21 promoter by ROS previously reported (Mowla et al., 2006; Salleh et al., 2012). However, with higher concentrations of H_2O_2 lateral root number was essentially the same in all the lines tested. Length of lateral roots was not consistently different in over-expressor lines compared to WT at any concentration of H_2O_2.

ROS functions upstream of the MAPK cascade during plant development and stress responses, and *mpk6* mutant plants produced more longer lateral roots after treatment with H_2O_2 (Wang et al., 2010). The lack of effect of increasing *SAG21* expression on lateral root length suggests that may be *SAG21* is not interacting with this redox signalling pathway in promoting root length, however the lines expressing SAG21 driven by its own promoter did have longer primary and more lateral roots that WT when grown on 1 mM H_2O_2. Han et al. (2015) provided evidence that loss of function mutant *atmpk6* seedlings showed an increase in root cell elongation when treated with H_2O_2 and ABA. Addition of calcium inophore to roots containing H_2O_2 showed enhanced root cell elongation and growth which supports that Ca^{+2} is required for the H_2O_2 induced elongation of root cells in *atmpk6* (Han et al.,2015).Expression of ROS generating enzymes like RBOH and oxidases when measured in roots showed an increase in transcription and peroxidase activity (Han et al., 2015). As *SAG21* is a ROS -inducible gene, It might be interesting to study the role of calcium on over-expressors of *SAG21* to know whether the increase in primary root length with oxidative stress is due to the H_2O_2 induction of SAG21 expression and to understand the interconnection of ROS and calcium in root cell elongation of *SAG21*.

A microarray array time course analysis on Arabidopsis root tips after treatment with H_2O_2 identified a *MYB30* as a transcription factor which showed a strong response to the ROS treatment and functions downstream of ROS signalling. It controls root development and also functions as a key regulator of the gene network which leads to the H_2O_2 dependent

inhibition of root cell elongation (Mabuchi et al., 2018). Transcriptome analysis of the *SAG21* over-expressor roots after exposure or treatment with H_2O_2 may provide an insights into the transcription factors involved in regulation of *SAG21* expression in roots and which might function as possible regulators of ROS signalling in roots.

Chapter 6 General Discussion and future work

The work presented in this thesis investigated the transcription factor networks regulating the expression of *SAG21* in response to development, senescence, and stress responses. One component of the research focused on understanding the transcriptional regulatory network involved in regulating *SAG21* expression (Chapter 3). Another aspect of my research was focused on understanding the function of transcriptional regulatory *cis*-elements in relation to development, senescence and stress response. Also studied was the involvement or role of the plant hormone kinetin in regulation of the senescence and wounding response in the promoter-reporter deletion constructs of *SAG21* (Chapter 4). The last part of the work investigated if the over-expression of *SAG21* under its own promoter had different effects when compared to over-expression of *SAG21* driven by the 35S promoter on development and stress response, mainly oxidative stress, as *SAG21* is a ROS induced gene (Chapter 5). This final chapter describes the main conclusions from different chapters of this thesis pointing out the directions for future work.

6.1 Transcription factor regulation of expression of *SAG21*

Before the start of the work described in this thesis, the yeast one-hybrid (Y1H) technique was applied to identify transcription factors and *cis*-elements involved in the regulation of the *SAG21* promoter. The Y1H technique is a variant of the yeast two-hybrid technique and is used generally for the detection of DNA-protein interactions (Sun et al., 2017). Yeast one-hybrid is based on the detection of the interaction of the transcription factor (prey) with a bait DNA sequence upstream of the reporter gene (Ouwerkerk et al., 2001). Transcription factors are linked to an activation domain that induces the reporter gene expression when the transcription factor DNA binding domain recognizes and interacts with a *cis*-acting element (Brent & Ptashne, 1985).

Seven overlapping promoter fragments of *SAG21* were screened against a library of 75 WRKY (Hickman et al., 2013) and 96 NAC TFs. Using this approach 13 WRKY TFs which play a role in different stress-related signalling, and four NAC TFs were found to be interacting with the promoter region (Rogers lab, unpublished). Among them, *WRKY15*, *63*, *67* and *NACO42* were chosen here as they are found to be responsive to stress (Ren et al., 2010; Vanderauwera et al., 2012; Wu et al., 2012). The Y1H analysis, showed a strong

interaction of WRKY15 and it required two W-boxes for its binding, whereas WRKY63 and WRKY67 showed strong interaction with the first W-box (Rogers lab, unpublished), closest to the ATG. Real-time PCR was used here to understand the role of these transcription factors in regulating *SAG21* expression. The expression of *SAG21* was determined in T-DNA mutants and an artificial micro RNA line that knockout or knockdown expression of the individual transcription factors. Expression of *SAG21* was assessed with and without abiotic stress treatment.

Real-time PCR analysis indicated that *WRKY15* was not required for *SAG21* expression, under optimal conditions or following oxidative stress treatment since expression in a *wrky15* amiRNA line and WT remained comparable. With drought treatment (ambient dehydration-stress) *SAG21* expression was upregulated in the *WRKY63* mutant and wild type but the effect was not significant, as the *SAG21* expression was highly variable in mutant seedlings under both conditions. Further experiments may help to establish whether the stress was sufficient to fully induce the *SAG21* in WT. However, under non-stressed conditions *WRKY63* appears to function as a negative regulator of *SAG21*. *WRKY67* appeared to function as a positive or negative regulator depending on the stress status but the method of salt treatment used in this study indicates *WRKY67* as a negative regulator under salt stress as there was upregulation of *SAG21* expression in mutant seedlings under long term salt stress treatments.

NAC042 was found to be not needed for the *SAG21* expression under oxidative stress or non-stress conditions since there was a lack of change in *SAG21* expression, although there was an upregulation in wild-type and mutant seedlings from the oxidative stress as expected.

Bioinformatic analysis of 1700 bp of the *SAG21* promoter identified four W-Boxes at positions 316, 360, 1023 and 1623 bp upstream from the ATG (Salleh, 2011), and this information was used to design the fragments for the Y1H study. However, I decided to re-assess the *cis*-elements in the *SAG21* promoter by bioinformatic analysis using Plant Pan software to identify the binding positions of transcription factors. This showed that different WRKY TFs might bind to the same position or *cis*-element present on the *SAG21* promoter region. Indeed the Plant Pan software identified 107 potential W-box elements, however, very few showed a high score (Figure 3.1, Chapter 3). Using this analysis, WRKY15 and 67 were predicted to interact with the *SAG21* promoter at 200-400 bp upstream from the ATG and also at positions 1000-1200 bp upstream from the ATG. At the 1600 bp position, it was predicted that all the three WRKYs 15, 63, 67 bind at the same position. Regulatory

proteins function not only independently and often they physically interact either transiently or permanently with each other (Chi et al., 2013). For example, group IIa WRKY proteins, AtWRKY18, AtWRKY40, and AtWRKY60 interact among themselves and also with each other forming homo or heterodimers with the help of the leucine zipper motifs situated at the N termini of this subclass of the WRKY proteins (Chi et al., 2013; Xu et al., 2006). Group IIb WRKY proteins in Arabidopsis AtWRKY42 and AtWRKY6 also interact with each other (Chen et al., 2009) W-boxes in the promoter sequences are sometimes clustered and organized in closely spaced direct or inverted repeats (Chi et al., 2013; Maleck et al., 2000). Based on the orientation and number of the intermediate nucleotides these W boxes function as target sequences for WRKY protein complexes. WRKY63 and 67 also belong to the same WRKY subfamily (IIIa) and are phylogenetically similar (Kalde et al., 2003). Because of their similarity in their protein structure and binding motif, these might function as heterodimers eliciting a regulatory response in *SAG21*, as it was found that most of the heterodimers in the WRKY family are formed between phylogenetically similar WRKYs (Chen et al., 2009). The W boxes at 200-400 bp on the *SAG21* promoter are very closely spaced with each other (32 bp) making it a possibility for WRKY15 and 67 to function as a heterodimer complex through the formation of a DNA loop and be involved in the transcriptional regulation of *SAG21* (Chi et al., 2013). So although the real time PCR did not provide definitive evidence for WRKY15 as a regulator for *SAG21*, it is still possible that it does have a role as part of a complex. More studies on understanding the protein-protein interactions using the yeast 2 hybrid technique would be required. It would also be interesting in the future to understand the role of the other WRKY TFs identified in regulating *SAG21* by Y1H.

Y1H is a useful technique to screen promoter fragments against libraries of TFs, however it has disadvantages. The major disadvantages of this technique are the identification of false positives which are usually caused by the recognition of the bait sequence by the endogenous yeast TFs and may activate a reporter gene in the absence of the prey (Sun et al., 2017). As the technique is done in an artificial environment it may also not precisely represent the interactions which take place *in vivo* and sometimes the strength of the TF binding might be misinterpreted because of incorrect processing by the yeast translational machinery (Hickman et al., 2013). So the other methods should be employed to confirm protein-DNA interactions like ChIP sequencing, and promoter cross-linking studies. Chromatin immunoprecipitation is a technique used for the characterization of protein and DNA interactions (Yamaguchi et al., 2014). In this technique cross linking of protein-DNA

interactions is done using formaldehyde, and tissue is then homogenized and cells are lysed. The chromatin is sheared by sonication and incubated with magnetic beads coupled to an antibody specific for the target protein that is used to precipitate the DNA -protein complex (Park, 2009). The cross-links are then reversed and the released DNA is evaluated to determine sequences bound by the protein (Park, 2009). However, the success of this technique is based on the antibody used (Schmidt et al., 2014). Other methods such as EMSA (electrophoretic mobility shift assay) also identifies the DNA-protein interactions where the purified protein is incubated with labelled DNA. The limitation of this method is that some proteins may bind weakly to DNA and also the conditions need to be perfect for the optimal binding (Murarka & Srivastava, 2018). Apart from the above-mentioned techniques, a very recent technique developed by Murarka et al. (2018) involves the cross-linking of the proteins to the promoter DNA with formaldehyde followed by the sonication of the cells. The promoter DNA is then digested with exonuclease generating overhangs which bind to a biotinylated primer attached to streptavidin beads and the bound proteins are eluted and separated on an SDS page gel and protein bands are gel digested and analyzed by ToF MS/MS. Studies on the promoter region of *SAG21* applying these kind of techniques may provide more experimental evidence of *SAG21* promoter and TF interactions.

6.2 Function of *SAG21* during plant development and stress responses

Overexpression of *SAG21* under its own promoter showed an interesting effect on root architecture under optimal conditions. There was an increase in primary root length, mean number of the lateral roots, and length of the lateral roots when compared to WT and in most cases effects were stronger than the *SAG21* expressed under a constitutive promoter. However, when the length of root hairs were compared, SAG21P::SAG21 ORF produced shorter root hairs than WT and 35S:SAG21 ORF seedlings.

Root growth is controlled by the balance between cell proliferation and expansion and ROS maintain this balance and act as signalling molecules (Tsukagoshi, 2016). ROS moieties accumulate in different zones of roots and control this balance promoting root growth. As over-expressors of *SAG21* under the 35S promoter and also under its own promoter had longer primary roots it may be that *SAG21* affects the local ROS levels or the effects of the

ROS on the cellular machinery which in turn shows an effect on the regulation of root development. Lateral root development is also controlled by the hormone auxin, and ROS are also thought to function as important signals during auxin-regulated lateral root formation although the mechanism behind it is not fully understood (Orman-Ligeza et al., 2016). The increase in lateral root number when *SAG21* over-expression was driven by its own promoter compared to the 35S promoter suggests an activation of the cell division process for creating more lateral meristems known to be mediated by ROS and auxin (Orman-Ligeza et al., 2016). It might be interesting to understand the process of cellular division and expansion along with ROS homeostasis and distribution in *SAG21* over expressors to determine the transition of cells in the root tip.

A reduction in root hair length in over expressors under *the SAG21* promoter indicates again a link to the involvement of ROS (Tsukagoshi, 2016). This process of regulation of root hair growth by ROS is interconnected with Ca^{+2} and pH. Increased levels of calcium in root hair tips activates the production of apoplastic ROS by NADPH oxidases (NOXs)(Mangano et al., 2017) which in turn causes an increase in auxin-responsive transcription factors which targets the genes involved in ROS production leading to cell wall loosening and thickening causing the root hair growth. So the overexpression of *SAG21* might interact with this auxin-ROS signalling pathway for promoting root hair growth. MAPK signal transduction cascades are known to function in stress and developmental responses (López-Bucio et al., 2014). Even though *SAG21* expression is independent of *OXI1* (Mowla et al., 2006) it might be regulated through the MEKK1-MKK1/2-MPK4/6 which plays a role in the oxidative burst mediated signalling process (Pitzschke & Hirt, 2009). Mutants of *MPK6*, a downstream regulator of MEKK1, also produced similar root phenotypes to *SAG21* over-expressors (López-Bucio et al., 2014). So, Understanding of how *SAG21* is regulated in *mpk6* mutants may help to assess whether MPK6 contributes to the *SAG21* over-expressor phenotype.

Under oxidative stress conditions with different concentrations of H_2O_2, over-expressors of *SAG21* driven by its own promoter produced a similar root length to the 35S over expressors but showed a longer primary root length than wild type. Even with increased concentrations of H_2O_2 the primary root length was still found to be increased by *SAG21* over-expression. At low concentration of H_2O_2 over-expressors of *SAG21* driven under its own promoter also produced an increase in number of lateral roots compared to WT, although this was less

clear at higher H_2O_2 concentrations. The mean length of the lateral roots was not usually different in over expressor lines compared to WT with any of the concentrations of H_2O_2. ROS also functions upstream of the MAPK cascade and *mpk6* mutant plants also produced a similar phenotype of long lateral roots after treatment with H_2O_2 (Wang et al., 2010).

Arabidopsis roots treated with ROS were able to restore the lateral root formation in mutant lines *aux1 lax3* in which auxin-mediated cell wall remodelling in cells overlying the lateral root formation sites was disrupted (Orman-Ligeza et al., 2016). ROS were also found to be deposited in the apoplast of these cells overlying the developing lateral roots and GUS expression driven by *RBOH* gene promoters overlaps with H_2O_2 localization in the peripheral cells. So lateral root production could be promoted by the RBOH mediated ROS production by the cell wall remodelling of peripheral cells (Orman-Ligeza et al., 2016). Lateral root production in *SAG21* over-expressors might be controlled by auxin-ROS signalling mediated by the RBOH genes. In this study the effect of *SAG21* over-expression on root architecture has been studied under the control of its own promoter or the 35S constitutive promoter. Although the 35S promoter is a widely utilized promoter, it might be also interesting to see if the effect on root architecture is similar even when *SAG21* was expressed under other strong constitutive promoters like ubiquitin, actin, tubulin and EIF (eukaryotic initiation factor) whose promoters are highly active in all organs and tissues throughout the life cycle of a plant (Hernandez-Garcia & Finer, 2014).

It would be also interesting to understand the effect of overexpression of *SAG21* under its own promoter compared to the 35S promoter with other abiotic stresses like salt and drought, as *SAG21* GUS constructs showed expression in roots under these stresses (Salleh, 2011). Transcriptomic analysis using roots of over-expressor lines after the treatment with H_2O_2 would also be interesting to understand the transcription factors involved in *SAG21* expression regulation in roots. Measurement of the level of ROS in roots using imaging tools like the GFP based redox probe, roGFP fluorescent dyes, and luciferase also could provide more understanding of localization of ROS in root tissues in the *SAG21* over-expressor lines. Monitoring the cellular redox changes using the redox-sensitive yellow fluorescent protein (rxYFP), roGFPs and the YFP based probe Hyper have been used in many biological systems (Mittler et al., 2011).

Finally there remains the question of the function of the SAG21 protein. It would be interesting to identify the proteins interacting with the *SAG21* protein by the yeast two-

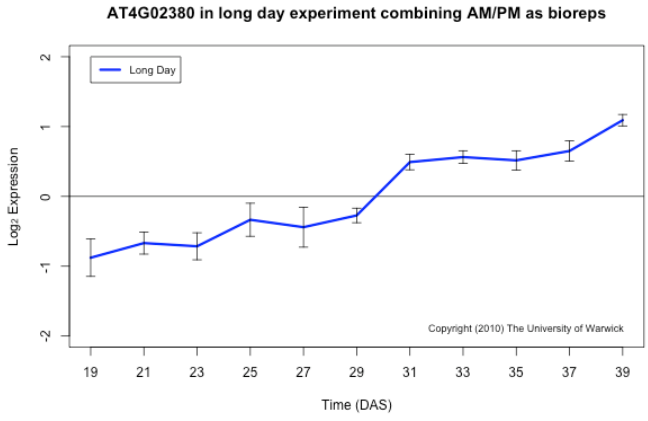
hybrid technique. This might help to understand any protein-protein interactions which may provide more insights into the understanding of the regulation and signalling pathways of *SAG21.* The Yeast 2 hybrid technique works on the principle where the protein of interest is fused to the DNA binding domain and the construct is named as bait. Interacting proteins are fused to the activation domain and called prey. the interaction of bait and prey makes a functional transcription factor leading to the transcription of a reporter gene (Brückner et al., 2009).

6.3 Functional Analysis of cis-regulatory elements of SAG21 in response to senescence, wounding and floral development

Cotyledon senescence is a process which leads to nutrient recycling and ultimately ends in cell death (Du et al., 2014). Phytohormones affect cotyledon senescence with cytokinin preventing chlorophyll breakdown and ethylene promoting senescence (Jing 2008; Du et al., 2014). The regulatory network underlying cotyledon senescence is not fully understood. In this study to understand *SAG21* expression in cotyledons and how kinetin affects its expression in them, cotyledons of two ages 14 and 24 days were selected for the study. At 14 days old, cotyledons of the promoter deletion constructs p*SAG21* (1700)::GUS-GFP, and p*SAG21* (737)::GUS-GFP displayed expression at the wound site and also across the cotyledons but the deletion construct p*SAG21* (489):: GUS-GFP showed intense expression at the wound site and also across the surface of the cotyledon. All this *SAG21* expression was abolished by treatment with kinetin. These data indicate that kinetin inhibits expression activated both by senescence and wounding. It was likely that the cotyledons might be just entering senescence at this age, but understanding of natural senescence parameters like measuring the chlorophyll content, and yellowing of the cotyledons would be important to study in the future to verify their senescence status. In the 24 day old cotyledons with all three constructs, senescence-related expression was inhibited by kinetin treatment but not the wound-related expression. In this work as the intermediate constructs were not studied it would also be useful to include them in the same experiments. The aim is to understand the promoter elements that might be affecting the senescence-related and wound-related expression of *SAG21* in cotyledons.

Promoter deletion constructs of *SAG21* showed an effect with rosette age and when the rosettes of different ages were wounded, 22 day old rosettes of the *pSAG21* (1700):: GUS-GFP construct showed a very weak expression in the wounded leaves but by 29 days the expression increased. Mature leaf senescence involves a series of events depending on the age of the plant. WT Arabidopsis plants show senescence phenotype by 39 days and leaf yellowing is visible from 31- 37 days (Breeze et al., 2011). The important change in gene expression takes place between 29 and 33 days after sowing (DAS). Many genes show downregulation as the senescence process progresses. Genes involved in chlorophyll biosynthesis show down regulation at 23 days and photosynthetic related genes also show downregulation from 29-33 days. Genes which are induced by oxidative stress, dehydration and ABA responsive genes are upregulated between 19-21 days. Genes involved in caspase activity are upregulated at 27 DAS. Caspases play an important role in programme cell death indicating an activation of PCD processes at this stage in senescence. At 29 DAS senescence genes involved in cell wall degradation are upregulated, as senescence involves cell wall degradation at this stage. As the leaf becomes more senescent, after 31 days, genes involved in nutrient mobilization and degradation, ethylene signalling and catalytic activity are upregulated (Breeze et al., 2011). The well-studied *SAG12* (senescence associated gene) gene shows an increased expression around 31 DAS which indicates the time where most or rapid changes occur in gene expression and shows a maximum expression between 33 and 35 DAS. Similarly, the expression profile of *SAG21* from the Presta arrays (Breeze et al., 2011; Figure. 6.1) shows that it is expressed at about 31 days onwards and the expression seem to increase until 39 days.

Figure: 6.1 expression profile of SAG21 (At4g02380) from the Presta array data (kindly provided by V. Buchanan Wollaston)



This expression pattern in mature leaves also agrees with expression pattern reported by Weaver et al (1998) in *SAG21* Arabidopsis leaves where they have noticed that the

expression peaks just before senescence and then declines thereafter. Thus, the increase in the response of *SAG21* to wounding in older rosettes may fit with the expression profile of *SAG21* in making *SAG21* more responsive to stresses such as wounding as leaves approach senescence. However, *pSAG21*(737):: GUS-GFP showed expression of *SAG21* only in 36-day old rosettes which indicates that there may be cis-elements between 737-1685 bp upstream of the ATG that influence the response of *SAG21* as related to leaf age.

Cotyledon senescence is not directly comparable to mature leaf senescence events but is likely to involve many similar molecular and developmental changes (Du et al., 2014). *SAG21* is expressed in the 14 day old cotyledons which again indicates an early senescence expression activation. However, age effects may differ in cotyledons because in the mature leaves cytokinin abolishes the wound response while in the older cotyledons it does not, which suggests different wound responses in the two tissues. When timing of cotyledons of 14 days used in this study are compared to mature leaf senescence timing it is likely that cotyledons have not reached senescence stage at 14 days, but the 24 days old cotyledons should be entering the senescence stage. Experiments on cotyledons throughout their development and senescence might be interesting to study in the future to fit with the timings of the leaf senescence or developmental age of the plant.

In this study the cytokinin treatment of the cotyledons was carried out in the light. It would also be interesting to study effects the dark induced senescence and dark treatment and whether we can get a similar kind of inhibition of expression of *SAG21* in younger and older cotyledons in the dark given that expression of *SAG21* is induced in the dark (Weaver et al., 1998; Mowla et al., 2006; Salleh et al., 2012). Cytokinin inhibits the expression of the SAGs along with the yellowing of the leaves and this inhibition is stronger in young leaves than older leaves which are about to senesce (Weaver et al., 1998). This may explain the effect of cytokinin in this study where the younger cotyledons showed a complete inhibition of the expression whereas in older cotyledons the cytokinin fails to inhibit the wound related expression.

Wound induced expression of *SAG21* is likely to be regulated by many signal transduction events which include ROS/Ca^{+2} signalling, MAPKs, activation by hormones like JA and also through the transcription factors like WRKYs and NACs as discussed in Chapter 4. Promoter deletion constructs *pSAG21* (1700):: GUS-GFP, *pSAG21* (1225):: GUS-GFP and *pSAG21* (489):: GUS-GFP showed a weaker expression of *SAG21* with wounding while constructs *pSAG21* (1439):: GUS-GFP and *pSAG21* (737):: GUS-GFP displayed stronger

expression of *SAG21* in response to wounding which suggests that *cis*-elements across the SAG21 promoter function as both activators and repressors of expression in response to wounding in adult leaves.

ROS production or oxidative burst (O_2^-, H_2O_2) is one of the early responses produced by wounding plants (Savatin et al., 2014) and ROS are also formed by the interaction between pathogens and plants and is produced by the activation of the membrane bound NADPH oxidases (Orozco-Cardenas & Ryan, 1999). It was shown that tomato leaves when wounded generate ROS or accumulate ROS in response to wounding and H_2O_2 was detected immediately after an hour of wounding with an increase after 4-6 hr followed by a decrease (Orozco-Cardenas & Ryan, 1999). It would be interesting to understand the interaction of ROS and wounding in the promoter reporter deletion constructs of *SAG21*. Although many methods are available for the detection of ROS, Prasad & Balukova et al. (2020) have applied the use of the confocal laser scanning microscopy. As the promoter reporter deletion constructs contain GFP as a reporter gene, and superoxide anion radical formation in wounded leaves can be detected using the fluorescent probe, DHE with confocal laser scanning microscopy (Prasad & Balukova, 2020) it might be possible to co-localise *SAG21* expression with ROS following wounding at a cellular level. Single oxygen generated in mechanically injured leaves can also be detected using a single oxygen sensor green probe (Prasad & Balukova, 2020). These fluorescent probes are considered as the best method for imaging to understand ROS signalling in plants.

Cytokinin generally originates in roots and is transported to the shoots. Priming by the cytokinin might be followed by the coordination of the signals generated above and below ground (Dervinis et al., 2010). Dervinis et al (2010) reported that the treatment of leaves with cytokinin and followed by wounding showed that the cytokinin increased the wound generated accumulation of JA and linolenic acid which indicates that cytokinin priming takes place upstream of JA biosynthesis in wounded leaves. *SAG21* appears to show both local and systemic wound responses in leaves (Salleh, 2011, results in Chapter 4) it would be interesting to see whether the treatment of leaves with cytokinin followed by wounding shows a similar priming effect. It would also be interesting to see whether a cytokinin increase in wound response in the leaves is caused by the accumulation of JA both locally and systemically. Wounding and cytokinin treatment in leaves of *Populus* reduced the weight of gypsy moth larvae which was in turn dependent on the position of the feeding

leaf. This effect in wounded leaves might be due to the cytokinin priming when compared to controls. However, when the assimilate movement was measured in wounded source leaves to sink leaves, cytokinin did not increase the assimilate transport into the sink leaves compared to controls but was found to act upstream of the JA biosynthesis pathway (Dervinis et al., 2010). With regards to *SAG21* future work involves understanding the interaction of cytokinin with ROS and wounding.

Wounding the plants by soft mechanical stress causes the accumulation of reactive oxygen species at the wound site within minutes and production of ROS at the wound site causes wound induced resistance to the *B. cinerea* (Benikhlef et al., 2013). Inoculation of spores of *B. cinerea* quickly after the mechanical stress caused a decrease in the size of the lesion and accumulation of ROS was seen in the leaves (Benikhlef et al., 2013). The mechanism behind this resistance might be due to the perception of DAMPs or MAMPs by membrane receptors which causes a change in calcium and ROS leading to the activation of the defence response, or might be sensed by the mechanoreceptors present on the cell membrane leading to the defence response activation (Benikhlef et al., 2013). It would be interesting to understand if *SAG21* protects the cells from oxidative damage at the wound site and also to know if the over-expressors under its own promoter offer any protection from pathogens and insect damage. Over-expression of SAG21 from the 35S promoter did reduce pathogen growth (Salleh et al., 2012) so it is possible that it switches on the defence pathway in transgenic plants which might be mediated by the above mentioned defence mechanism.

Deletions of the promoter region from -1700 to -1439 caused an increase in expression in petals and filaments in the *pSAG21*(1439)*::*GUS-GFP construct which suggests the cis-elements between 1439 and 1700 bp as repressors of expression. There were 225 potential cis elements in this region identified by the Plant Pan software, representing 24 TF families of which transcription factors belonging to the family BHLH, BZIP, MYB and Dof are active in floral organs. Pollen specific cis elements like TGTGGTT (PB core motif), AGAAA, TCCACCATA and GTGA were identified in other plant species (Bate & Twell, 1998; Eyal et al., 1995; Hamilton et al., 1998; Rogers et al., 2001; Twell et al., 1990). Previous analysis of the 1685 bp *SAG21* promoter region identified eight POLLEN1LELAT52 *cis*-elements (AGAAA) (Salleh, 2011; Twell et al., 1989), and five are found in the 1439 and 1700 bp, which may be important in pollen directed expression of *SAG21*. Pollen development in Arabidopsis starts with the division of an initial cell which

gives rise to a pollen mother cell. Meiotic division of the pollen mother cell results in the formation of four microspores. Microspores develop and are enclosed in an extra layer called primexine. During the process of maturation, layers surrounding the microspores degrade causing the release of the individual microspores into the anther locules and inside the locules the microspore primexine matures and forms exine accumulated by the tapetum (Francis et al., 2006). Within the microspores through the process of the mitosis a single pollen grain is produced which contains two nuclei called vegetative and generative nuclei. Pollen specific genes are categorised into two groups on the basis of the expression stage. For example, the *AMS* gene in Arabidopsis (Sorensen et al., 2003), is active very soon after meiosis and its expression decreases after pollen maturation (Jeon et al., 1999). *LAT52* is a pollen specific gene in tomato and the transcription of this gene occurs after meiosis (Twell et al., 1990). It would be interesting to know exactly when *SAG21* is expressed during the pollen development: whether it is before or after meiosis. This can be determined by looking at GUS expression in heterozygote lines and if expression is pre-meiosis then GUS expression is seen in all pollen grains and if it is post-meiosis only 50% of the pollen grains would stain blue. To understand the function of the pollen-related *cis*-elements more deletion constructs of *SAG21* could be transiently expressed in pollen (Rogers et al., 2001). Another approach to understand the individual cis elements or motifs present in the deletion constructs is that fragments containing the motifs can be placed downstream of constitutive promoters like CaMV35S to generate reporter fusion constructs. As the CaMV35S promoter does not show any promoter expression in pollen grains (Twell et al., 1990) then it is easy to conclude which region of the promoter drives the expression in pollen grains. Individual pollen specific *cis*-elements present in promoter reporter deletion constructs can be studied by the process of bombardment into the pollen grains to study the transient GFP and GUS expression by GUS staining and fluorescence microscopy (Zhou et al., 2010). The other method to understand the regulatory elements involves carrying out a point mutation or site directed mutagenesis of the *cis*-element to see if the mutation abolishes the expression and also to see the transient expression using reporter genes GFP and GUS (Rogers et al., 2001; Zhou et al., 2010). Expression could be compared to marker lines. Several marker lines were used in the literature one of them is a *pLAT52:UidA* construct which shows GUS expression before the first gametophytic mitotic division and also in the tricellular and bicellular stage of the pollen grains. Another promoter marker pLAT52: GFP shows fluorescence in pollen grains and pollen tubes. pAt5g17340: UidA: GFP marker lines exhibit a blue staining in uninucleate microspores, and also shows GFP and GUS expression

in all the stages of pollen development so it also functions as good marker line in all stages of male gametophyte along with pollination (da Costa-Nunes, 2013).

Deletion of the promoter region from -1225 to -965 caused a reduction in expression in sepals, petals and filaments suggesting the *cis*-elements act as activators of expression in all three of these tissues. Transcription factors have been identified in *Arabidopsis thaliana* which control the transition of floral organs to flower formation. *APETALA1*(AP1) and *LEAFY*(LFY) floral meristem genes function together to define the identity of the four of the floral organs sepals, petals, stamens and carpels (Chen et al., 2018). APETALA3(AP3) and PISTIL-LATA(PI) genes which encode MADS transcription factors are required for determining the stamen and petal identification in Arabidopsis (Mara & Irish, 2008). Growth and size of the plant organs is then controlled by the cell division and proliferation. *BIGPETALp*, BHLH transcription factor controls Arabidopsis petal growth by the cell expansion process and also interacts with auxin response factor (*ARF8*) which affects growth of the petal (Varaud et al., 2011). *TGA9* and *TGA10*, Arabidopsis BHLH transcription factors are required for the anther development and the plants lacking these transcriptions factors show defects in the process of male gametogenesis (Murmu et al., 2010). MYB transcription factors *MYB21*, *MYB24* and *MYB57* are required for stamen development and are also found to be upregulated by JA dependent on COI1. JA is biosynthesized during flower development and is recognized by COI1 which initiates the Jaz protein degradation and activates the expression of *MYB* TFs (Song et al., 2013). Promoter reporter deletion constructs of *SAG21* contain GFP so more accurate localization of the expression in floral organs can be carried out in the future.

Deletion from -965 to -737 and -737 to -490 region resulted in further loss in expression in sepals, petals and filaments indicating *cis*-elements as activators of gene expression. The region between 1439 and 1700 lost in the deletion from *pSAG21*(1700) :: GUS-GFP to *pSAG21*(1439):: GUS-GFP also contains binding sites for different transcription factor families mainly showing binding sites for dof and zinc finger transcription factor families. It might be interesting to understand the role of the *cis*-elements in the *SAG21* promoter by the construction of synthetic promoters. Synthetic promoters consist of a stretch of DNA containing multiple copies of a specific cis element upstream of a minimal promoter to bind the transcription factors in response to the specific stimuli (Dey et al., 2015; Rushton, 2016). The CaMV35S promoter was studied as the best characterized minimal promoter for the

construction of the synthetic promoters. The advantage of generating a synthetic promoter over native promoters is that the strength of the promoter can be altered to produce a weaker or stronger response depending on the numbers of copies of the *cis*-element and also can be used to eliminate undesired expression (Rushton, 2016).

To summarise here are some of the major experiments which could be carried out in future studies-

- Understand protein-protein interactions of SAG21 using the yeast 2 hybrid technique (Y2H).
- Study the role of other WRKY transcription factors identified by the yeast one hybrid technique (Y1H) in regulating the expression of *SAG21.*
- Understand the effect of overexpression of *SAG21* under its own promoter when compared to the 35S promoter with other abiotic stresses like drought, salt, and cold treatment as *SAG21*::GUS constructs show expression in roots with several abiotic stresses.
- Transcriptomic analysis of roots of over-expressor lines after the treatment with H_2O_2 would be interesting to identify the transcription factors involved in *SAG21* expression.
- Measure the ROS scavenging activity in over-expressor lines.
- Measurement of the level of ROS in roots using imaging tools like the GFP based redox probe, roGFP fluorescent dyes, and luciferase to provide more understanding of the localization of ROS in root tissues in the *SAG21* over-expressor lines.
- As *SAG21* expression was abolished by treatment with kinetin in cotyledons understanding of natural senescence parameters like measuring the chlorophyll content, and yellowing of the cotyledons would be important to study in the future to verify their senescence status.
- Experiments on the cotyledons throughout their development and senescence would be interesting to fit with the timings of leaf senescence or developmental age of the plant.
- Studying the effects of dark induced senescence and dark treatment on cotyledons to check if there is a similar kind of inhibition of expression of *SAG21* in young and old cotyledons.

- Understand the interaction of ROS and wounding in the promoter reporter deletion constructs of *SAG21*.
- It would also be interesting to see whether a cytokinin increase in wound response in the leaves is caused by the accumulation of JA both locally and systemically.
- Understanding the interaction of cytokinin with ROS and wounding.
- Understand if *SAG21* protects the cells from oxidative damage at the wound site and also to know if the SAG21 over-expressors under the SAG21 promoter offer any protection from pathogens and insect damage.
- To understand the function of the pollen-related *cis*-elements more deletion constructs of *SAG21* could be transiently expressed in pollen.
- Individual pollen specific *cis*-elements present in promoter reporter deletion constructs can be studied by the process of bombardment into the pollen grains to study the transient GFP and GUS expression by GUS staining and fluorescence microscopy.
- The other method to understand the regulatory elements involves carrying out a point mutation or site directed mutagenesis of the *cis*-elements to see if the mutation abolishes the expression and also to see the transient expression using reporter genes GFP and GUS.
- Promoter reporter deletion constructs of *SAG21* contain GFP so more accurate localization of the expression in floral organs can be carried out in the future.
- Further experiments could also include crossing the SAG21::GUS lines with a wide range of mutants in transcription factors and other regulators to further explore the signalling cascade that activates *SAG21* under different circumstances.
- It might be interesting to understand the role of the *cis*-elements in the *SAG21* promoter by the construction of synthetic promoters. Synthetic promoters could be used to enhance *SAG21* expression further in specific organs or in response to ROS or specific groups of stresses. This may provide more information to help understanding of the role of reactive oxygen species in roots and other organs.

Overall, the work described in this thesis provides further information on the promoter-regions and *cis*-elements that play major roles in regulating the expression of the *SAG21.* Due to time constraints, experiments focussed only on a limited number of plant organs and stresses, and its regulation by the WRKY and NAC transcription factors. Further work

would be aimed at exploiting more fully the transgenic lines generated here to examine the promoter segments important in responses e.g. to oxidative stresses, drought, cold, and salt known to affect *SAG21* expression (Mowla et al., 2006; Salleh et al., 2012; Salleh, 2011) and also assess effects of stress on *SAG21* expression in different tissues. The constructs also contained GFP hence enabling the use of the lines to assess expression in detail e.g. in different root and floral tissues. Further experiments could also include crossing the lines with a wide range of mutants in transcription factors and other regulators to further explore the signalling cascade that activates *SAG21* under different circumstances. Other work could be aimed at understanding protein-protein interactions by using the techniques discussed earlier in this chapter to understand whether the transcription factors studied here function as individuals or as a complex to regulate the expression of the *SAG21*. As the *cis*-elements in this study seem to regulate responses related to both development, senescence and wounding, a more detailed study by the construction of synthetic promoters may shed light on individual elements. This would greatly benefit from high throughput methods using microprojectile bombardment to assess promoter function transiently in specific organs.

When overexpressed under its own promoter *SAG21* seemed to induce a change in root phenotype under optimal conditions and when exposed to ROS. Synthetic promoters could be used to enhance *SAG21* expression further in specific organs or in response to ROS or specific groups of stresses. This may provide more information to help understanding of the role of reactive oxygen species in roots. It may also result in useful phenotypes suggesting value in transferring the technology to crop species to provide better growth under optimal or stressful conditions.

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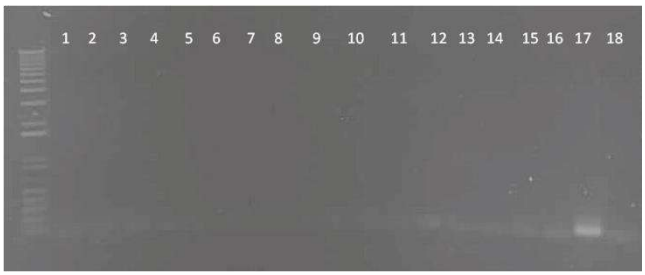
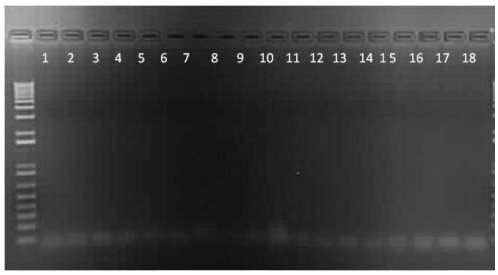
Appendix- 3

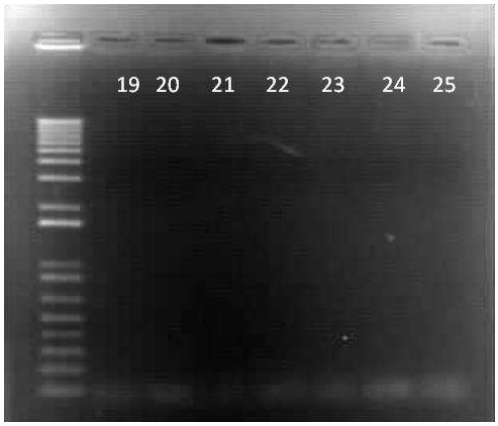
This Appendix section is relevant to Chapter 3 of the thesis.
Gel images of PCR amplification in preparation for real time PCR analysis. The lack of banding on the gels but band of correct size in the positive control in Fig A3.1 indicates successful removal of the genomic DNA from the RNA samples. The equal intensity banding across the different samples in Fig. A3.2 indicates that the amount of cDNA in the different samples is of equal amount.

(a)

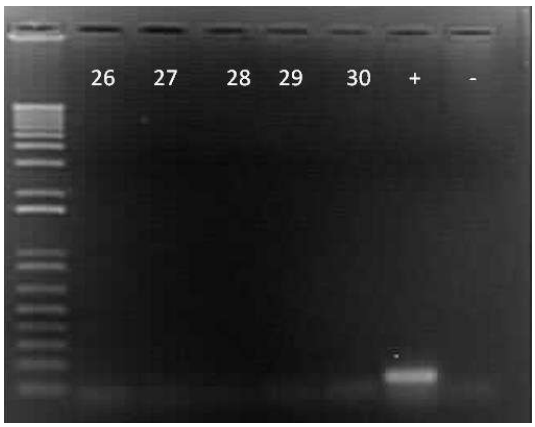


(b)





(c)



(d)

Figure A3.1 PCR on DNAse treated RNA – (a) PCR with *actin2* primers on DNase treated RNA samples. Lanes 1-3: WT H_2O_2 stressed. Lanes 4-6: WT non stressed. Lanes 7-9-: *nac042ko* stressed. Lanes 10-12: *nac042ko* non stressed. Lanes 13-15: *nac042*^{oe} stressed. Lanes 16-18: *nac042*^{oe} non stressed. (b), (c), (d): Lanes1-3: *wrky15* H2O*2* stressed samples. Lanes4-6: *wrky15* non stressed samples. Lanes 7-9: *wrky63* drought stressed. Lanes 10-12: *wrky63* non stressed samples. Lanes 13-15: *wrky67* drought stressed. Lanes 16-18: *wrky67* non stressed. Lanes 19-21: WT salt stressed. Lanes 22- 24: WT non stressed. Lanes 25-27: WT drought stressed. Lanes 28-30: WT non stressed.

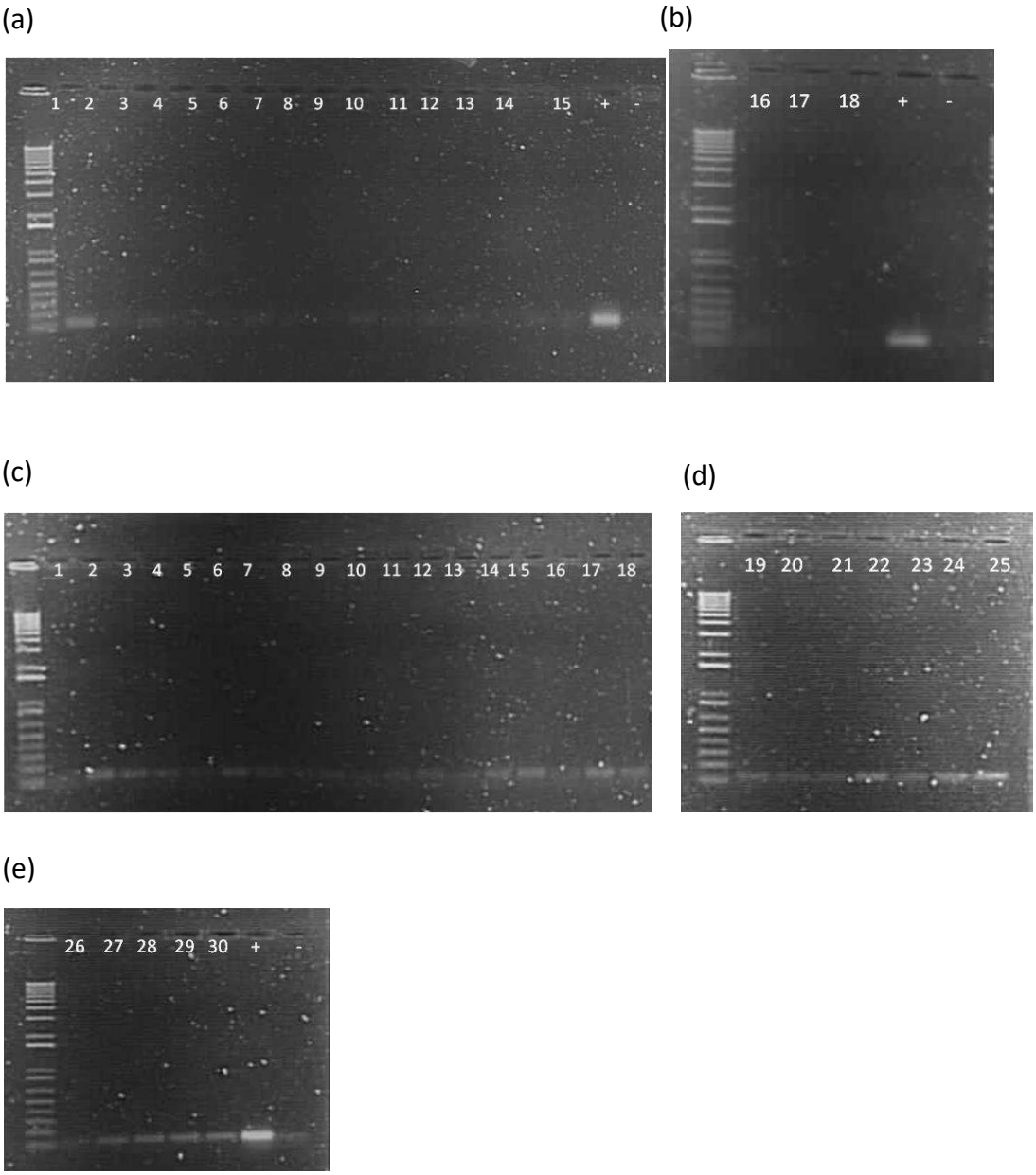


Figure A3.2: PCR on equalised cDNA samples (a), (b) PCR with *actin2* primers on 10 ng cDNA. Lanes 1-3: WT H_2O_2 stressed. Lanes 4-6: WT non stressed. Lanes 7-9-: *nac042ko* stressed. Lanes 10-12: *nac042ko* non stressed. Lanes 13-15: *nac042*^{OE} stressed. Lanes 16-18: *nac042*^{OE}non stressed. (+) WT genomic DNA, (–) negative control water. (c), (d), (e): lanes1-3: *wrky15* H_2O_2 stressed samples. Lanes 4-6: *wrky15* non stressed PCR with *actin2* primers on 10 ng cDNA samples. Lanes 7-9: *wrky63* drought stressed. Lanes 10-12: *wrky63* non stressed samples. Lanes13-15: *wrky67* drought stressed. Lanes 16-18: *wrky67* non-stressed. Lanes 19-21: WT salt stressed. Lanes 22-24: WT non stressed. Lanes 25-27: WT drought stressed. Lanes 28-30: WT non stressed. (+) WT genomic DNA, (–) negative control water.

Appendix-4

This section of Appendix is relevant to Chapter 4 of the thesis

Appendix 4.1 Gibson assembly

A4.1.1 Vector Preparation

Vector DNA (1 µg of pGgreenII0229) was digested with 1 µl (10 units) of restriction enzymes BamHI and NotI (Promega) individually in 1 X reaction buffer(D)(Promega) in 50 µl total volume. The reaction mix was incubated at 37 °C for 3 hours. Following incubation, digested product was checked on a 1 % agarose gel and gel images were visualized using Genesnap software. The reciprocal digestion with BamHI and NotI was then carried out in the same buffer for 3 hours at 37 °C to double digest the vector. The reaction mix was mixed and treated with 1 µl of TSAP (Thermo sensitive alkaline phosphate, Promega) and incubated for 30 minutes at 37 °C to dephosphorylate the digest. Digests were purified using a QIAGEN QIA quick PCR purification kit. Eluted DNA was quantified using a Nanodrop UV spectrophotometer.

A4.1.2 Insert Preparation

A 3 kb fragment comprising the GUS-GFP-NOS cassette was amplified from plasmid pKGWFS7 using GibF1R1 primers (Table A4.1) in a Gene Amp 3700 Applied Biosystems thermocycler. A total reaction volume of 25 µl contained: 1 µl (5 ng) template, Q5 Reaction buffer (5X), 10 mM dNTP's, 10 µM Forward & Reverse primers, 0.02 U/µl of the Q5 polymerase. Cycling was 40 cycles of 94 °C- 2min, 94 °C -1 min, 62 °C-1 min, 72 °C-2 min for 2 cycles, 94 °C -1 min, 72 °C -1 min, 72 °C -2 min for 38 cycles. PCR product was analysed on a 1% agarose gel and was gel extracted using a QIAGEN qiaquick Gel extraction kit. The extracted DNA was quantified using a Nanodrop UV spectrophotometer.

A4.1.3 Reaction assembly

Gibson Assembly reactions were set up using 50-100 ng of vector with a 2-3 fold excess of insert DNA using 2X Assembly master mix (NEB) and sterile distilled water in a 20 µl total volume. Positive (supplied with the kit) and negative control (without insert) reactions were included. The mix was incubated in a thermocycler at 50°C for 15 minutes, then 2 µl of the mix was transformed into DH5-α chemically competent cells

(NEB) according to the kit protocol Each transformation mix (100 µl) was spread on Petri dishes containing LB agar with the appropriate antibiotic and incubated overnight at 37 °C. Transformed colonies were then screened by PCR using Seq GNF- Forward and GUS -Reverse primers (Table 2-3)

A4.1.4 Gibson cloning

Digestion of the pGreenII0229 vector (Figure A4.1 a) and PCR amplification (Figure 4.1 b) of the GUS-GFP- NOS cassette were successful showing the expected banding patterns.

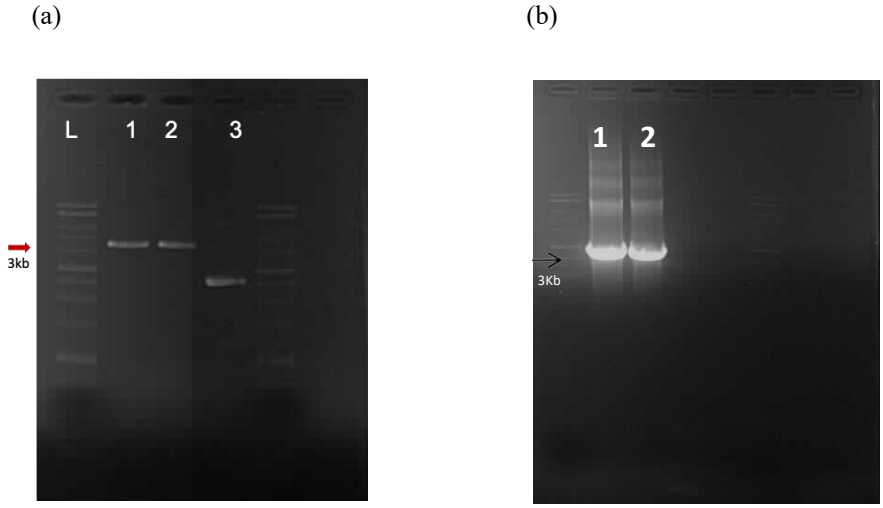


Figure A4.1 (a) restriction digestion of vector pGreenIIo229. lane1-BamHI digestion lane2- NotI digestion lane3-uncut plasmid pGreenII0229. (b) amplification of the insert showing a 3kb fragment by PCR using GibF1R1 primers.(+) 1685:: GUS was used as positive control.(-) indicates negative control water.

Following transformation, colony screening by PCR using Seq GNF- Forward and GUS- Reverse primers revealed five positive colonies (Figure.A4.2) through the amplification of the predicted 630 bp fragment.

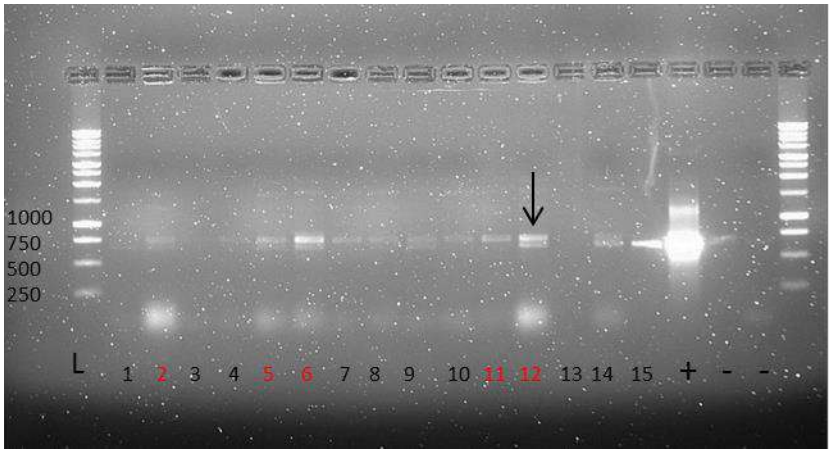


Figure A4.2 Ethidium bromide stained agarose gel of colony PCR products with Seq GNF- forward & GUS reverse primers. lanes 1-15 shows PCR products from ligation reaction.(+) pKGWFS7 DNA used as positive control.(-) indicates pGreenIIO229 DNA and sterile distilled water as a negative control .l- 1 kb ladder.

Plasmid DNA was isolated from the five positive colonies (2,5,6,11,12) and PCR was carried out again with same primers to confirm them as positive clones. However, no amplification product of the correct size was detected (Figure A4.3), indicating that the Gibson cloning was not in fact successful.

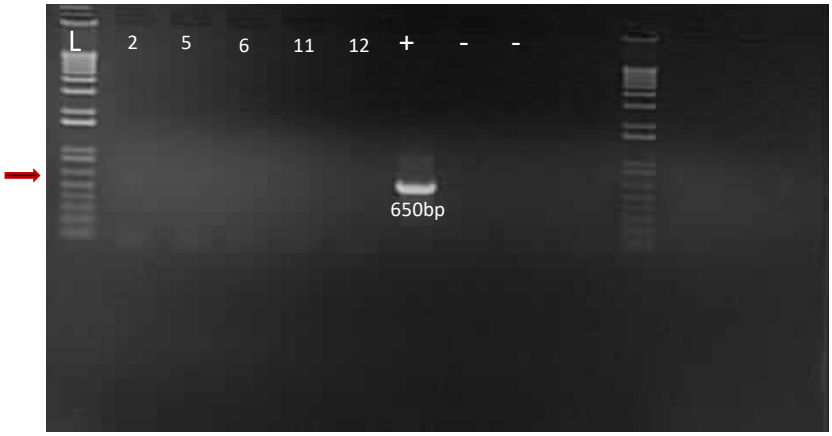


Figure A4.3. colony PCR on positive colonies with Seq GNF-forward & GUS reverse primers. lanes 2,5,6,11,12- shows positive colonies from above PCR. (+) pKGWFS7 used as positive control, (-) shows pgreenII0229 DNA and sterile distilled water. L-1 kb plus ladder.

Several modifications to the Gibson protocol were attempted which included trying different molar ratios of vector and insert. The primers were also redesigned (GibF2R2 primers, Table A4.1) for amplification of the insert and digested the vector with a single restriction enzyme (SpeI) instead of BamHI and NotI. However, it was not possible to obtain positive colonies through this approach.

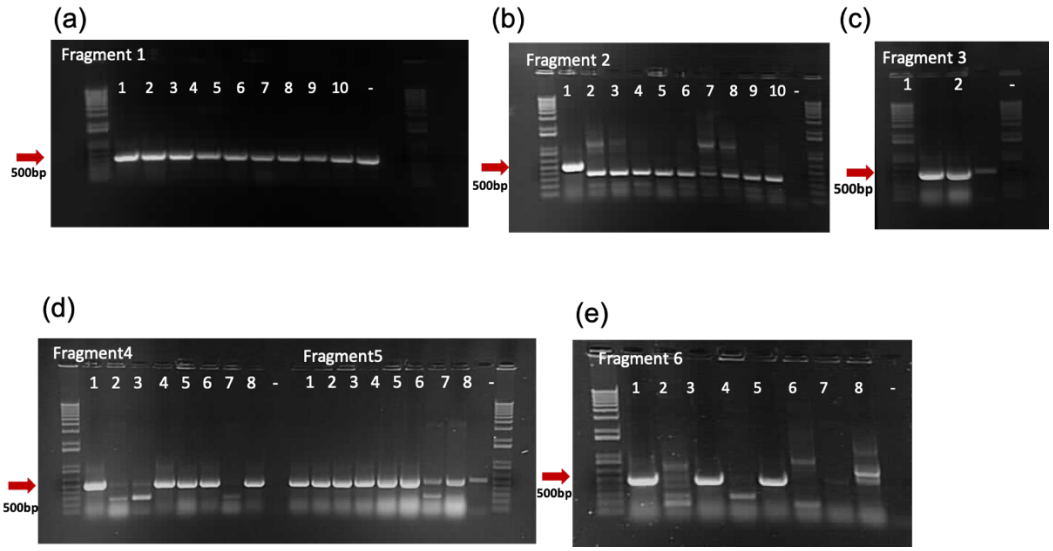


Figure A4.4 (a) (b) (c) (d) (e) Agarose gels showing colony PCR products with CP-FR primers. Lanes 1-10 show colonies from ligation reaction. (-) indicates a reaction with water. PCR products showing a band of 500 bp are considered as positive clones

This PCR screening was repeated and resulted in the selection of around 10 positive clones with fragment 1, one positive clone for fragment 2, two positive clones for fragment 3, five for fragment 4, six for fragment 5 and three for fragment 6 (Figure A4.5).

Table A4.1 Primer sequences used for Gibson cloning.

Primer name	**Oligonucleotide Sequence**
GibF1R1	F1-CGAATTCCTGCAGCCCGGGTCTAGAGGATCCccatggtgagcaaggcgagg R1- CTCCACCGCGGTGGCcctgcaggtcactggattttggttttagg
GibF2R2	F2-GAATTCCTGCAGCCCGGGGGATCCAATGGTGAGCAAGGGCGAGG R2-CCGCGGTGGCGGCCGCTCTAGAAAGGTCACTGGATTTTGGTTTTAGG
GUS-1	1-GAAACCCCAACCCGTGAAATCA
GUS-2	2-AACCTTCACCCGGTTGCCAGA

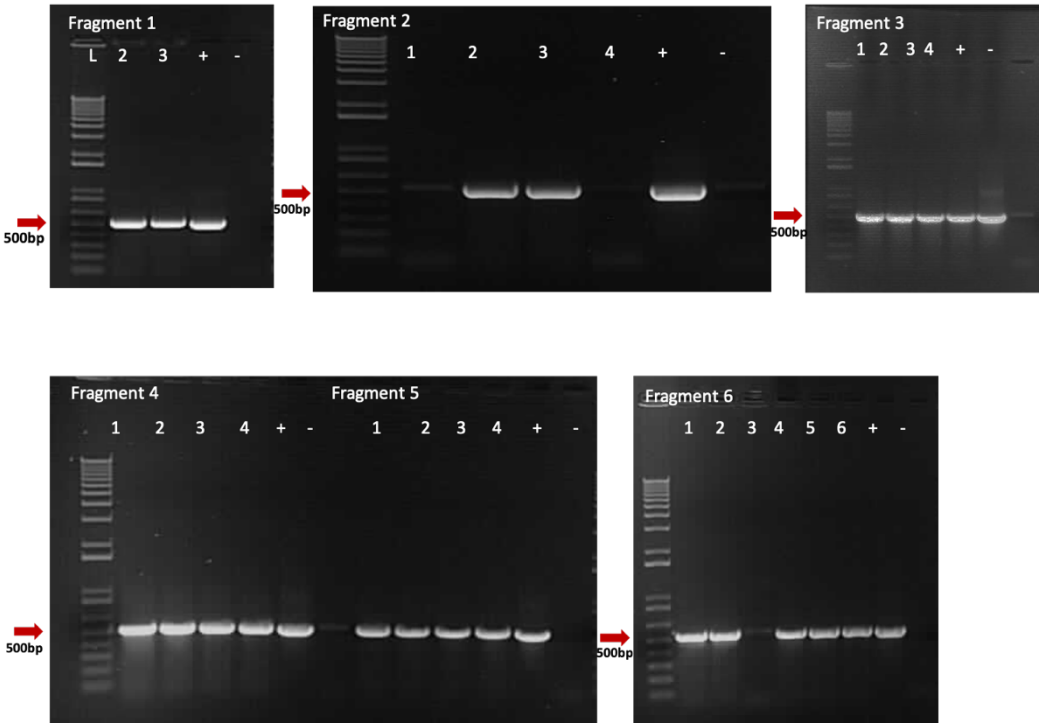


Figure A4.5- (a) (b) (c) (d) (e) Agarose gel showing Agrobacterium colony PCR products on liquid culture with cp-FR primers. lanes 1-4 show colonies from ligation reaction. (+) plasmid DNA of *E.coli* clone for fragments. (-) indicates a reaction with water.L:1 kb plus DNA ladder.

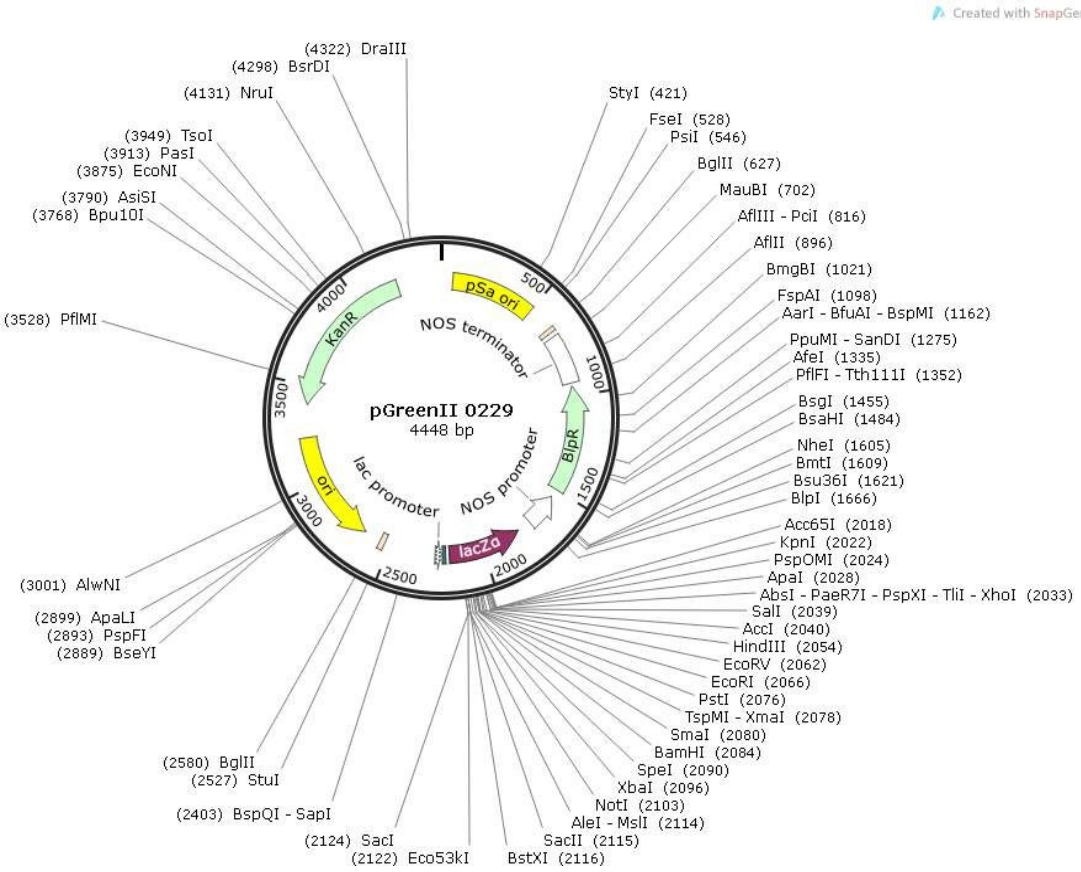


Figure A4.2 (a)- map of pGreenII0229 plasmid

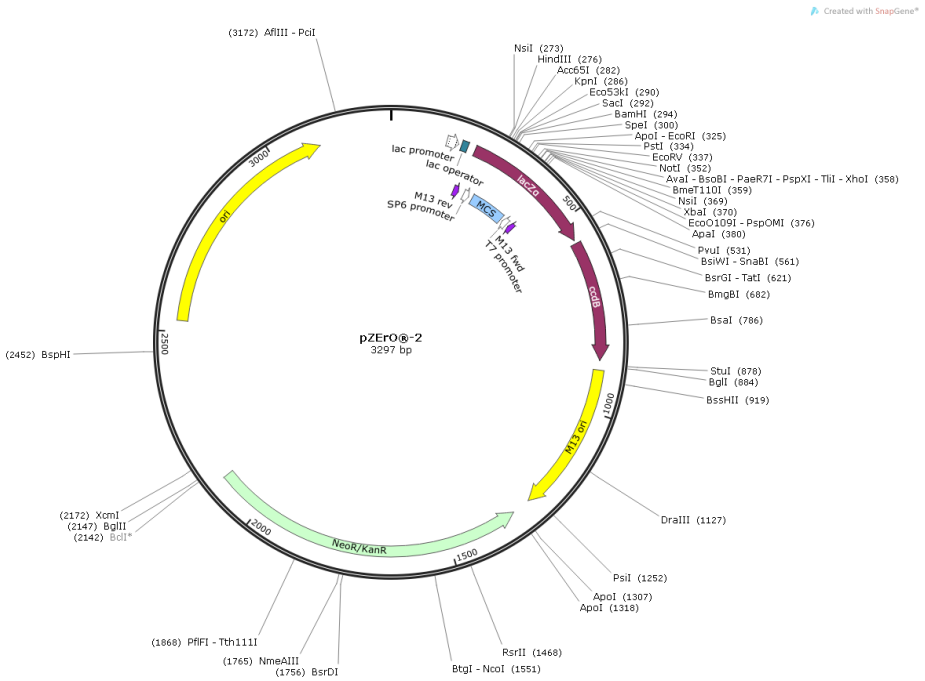
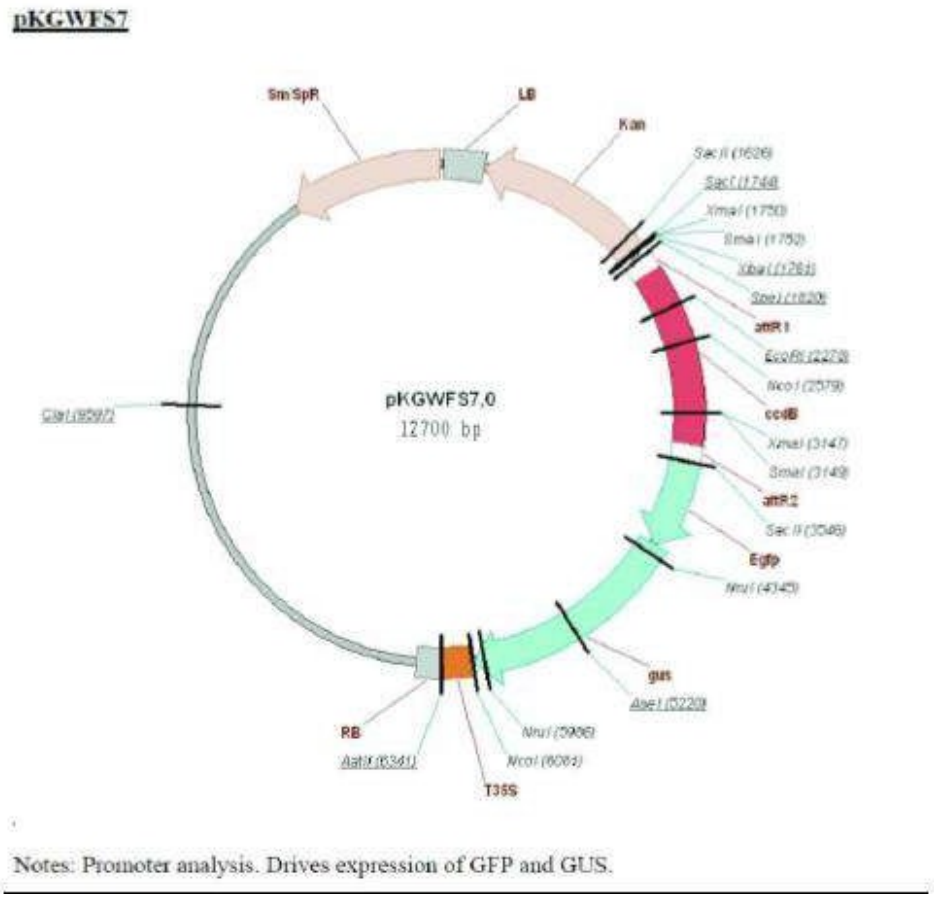


Figure A4.2(b)-map of pZErO-2 plasmid



Notes: Promoter analysis. Drives expression of GFP and GUS.

Figure A4.2(c)- map of pKGWFS7 plasmid

The GFP-GUS-NOS fragment (3kb) starts from 3548-6325bp so M13 forward primer sequenced region from 3470-4550 bp and M13 Reverse primer sequenced region starting from NOS terminator towards GUS region from 6450-5350bp Region between 4550-5350 bp was sequenced using SeqGNF-F and GUS F-4600 primers.

Appendix 4.3 Transcription factor families and cis element sequences in the *SAG21* 1700 upstream region

Table A4.3.1 deletion region of the 1700-1439 bp

TF ID	position	strand	Score	Sequence	Matrix ID	TF Family
TFmatrixID_0079	1628	-	1	gcgGCGGCga	TFmatrixID_0130	BES1
TFmatrixID_0101	1628	-	1	gcgGCGGCga	TFmatrixID_0130	BES1
TFmatrixID_0102	1629	-	1	cgGCGGCgac	TFmatrixID_0131	BES1
TFmatrixID_0110	1629	-	1	cggCGGCG	TFmatrixID_0131	BES1
TF_motif_seq_0390	1511	+	0.86	TCATCtc	TFmatrixID_0495	BES1
TFmatrixID_0794	1539	-	0.82	ttgagAGAGAgaaca	TF_motif_seq_0300	bHLH
TFmatrixID_0794	1663	+	0.82	tgtttTCTCTctttt	TF_motif_seq_0300	bHLH
TFmatrixID_0925	1654	+	0.85	taAAAAGtctgtttt	TF_motif_seq_0444	bHLH
TFmatrixID_0131	1450	-	1	TTTATttcc	TFmatrixID_0137	bHLH
TFmatrixID_0131	1649	+	1	aagaATAAA	TFmatrixID_0137	bHLH
TFmatrixID_0136	1650	+	1	agaATAAA	TFmatrixID_0140	bHLH
TFmatrixID_0061	1629	-	0.98	cggCGGCG	TFmatrixID_0289	bHLH
TFmatrixID_0243	1654	+	1	taAAAAGt	TFmatrixID_0289	bHLH
TF_motif_seq_0243	1559	+	1	GATAC	TFmatrixID_0720	bHLH
TFmatrixID_0369	1612	+	0.98	atATATCtc	TFmatrixID_0748	bHLH
TFmatrixID_0748	1616	-	0.98	atctctccttctgcGGCGGcgacaagaa	TFmatrixID_0949	bHLH
TFmatrixID_0794	1541	-	0.83	gagagAGAGAacaca	TFmatrixID_1035	bHLH
TFmatrixID_0904	1647	-	0.91	acaagaataAAAAGtctg	TFmatrixID_1189	bHLH
TF_motif_seq_0261	1441	-	0.8	GTCAC	TF_motif_seq_0271	bZIP
TF_motif_seq_0261	1458	-	0.8	CTCTC	TF_motif_seq_0271	bZIP
TF_motif_seq_0261	1471	-	0.8	CTCTC	TF_motif_seq_0271	bZIP
TF_motif_seq_0258	1630	-	0.8	GGCGG	TF_motif_seq_0271	bZIP
TF_motif_seq_0258	1634	+	0.8	GCGAC	TF_motif_seq_0271	bZIP
TF_motif_seq_0258	1660	-	0.8	GTCTG	TF_motif_seq_0271	bZIP
TFmatrixID_0676	1619	-	0.9	tctccttctgCGGCGgcgaca	TFmatrixID_1055	bZIP
TFmatrixID_0989	1578	+	0.92	ctcgttgCCGCAgta	TFmatrixID_1193	bZIP
TFmatrixID_1046	1663	-	0.99	tgTTTTCtc	TFmatrixID_1368	bZIP
TFmatrixID_0030	1611	-	0.99	gataTATCTc	TFmatrixID_0224	CPP
TFmatrixID_0062	1627	-	0.99	tgcggCGGCG	TFmatrixID_0224	CPP
TFmatrixID_0077	1627	-	0.99	tgcgGCGGCg	TFmatrixID_0224	CPP
TFmatrixID_0122	1629	-	0.99	cgGCGGCg	TFmatrixID_0224	CPP
TFmatrixID_0900	1646	-	0.94	tacaagaataAAAAGtctg	TFmatrixID_1001	CPP
TFmatrixID_0216	1467	+	1	tTCACTc	TFmatrixID_0233	Dof
TFmatrixID_0222	1652	+	1	aATAAAaa	TFmatrixID_0233	Dof
TFmatrixID_0233	1654	+	1	taAAAAGtct	TFmatrixID_0235	Dof
TFmatrixID_0146	1595	-	0.99	ATTATctca	TFmatrixID_0237	Dof
TFmatrixID_0234	1655	+	1	aAAAAGt	TFmatrixID_0238	Dof
TFmatrixID_0235	1654	+	1	taAAAAGt	TFmatrixID_0243	Dof
TFmatrixID_0238	1653	+	1	ataAAAAGt	TFmatrixID_0284	Dof
TFmatrixID_0724	1618	+	0.93	ctctccttctgCGGCGgcg	TFmatrixID_0892	Dof
TFmatrixID_0700	1622	+	0.93	ccttctgCGGCGgcg	TFmatrixID_0892	Dof
TFmatrixID_0664	1629	-	0.94	cGGCGGcgacaagaagctacaaga	TFmatrixID_0893	Dof
TFmatrixID_0672	1622	+	0.94	ccttctgCGGCGgcg	TFmatrixID_0895	Dof

TFmatrixID_0675	1624	+	0.94	ttctgcggCGGCGacaagaa	TFmatrixID_0900	Dof
TFmatrixID_0698	1627	-	0.94	tgcGGCGGcgacaagaagcta	TFmatrixID_0900	Dof
TFmatrixID_0767	1618	-	0.93	ctctccttctgCGGCGgcgac	TFmatrixID_0902	Dof
TFmatrixID_0721	1626	+	0.94	ctgCGGCGgcgacaa	TFmatrixID_0903	Dof
TFmatrixID_0753	1626	-	0.94	ctgcGGCGGcgacaagaagct	TFmatrixID_0904	Dof
TFmatrixID_0686	1627	-	0.96	tgcGGCGGcgacaag	TFmatrixID_0906	Dof
TFmatrixID_0671	1628	-	0.96	gcGGCGGcgacaaga	TFmatrixID_0906	Dof
TFmatrixID_0704	1625	+	0.96	tctgCGGCGgcgaca	TFmatrixID_0907	Dof
TFmatrixID_0744	1626	+	0.96	ctgCGGCGgcgacaa	TFmatrixID_0910	Dof
TFmatrixID_0745	1620	-	0.96	ctccttctgcGGCGGcgacaa	TFmatrixID_0911	Dof
TFmatrixID_0723	1626	+	0.92	ctgcGGCGGcgacaa	TFmatrixID_0913	Dof
TFmatrixID_0656	1628	-	0.98	gcgGCGGCgacaagaagc	TFmatrixID_0914	Dof
TFmatrixID_1301	1669	+	0.82	ctctcttttCAAGAaac	TFmatrixID_0915	Dof
TFmatrixID_0663	1621	-	0.98	tccttctgcGGCGGcgac	TFmatrixID_0917	Dof
TFmatrixID_0763	1626	-	0.97	ctgCGGCGgcgacaa	TFmatrixID_0917	Dof
TF_motif_seq_0268	1575	-	1	AATCT	TFmatrixID_1527	Dof
TF_motif_seq_0458	1581	+	0.94	gTTGCCgc	TFmatrixID_0476	E2F/DP
TFmatrixID_0687	1626	-	0.95	ctgCGGCGgcgacaa	TFmatrixID_0750	EIL
TFmatrixID_0979	1462	+	0.99	cttcctTCACTctct	TFmatrixID_1208	FAR1
TFmatrixID_0985	1654	+	0.94	taAAAAGtctg	TFmatrixID_1236	FAR1
TFmatrixID_0953	1463	-	1	ttcctTCACTc	TF_motif_seq_0237	GATA
TFmatrixID_0689	1628	-	1	gCGGCG	TF_motif_seq_0237	GATA
TFmatrixID_0720	1629	+	1	cGGCGGcg	TF_motif_seq_0237	GATA
TFmatrixID_0689	1631	-	1	gCGGCG	TF_motif_seq_0237	GATA
TFmatrixID_0977	1674	-	1	ttTTCAA	TF_motif_seq_0237	GATA
TF_motif_seq_0239	1447	-	1	TCCTT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1448	-	1	CCTTT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1464	-	1	TCCTT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1486	-	1	ACCTT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1533	+	1	AAAGC	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1536	-	1	GCCTT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1566	-	1	ACTTT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1621	-	1	TCCTT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1657	+	1	AAAGT	TF_motif_seq_0243	GATA
TF_motif_seq_0239	1672	-	1	TCTTT	TF_motif_seq_0243	GATA
TFmatrixID_0638	1669	-	0.99	ctcTCTTTtc	TFmatrixID_0952	GATA
TFmatrixID_0031	1654	+	0.97	taAAAAGtct	TFmatrixID_0282	GeBP
TFmatrixID_0749	1626	-	0.9	ctgcGGCGGcgacaa	TFmatrixID_1064	GeBP
TFmatrixID_0920	1654	+	0.96	taAAAAGtctg	TFmatrixID_1064	GeBP
TFmatrixID_0989	1624	-	0.96	ttcTGCGGcggcgac	TFmatrixID_1142	GRAS
TFmatrixID_0951	1552	+	0.91	cacagacgatACCAActtt	TFmatrixID_1275	GRAS
TFmatrixID_1071	1628	+	0.92	gcGGCGGcgacaagaagctac	TFmatrixID_1275	GRAS
TFmatrixID_0681	1626	-	0.95	ctgCGGCGgcgacaa	TFmatrixID_0733	HSF
TFmatrixID_1045	1648	-	0.88	caaGAATAaaaagtc	TFmatrixID_1060	HSF
TFmatrixID_0914	1649	+	0.98	aagaataAAAAGtct	TFmatrixID_1158	HSF
TF_motif_seq_0246	1440	-	1	AGTCA	TFmatrixID_0988	LBD
TFmatrixID_0911	1654	+	0.95	taAAAAGtctgtt	TFmatrixID_0988	LBD
TFmatrixID_0891	1654	+	0.96	taAAAAGtctgttt	TFmatrixID_0988	LBD
TF_motif_seq_0261	1473	-	0.8	CTCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1492	-	0.8	CTCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1503	-	0.8	CTCTC	TF_motif_seq_0248	MYB_related

TF_motif_seq_0261	1508	-	0.8	TTCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1513	-	0.8	ATCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1541	+	0.8	GAGAG	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1543	+	0.8	GAGAG	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1545	+	0.8	GAGAG	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1547	+	0.8	GAGAA	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1554	+	0.8	CAGAC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1559	+	0.8	GATAC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1576	-	0.8	ATCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1598	-	0.8	ATCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1616	-	0.8	ATCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1618	-	0.8	CTCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1634	+	0.8	GCGAC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1660	-	0.8	GTCTG	TF_motif_seq_0248	MYB_related
TF_motif_seq_0261	1667	-	0.8	TTCTC	TF_motif_seq_0248	MYB_related
TF_motif_seq_0257	1477	+	0.8	CCTAT	TF_motif_seq_0258	MYB_related
TF_motif_seq_0257	1524	+	0.8	CCATT	TF_motif_seq_0258	MYB_related
TF_motif_seq_0257	1563	+	0.8	CCAAC	TF_motif_seq_0258	MYB_related
TF_motif_seq_0257	1573	+	0.8	TCAAT	TF_motif_seq_0258	MYB_related
TF_motif_seq_0267	1441	-	0.8	GTCAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0267	1441	+	0.8	GTCAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0267	1559	+	0.8	GATAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0267	1581	+	0.8	GTTGC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0267	1644	+	0.8	GCTAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0267	1681	-	0.8	GAAAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0263	1684	+	0.8	ACCAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0267	1688	+	0.8	CTTAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0271	1440	-	0.8	AGTCA	TF_motif_seq_0263	MYB_related
TF_motif_seq_0271	1466	-	0.8	CTTCA	TF_motif_seq_0263	MYB_related
TF_motif_seq_0271	1517	-	0.8	CTTCA	TF_motif_seq_0265	MYB_related
TF_motif_seq_0271	1555	+	0.8	AGACG	TF_motif_seq_0265	MYB_related
TF_motif_seq_0271	1571	-	0.8	CTTCA	TF_motif_seq_0265	MYB_related
TF_motif_seq_0271	1583	+	0.8	TGCCG	TF_motif_seq_0265	MYB_related
TF_motif_seq_0271	1635	-	0.8	CGACA	TF_motif_seq_0265	MYB_related
TF_motif_seq_0275	1441	-	0.8	GTCAC	TF_motif_seq_0275	MYB_related
TF_motif_seq_0275	1467	+	0.8	TTCAC	TF_motif_seq_0275	MYB_related
TF_motif_seq_0275	1518	-	0.8	TTCAA	TF_motif_seq_0275	MYB_related
TF_motif_seq_0275	1539	+	0.8	TTGAG	TF_motif_seq_0275	MYB_related
TF_motif_seq_0275	1572	-	0.8	TTCAA	TF_motif_seq_0275	MYB_related
TF_motif_seq_0275	1582	+	0.8	TTGCC	TF_motif_seq_0275	MYB_related
TF_motif_seq_0275	1636	-	0.8	GACAA	TF_motif_seq_0275	MYB_related
TF_motif_seq_0275	1676	-	0.8	TTCAA	TF_motif_seq_0275	MYB_related
TF_motif_seq_0321	1453	-	1	aTTTCC	TF_motif_seq_0321	MYB_related
TF_motif_seq_0321	1526	-	1	aTTTTC	TF_motif_seq_0321	MYB_related
TF_motif_seq_0321	1595	-	1	aTTATC	TF_motif_seq_0321	MYB_related
TF_motif_seq_0318	1630	-	1	gGCGGC	TF_motif_seq_0321	MYB_related
TF_motif_seq_0321	1696	+	1	GAAAAt	TF_motif_seq_0321	MYB_related
TF_motif_seq_0343	1661	-	0.86	tcTGTTT	TF_motif_seq_0354	MYB_related
TF_motif_seq_0369	1467	-	0.86	ttCACTC	TF_motif_seq_0445	MYB_related
TFmatrixID_0601	1629	-	0.95	cggCGGCGac	TFmatrixID_0708	MYB_related
TFmatrixID_0677	1626	-	0.95	ctgCGGCGgcgaca	TFmatrixID_0719	NAC

TFmatrixID_0339	1607	+	0.93	ctcGGATAtat	TFmatrixID_0767	NAC
TF_motif_seq_0243	1596	-	1	TTATC	TFmatrixID_0790	NAC
TF_motif_seq_0243	1611	+	1	GATAT	TFmatrixID_0790	NAC
TF_motif_seq_0243	1614	-	1	ATATC	TFmatrixID_0790	NAC
TFmatrixID_1263	1592	+	0.93	ataaTTATCtcatt	TFmatrixID_1704	NAC
TF_motif_seq_0254	1576	+	0.8	ATCTC	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	1598	+	0.8	ATCTC	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	1612	+	0.8	ATATA	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	1613	-	0.8	TATAT	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	1616	+	0.8	ATCTC	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	1643	+	0.8	AGCTA	TF_motif_seq_0257	NF-YB
TFmatrixID_0412	1590	+	1	gtaTAATTat	TFmatrixID_0419	NF-YC
TFmatrixID_0412	1592	-	1	atAATTAtct	TFmatrixID_0419	NF-YC
TFmatrixID_0156	1633	+	0.96	ggCGACAag	TFmatrixID_0585	NF-YC
TFmatrixID_1550	1629	-	0.99	cgGCGGCg	TFmatrixID_0007	Nin-like
TFmatrixID_0237	1655	+	0.99	aAAAAGtct	TFmatrixID_0299	Nin-like
TFmatrixID_0688	1625	+	0.95	tctgcggCGGCGacaaga	TFmatrixID_0787	Nin-like
TFmatrixID_0693	1626	-	0.95	ctgCGGCGgcgacaa	TFmatrixID_0787	Nin-like
TFmatrixID_0719	1619	+	0.95	tctccttctgCGGCGgcgacaagaa	TFmatrixID_0791	SRS
TFmatrixID_0712	1626	+	0.95	ctgCGGCGgcgacaa	TFmatrixID_0791	SRS
TF_motif_seq_0066	1580	+	0.73	CGTTGccgcag	TF_motif_seq_0072	TCP
TF_motif_seq_0421	1443	+	0.88	CACTTcct	TFmatrixID_0648	TCP
TF_motif_seq_0249	1443	-	0.8	CACTT	TF_motif_seq_0254	Trihelix
TF_motif_seq_0248	**1448**	**-**	**0.8**	**CCTTT**	TF_motif_seq_0254	Trihelix
TF_motif_seq_0254	1479	-	0.8	TATAT	TF_motif_seq_0254	Trihelix
TF_motif_seq_0254	1480	+	0.8	ATATA	TF_motif_seq_0254	Trihelix
TF_motif_seq_0254	1513	+	0.8	ATCTC	TF_motif_seq_0254	Trihelix
TF_motif_seq_0248	**1524**	**-**	**0.8**	**CCATT**	TF_motif_seq_0254	Trihelix
TF_motif_seq_0249	1578	-	0.8	CTCGT	TF_motif_seq_0254	Trihelix
TF_motif_seq_0248	**1579**	**-**	**0.8**	**TCGTT**	TF_motif_seq_0254	Trihelix
TF_motif_seq_0248	**1662**	**-**	**0.8**	**CTGTT**	TF_motif_seq_0254	Trihelix
TF_motif_seq_0249	1686	-	0.8	CACTT	TF_motif_seq_0254	Trihelix
TF_motif_seq_0258	1554	+	0.8	CAGAC	TF_motif_seq_0267	Trihelix
TF_motif_seq_0258	1563	+	0.8	CCAAC	TF_motif_seq_0267	Trihelix
TF_motif_seq_0258	1607	-	0.8	CTCGG	TF_motif_seq_0267	Trihelix
TF_motif_seq_0257	1685	+	0.8	CCACT	TF_motif_seq_0267	Trihelix
TFmatrixID_0781	1623	+	0.99	cttctgCGGCGgcga	TFmatrixID_0986	Trihelix
TFmatrixID_0969	1468	-	0.99	TCACTctctcc	TFmatrixID_1182	Trihelix
TFmatrixID_0418	1480	+	1	ATATAaa	TFmatrixID_0451	WRKY
TFmatrixID_0988	1654	+	0.95	taAAAAGtctg	TFmatrixID_1080	WRKY
TF_motif_seq_0252	1575	-	1	AATCT	TFmatrixID_1085	WRKY
TFmatrixID_0917	1654	+	0.97	taAAAAGtctg	TFmatrixID_1090	WRKY
TFmatrixID_0892	1647	-	0.93	acaagaataAAAAGtctgttt	TFmatrixID_1152	WRKY
TF_motif_seq_0318	1584	+	0.83	GCCGCa	TFmatrixID_1455	WRKY
TF_motif_seq_0318	1627	-	0.83	tGCGGC	TFmatrixID_1463	WRKY
TFmatrixID_1275	1589	-	0.91	agtataaTTATCtcattcctc	TFmatrixID_1480	WRKY
TFmatrixID_1274	1592	-	0.92	ataaTTATCtcattc	TFmatrixID_1484	WRKY
TFmatrixID_1321	1667	-	0.86	ttctctcttttcAAGAAac	TFmatrixID_1498	WRKY
TFmatrixID_0949	1465	+	0.98	cctTCACT	TFmatrixID_1203	YABBY
TF_motif_seq_0237	1512	-	1	CATCT	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	1558	+	1	CGATA	TF_motif_seq_0241	ZF-HD

TF_motif_seq_0237	1575	-	1	AATCT	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	1597	-	1	TATCT	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	1610	+	1	GGATA	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	1615	-	1	TATCT	TF_motif_seq_0241	ZF-HD
TFmatrixID_0055	1625	-	1	tctgCGGCGg	TFmatrixID_0129	ZF-HD
TFmatrixID_0033	1628	-	1	gcggCGGCGa	TFmatrixID_0129	ZF-HD
TFmatrixID_0054	1629	-	1	cGGCGGcg	TFmatrixID_0129	ZF-HD
TFmatrixID_0078	1630	-	1	gGCGGCga	TFmatrixID_0129	ZF-HD
TFmatrixID_1211	1559	-	0.98	gatACCAActttctt	TFmatrixID_1510	ZF-HD
TFmatrixID_1535	1626	+	1	ctgcgGCGGC	TF_motif_seq_0239	#N/A
TFmatrixID_1544	1655	+	1	aAAAAGtct	TF_motif_seq_0239	#N/A
TFmatrixID_1369	1675	-	1	tTTCAA	TF_motif_seq_0239	#N/A
TF_motif_seq_0263	1441	+	0.8	GTCAC	TF_motif_seq_0249	#N/A
TF_motif_seq_0263	1581	-	0.8	GTTGC	TF_motif_seq_0249	#N/A
TF_motif_seq_0263	1584	+	0.8	GCCGC	TF_motif_seq_0249	#N/A
TF_motif_seq_0263	1628	-	0.8	GCGGC	TF_motif_seq_0249	#N/A
TF_motif_seq_0263	1631	-	0.8	GCGGC	TF_motif_seq_0249	#N/A
TF_motif_seq_0263	1634	+	0.8	GCGAC	TF_motif_seq_0249	#N/A
TF_motif_seq_0263	1644	+	0.8	GCTAC	TF_motif_seq_0249	#N/A
TF_motif_seq_0261	1669	-	0.8	CTCTC	TF_motif_seq_0249	#N/A
TF_motif_seq_0261	1681	+	0.8	GAAAC	TF_motif_seq_0249	#N/A
TF_motif_seq_0270	1440	-	1	AGTCA	TF_motif_seq_0268	#N/A
TF_motif_seq_0281	1649	+	1	AAGAAt	TF_motif_seq_0268	#N/A
TF_motif_seq_0241	1592	-	1	ATAAT	TF_motif_seq_0270	#N/A
TF_motif_seq_0241	1595	+	1	ATTAT	TF_motif_seq_0270	#N/A
TF_motif_seq_0341	1521	+	1	aAACCA	TF_motif_seq_0343	#N/A
TF_motif_seq_0341	1682	+	1	aAACCA	TF_motif_seq_0343	#N/A
TF_motif_seq_0434	1610	+	0.83	GGATAtat	TF_motif_seq_0353	#N/A
TFmatrixID_0239	1654	+	0.99	taAAAAGtct	TFmatrixID_0443	#N/A

Table A4.3.2 Transcription factor families and *cis* -elements sequences present in the deletion region of the 326-489.

ID	position	strand	Score	Sequence	Matrix ID	TF family
TF_motif_seq_0410	327	-	0.75	ataCTTAT	TF_motif_seq_0410	bHLH
TF_motif_seq_0410	327	+	0.75	ATACTtat	TF_motif_seq_0410	bHLH
TFmatrixID_0793	390	-	0.81	gatagtATATAaaata	TF_motif_seq_0300	bHLH
TFmatrixID_0525	472	-	0.82	gtcgagtctCGTGA	TF_motif_seq_0300	bHLH
TFmatrixID_0585	395	+	0.97	tatATAAAata	TFmatrixID_0808	bHLH
TF_motif_seq_0241	335	-	1	GTAAT	TF_motif_seq_0301	bHLH
TFmatrixID_0148	350	+	1	aaAAAATa	TFmatrixID_0176	bHLH
TFmatrixID_0137	351	+	1	aaaAATAT	TFmatrixID_0146	bHLH
TFmatrixID_0132	398	+	1	ataaAATAA	TFmatrixID_0138	bHLH
TFmatrixID_0131	399	+	1	taaaATAAA	TFmatrixID_0137	bHLH
TFmatrixID_0136	400	+	1	aaaATAAA	TFmatrixID_0140	bHLH
TFmatrixID_0138	400	+	1	aAAATAaa	TFmatrixID_0146	bHLH
TF_motif_seq_0239	415	+	1	AAAGA	TF_motif_seq_0246	bHLH
TFmatrixID_1066	331	+	0.87	ttatgtaATGATagt	TFmatrixID_0193	bZIP
TF_motif_seq_0258	374	+	0.8	CCAAC	TF_motif_seq_0271	bZIP
TFmatrixID_0176	424	-	0.93	cCAAGTtgt	TFmatrixID_0700	bZIP

TF_motif_seq_0258	472	-	0.8	GTCGA	TF_motif_seq_0271	bZIP
TF_motif_seq_0341	375	+	0.95	cAACCA	TFmatrixID_0225	CPP
TF_motif_seq_0341	421	+	0.95	gAACCA	TFmatrixID_0225	CPP
TFmatrixID_0808	423	-	0.88	acCAAGTtgtg	TFmatrixID_1002	CPP
TF_motif_seq_0243	360	-	1	CTATC	TFmatrixID_0896	Dof
TF_motif_seq_0268	371	-	1	AATCC	TFmatrixID_1544	Dof
TF_motif_seq_0243	390	+	1	GATAG	TFmatrixID_0905	Dof
TFmatrixID_0148	398	+	0.99	atAAAATa	TFmatrixID_0239	Dof
TFmatrixID_0806	423	+	0.94	acCAAGTtgtg	TFmatrixID_0922	Dof
TF_motif_seq_0490	429	-	0.88	ttgtGAAAC	TFmatrixID_0921	Dof
TF_motif_seq_0243	484	+	1	GATAG	TFmatrixID_0911	Dof
TFmatrixID_0803	422	-	0.99	aacCAAGTtgtgaa	TFmatrixID_1079	GATA
TF_motif_seq_0415	379	+	0.88	CAAAAtgc	TFmatrixID_0537	LBD
TFmatrixID_0812	423	-	0.96	acCAAGTtgtg	TFmatrixID_0988	LBD
TF_motif_seq_0267	335	-	0.8	GTAAT	TF_motif_seq_0261	MYB_related
TF_motif_seq_0267	344	+	0.8	GTAAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0434	353	-	0.83	aaaTATAC	TF_motif_seq_0399	MYB_related
TF_motif_seq_0434	394	+	0.83	GTATAtaa	TF_motif_seq_0399	MYB_related
TFmatrixID_0638	413	+	0.99	agAAAGAgga	TFmatrixID_0969	MYB_related
TF_motif_seq_0267	420	-	0.8	GGAAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0275	431	-	0.8	GTGAA	TF_motif_seq_0275	MYB_related
TF_motif_seq_0267	433	-	0.8	GAAAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0341	434	+	1	aAACCA	TF_motif_seq_0377	MYB_related
TF_motif_seq_0267	440	+	0.8	TTTAC	TF_motif_seq_0261	MYB_related
TF_motif_seq_0275	440	+	0.8	TTTAC	TF_motif_seq_0275	MYB_related
TF_motif_seq_0319	440	-	1	tTTACC	TF_motif_seq_0321	MYB_related
TF_motif_seq_0321	440	-	1	tTTACC	TF_motif_seq_0321	MYB_related
TF_motif_seq_0321	457	+	1	GAAAAa	TF_motif_seq_0321	MYB_related
TF_motif_seq_0343	462	+	0.86	AAACAag	TF_motif_seq_0363	MYB_related
TF_motif_seq_0275	472	-	0.8	GTCGA	TF_motif_seq_0275	MYB_related
TF_motif_seq_0300	479	-	0.83	cTCGTG	TF_motif_seq_0434	MYB_related
TF_motif_seq_0300	479	+	0.83	CTCGTg	TF_motif_seq_0434	MYB_related
TF_motif_seq_0323	480	-	1	tCGTGA	TF_motif_seq_0341	MYB_related
TF_motif_seq_0271	481	-	0.8	CGTGA	TF_motif_seq_0275	MYB_related
TF_motif_seq_0508	486	-	0.75	taGTATGaa	TF_motif_seq_0450	MYB_related
TFmatrixID_1063	331	-	0.86	ttatgtaATGATagt	TFmatrixID_1321	NAC
TF_motif_seq_0252	371	-	1	AATCC	TFmatrixID_1139	NAC
TFmatrixID_0569	396	+	0.97	atATAAAataaaatg	TFmatrixID_0763	NAC
TFmatrixID_1378	453	+	0.91	agaagAAAAAaacaa	TFmatrixID_1605	NAC
TF_motif_seq_0254	333	+	0.8	ATGTA	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	346	+	0.8	AACTA	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	355	+	0.8	ATATA	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	362	+	0.8	ATCTC	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	372	+	0.8	ATCCA	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	395	-	0.8	TATAT	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	396	+	0.8	ATATA	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	439	+	0.8	ATTTA	TF_motif_seq_0257	NF-YB
TF_motif_seq_0254	466	-	0.8	AAGAT	TF_motif_seq_0257	NF-YB
TFmatrixID_0357	352	+	0.97	aaAATATact	TFmatrixID_0585	NF-YC
TFmatrixID_0134	454	+	0.98	gaagaAAAAA	TFmatrixID_0291	Nin-like
TF_motif_seq_0239	411	+	1	AAAGA	TF_motif_seq_0244	SBP

TF_motif_seq_0243	340	+	1	GATAG	TFmatrixID_0791	SRS
TFmatrixID_0518	331	+	0.78	ttatgtaATGATagtaact	TFmatrixID_0542	TCP
TFmatrixID_0542	331	+	0.78	ttatgtaATGATagtaact	TFmatrixID_0108	TCP
TFmatrixID_0793	349	-	0.86	taaaaaATATActatc	TFmatrixID_1063	TCP
TFmatrixID_0818	423	-	0.98	acCAAGTtgtg	TFmatrixID_1070	TCP
TFmatrixID_0108	331	+	0.8	ttatgtaATGATagtaact	TF_motif_seq_0254	Trihelix
TF_motif_seq_0257	374	+	0.8	CCAAC	TF_motif_seq_0267	Trihelix
TF_motif_seq_0257	378	+	0.8	CCAAA	TF_motif_seq_0267	Trihelix
TF_motif_seq_0257	424	+	0.8	CCAAG	TF_motif_seq_0267	Trihelix
TF_motif_seq_0249	425	-	0.8	CAAGT	TF_motif_seq_0254	Trihelix
TF_motif_seq_0248	**437**	**-**	**0.8**	**CCATT**	TF_motif_seq_0254	Trihelix
TF_motif_seq_0257	437	+	0.8	CCATT	TF_motif_seq_0267	Trihelix
TF_motif_seq_0257	444	+	0.8	CCAAG	TF_motif_seq_0267	Trihelix
TF_motif_seq_0249	479	-	0.8	CTCGT	TF_motif_seq_0254	Trihelix
TF_motif_seq_0249	480	+	0.8	TCGTG	TF_motif_seq_0254	Trihelix
TFmatrixID_0418	396	+	1	ATATAaa	TFmatrixID_0451	WRKY
TF_motif_seq_0261	477	-	1	GTCTC	TFmatrixID_1477	WRKY
TF_motif_seq_0237	339	+	1	TGATA	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0267	344	-	1	GTAAC	TFmatrixID_1508	ZF-HD
TF_motif_seq_0237	361	-	1	TATCT	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	371	-	1	AATCC	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	389	+	1	TGATA	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	467	+	1	AGATG	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0237	483	+	1	TGATA	TF_motif_seq_0241	ZF-HD
TF_motif_seq_0261	362	-	0.8	ATCTC	TF_motif_seq_0249	#N/A
TF_motif_seq_0261	367	+	0.8	GAGAA	TF_motif_seq_0249	#N/A
TF_motif_seq_0261	433	+	0.8	GAAAC	TF_motif_seq_0249	#N/A
TF_motif_seq_0261	475	+	0.8	GAGTC	TF_motif_seq_0249	#N/A

Appendix-5.

This section is relevant to chapter 5 of the thesis

5.1 Construction of SAG21.:: SAG21-ORF-His construct

The SAG21::SAG21-ORF-His construct was assembled in the pGEM-T Easy (Promega) vector by amplification of a 1685 bp fragment upstream of the *SAG21* start codon using primers 5′-ATTGTCGACTAATCTCCAAAACATTGTG that incorporates a SalI restriction site and 5' –AGCCATGGTCGAAGTAAGTGG incorporating an NcoI site using a SAG21 promoter-GUS construct (Salleh et al., 2012) as template. The open reading frame was amplified separately using NcoIF 5'-CGACCATGGCTCGTTCTATCTCTAACG incorporating an NcoI site and 5'CTTGAACAACAAGCAGCATCACCATCACCATCACTGAGCGGCCGCTA incorporating a Not I site and a His-Tag sequence using a 35S:: SAG21-YFP fusion construct (Salleh et al., 2012) as template and was inserted into the NcoI and NotI sites of pGEM-T Easy containing the promoter fragment. The assembled construct was then excised from pGEM-T Easy using SalI and EcoRI and cloned into the pGREEN vector containing the Nos terminator. The pGREEN construct was then transformed into *Agrobacterium tumefaciens* GV3101, and then transformed into *Arabidopsis* plants using the floral dip method (Clough and Bent 1998) as adapted by (Logemann et al. 2006). Transformants after floral dipping were selected on 1 x MS medium containing 50 $\mu g ml^{-1}$ kanamycin. Twenty primary transformants were tested by PCR on genomic DNA extracted from young leaves using primers NcoIF and SAG21R 5'CCGGTTTCGGGTCTGTAATA which amplify both the endogenous gene (336 bp) and the transgene that lacks the intron (236 bp) and 19 independent transformants were obtained.