ABSTRACT

Title of Dissertation: FUNCTIONAL ANALYSIS OF THREE *ARABIDOPSIS* TRANSCRIPTIONAL CO-REPRESSORS LINKING FLORAL REGULATORS TO ABA HORMONE SIGNALING

Jayashree Sitaraman, Doctor of Philosophy, 2004

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Hormonal signaling and developmental patterning have mostly been studied as separate and independent processes. However, the highly plastic development of plants suggests that plant development must incorporate environmental and physiological signals and adapt accordingly. The question of how development and hormone signaling influence each other needs to be answered yet. This work shows that the regulators of flower development such as *LUG*, *LUH* and *SEU* also function in ABA (Abscisic Acid) hormone signaling. Further, it is shown that *LUG* and *LUH*, two paralogs in *Arabidopsis*, are partially redundant in function, but each plays a more prominent role in a different process. This study provides evidence that the basic mechanisms of plant developmental regulation and hormonal signaling are interconnected and utilize similar regulatory components.

LUG and *SEU* are two transcription co-repressors previously shown to regulate the spatial and temporal domains of expression of a key regulator of flower development *AGAMOUS* (*AG*) in *Arabidopsis*. LUG and SEU was shown to form a co-repression complex similar to the TUP1/Ssn6 co-repressor complex of yeast. In *Arabidopsis*, the one and only homolog of *LUG*, *LUH,* is 44% identical to *LUG*. However, its function is completely unknown. A putative *luh* null mutation was identified and *luh* mutants show reduced sensitivity to ABA and reduced expression of ABA-response genes. *LUH* is proposed to act to repress the transcription of *ERA1*, a negative regulator of ABA signaling. This is supported by increased *ERA1* expression in *luh-1* mutants. This study also suggests a possible role of *LUG* and *SEU* in ABA signaling.

Although *luh* mutant by itself does not have a floral phenotype, *luh* has been found to interact with *lug* and *seu* genetically during flower development, based on the enhanced floral phenotype in the double mutants between *luh* and *lug* and between *luh* and *seu*. Yeast two hybrid data suggests that, like LUG, LUH also physically interacts with SEU to form a co-repressor complex. Thus, while *LUG* plays a major role in flower development, *LUH* plays a more important role in ABA signaling.

FUNCTIONAL ANALYSIS OF THREE *ARABIDOPSIS* TRANSCRIPTIONAL CO-REPRESSORS LINKING FLORAL REGULATORS TO ABA HORMONE SIGNALING

By

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Dedication

Dedicated to my family, for always being there

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List of Abbreviations

ABA	Abscisic acid
ABI1	Abscisic acid insensitive
ABF	ABRE Binding Factor
ABH1	Abscisic acid hypersensitive 1
ABRE	ABA Response Elements
ACT	*ACTIN*
AG	*AGAMOUS*
ANT	AINTEGUMENTA
AP1	*APETALA1*
AP2	*APETALA2*
ATHB	*Arabidopsis thaliana* Homeobox protein
BLR	*BELLRINGER*
CADPR	Cyclic ADP Ribose
CAL	*CAULIFLOWER*
CO	*CONSTANS*
Col	Columbia
CRY	Cryptochrome
dCAPS	Derived Cleaved Amplified polymorphic sequence
DRE	Drought Response Element
ERA1	*Enhanced Response to ABA1*
EREBP	Ethylene Response Element Binding Protein
FLC	*Flowering Locus C*
FRI	*FRIGIDA*
FRY	*FIERY*
GI	*Gigantea*
GRO	GROUCHO
HAG	*Hyacinth AG*
HAT	Histone Acetyltransferase
HDAC	Histone Decetylases
HYL1	*Hyponastic Leaves 1*

IM	Inflorescence Meristem
Ler	Landsberg erecta
LFY	*LEAFY*
LUG	*LEUNIG*
LUH	*LEUNIG HOMOLOG*
MADS	MCM1, AG, DEFICIENS, SRF
PHY	PHYTOCHROME
RACE	Rapid Amplification of cDNA Ends
SAD1	Supersensitive to ABA and Drought1
TILLING	Targeting Induced Local Lesions IN Genomes
SAM	Shoot Apical Meristem
SEP	*SEPALLATA*
STY	*STYLOSA*
VRN	*Vernalization*
WUS	*WUSCHEL*
ZAG	*Zea AG*

Chapter one

LITERATURE REVIEW

A. FLOWER DEVELOPMENT

In the past decades, *Arabidopsis* has virtually been the workhorse of plant researchers worldwide. This tiny weed plant has risen to such a status due to the ease with which it can be handled and manipulated in the laboratory. From flowering to physiology, metabolism to microbe interaction, plant researchers have provided valuable insights using *Arabidopsis* as the model plant.

Arabidopsis thaliana is a dicotyledonous flowering plant that belongs to the mustard family, Brassicaceae. The small genome size, the relative ease of propagating the plant under laboratory conditions, short generation time, production of plenty of seeds and the ease of transformation by simple techniques are but a few of the aspects that has endeared *Arabidopsis* to the plant research community. *Arabidopsis* plant has a whorl of rosette leaves at its base and an inflorescence (flower stalk). Cauline leaves are borne on the flower stalk, subtending branches bearing flowers (Meyerowitz et al., 1994).

Flowers are composed of floral organs: sepals, petals, stamens and carpels (Fig 1-1), in four concentric whorls. There are 4 sepals, 4 petals, 6 stamens and 2

carpels. Two carpels are fused to form the central gynoecium or pistil, which is composed of the style, stigma and ovaries. The ovaries contain rows of ovules, each bearing an embryo sac (female gametophyte). A stamen is composed of an anther, which bears pollen grains (the male gametophytes), and a supporting filament.

Through pollination pollen grains gain entry into the gynoecium through the long tube-like style. Once inside the gynoecium, the diploid zygote is formed by the fusion of the pollen with a single egg cell. The triploid endosperm is formed by the fusion of the pollen with two polar nuclei in the female gametophyte. Endosperm is the protective, nutritive sac that nourishes the embryo (Howell S, 1998).

The propagation of its species through reproduction and procreation is inherent in any living organism. Flowers are the main organs of the plants through which reproduction and formation of seeds for the next generation takes place. Therefore, flowers are important parts of the plant and hence warrant a detailed study of its development. Research on flowering in *Arabidopsis* spans many aspects of flowering, from the perception of the flowering signal to the inception of the floral bud and the subsequent development of the flower. The first part of this chapter focuses on what is known about flowering in general and the important players identified so far.

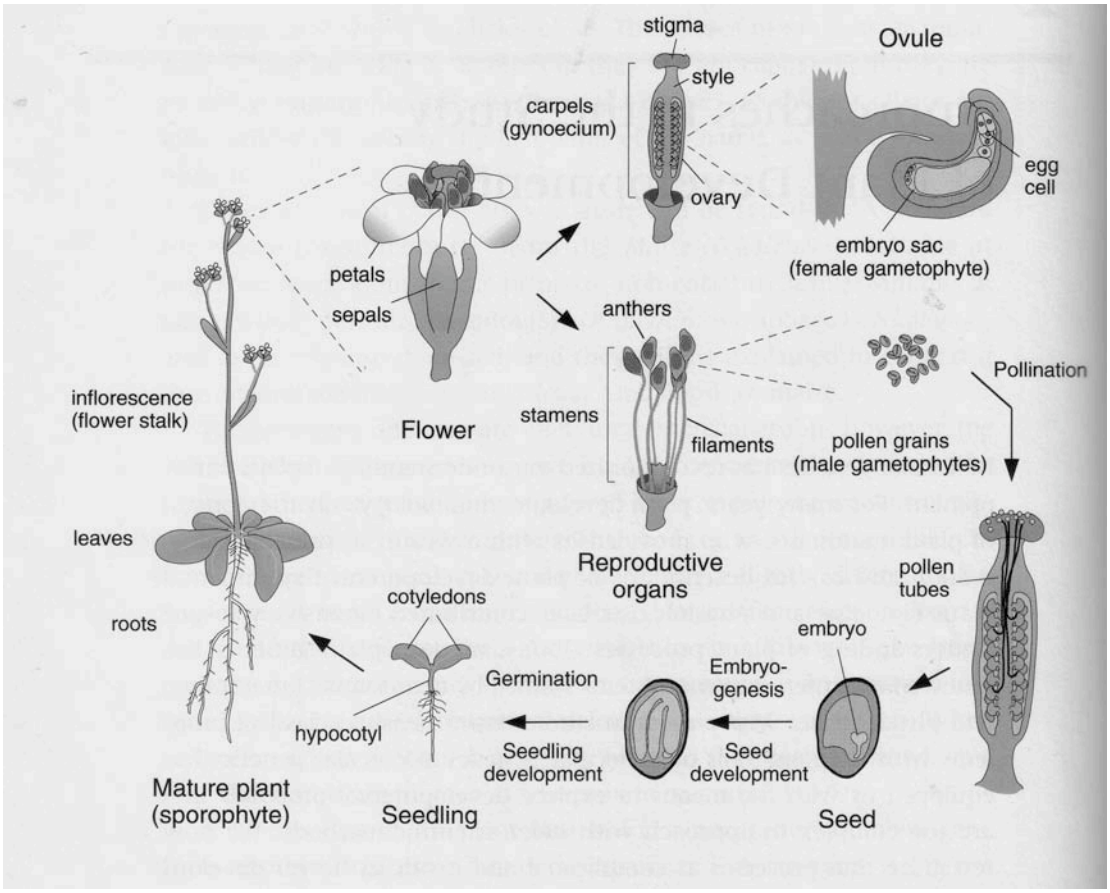


FIG 1-1 Life cycle of *Arabidopsis thaliana* (Adapted from Howell S, Cambridge University Press)

TO FLOWER OR NOT TO FLOWER- the flowering pathway

The commitment to flowering in plants is a terminal event and as such requires the input of many factors both internal and external. Therefore, it is not surprising that there are a variety of pathways involving a plethora of genes that control flowering. In effect, flowering is the cessation of the vegetative phase and the commencement of the reproductive phase.

Environmental factors such as light, day length period and prolonged periods of cold (vernalization) work in conjunction with internal cues like hormones to set flowering in motion. These stimuli converge on the shoot apical meristem (SAM). Shoot apical meristem is a rapidly dividing mass of cells that are organized in three different layers L1, L2 and L3. SAM cells can self-renew and give rise to lateral organs (Fig 1-2F). During vegetative growth, SAM gives rise to leaves and secondary shoots. Upon receiving proper environmental stimuli and developmental stage, SAM switches to produce floral meristems instead of leaves and shoots. This switch from the vegetative phase to the reproductive phase results in the formation of inflorescence meristem (IM). Once the floral meristems are formed, flower organs are formed by the action of organ identity genes (FIG 1-2) (Zik and Irish, 2003).

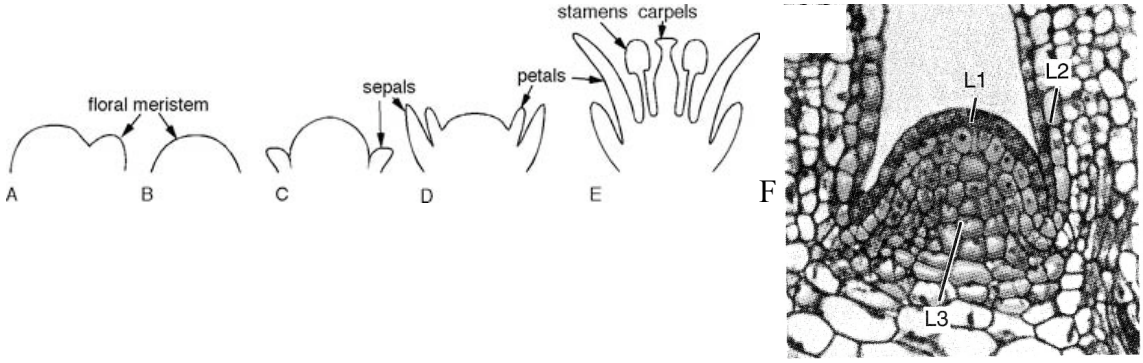


FIG 1- 2. Development of the flower. Flowers develop from floral meristems, which often arise on the flanks of an inflorescence meristem (A). The floral meristem is a small domed population of cells consisting of several layers of cells (B). Cells on the flanks of the floral meristem proliferate to first form the sepal primordia (C), followed by the petal primordia (D). Eventually, the entire floral meristem forms the determinate floral structure, consisting of sepals, petals, stamens, and carpels (E). (F). Organization of SAM in L1, L2 and L3 layers. (Zik and Irish., 2003)

PHOTOPERIOD PATHWAY

Arabidopsis is a facultative long-day plant, which flowers earlier under long days (such as 16 hours light and 8 hours dark). Based on mutants that are delayed in flowering in long days, the genes that function in the PHOTOPERIOD PATHWAY of flowering have been identified and their functions elucidated. The genes in this pathway are thought to be involved in promoting flowering under long day conditions. Light is perceived through receptors called phytochromes (red light and far red light receptors) and cryptochromes (blue light receptors).

Five types of phytochromes – *PHYA-PHYE* (Hirshfeld et al., 1998) and two cryptochromes *CRY1* and *CRY2* (Cashmore, 1998; Cashmore et al., 1999) have been identified in *Arabidopsis*. *phyB* (red light receptor) mutants exhibit early flowering while *cry1* (blue light receptors) mutants exhibit delayed flowering (King and Bagnall, 1996). In contrast, *phyA* (far red light receptor) mutants are late flowering in long days (Johnson et al., 1994). These data suggest that *PHYA, CRY1* promote flowering; *CRY2* has been shown to act as positive regulator of flowering by inhibiting the *PHYB* mediated repression of flowering (Guo et al., 1998). The other genes that are involved in this pathway are *CONSTANS* (*CO*), *GIGANTEA* (*GI*), *FT* and *FWA* (Koornneef et al., 1991). *CO* encodes a protein consisting of two Zinc fingers (Putterill et al., 1995) and a carboxy-terminal domain called CCT. CCT is essential for the nuclear localization of CO (Strayer et al., 2000; Robson et al., 2001). *CO* has been shown to promote flowering and *CO* mRNA levels are elevated under long-day conditions (Putterill et al., 1995; Simon et al., 1996). In addition, *CO* transcript level shows a diurnal rhythm in long days, indicating that *CO* is part of the circadian clock (Suarez-Lopez et al., 2001). An early target of *CO*, *FT* also is part of the circadian clock, as *FT* mRNA levels follow a circadian rhythm in long days (Samach et al., 2000). *GI* is another player in the photoperiod pathway of flowering, that encodes a protein with six transmembrane domains and functions upstream of *CO* and *FT* (Fowler et al., 1999; Park et al., 1999). *gi* mutants are late flowering

under long day conditions and has been suggested to function in the *PHYB* mediated signal transduction pathway (Huq et al., 2000).

VERNALIZATION PATHWAY

The winter annual ecotypes of *Arabidopsis* do not flower until they have had an exposure to low temperatures for several weeks. They tide over unfavorable winter conditions and flower in spring or summer. This is in contrast to the summer annual varieties, which germinate and flower in the same season. The difference between these ecotypes is attributed to the *FLC* locus and *FRIGIDA* (*FRI*). The summer annual varieties were found to have loss-of-function mutations at the *FRI* locus (Johanson et al, 2000; Gazzani et al., 2003). The product of the gene *FRIGIDA* (*FRI*), is an activator of FLC and increases FLC mRNA abundance (Michaels and Amasino, 1999; Sheldon et al., 1999). Loss of FRI results in low FLC, the repressor of flowering. Hence, summer annuals can flower without vernalization. The vernalization-requiring winter annual ecotypes, have functional *FRI* and thus higher levels of *FLC* mRNA and protein, which inhibits flowering. *FLC* mRNA levels fall, when plants are exposed to low temperatures (Sheldon et al., 2000; Michaels and Amasino, 1999). Thus, the repressive effect of *FLC* can be overcome by vernalization. It has been proposed that vernalization is regulated by an epigenetic mechanism through regulation of gene expression by methylation. Demethylating

agents cause early flowering of late- flowering mutants of *Arabidopsis,* and transient reduction in methylation in vernalized plants indicate that epigenetic regulation plays a role in flowering (Finnegan et al., 1998).

Recent data suggest that the *FLC* repression of flowering is mediated by histone modification at *FLC* chromatin. Acetylation or deacetylation at the histone tails is associated with activation or repression of gene expression, respectively. Histone H3 deacetylation, following vernalization, has been detected at the 5' regulatory region of *FLC* (Sung and Amasino, 2004). Vernalization also increases methylation at H3 K9 and H3 K27, which maintain the repressed state of FLC (Sung and Amasino, 2004). Upstream trans-activating factors that are involved in the repression of *FLC* in response to cold have been identified. One such factor is *VERNALIZATION INSENSITIVE 3 (VIN3*), which encodes a protein containing the Plant Homeodomain (PHD) and fibronectin type III repeats. The histone deacetylation and methylation at the *FLC* chromatin associated with vernalization is affected in *vin3* mutants (Sung and Amasino, 2004). However, how *VIN3* is upregulated in response to cold is not known yet. Other genes that are involved in this pathway are *VRN1* and *VRN2*. In contrast to *VIN3*, which is required for the repression of *FLC*, *VRN1* and *VRN2* seem to be required for maintaining repression of *FLC*. In *vrn1* and *vrn2* mutants, *FLC* mRNA levels have been shown to increase,

indicating a lack of repression (Chandler et al., 1996; Gendall et al., 2001; Levy et al., 2002). VRN2 protein is homologous to the Polycomb-group protein, Suppressor of Zeste 12 (Su(z)12), of *Drosophila*, which is involved in maintaining repression of homeotic genes (Orlando, 2003).

AUTONOMOUS PATHWAY

Mutants that are late flowering under both long and short days fall under the class of flowering mutants – the AUTONOMOUS PATHWAY mutants. *FCA, FY, FPA, LD, FVE, FLD* have been shown to function in this pathway. A common feature of the autonomous pathway mutants is that they are responsive to vernalization (Koornneef et al., 1991; Sheldon et al., 2000; Michaels and Amasino, 2001) . An interesting feature in all these mutants is an elevated level of *FLC* (*FLOWERING LOCUS C)* mRNA. This suggests that the autonomous pathway genes act in parallel to vernalization pathway genes to repress *FLC* expression. The FVE/FLD proteins function with a histone deacetylase (HDAC) to bring about the repression of *FLC* (Ausin et al., 2004).

Other factors like the hormone Gibberellic acid (GA) and sugar have also been implicated in orchestrating flowering responses (Wilson et al., 1992; Zeevart et al., 1983; Bernier et al., 1993). Gibberellic acid has been shown to accelerate flowering through activation of *LFY* (Blazquez et al., 1998; Blazquez and Weigel,

2000). Fig 1-3 shows a schematic representation of the integration of various flowering pathways.

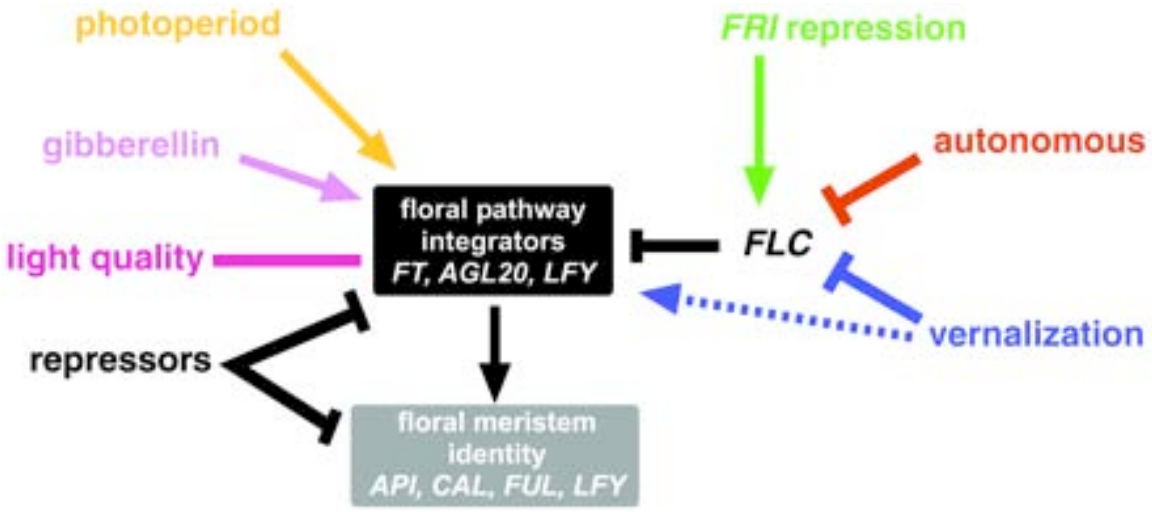


FIG 1-3. Integration of Flowering Pathways (Adapted from Simpson and Dean, 2000)

MERISTEM AND FLORAL ORGAN IDENTITY GENES

Once the plant is committed to flowering, the vegetative SAM is converted to the inflorescence meristem (IM) by the concerted action of at least three genes, *LEAFY* (*LFY*), *AP1* and *CAULIFLOWER* (*CAL*) with *LFY* playing the leading role of an activator. In *lfy* mutants, *AG* expression is delayed, suggesting that *LFY* activates *AG* (Weigel and Meyerowitz, 1993). Transgenic plants harboring LFY::VP16, a viral transcriptional activator, exhibited both ectopic and precocious *AG* expression (Parcy et al., 1998), suggesting a direct role for *LFY* on *AG* activation. In *ap1* mutants, leaf-

like organs are formed in the first whorl instead of sepals and there is a lack of petals (Gustafson-Brown et al., 1994). In *ap1 lfy* double mutant, flowers are completely transformed into leaves and shoots indicating that *AP1* and *LFY* together act to promote flower identity (Weigel and Meyerowitz, 1993). Mutants of *CAL* do not have a phenotype. However, *cal ap1* double mutants have a more dramatic phenotype than *ap1*, indicating that the function of *CAL* is redundant to that of *AP1* (Kempin et al., 1995), but *CAL* function is needed in *ap1* background.

THE ABCE MODEL

Dicotyledon flowers are organized into four concentric rings or whorls. Starting from the outermost whorl, four sepals (also known as the calyx) are formed. Sepals are green in color and serve to enclose the inner and the reproductive parts of the flower. The second whorl is occupied by four petals otherwise known as the corolla. The first and second whorls are together referred to as the perianth. The third and the fourth whorls are occupied by the most important parts of the plant, the stamens and the carpels, respectively. The stamens, numbering six, are the male reproductive organs and the two fused carpels are the female reproductive organs.

How does a flower know which organs should be produced in which whorl? In other words, what would happen if the flower organs were not produced in the

proper whorls? The answers to these questions came in the form of the ABC model. The idea of this model is that a flower needs to know its ABCs to be a flower! In its simplest form, the model proposes that the correct organs are formed in the right positions through the combinatorial actions of three classes of genes. These genes are called the homeotic genes or organ identity genes. The A class genes are *APETALA 1* (*AP1*) and *APETALA 2* (*AP2*) that function in the first 2 whorls to ensure the formation of sepals and petals. *APETALA 3* (*AP3*) and *PISTILLATA* (*PI*) are the B class genes genes that function in the second and third whorls, to specify petal (with A) and stamens (with C). *AGAMOUS* (*AG*) is the lone C class gene that acts in the third and the fourth whorls of the flower to specify stamens and carpels, respectively. The results of mutations in the A, B, C class genes could be predicted from the model.

The actions of A and C class genes are mutually antagonistic and thereby prevent each other from encroaching the other's territory. Thus, in the class A mutant, C is ectopically expressed in the first two whorls and in the class C mutant, A is expressed in inner two whorls. In a class A mutant, third and fourth whorls develop normally. However, sepals are converted to carpels and petals are converted to stamens due to ectopic C activity spreading into first two whorls. In a plant lacking a B class gene, the first two whorls are sepals and the inner two whorls develop into carpels. In a C class mutant, sepals and petals are formed in a reiterative fashion,

resulting in the formation of a flower within a flower. This suggests that *AG* is required for maintaining floral meristem determinacy. When all the A B C class genes were mutated, only leaves were formed in all four whorls. Thus, leaves are the ground state, when no organ identity gene is active (Bowman et al., 1991; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; Weigel and Meyerowitz 1994).

In a recent study, another class of genes, namely the E class, have been added to this model. This class comprises of *SEPALLATA1* (*SEP1*), *SEP2* and *SEP3* genes. Through elegant mutant studies, it has been shown that together with the A B C genes, *SEP* genes are required to specify the floral organ identity (Fig 1- 4). The three *SEP* genes function redundantly and are necessary for the formation of petal, stamen and carpel. In triple *sep1, sep2* and *sep3* mutants, only sepals are made in all four whorls (Pelaz et al., 2000; Honma and Goto, 2001).

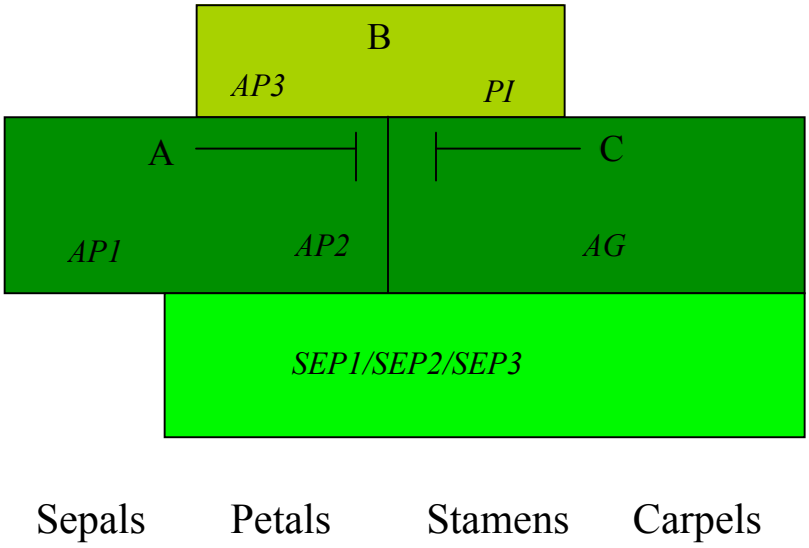


FIG 1-4. The ABCE genes specify floral organ identity. The three *SEP* genes function redundantly and are necessary for petal, stamen and carpel development. In whorl 1, A class activity specifies sepals; in whorl 2, A+B+*SEP* activities specify petals; in whorl 3, B+C+*SEP* activities specify stamens; and in whorl 4, C+*SEP* activities specify carpels.

All of the ABCE genes except *AP2* are known to encode MADS box transcription factors. MADS domain stands for MCM1 (yeast), AG (Arabidopsis), DEFICIENS (Antirrhinum) and SRF (Human). The basic N-terminal half of the MADS-domain is required for DNA binding and the C-terminal half for dimerization (Reichmann et al., 1996). A second conserved domain, the K box, was identified based on its similarity to the coiled-coil domain in keratin (Ma et al., 1991). Between the MADS domain and the K box region lies a less conserved linker (L) region which, together with the K box, is important for partner specificity in dimer formation (Reichmann et al., 1996). The MADS-box proteins bind to consensus sequence

$CC(A/T)_6GG$, which is known as the CArG-box (Schwarz-Sommer et al., 1992; Wynne and Treisman, 1992; Huang et al., 1993; Shiraishi et al., 1993). MADS box proteins function in complexes as multimers to specify floral organ identities (Honma and Goto, 2001; Jack, 2001).

In *Antirrhinum* and petunia, two dicot plants that belong to the Scrophulariaceae and Solanaceae family respectively, *SQUAMOSA* is the *AP1* ortholog (Huijser et al., 1992) and *BLIND* is an ortholog of *AP1* in petunia. *DEF* and *GLO* are orthologous to *AP3* and *PI* of *Arabidopsis*, and are essential for B function in *Antirrhinum* (Sommer et al, 1990; Trobner et al., 1992). In petunia, *FBP1* has been shown to be a B class gene similar to *PI* gene in *Arabidopsis* required for petal and stamen development (Angenent et al., 1992, 1994). The *AG* orthologs *PLENA (Antirrhinum*) and *pMADS3* and *FBP6* (petunia) have functions similar to *AG* in specifying the reproductive structures of the flowers (Bradley et al., 1993; Tsuchimoto et al., 1993; Kater et al., 1998). Although the arrangement of flowers in the monocots is different from that of the dicots, the function of A B C genes is largely conserved. *ZAP*, the maize ortholog of *AP1*; *SILKY* (maize) and *OsMADS16* (Rice), similar to B class genes and maize C class gene *ZAG1*, are few genes that function similar to A B C class genes. In contrast to *AG*, which is required for both stamen and carpel development, *zag1* mutation does not affect stamen development. *zag1* mutants exhibit supernumerary carpel formation, indicating a lack of floral -

meristem determinacy. This suggests that the basic mechanism of regulation of flower development is conserved across species (Ambrose et al., 2000; Schmidt et al., 1993; Moon et al., 1999).

AG is involved in maintaining floral meristem determinacy as shown by the formation of flower- within- flower in *ag* mutant (Bowman et al., 1989). The expression of *AG* in the floral meristem requires *LFY*, and *WUSCHEL* (*WUS*), a homeodomain containing protein known to stimulate stem cell proliferation (Busch et al., 1999; Lenhard et al., 2001; Lohmann et al., 2001). Once *AG* expression is initiated, *WUS* expression is repressed by *AG*, to ensure the determinacy of the floral meristem (Lenhard et al., 2001; Lohmann et al., 2001).

If the A,B,C,E genes are only expressed in specific whorls in order to specify proper floral organs in proper whorls, what determines the spatial domains of A, B, C, E gene expression? The cadastral genes function in limiting the homeotic gene expression to their respective whorls. Many negative regulators of the C class gene *AG* have been studied in detail. The absence of these negative regulators or repressors of *AG* lead to the ectopic expression of *AG* in the first two whorls of a flower. This results in the homeotic conversion of the sepals to carpels and the petals to stamens. *AP2* (Drews et al., 1991), *AINTEGUMENTA* (*ANT*) (Krizek et al., 2000), *STERILE APETALA* (*SAP*) (Byzova et al., 1999), *CURLY LEAF* (*CLF*) (Goodrich et al., 1997)

are a few such negative regulators. Recently, *LEUNIG* (*LUG*), *SEUSS* (*SEU*) and *BELLRINGER* (*BLR*) have been identified and characterized in our lab. They also belong to this class of cadastral genes.

APETALA2 and *AINTEGUMENTA*

APETALA2 (*AP2*), an A class gene was identified as a negative regulator of *AG*, based on ectopic expression of *AG* in *ap2* mutant flowers. AP2 is the only A B C class protein that is not a MADS–box protein. AP2 has two 68 amino acid repeat sequences, known as the AP2 domain. Recently, a microRNA (miRNA) has been shown to function as a translational repressor of *AP2* in flower development (Chen, 2004). miRNAs are a class of small (20nt-24nt) non-coding RNA species produced from large precursor RNAs with hairpin structure (Hutvagner and Zamore 2002). These miRNAs are formed by the chopping up of larger precursor RNAs by *DICER-LIKE1* (*DCL1*), a homolog of *DICER*, an RNA-specific endonuclease required for production of mRNA in animals (Jacobsen et al., 1999; Golden et al., 2002). This miRNA (miRNA172), can base-pair with the mRNA of *AP2*, thus regulating the expression of *AP2* through translational inhibition.

AINTEGUMENTA (*ANT*), though not an ABCE class gene, has been found to show characteristics of A class gene. *ant* mutants exhibit narrow floral organ shape

and a decrease in floral organ number (Elliott et al., 1996; Klucher et al., 1996). *ANT* is a member of the AP2/ EREBP family of transcription factors, and acts as a negative regulator of *AG* (Krizek et al., 2000). Furthermore, *ANT* is involved in ovule and female gametophyte development (Elliott et al., 1996; Klucher et al., 1996). ANT has been shown to bind sequences in the *AG* regulatory region in vitro, suggesting that *ANT* may be involved in direct regulation of *AG* (Nole-Wilson and Krizek, 2000).

LEUNIG, THE CO-REPRESSOR

LUG was the first negative regulator of *AG* isolated in our lab. The *lug* mutant was identified in a genetic screen for enhancer mutations of *ap2* (Liu amd Meyerowitz, 1995*)*. In the *lug* mutant, ectopic expression of *AG* in the first two whorls of a flower lead to the homeotic transformation of sepals to carpels and petals to stamens. This identified *LUG* as a repressor of *AG* and *LUG* restricts *AG* transcription to the inner two whorls of a flower (Liu and Meyerowitz, 1995).

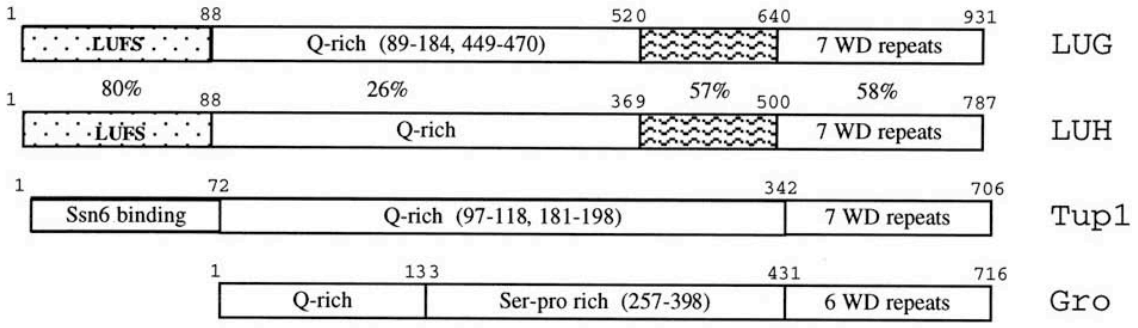


FIG 1-5. Schematic diagram of LUG and LUH structure, together with Tup1 in yeast and *Groucho* (Gro) in Drosophila. (Modified from Conner and Liu, 2000).

LUG encodes a protein containing a LUFS domain, two Glutamine (Q) rich regions and 7 WD repeats (Fig 1- 5). The name LUFS is derived from LUG, LUH, FLO8 and ssDP. All four proteins have this motif. FLO8 is a yeast transcriptional activator and ssDP is a single strand DNA binding protein from humans. LUFS and WD motifs are involved in protein-protein interactions. LUG has a putative nuclear localization signal and has been shown to localize to nucleus in onion epidermal cells (Conner and Liu, 2000). *LUG* has been proposed to be a co-repressor based on its structural similarities to Tup1 (yeast) and *Groucho* (*Drosophila*) (Conner and Liu, 2000). The mechanism of how these co-repressors repress target gene expression has been studied in details in yeast and *Drosophila* (Williams and Trumbly, 1990) and could serve as a guide for our study of *LUG*. The Tup1/Gro proteins do not have a DNA-binding domain. The Tup1/Gro co-repressors are recruited by DNA-binding transcription factors to repress target gene expression. In the case of yeast, Tup1 interacts with an adaptor protein Ssn6 that directly interacts with transcription factors (Smith and Johnson, 2000). Although the exact mechanism underlying Tup1-Ssn6 mediated repression is not known, it is proposed that Tup1 interacts with chromatin modifying enzymes such as Rpd3 as well as transcription machinery to bring about repression (Davie et al., 2003). It has been shown that Tup1 can organize repressive chromatin structure through direct interaction with the N-terminal regions of histones H3 and H4 (Edmondson et al., 1996).

Interestingly, recent studies in another flowering plant, *Antirrhinum*, have shown the presence of a *LUG* orthologue named, *STYLOSA (STY*). Similar to LUG, STY has a N-terminal LUFS domain, centrally located Q-rich region and WD repeat domain at the C-terminus. However, STY has shorter Q-rich regions and an additional WD repeat (Navarro et al., 2004). A STY-like protein, STY-L, has sequence similarity to LUH in *Arabidopsis*. Yeast two hybrid results showed that STY interacts with GRAMINIFOLIA (GRAM), a member of the plant –specific YABBY protein family. YABBY proteins have a highly conserved N-terminal domain Zinc-finger domain and the YABBY domain. This indicates that both in *Arabidopsis* and in *Antirrhinum*, similar proteins function in similar regulatory patterns suggesting the possible conservation of regulatory mechanisms across species. Similar to *lug* mutants, *sty* mutants also show pleiotropic phenotypes such as alteration in leaf venation patterns and hypersensitivity to auxin (Navarro et al., 2004).

SEUSS acts in the same complex as *LUG*

SEUSS (SEU) has been shown to be another negative regulator of *AG* repression. *seu* mutants display a phenotype similar to that of *lug*. *seu lug* double mutants show an enhanced phenotype with narrow and reduced number of floral organs and more

complete conversion from sepals to petals. Like in *lug* mutants, *seu* mutants also show precocious and ectopic expression of *AG* (Franks et al, 2001).

SEU has been cloned and has been shown to encode a protein containing Q-rich regions and a dimerization domain. This dimerization domain is similar to the Ldb proteins found in animals. Ldb proteins are transcriptional co-regulators that mediate their activity through physical interactions with LIM domain containing homeobox proteins (Agulnick et al., 1996; Bach et al., 1997; Jurata and Gill, 1997). Ldb proteins contain LIM Interacting Domain or LID. However, there is no region that is homologous to LID in SEU (Franks et al., 2001).

The genetic interaction between *SEU* and *LUG* has been further corroborated by a physical interaction between these two proteins by yeast two hybrid assay and *in vitro* pull down (Sridhar et al, 2004). Yeast two hybrid experiments performed in our lab showed that it is through the LUFS region that LUG interacts with SEU indicating that the LUFS motif might play an important role in protein-protein interaction (Sridhar et al., 2004). *In vivo* assays using *Arabidopsis* protoplasts have shown that *LUG* can repress any gene when LUG is tethered to their promoters. On the contrary, SEU does not have any repressor activity when tethered to the promoter of target genes, but can bridge the interaction between LUG and DNA-binding transcription factor (Sridhar et al., 2004). However, since SEU does not possess any DNA binding

domain, the LUG-SEU co-repressor complex may interact with as yet unidentified DNA-binding partners, which direct the LUG/SEU co-repressor complex to the target genes including *AG*.

BELLRINGER, a direct regulator of *AG*

The much elusive DNA binding partner of *LUG* and *SEU* in the transcriptional repression of *AG*, was cloned in the form of *BELLRINGER* (*BLR*). BLR belongs to the BELL-type homeobox protein family. The BELL family members are known to heterodimerize with *KNOTTED* type homeodomain protein. *blr* mutants do not have a floral phenotype on their own under normal conditions. However, at higher temperatures, *blr* mutants exhibit a phenotype similar to that of *lug* and *ap2*.

Moreover, *blr* enhances *lug* mutation at normal temperature (Bao et al., 2004). *blr lug* double mutants exhibit a much more severe phenotpye than *lug* single mutant at room temperature. It has already been shown that the cis-regulatory sequences for *AG* lies in its second intron (Deyholos and Sieburth, 2000). Electrophoretic Mobility Shift Assay (EMSA) has been used to demonstrate that BLR binds to *AG* in its second intron (Bao et al., 2004). The mechanism of *AG* repression could thus be similar to yeast in that BLR recruits *LUG* and *SEU* to the *AG* second intron. *LUG* then executes transcriptional repression by further recruiting histone deacetylases and other

chromatin modifying enzymes. However, direct physical interactions between BLR and SEU or LUG are yet to be established.

THE *LUG HOMOLOG*

By BLAST search, *LEUNIG* has been shown to have only one homolog in *Arabidopsis* (Conner and Liu, 2000). This homolog referred to hereafter as the *LEUNIG HOMOLOG* (*LUH*), encodes a protein of 787 amino acids (Fig 5). The gene has 17 exons and 16 introns. LUH has overall sequence identity of 44% to LUG and is similar to LUG throughout the entire sequence (Fig 5). Specifically, LUH also has a Q-rich region and 7 WD repeats at the C -terminus. In addition, there is a highly conserved motif called the LUFS domain at the N-terminus of LUH, that shows 80% identity between LUG and LUH. However, the function of *LUH* is not known. This thesis is focused on identifying the function of *LUH*. Since LUG and LUH share structural similarities, it is possible that *LUH* might also function as a co-repressor similar to *LUG*. Does LUH have a role similar to that of *LUG*? Is the function of *LUH* redundant to that of *LUG* in flower development? Or does *LUH* have a different function altogether? Do *LUH* and /or *LUG* and *SEU* have roles outside flower development? I hope to answer these questions in my thesis.

The central role of *AGAMOUS* in floral organ identity determination and in maintaining the determinacy of the floral meristem have made it a well-studied gene in *Arabidopsis*. Therefore, it is not surprising that many negative regulators of *AG* have been identified and cloned in recent years. The emerging picture is the formation of multi-protein complexes, some of which can interact with *AG* by binding to *AG* cis-regulatory elements while others act indirectly as transcriptional co-repressors.

Transcription factors repress and regulate a variety of genes involved in different cellular processes. For example, Tup1/Ssn6 complex functions in glucose utilization, DNA damage, hypoxia, mating-type specificity. It has been shown that for each of these functions, there is a specific DNA-binding protein that recruits the Tup1/Ssn6 complex to specific upstream sequences of target genes (Smith and Johnson, 2000). Similarly the GRO/TLE repressor in *Drosophila* is also an important transcription factor that is involved in many developmental processes such as dorsal/ ventral pattern formation, segmentation, sex determination and eye development (Chen and Courey, 2000). Like Tup1 and Gro, plant transcriptional factors can also function in different tissues and/or at different developmental stages to bring about the regulation of the target genes. Therefore, the floral regulators *LUG*, *LUH* and *SEU* can act as repressors in tissues other than flowers, thereby acting as global transcriptional regulators. In my studies, I have shown that flower development genes *SEU*, *LUG* and *LUH* also function in ABA signaling.

CHROMATIN MODIFICATION AND TRANSCRIPTIONAL REGULATION

Transcriptional repressors may bring about the repression of target genes by establishing a repressive chromatin structure. Such a repressive structure can prevent access for basal and gene-specific transcription factors and might prevent chromatin remodeling. Alternatively, these repression complexes could interact with and inhibit the basal transcriptional machinery. Chromatin modifications in light of transcriptional activation or repression have been gaining increasing interest and importance.

Nucleosomes are the basic structures of nuclear DNA packaged in repetitive units. These consist of ~145 bp of DNA wrapped around an octamer of basic proteins, histones. The octamer is composed of two molecules each of H2A, H2B, H3 and H4. That these higher order chromatin structures contain the key to gene regulation, has triggered much interest and fueled intense research in this field. It has been known for a long time that histones are capable of being post-translationally modified. Such modifications include methylation, acetylation, phosphorylation, glycosylation and ubiquitination. Based on such modifications, it has been proposed that there exists what is called the histone code. It hypothesizes that multiple histone modifications act in a sequential or combinatorial fashion to orchestrate specific downstream functions.

Acetylation of the lysine moieties of histone tails has been studied in great detail. The enzymes involved in this process namely, *HAT* (Histone Acetyl transferase) and *HDAC* (Histone Deacetylases) have been characterized in plants and animals (Kornberg et al., 1999; Struhl K, 1999). The striking feature of histone acetylation is that there is a fixed pattern of the residues that are acetylated. Thus, in H3, the main acetylation sites are lysines 9, 14, 18 and 23 (Van Holde, KE, 1988; Thorne et al., 1990) and 5, 8, 12 and 16 in H4 (Grunstein M, 1997). Such histone modifications could serve two purposes. One is that the modifications on the histone tails affect the chromatin structure directly. Alternatively, these modifications act as specific receptors to recruit unique transcriptional regulators that mediate downstream functions. Depending on whether the target is switched 'on' or 'off', co-repressors or co-activators could be recruited at such sites to bring about activation or repression, respectively.

In general, acetylation is associated with transcriptional activation and deacetylation is associated with repression. The yeast co-repressor Tup1 has been shown to bring about repression through recruitment of Rpd3, a member of the class I HDAC (Davie et al, 2003). In *Arabidopsis*, antisense inhibition of Rpd3 –type HDAC has shown to result in various growth and developmental defects (Wu et al., 2000; Tian and Chen, 2001; Tian et al., 2003). Recently, it has been shown that the

repression effect of *LUG* is abolished when TSA, a HDAC inhibitor, is added to the repression assay mix. This data indicates that LUG may bring about transcriptional repression by recruiting HDACs (Sridhar et al., 2004). In animals, interesting connections have been made between the Alzheimer's protein Amyloid precursor protein (APP) and a HAT. A cleaved fraction of APP and HAT interact to form a complex that activates target genes (Cao and Sudhof, 2001). Similarly, it was shown in Drosophila that the Huntington disease protein can inhibit HAT activity resulting in the mutant phenotype (Steffan et al., 2001). Thus, more and more research points to the fact that histone acetylation is an important modification that has widespread consequences on gene regulation, and in the growth and development of organisms.

Similar to histone acetylation, histone methylation has been gaining grounds rapidly. Histone methyl transferases catalyze the transfer of methyl groups to lysine or arginine on histones H3 and H4. Like histone deacetylation, histone methylation has been correlated with transcriptional silencing. Moreover, there is evidence indicating that chromatin modification and RNA silencing are interdependent. For example, DNA methylation, mediated by RNA-directed transcriptional silencing, is enhanced by *HDA6*, an Rpd3 type HDAC, in *Arabidopsis* (Aufsatz et al, 2002). Such interdependent mechanisms of chromatin modification prove that the mechanisms underlying gene regulation is highly complex. Thus, deciphering the secrets in the

histone code and unlocking them is one of the major challenges in chromatin research.

B. PLANT HORMONE ABSCISIC ACID AND ABA SIGNALING PATHWAY

Plants, being sessile, need to have a robust defense mechanism in order to circumvent adverse conditions. These range from morphological adaptations, like the sensitive mimosa that closes its leaves upon touch, to the expression of various genes specifically expressed under such conditions. Like animals, plants are also able to sense stress and produce hormones that can induce or repress the expression of effector genes. Like for most other functions, *Arabidopsis* has been adopted as the model system for studying the stress responses of plants. In recent years, many important players in the stress response pathways of *Arabidopsis* have been identified. These include the genes that function in the ABSCISIC ACID signal pathway.

ABA STRUCTURE AND BIOSYNTHESIS

ABSCISIC ACID (ABA) is the most important stress hormone in plants. The name abscisin was given to compounds accumulating in abscising cotton fruit. Similar compounds found in sycamore leaves were named dormin since they induced

dormancy. Later, when the structures of these compounds were elucidated, they were shown to be similar and were called ABSCISIC ACID (ABA) (Addicott, 1983). ABA is a sesquiterpene, a C-15 compound, and has been shown to be present in lower and higher plants, in some phyto pathogenic fungi (Assante et al., 1977; Neill et al., 1982; Kitagawa et al., 1995), and even in mammalian brain tissues (Le page-Degivry et al., 1986). Structurally, ABA has one optically active carbon atom, which gives rise to the production of stereospecific enantiomers. The naturally occurring form is the (S)-enantiomer (Fig 1-6).

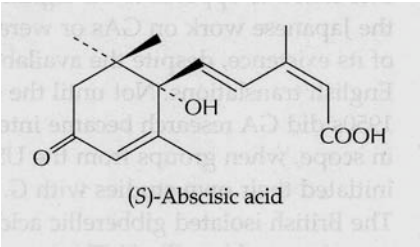


FIG 1-6. Structure of ABA.

ABA was shown to be synthesized in higher plants indirectly from carotenoids. Oxidative cleavage of a C40 carotenoid precursor results in the formation of a C15 intermediate, which is then converted to ABA by a two-step reaction involving ABA-aldehyde (Sindhu et al., 1990; Taylor et al., 2000; Finkelstein and Rock, 2002). The formation of the C15 intermediate Xanthoxin is the first committed step in the biosynthetic pathway (Schwartz et al., 1997). Xanthoxin is

then exported from the plastid to the cytosol, where it is converted to ABA-aldehyde, by the action of *ABA2* gene product (Rook et al., 2001; Cheng et al., 2002; Gonzalez-Guzman et al., 2002). ABA- aldehyde is converted to ABA by the action of *ABA aldehyde oxidase* (AAO) (Fig 1- 7). Biosynthetic genes may be activated through Ca^{2+} mediated processes triggered by abiotic stresses or feedback activation of ABA (Fig 1-7).

Transcriptional regulation plays an important role in stress-mediated activation of ABA biosynthesis genes since inhibition of transcription by inhibitors impairs stress-induced ABA biosynthesis. Under stress conditions, an increase in ABA levels is due to increased de novo biosynthesis. All the biosynthetic genes have been shown to be upregulated by salt and drought stress (Seo et al., 2000; Iuchi et al., 2001; Xiong et al., 2001; Xiong et al., 2002). Like many other biosynthetic pathways, ABA itself can negatively regulate ABA accumulation by activating catabolic enzymes (Cutler and Krochko, 1999). In ABA-deficient mutants, the transcription levels of ABA-biosynthetic genes, was shown to be reduced under stress conditions (Xiong et al, 2002). These genes were shown to be up regulated by ABA, supplied both exogenously and endogenously (Xiong et al., 2001, 2002)

Although originally identified in abscising cotton fruits, ABA was later shown not to be involved in abscission. Rather, the high levels of ABA in such tissues are due to

senescence related stress responses. ABA levels in plants are increased during seed maturation and during environmental stresses. Even under non-stressed conditions, a low level of ABA is required to maintain plant vigor, Since ABA-deficient mutant plants are severely stunted. However, these can be rescued by applying exogenous ABA (Finkelstein and Rock, 2002). The physiological roles of ABA are not limited to safeguarding plants during environmental stresses, but is also important in seed development, seed germination, seed dormancy, cell division and elongation, and environmental stresses such as drought, salinity, cold and pathogen attacks (Leung and Giraudat, 1998; Rock, 2000). ABA is involved in the early phase of seed development by promoting reserve accumulation During the maturation/desiccation phase, ABA is required for the synthesis of Late Embryogenesis Abundance (LEA) proteins, for initiating seed dormancy, for inducing desiccation tolerance and for the inhibition of seed germination. During drought or other water deficient conditions, ABA level increases to induce stomatal closure. Stomata are pores present on the abaxial side of the leaf surface, essential for gas and water exchange with the environment. Stomatal closure reduces transpiration or water loss.

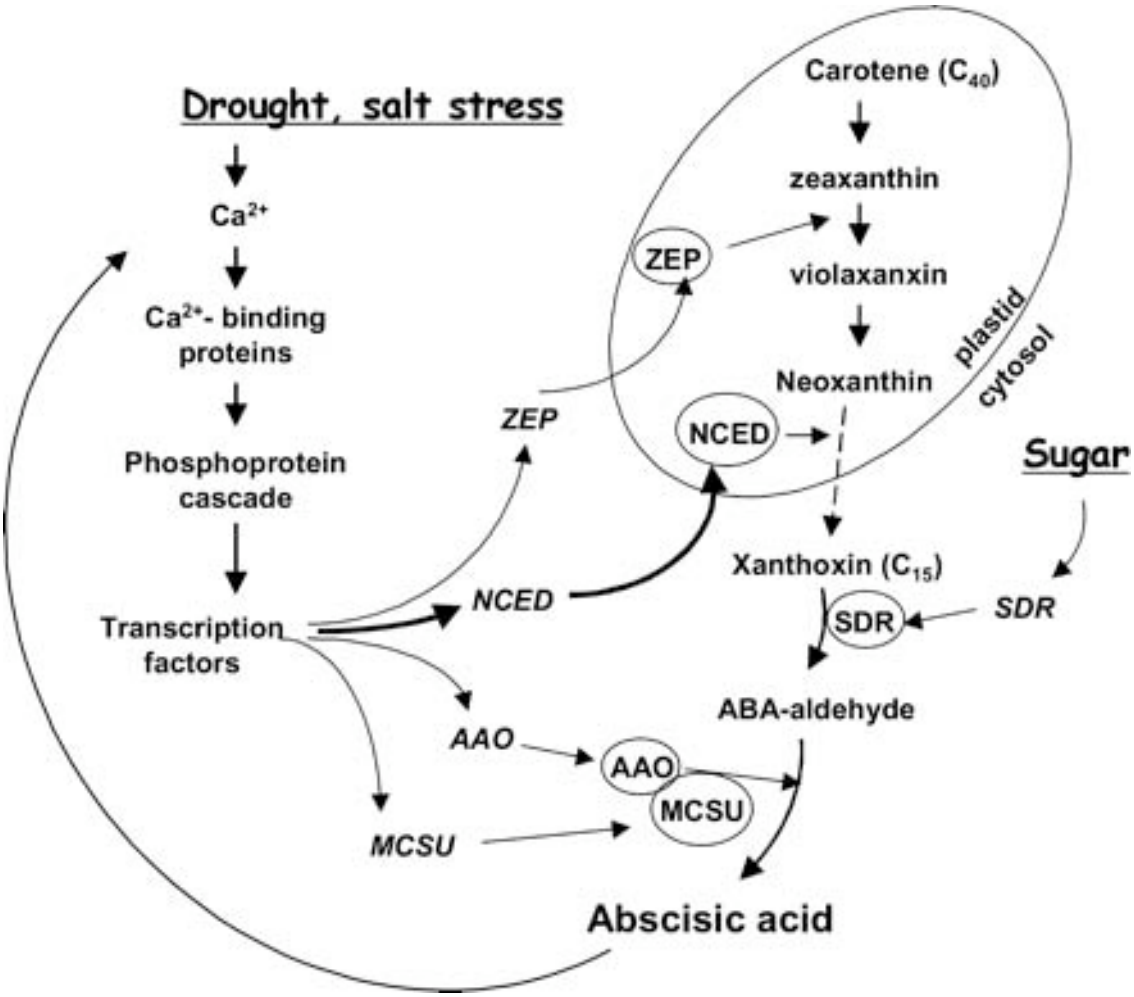


FIG 1-7. ABA biosynthetic pathway. *SDR*-Short-chain alcohol Dehydrogenase reductase; *ZEP*-Zeaxanthin epoxidase; *NCED*-9-cis-epoxycarotenoid dioxygenase; *AAO*-ABA- aldehyde oxidase; MCSU-MoCo sulfurase. (Adapted from Xiong and Zhu, 2003).

Although the ABA signaling pathway or mechanism has not been unraveled as yet, nevertheless, many genes involved in this pathway have been identified and isolated (Leung and Giraudat, 1998; Rock, 2000). The protein products that these genes encode include protein phosphatases, homeodomain proteins, G-proteins, leucine zipper proteins, inositol polyphosphatase, protein kinases, RNA binding proteins and proteins involved in RNA metabolism. This array of molecules of different biochemical properties indicate the complexity involved in ABA signal transduction. Moreover, the presence of cross talk between ABA and other hormones such as ethylene and auxin only makes it a more complicated but important hormone to study.

ABSCISIC ACID INSENSITIVE (ABI) GENES

Based on the inhibitory effect of ABA on germination, genetic screens have identified the so-called abscisic acid insensitive (*abi)* mutants (Koornneef et al., 1989). These mutants are resistant to ABA mediated inhibition of germination and can germinate in higher concentrations of ABA. There are 5 *ABI* genes identified by this type of screens.

ABI1 and ***ABI2***

Based on insensitivity to ABA mediated inhibition of germination, *abi1-1* and *abi2-1* mutants were identified (Koornneef et al., 1984). These mutants exhibited reduced dormancy and sensitivity to ABA mediated inhibition of germination and impaired stomatal regulation). Both *ABI1* and *ABI2* genes encode type 2C serine/threonine protein phosphatase (PP2Cs) (Leung et al., 1994; Meyer et al., 1994; Rodriguez et al., 1998). The mutations in *abi1-1* and *abi2-1* both result in the conversion of a conserved Gly to Asp, leading to decreased phosphatase activity (Leung et al., 1997; Rodriguez et al., 1998; Sheen, 1998). Since there were no null alleles available for these two genes, it was not possible to decipher the role of *ABI1* or *ABI2* in ABA signaling based on the dominant *abi-1* or *abi2-1* mutants. Later, intragenic suppressors for *abi1-1* and *abi2-1* mutants were shown to be hypersensitive to ABA mediated inhibition of germination and stomatal closure. These revertants also showed reduced phosphatase activity. Since the reduced phosphatase activity resulted in hypersensitivity, the WT gene was designated as a negative regulator of the ABA signaling pathway (Gosti et al., 1999, Merlot et al., 2001).

Two models were proposed as to how the *ABI1* gene functions in the ABA signaling pathway. One is that *ABI1/ABI2* acts as a repressor of a transcriptional activator of ABA responses by removing a phosphate group thereby inactivating the transcription factor and hence turning off the downstream genes. In the presence of ABA, the *ABI1/ABI2* phosphatase activity is reduced, leading to the expression of

ABA response genes (Fig 8). This model was later shown to be not the case since the phosphatase activity is actually increased in the presence of ABA (Leung et al., 1997).

Alternatively, it was hypothesized that *ABI1* acts in the desensitizing (resetting) pathway. In the presence of ABA, the signal transduction takes place leading to ABA response (Fig 1-8). At the same time, the increased *ABI1* activity acts to attenuate the response. Recent reports have shown that over expression of *ABI1* gene does not shut off the ABA response (Wu et al., 2003). Thus, it is still not clear whether *ABI1* functions as a positive or negative regulator.

ABI3, ABI4, ABI5

ABI3, ABI4 and *ABI5* all encode transcription factors of the B3, APETALA2 (AP2) and basic leucine zipper (bZIP) domain respectively (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). *ABI3* is homologous to the maize *VIVIPAROUS1 (VP1)* (McCarty et al.,1991) and is shown to be a seed specific transcriptional activator. *abi3, abi4* and *abi5* mutants show seed specific defects in ABA responses but did not exhibit defects in ABA responses in vegetative tissues (Finkelstein, 1994; Finkelstein and Lynch, 2000; Finkelstein et al., 1998). *ABI3* is involved in both phases of seed development, ie, the

reserve accumulation phase and the maturation/desiccation phase. *ABI3* is positively regulated by *LEAFY COTYLEDON* (*LEC1*), and *FUSCA* (*FUS3*) which are also seed specific transcriptional factors (Parcy et al., 1997; Rhode et al., 2000). Although mutations in *ABI3*, *ABI4* and *ABI5* loci have similar effects on seed development and ABA sensitivity, *abi3* mutants have a more severe mutant phenotype than *abi4* or *abi5* (Parcy et al., 1994; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). *ABI3, ABI4* and *ABI5* were proposed to act in a combinatorial fashion to control gene expression in seed specific development. *ABI3* and *ABI5* were shown to actin the same genetic pathway by genetic analysis and which was corroborated by a physical interaction between these two proteins in a yeast two-hybrid assay through the B1 domain of ABI3 (Mary-Brady et al., 2003; Nakamura et al., 2001). In addition to their roles in ABA signaling, *ABI3*, *ABI4* and *ABI5* have been shown to participate in cross talks with other hormones and signaling molecules such as sugar. For example, *ABI3* has also been shown to be involved in auxin signaling and lateral root formation (Mary-Brady et al., 2003). *ABI4* was shown to be involved in salt- resistant germination or sugar-insensitive seedling growth (Huijser et al., 2000; Soderman et al, 2000). Similarly, over expression of *ABI5* led to hypersensitivity to inhibition of growth by sugar, implicating a role in sugar response (Brocard et al., 2002). There is considerable overlap between plant sugar sensing pathways and ABA signaling pathways. There is evidence supporting that sugar signaling could be mediated by

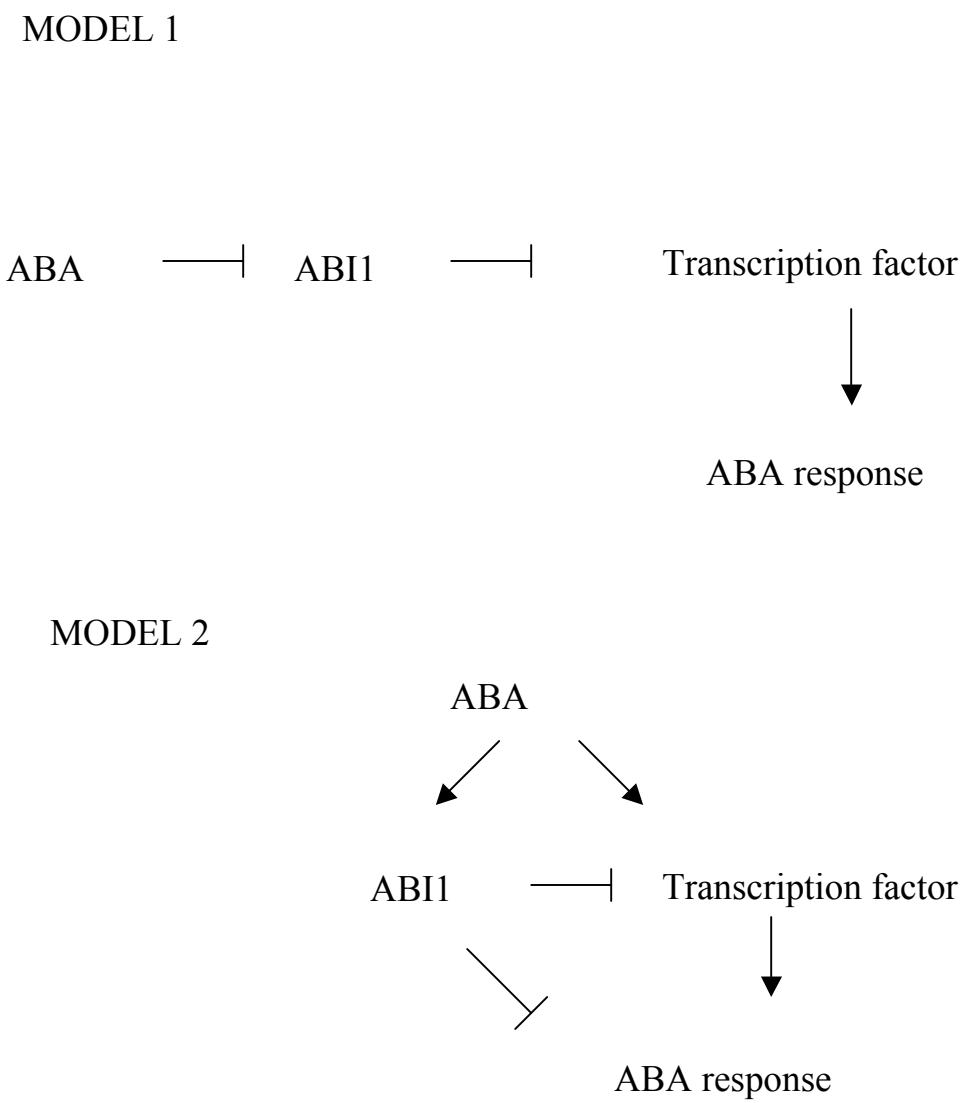


FIG 1-8. Models representing the action of *ABI1*

ABA. Exposure to high glucose induced ABA synthesis as well as the expression of *ABI4* and *ABI5* (Arroyo Becerra et al., 2001; Brocard et al., 2002).

ABREs and ABFs

Although the exact mechanism of ABA signaling is not yet known, the mystery is being unravelled slowly. A putative receptor for ABA action has not been found. However, downstream processes, especially, the mechanism of transcriptional activation of ABA-inducible genes are beginning to be understood. The characterization of promoters of these ABA-responsive genes revealed the presence of specific cis-regulatory sequences called the ABA Responsive Elements, (ABREs). ABREs are composed of sequences 8-10 base pairs in length having an ACGT core. Expression studies indicate that (C/T)ACGTGGC is a strong ABRE, typified by the Em1a element of the wheat Em gene (Guiltinan et al, 1990). These ABREs are a subset of a larger group of elements called 'G-box' (Busk and Pages, 1998; Guiltinan et al, 1990).

ACGT elements are found in many promoters mediating the effect of light, anaerobiosis and UV light. Therefore, the sequences flanking the core ACGT is important for functional specificity. Other sequences, called the "coupling elements", function only in conjunction with ABRE but not alone. The promoters of barley genes, *HVA22* and *HVA1,* contain these coupling elements called CE1 and CE3 (Shen

and Ho, 1997). The sequences of these elements, which share a CGCGTG consensus, are similar to ABREs, except that G replaces the A of the ACGT core. The single base pair change is critical for the binding of plant bZIP factors, which cannot bind to the GCGT element (Izawa et al., 1990). In addition to the ABREs, other cis elements involved in ABA signaling are Sph/RY elements, which are bound by B3 domain proteins such as ABI3 and the Myb/Myc recognition elements bound by MYB and MYC transcription factors (Abe et al., 1997; Busk and pages, 1998; Rock, 2000).

A well-characterized ABA-response gene, *RD29A*, is induced by both ABA and drought. *RD29A* promoter has, in addition to ABREs, the DRE or Drought Responsive Element (DRE) (Thomashow, 1999; Liu et al., 1998; Rock, 2000). Both ABREs and DREs are required for the induction by ABA and drought or dehydration.

ABRE BINDING FACTORS (ABF)

Many transcription factors that bind to ABREs have been cloned to date. Most of these transcription factors are bZIP type with the ability to bind ABREs. Although there are about 81 bzip factors in *Arabidopsis*, not all of them are involved in ABA responses. Only those that belong to the *ABI5* family and the AtDPBF (Arabidopsis thaliana Dc3 promoter binding factors) have been implicated in ABA responses (Choi et al., 2000; Uno et al., 2000). Genetic and molecular studies showed that these bZIP factors regulate diverse biological functions such as pathogen defense, light and stress

signaling, seed maturation and flower development (Jakoby et al., 2002). The bZIP domain consists of two structural features: a basic region of ~16 amino acid residues containing a nuclear localization signal followed by an invariant motif that contacts the DNA; a heptad repeat of leucines or other bulky hydrophobic amino acids positioned exactly nine aminoacids towards the C-terminus, creating an amphipathic helix (Hurst, 1995; Izawa et al., 1993). Plant bZIP proteins preferentially bind to DNA sequences with an ACGT core. Binding specificity is regulated by flanking sequences (Kim et al., 1997; Choi et al., 2000; Finkelstein et al., 2002). Four of these bZIP factors have been shown to bind ABRE sequences in vitro and that mutation in the binding site prevents binding (Choi et al., 2000). The ABRE binding bZIP factors are also known as ABF (ABRE Binding Factors). ABA and abiotic stresses induce ABF.

To date, four ABFs (ABF1 to 4) have been isolated (Choi et al, 2000; Uno et al., 2000; Kang et al., 2002). Recently, it has been shown that over expression of *ABF3* and *ABF4* leads to ABA hypersensitivity, indicating that these transcription factors have a role in ABA signaling in vivo (Kang et al., 2002). The *ABA INSENSITIVE 5* (*ABI5*) gene mentioned above is also an ABF capable of binding an ABRE. The mutant *abi5-1* allele lacks the DNA binding and dimerization domain required for normal function and hence becomes insensitive to ABA in seeds (Finkelstein and Lynch, 2000).

ATHB PROTEINS

A novel class of ABA signaling proteins has been isolated recently. These are the *Arabidopsis thaliana* Homeobox proteins or ATHB proteins. These proteins have a conserved homeobox domain and also a leucine zipper domain and are thus called the HD-Zip proteins (Ruberti et al., 1991). There are about a dozen members so far identified in this gene family in *Arabidopsis* and there seems to be a lot of functional redundancy among family members.

Over expression of *ATHB5* in *Arabidopsis* causes an enhanced sensitivity to inhibition of germination by ABA and an increase in ABA-responsive gene expression (Johannesson et al., 2003). Exogenous ABA induces the expression of *ATHB7* transcripts (Soderman et al., 1996). Yeast two hybrid assays have shown that ATHB6 and ABI1 physically interact and that an intact catalytic domain of ABI1 is essential for the binding of ATHB6 (Himmelbach et al., 2002). The ability of ABI1 to interact with ATHB6 was dependent upon the PP2C activity of ABI1, thus suggesting that ATHB6 could be the substrate of ABI1. *ATHB6* was shown to be involved in regulating ABA response in vegetative tissues (Himmelbach et al., 2002; Soderman et al., 1999).

NEGATIVE REGULATORS OF ABA SIGNALING

A common theme is that loss of function mutations in these ABA signaling mutants cause hypersensitivity to ABA. Many repressors or negative regulators of ABA signaling have been identified. These include the inositol phosphatase *FIERY1*, the double-stranded RNA-binding proteins *FIERY2* and *HYL1*, the farnesyl transferase *ERA1*, the mRNA CAP binding protein *ABH1*, the Sm-like SnRNP protein *SAD1*, the GTPase *ROP10* (Hugouvieux et al., 2001; Lu and Federoff, 2000; Xiong et al, 2001; Xiong et al., 2002; Cutler et al., 1996; Zheng et al., 2002).

FIERY GENES

fiery (*fry*) mutants were identified in a screen for mutants with altered responses to ABA, drought and cold stress. *Arabidopsis* plants containing the firefly luciferase (*LUC*) gene under the control of the *RD29A* promoter emit bioluminescence in response to ABA or abiotic stresses (Ishitani et al., 1997). EMS mutagenized bioluminescent plants were screened for mutants that showed altered *LUC* expression in response to various stresses (Xiong et al., 2001). Two such mutants *fry1* and *fry2* were found to have increased expression of ABA-responsive genes. Although *fry1* mutant is more sensitive to inhibition of seed germination by ABA, *fry2* mutant is less sensitive than the WT (Xiong et al., 2001). However, *fry2* mutant seedlings are more sensitive during root elongation (Xiong et al., 2002). *FRY1* has encodes an inositol phosphatase, while *FRY2* encodes a ds-RNA binding protein. FRY2 also contains a region homologous to the catalytic domain of the RNA

polymerase II C-terminal domain phosphatase involved in transcriptional regulation (Koiwa et al., 2002). Therefore, *FRY2* is also called *CPL-1*C-terminal domain phosphatase like 1). *FRY2* specifically controls the transcription of the DRE/CRT class of stress responsive genes (Xiong et al., 2002). *FRY2*, therefore seems to be specific to one particular type of stress.

SAD1

Similar to fry mutants, the ABA hypersensitive *Arabidopsis* mutant Supersensitive to ABA and Drought1 (*SAD1*) was identified as an ABA induced bioluminescence activation mutant in the same screen described for *fry1*, *fry2* mutants. *sad1* mutant shows ABA hypersensitivity during seed germination and root growth, and has increased expression of stress-response genes. Interestingly, *SAD1* mRNA expression itself is not changed by ABA, which is similar to that observed for *ABA Hypersensitive1* (*ABH1)* (Xiong et al., 2001; Hugouvieux et al., 2002), suggesting regulation by ABA at post-transcriptional level.

The SAD1 protein exhibits a high level of sequence similarity to Sm-like small nuclear ribonucleoproteins, especially to the human and yeast proteins. These Sm-like proteins assemble the components of spliceosomal SnRNPs and participate in several steps of RNA metabolism such as splicing, export and degradation (He et al.,

2000). That both *ABH1* and *SAD1* encode proteins with function in mRNA metabolism and processing, indicates that ABA signal transduction maybe modulated through RNA- mediated step(s).

ABH1

The *ABA-hypersensitive1* (*abh1*) mutant, as the name implies, was identified in a screen for mutants with enhanced response to ABA during seed germination (Table 1-1). These mutants show hypersensitivity to ABA in stomatal closure and hence exhibit reduced wilting during drought (Hugouvieux et al., 2001). Other phenotypes of *abh1* mutant plants include slow growth and serrated leaves (Hugouvieux et al., 2002). The *ABH1* gene encodes the large subunit of a dimeric *Arabidopsis* nuclear cap-binding complex (CBC) (Hugouvieux et al., 2002). The CBC has been shown to participate in several steps of mRNA processing, nuclear export, and mRNA decay in yeast and human HeLa cells. *ABH1* is likely to function in various RNA processing events, which points to a link between mRNA metabolism and ABA signal transduction.

ERA1

ENHANCED RESPONSE TO ABA (*ERA1*) has been identified recently in a screen for mutants that do not germinate at the concentrations of ABA in which WT can. *ERA1*

has been shown to encode a farnesyl transferase and has been proposed to be a negative regulator of ABA signaling, based on its hypersensitive seed germination phenotype (Table 1-1) (Cutler et al., 1996). A farnesyl transferase transfers a farnesyl pyrophosphate group to the substrate (Shafer and Rine, 1992). A farnesylation assay with *era1* mutant flower extracts could not detect farnesylation activities in the *era1* mutants (Cutler et al., 1996). The increased ABA sensitivity of the *era1* mutants and a lack of farnesylation activity suggests that ABA signaling involves farnesylation of signaling molecules. *era1* mutants show increased drought resistance due to decreased transpiration (Pei et al., 1998). Also known as *WIGGUM* (*WIG*), *era1* mutants exhibit developmental phenotypes including increased number of petals, larger meristem size and protruding carpels (Bonetta et al., 2000; Zeigelhoffer et al., 2000).

ROP10

Recent studies provided evidence for a role of heterotrimeric G proteins in ABA response (Lemichez et al., 2001; Zheng et al., 2002). The Rho-like small G protein *ROP10* negatively regulates ABA-mediated stomatal closure, germination and growth inhibition. The recruitment of *ROP10* to the plasma membrane requires a functional farnesylation site and is a prerequisite for altering ABA responses (Zheng et al.,

2002). This finding provides a strong link to the observed role of a farnesyl transferase beta subunit *ERA1*, as a signaling step in ABA responses.

POSITIVE REGULATORS OF ABA SIGNALING

RCN1

Although several negative regulators of ABA signaling have been identified information about positive regulators is still lacking. The roots curl in Naphthylthalamicacid1 (NAA1) (*rcn1*) mutant exhibits reduced ABA-responsive gene expression. *RCN1* encodes the regulatory subunit of a protein phosphatase 2A and acts in guard cell signal transduction. The *rcn1* mutation impairs ABA-induced stomatal closure, and seed germination of the mutant is insensitive to ABA (Kwak et al., 2002). These data suggest a positive role of *RCN* in the ABA signaling pathway. Although a host of genes and their products have been identified, it is still unclear how they interact to mediate the ABA responses. This may be due to the complexity involving multiple levels of regulation through multiple pathways. Cross-talks between different signaling pathways also add additional levels of complexity. The phenotypes and the products of some of the genes mentioned above are summarized in Table 1-1.

INTRACELLULAR MESSENGERS

Calcium serves as an intracellular messenger in many signaling events, including ABA signaling, in which Ca signaling is triggered by secondary messengers such as cyclic ADP Ribose (cADPR), inositol 1,4,5 triphosphate ($InsP_3$) or hydrogen peroxide (Wu et al., 1997; Klusener et al., 2002; Allen et al., 2001; Burnette et al., 2003; Grill and Himmelbach, 1998).

Several phospholipid derived intracellular messengers are involved in ABA responses. The activation of Phospholipase C and phospholipase D during ABA signaling generates the secondary messengers $InsP_3$ and phosphatidic acid respectively (Sanchez and Chua, 2001, Ritchie et al., 2002). Reduced PLC expression in the guard cells of transgenic tobacco cells impaired ABA mediated activities (Hunt et al., 2003; Himmelbach et al., 2003). The phospholipid Sphingosine-1-phosphate was shown to stimulate stomatal closure in a G protein dependent manner (Coursol et al., 2003). ABA induction of Reactive Oxygen Species (ROS) has been shown to be a Rho-protein dependent response (Lee et al., 1999). Pei et al (2000) have shown that ABA induces the production of H_2O_2 , which is a Reactive Oxygen molecule, inguard cells because when H_2O_2 production is blocked, ABA induced stomatal closure is inhibited.

TABLE 1-1. Mutants involved in ABA signaling.

Mutants	Phenotype	Gene product	Reference
abi1-1	ABA insensitivity	Protein Phosphatase 2C	Koornneef et al., (1984); Leube et al., (1998); Gosti et al., (1999)
abi2-1	ABA insensitivity	Protein Phosphatase 2C	Koornneef et al., (1984); Leung et al., (1997); Rodriguez et al., (1998)
abi3	ABA insensitivity in seeds	B3 domain Transcription Factor	Koornneef et al., (1984); Giraudat et al., (1992)
abi4	ABA insensitivity in seeds; sugar and salt insensitive	AP2 domain Transcription Factor	Finkelstein et al., (1994), (1998); Huijser et al., (2000)
abi5	ABA insensitivity in Seeds	BZIP domain Transcription factor	Finkelstein (1994); Finkelstein and Lynch (2000)
abh1	ABA hypersensitive; enhanced drought tolerance	mRNA CAP binding protein	Hugouvieux et al., (2001)
era1	ABA hypersensitive enhanced stomatal response; meristem defects	Farnesyl Transferase β-Subunit	Cutler et al., (1996)
fry1	ABA hypersensitive	Inositol polyphosphate-1-phosphatase	Xiong et al., (2001)
fry2	ABA insensitive	Ds-RNA binding	Xiong et al., (2002)
hyl1	ABA hypersensitive	Ds-RNA binding	Lu and Federoff, 2000
rcn1	ABA insensitive in seeds and guard cells	Protein phosphatase 2A	Kwak et al., (2002)
rop10	ABA hypersensitive	Rho-protein like	Zheng et al., (2002)
sad1	ABA hypersensitive; Reduced ABA biosynthesis	Sm-like small ribo nucleo protein	Xiong et al., (2001)

H_2O_2 induces the activation of Ca^{2+} channels in the membrane of *Arabidopsis* guard cells. These data suggest that ABA induced H_2O_2 production is required for H_2O_2 mediated activation of ca^{2+} channels for ABA induced stomatal closing (Pei et al., 2000; Zhang et al., 2001).

Ca^{2+} SIGNALING AND SIGNAL TRANSDUCTION

Ca^{2+} plays a central role in signal transduction cascades, as an important second messenger. The processes that involve Ca^{2+} in plants are varied and include touch, wind, temperature shock, fungal elicitors, wounding, oxidative stress, ABA and osmotic stress. The resting cytosolic concentrations of Ca^{2+} in a plant cell are very low, of the order of nanomolar levels. However, upon perception of a signal, this concentration can shoot up transiently, thereby facilitating signal transduction (Sanders et al., 1999). The major intracellular Ca^{2+} reserves are vacuoles and rough ER, from which Ca^{2+} can be mobilized by other intracellular messengers such as Inositol triphosphate (IP_3) and cADPR (cyclic ADP-ribose) as in the case of ABA signaling. Apart from these storage organelles, Ca^{2+} can also enter the cytosol from the extracellular medium through opening of Ca^{2+} channels located on plasma membrane. The increase in cytosolic calcium leads to the activation of Ca^{2+} binding proteins such as Calmodulin and other Ca^{2+} dependent kinases (Zielinski RE, 1998). Once the signal is transduced, the internal Ca^{2+} level is restored by the action of Ca^{2+}

ATPases. The specificity of Ca^{2+} signaling is dependent upon the duration as well as the frequency of oscillation.

The physiology and role of calcium in ABA signaling in general, and ABA mediated stomatal closure in particular, has been well characterized. Under drought and other water stress conditions, ABA mediates the closure of stomatal pores in the guard cells thereby reducing loss of water through these pores. Although no receptors for ABA signal transduction has yet been discovered, both extracellular and intracellular receptors are thought to play a role (Jeanette et al., 1999; Allan et al., 1994). Following the perception of ABA in guard cells, there is an increase in the cytosolic Ca^{2+}. This increase is mediated by the opening of plasma membrane Ca^{2+} channels or release from intracellular storage reserves. Consequently, the membrane is depolarized because the increased Ca^{2+} inhibits influx of K^+ and H^+ pumps and activates the efflux of Cl^- channels. Various other changes such as the increase in cytosolic pH and the production of phosphatidic acid (both of which are caused by ABA), lead to the inhibiton of K^+ influx and activation of K^+ efflux. Once the anions and K^+ are released from the guard cells, the guard cell turgor decreases resulting in osmotic water loss and stomatal closure (Schroeder et al., 2001). The signal transduction that occurs in response to ABA is a concerted effect involving many other secondary messengers, kinases, phosphatases.

Kinases and phosphatases are emerging as ubiquitous signaling messengers. There are a lot of phosphatases that have been shown to have an active role in ABA signaling such as *ABI1* and *ABI2*, *RCN1*. ABA-induced protein Kinase1 and ABA activated protein kinase are positive regulators of ABA-mediated gene expression and stomatal response (Li et al., 2002; Kuhn and Schroeder, 2003). The specificity of the kinases and phosphatases are determined by substrate specificity as well as transcriptional and post-translational modifications.

INTEGRATION OF HORMONE SIGNALING AND FLOWER PATTERNING

The role of hormones in flowering and flower development has not been studied in great details. Although it is known that Gibberellic acid accelerates flowering, the part played by hormones in the formation of floral organs is not known. For example, the A B C E model specifies different organ identity genes that form the foundation for floral organ identity specification. However, whether hormones play any role in such floral organ specification still remains a mystery.

Recent research has shown that some mutants involved in hormone signaling also exhibit defects in flower development. The *HYPONASTIC LEAVES1* (*HYL1*) gene encoding a dsRNA binding protein was shown to play a role in Abscisic acid, auxin and cytokinin hormone responses. Moreover, (*hyl1*) mutants are delayed in

flowering, produce flowers that are smaller than wild type and have reduced fertility (Lu and Federoff, 2000). *HYL1* was shown to be involved in microRNA accumulation. In *hyl1* mutants, the microRNA targets are not down regulated due to reduced accumulation of microRNAs. These target mRNAs include meristem and auxin-related genes, thereby indicating that the hormonal and floral phenotypes in *hyl1* mutants are due to the role of *HYL1* in miRNA pathway (Vazquez et al., 2004). So far, only one ABA signaling mutant has been shown to have a floral phenotype. *ERA1* (also known as *WIGGUM*) encodes a farnesyl transferase and has been shown to be a negative regulator of ABA response. *era1* mutants show an increased number of floral organs especially, sepals and petals. This increased floral organ number is due to an increase in the size of floral meristems (Bonetta et al., 1999; Zeigelhoffer et al., 2000). In addition to increased floral organs, *era1* mutants also show delayed flowering, precocious opening of flower buds, and aberrant sepal development (Zeigelhoffer et al., 2000). Furthermore, *abh1* mutants are delayed in flowering (Hugouvieux et al., 2002).

Although mutants in hormone signaling have been shown to have defects in flowering time and floral organ number, a link between hormone signaling and floral organ identity is yet to be established. The application of the hormones cytokinin and auxin has been shown to alter the organ identity in Hyacinth. The expression of Hyacinth *AG* (*HAG*) mRNA seems to depend on the amount of hormone in the

medium thus implying that hormone may regulate *HAG* activity to regulate floral organ identity (Li et al., 2002). Yu et al., (2004), showed that the hormone gibberellic acid (GA) promotes flower development by opposing the function of several DELLA repressors and thereby promoting the expression of floral homeotic genes *AP3*, *PI* and *AG*. DELLA proteins are nuclear repressor proteins that are suppressed by GA, to regulate plant developmental processes (Yu et al., 2004). *ga1-3*, a GA-deficient mutant produces flowers with normal floral identities but the floral organs exhibit abnormal growth (Wilson et al., 1999; Goto and Pharis, 1999). These defects in *ga1-3* mutants can be restored by removing the DELLA proteins; in addition, the expression of floral homeotic genes *AP3*, *PI* and *AG* are up regulated by treatment of *ga1-3* with GA (Yu et al., 2004).

An orthologue of *LUG* in *Antirrhinum*, *STYLOSA* (*STY)*, was shown to have a role in auxin response (Navarro et al., 2004). Similar to *lug* mutants, *sty* mutants show homeotic transformation in the first two whorls of floral organs resulting in the formation of petaloid sepals and stamenoid petals. In the presence of exogenous auxins, *sty* mutant plants are severely retarded in growth suggesting hypersensitivity to auxins. In the presence of auxin transport inhibitors, the main shoot of *sty* mutant seedlings develops a pin-like structure (Navarro et al., 2004). It is possible that *STY* is a common component that functions in both auxin signaling and floral organ specification.

seu mutants were shown to have phenotypes similar to those found in auxin mutants. In fact, *SEU* has been shown to be involved in auxin signaling (Pfluger et al., 2004). *seu* mutants show reduced apical dominance, lateral root initiation and reduced sensitivity to exogenous auxin. A double mutant of *seu pinoid* (an auxin response mutant), exhibits reduced outer whorl floral organs (Pfluger et al., 2004). Mutants that alter the floral organ specificity such as *seu*, *sty*, *lug* are now being shown to have roles in hormone signaling thus linking hormone signaling with floral organ development. Since *lug* and *seu* mutants have so far been extensively studied only in light of flower development, it would be interesting to reveal other functions of these genes. Another important aspect of such studies is that it will shed light on how the same protein functions in different tissues or under different growth conditions.

SUMMARY & GOAL OF THESIS

Transcriptional regulation is a complex mechanism involving multi-protein complexes. It is an act of fine balance between transcriptional activators and repressors. Although much is known about transcriptional activators, the study of the mechanism of transcriptional repression is slowly gaining significance. Emerging studies show that the basic mechanism of transcriptional repression is conserved

across different species. For instance, Tup1 protein is a well-studied protein in yeast that functions as a global repressor (Williams and Trumbly, 1996). Tup1 interacts with Ssn6, a tetratrico peptide repeat protein that has no DNA-binding domains (Smith and Johnson, 2000). Goucho (Gro) is the Tup1 homolog in Drosophila, which brings about repression of segmentation genes, sex determination and dorsal/ventral specification genes (Chen and Courey, 2000). Recently, the plant co-repressor *LUG* has been shown to have structural and functional similarities to Tup1 and Gro and *LUG* is involved in repressing the expression of floral homeotic gene *AG* expression. SEU, has been shown to be the protein binding partner of LUG. Both LUG and SEU do not have any DNA binding domain. Therefore, it is possible that LUG and SEU function in a multi-protein complex that contains DNA-binding partners, capable of recruiting *LUG* and *SEU* to bring about transcriptional repression to specific target genes.

Transcriptional repression of *LUG* and *SEU* have been studied so far in flower development in the context of repression of *AG* expression. The goal of this thesis is to identify additional functions of *LUG* and *SEU* as well as the sole homolog of *LUG* in *Arabidopsis* named *LUH*. My study showed that these three genes play important roles in ABA responses. Thus, floral pattern formation and hormone signaling utilize similar co-repressor molecules. By utilizing similar regulators, these two processes

(floral patterning and ABA signaling) may be coupled to some extent. The significance of shared regulators by different processes remains to be revealed.

Chapter Two

LUH plays a redundant role with *LUG* in flower development

ABSTRACT

Previous molecular genetic studies led to the isolation and characterization of two *Arabidopsis* co-repressors *LUG* and *SEU*, which form a co-repression complex recruited to repress floral homeotic gene *AG* in the outer two whorls of flowers. *LUH*, the only gene in *Arabidopsis* with a high level of sequence similarity to *LUG*, is the focus of this thesis. In this chapter, I aim to determine the role of *LUH* in flower development and the genetic interactions between *LUH* and *LUG* or between *LUH* and *SEU*. Genetic studies indicate that *luh* can enhance *lug* and *seu* floral phenotypes indicating a redundant role for *LUH* in flower development. Since LUG and SEU physically interact to form a co-repression complex, a physical interaction between LUH and SEU was also revealed by yeast two-hybrid assay. The data presented in this chapter suggest that, like *LUG*, *LUH* may also form a co-repression complex with *SEU* and participate in the regulation of *AG* in flower.

INTRODUCTION

Flowers are both aesthetically and reproductively important parts of plants. Therefore, it is not surprising that their development and regulation involves intricate and complex processes. However, the organization of *Arabidopsis* flowers is deceptively

simple. The *Arabidopsis* flowers are organized into four concentric whorls or organs. The outermost whorl is the sepal, followed by the petal, stamen and carpel in the second, third and innermost whorls respectively. The development of these organs in their respective whorls is specified by the interaction of a host of genes including the floral homeotic genes (Weigel and Meyerowitz, 1994).

The C class gene *AGAMOUS* (*AG*) has been in the center of attention by plant biologists due to its pivotal functions in flower development. *AG* plays a key role in specifying stamen and carpel identity. In *ag* loss-of-function mutants, stamens are replaced by petals and carpels are replaced by a new flower. The generation of flowers within a flower phenotype in *ag* mutants suggests that *AG* is responsible for the determinacy of the floral meristem. A third role of *AG* is to prevent the expression of A class genes in the third and fourth whorls.

AG has been shown to encode a MADS-domain containing protein (Yanofsky et al., 1990). MADS domain is named after its four founding genes MCM1 (yeast), AG (*Arabidopsis*), *DEFICIENS* (Antirrhinum) and SRF (Human). The basic N-terminal half of the MADS-domain is required for DNA binding and the C-terminal half of the MADS domain is for dimerization (Reichmann et al., 1996). A second conserved domain in AG is the K box, which was identified based on its similarity to the coiled-coil domain in keratin (Ma et al., 1991). Between the MADS domain and the K box domains lies a less conserved region linker (L) region which, together with

the K box, is important for the partner specificity in dimer formation (Reichmann et al., 1996). MADS-box proteins bind to the consensus sequence $CC(A/T)_6GG$ known as the CArG-box (Schwarz-Sommer et al., 1992; Wynne and Treisman, 1992; Huang et al., 1993; Shiraishi et al., 1993).

AG expression is initiated early (at stage 3) in floral meristem and its activation is dependent on atleast two transcription factors, *LEAFY* (*LFY)* and *WUSCHEL* (*WUS*). Both *LFY* and *WUS* are needed to activate *AG* expression and both have been shown to bind to adjacent sequences in *AG* intronic sequences. (Busch et al., 1999; Lenhard et al., 2001; Lohmann et al., 2001). However, the determinacy of the floral meristem depends on the negative regulation of *WUS* by *AG* at later stages of flower development.

A large number of negative regulators for *AG* have been identified over the years. Many of these gene products may function in a complex to bring about the repression of *AG*. *AP2* was identified as a negative regulator of *AG*, based on the ectopic expression of *AG* in the first two whorls of *ap2* mutant flowers (Bowman et al., 1991; Drews et al., 1991). Unlike other ABC genes, AP2 is not a MADS-box protein. Instead, it has two 68 amino acid repeat sequences, known as the AP2 domain. Together with the EREBP (Ethylene Response Element Binding Protein) class of transcription factors involved in ethylene response (Riechmann and Meyerowitz, 1998), AP2-domain class transcription factors constitute a large gene

family of over 100 members in *Arabidopsis* genome (Riechmann et al., 2000). *AP2* mRNA is found throughout the four whorls of flowers implying that other region specific factors might be involved in delimiting *AP2* activity to the first two whorls. A recent study by Chen (2004) and Aukermann and Sakai (2003) suggest that the outer whorl-specific *AP2* activity is dependent on outer whorl-specific translation of AP2. MicroRNA (miR172) is responsible for this outer whorl-specific translation of AP2.

A genetic screen for enhancer of *ap2* identified a mutant named *leunig* (*lug)* which also showed ectopic *AG* expression in outer two whorls, causing homeotic transformation from sepals to carpels (Liu and Meyerowitz, 1995). However, unlike *AP2*, *LUG* is a cadastral gene meaning that, it is involved in delimiting the expression of *AG* in the first two whorls with no direct role in sepal and petal identity specification. In addition, *lug* mutants show pleiotropic defects with narrow leaves and flower organs, split carpels and the formation of horn-like structures on top of carpels (Liu and Meyerowitz, 1995).

LUG has been cloned and shown to encode a protein similar in structure to transcriptional co-repressors Tup1 (yeast) and Groucho (Drosophila) (Hartley et al., 1988; Williams and Trymbly, 1990). These proteins are characterized by the presence of centrally located Q-rich regions and WD repreats in the C-terminus (Conner and Liu, 2000). Tup1 is a well-studied transcriptional co-repressor, which interacts with

Ssn6, a tetratrico peptide repeat protein (Smith and Johnson, 2000). Tup1-Ssn6 interacts with Histone deacetylase (HDAC) and this interaction is required to mediate repression of target genes (Davie et al., 2003).

Since LUG shows a high level of structural similarity to Tup1 and Gro, *LUG* may act to bring about *AG* repression through chromatin modification. The work on *Arabidopsis* protoplasts have shown that the addition of Trichostatin A (TSA), an inhibitor of HDAC, inhibits the repression mediated by *LUG*, thus implicating histone modification as a mode of LUG mediated repression (VV Sridhar et al., 2004). The pleiotropic phenotype mentioned above and the presence of *LUG* mRNA in all the organs of flowers suggests that *LUG*, like Tup1, could be a global repressor, interacting with specific partners in different parts of the plant to regulate different developmental processes. In addition to the Q-rich and WD repeats, LUG has an additional conserved domain that is not found in Tup1 or Gro. This is the 88 amino acid LUFS domain at the N-terminus of LUG. This motif is highly conserved between LUG, its only homolog in *Arabidopsis* (LUH), Flo8 (Yeast transcriptional activator) and SsDNA binding protein (Humans) (Conner and Liu, 2000). Recently, it has been shown that this LUFS region is necessary and sufficient for LUG to interact with SEUSS (SEU), another negative regulator of *AG* (VV Sridhar et al., 2004).

Yeast two hybrid data showed that LUG and SEU interact physically as well through the LUFS domain (Franks et al., 2002; Sridhar et al., 2004). *SEU* encodes a protein containing a highly conserved dimerization domain found in LIM-domain binding transcriptional co-regulators, and Q-rich regions. *SEU* also has a nuclear localization signal. However, SEU like LUG does not have any DNA-binding domain. Recently, it has been shown that *SEU*, by itself, does not have any repression activity. SEU, rather, acts as an adaptor protein to facilitate the interaction of other transcriptional factors with LUG (Sridhar et al., 2004).

The only homolog for *LUG*, in *Arabidopsis*, termed the *LUG HOMOLOG (LUH*), is about 44% identical to LUG at the amino acid level. LUH also has the LUFS domain, Q-rich regions and 7 WD repeats. The Q-rich regions in LUH are not as continuous or as long as in LUG. Because of the high level of similarity between LUG and LUH, these two genes may perform similar and redundant functions. Alternatively, as mentioned in chapter two, *LUH* may differ in its function from *LUG* with no role in flower development. Finally, *LUH* may have a more prominent role in ABA signaling, while *LUG* may have a prominent role in flower development. In other words, the function of *LUH* might be redundant to that of L*UG* in flower development while the function of *LUG* might be redundant to that of *LUH* in ABA signaling. While the basic molecular mechanism for *LUG* or *LUH* functions may be similar, they may act upon different downstream targets.

LEUNIG HOMOLOG is the only homolog of *LEUNIG* in *Arabidopsis*. Since there is high homology between these proteins, it would be interesting to analyze the functions of *LUH*. Does *LUH* have a role similar to that of *LUG*? i.e., Does it have a role in flower development? If so, is it similar to that of *LUG*? We already saw that *LUH* does have additional roles in plant development. In the second chapter, I have shown that *LUH* is involved in ABA signaling. This chapter focuses on the role of *LUH* in flower development. The genetic interactions between *LUG* and *LUH* and also between *LUH* and *SEU* are described in detail in this chapter.

Materials and Methods

(see Chapter five)

RESULTS

luh-1 single mutants form wild type flowers

In order to characterize the function of *LUH*, reverse genetic approach was used to obtain knockout mutants in *LUH*. The search for *luh*- knockout mutants yielded a lot of mutants with T-DNA insertions, deletions, splice variations and missense mutations (Table 2-1). Most of these mutants were not studied further because they lacked clear phenotype, or they did not affect *LUH* RNA level (downstream of T-DNA insertion) or it is not possible to determine if they cause a loss of function (such as the missense mutations. The TILLING mutant collection contains mutants

generated by EMS mutagenesis (McCallum et al., 2000). Several missense mutations and a single nonsense mutation in *LUH* were identified. The nonsense mutation named *luh-1* converts a tryptophan (W) to a stop codon. The *luh-1* mutant protein is truncated after 55 amino acids. The WT LUH protein is 787 amino acids long, it is likely the 55 amino acid long mutant protein does not have any function. *luh-1* is therefore a putative null, with a strong loss of function and was used in analyses described in this thesis.

Unlike *lug* mutants, *luh-1* mutant flowers are morphologically wild type. The flowers did not exhibit any homeotic transformation of sepals or petals; nor did they exhibit any reduced organ number or size. It is possible that the function of *LUH* is not necessary when *LUG* is intact, but will become necessary when *LUG* is absent.

Table 2-1: Summary of different *LUH* mutations found in databases in Arabidopsis

SOURCE OF MUTANT LINES	NATURE OF MUTATION	HITS IN *LUH*	WEBSITES	# OF LINES	TOTAL # OF HITS
COLD SPRING HARBOR	Ds TRANSPOSON	NONE	http://genetrap.cshl.org/	50000	ZERO
SALK INSTITUTE	T-DNA INSERTION	2 INSERTIONS 1 EXON / INTRON JUNCTION 1 INTRON	http://signal.salk.edu/	75000	TWO
SLAT LINES (SAINSBURY LAB)	DSpm TRANSPOSON	NONE	http://www.jic.bbsrc.ac.uk/sainsbury-Lab/	60000	ZERO
SYNGENTA	T-DNA INSERTION	1 INSERTION AFTER POLY A TAIL	http://www.nadii.com/	98000	ONE
TILLING	EMS MUTAGENESIS	4 MUTATIONS 2 INTRONS 2 EXONS	http://tilling.fhcrc.org:9366/		FOUR
VERSAILLES	T-DNA INSERTION	NONE	http://weedsworld.arabidopsis.org.uk/	50000	ZERO
U OF WISCONSIN	T-DNA INSERTION	3 INSERTIONS 2 PROMOTER, 1 AFTER POLY A TAIL	http://www.biotech.wisc.edu/arabidopsis/	60000	THREE
ZIGIA	En TRANSPOSON	NONE	http://www.mpiz.koeln.mpg.de/~Zigia/	66000	ZERO

luh-1 enhances *lug* phenotype

Earlier reports have shown that mutant of the floral meristem identity gene *CAULIFLOWER (CAL)*, by itself, does not exhibit any phenotype (Kempin et al., 1995). However, it enhances the phenotype of weak *ap1* alleles and exhibited a dramatic phenotype in *cal ap1* double mutant plants. Similarly, *luh-1*, might exhibit a dramatic phenotype in *lug* background. In order to see if *luh-1* could enhance the floral phenotype of *lug* mutants, a cross was made between *luh-1* and a weak *lug* mutant (*lug-16*) and a strong *lug* mutant (*lug-3*), respectively.

Seeds from F1 wild type plants were collected and segregation of mutants in the F2 population was analyzed. In F2 generation, plants that showed a more severe phenotype than *lug* single mutants (*lug-16* or *lug-3)* were first genotyped. Out of 71 F2 plants, 9 plants showed severe or enhanced *lug-16* floral phenotype. All 9 plants with stronger floral phenotypes were genotyped by for *luh-1* and sequencing for *lug-16*, confirming that they are all *luh-1*/+. Out of these 9 plants, 5 were sequenced for *lug-16* mutation and all 5 were homozygous for *lug-16*. For the double mutants between *lug-3* and *luh-1*, out of 27 plants, 2 showed severe than *lug-3* phenotype. Genotyping for *luh-1* showed that all 3 were heterozygous for *luh-1* and sequencing for *lug-16* indicated that they were homozygous for *lug-3*.

The *lug-3 / lug-3; luh-1/+* and *lug-16/lug-16; luh-1/+* plants were sterile and showed severe reduction in the number of floral organs. The numbers of petals were reduced from 4 to zero. Stamens were also reduced and were either completely absent (*lug-3/lug-3; luh-1/+)* or were only 3 to 4 in number (*lug-16/lug-16; luh-1/+*) (Fig 2-1C and F; 2-2C and F). The sepals were more carpelloid than *lug* single mutants suggesting a more severe homeotic transformation possibly due to ectopic expression of *AG*. The l*ug-3/lug-3; luh-1/+* plants showed a more severe phenotype.

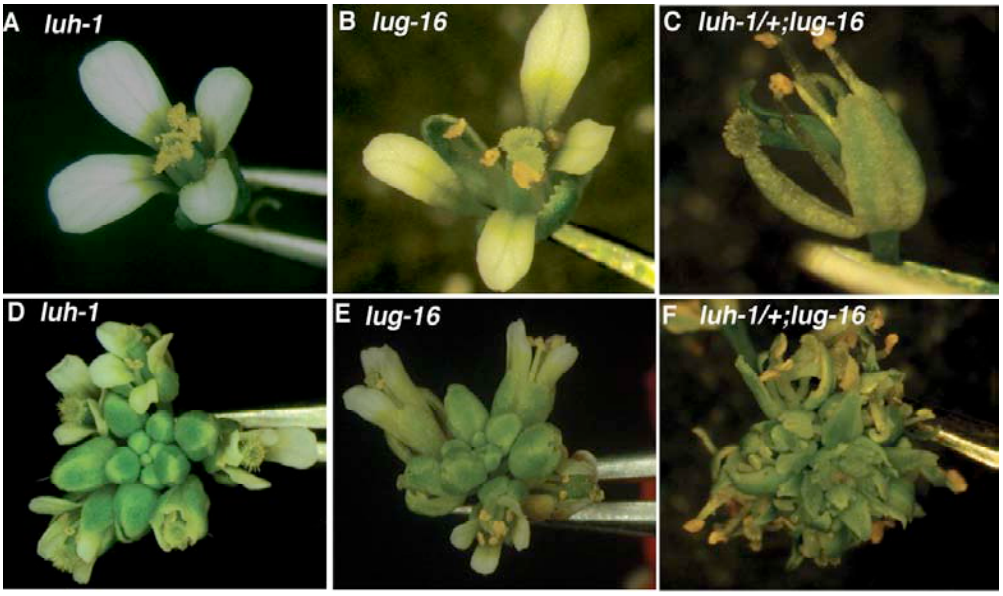


FIG 2-1. *luh-1* enhances *lug-16* phenotype A) *luh-1* flower B) *lug-16* flower C) *luh-1/+, lug-16* mutant flower D) *luh-1* inflorescence E) *lug-16* inflorescence F) *luh-1/+, lug-16* inflorescence.

The overall height of the plants is much reduced**.** Flowers have significantly reduced number of floral organs and the gynoecium is often absent. The stamens are completely absent and so are the petals. A few carpelloid sepals and sepal-like organs, which are rod-shaped with curved horns, are the only organs seen in the flowers (Fig 2-2C, F).

The wild- type- looking plants in the F2 generation from the cross between *luh-1* and *lug-16* cross, were analyzed, to see if they segregate double homozygous plants for both *luh-1* and *lug-16*. 20 WT F2 plants were genotyped with *luh-1* dCAPS and 4 of these were *luh-1/luh-1*. Two of these four F2 *luh-1/luh-1* plants segregated *lug-16* in F3, which were analyzed by sequencing of *lug-16* locus. No double homozygous plants in the F3 generation, was observed. Nevertheless, 2 out of 4 F3 plants that were homozygous for *luh-1* produced smaller flowers (Fig 2-3B). These plants were genotyped and shown to be heterozygous for *lug-16*. When some of the siliques from these *lug-16/+; luh-1/luh-1* plants were opened, they were found to contain dead embryos in roughly one quarter of the total embryos (Fig 2-3D). The absence of double homozygous plants among F3 plants and the presence of dead embryos in the silique of *luh-1/luh-1; lug-16/+* plants suggests that the *lug-16; luh-1* double homozygous plants are embryo lethal. This result strongly supports the significant functional redundancy between *LUH* and *LUG* during embryo development as well. as flower development. Either one of them is sufficient to support embryo

development, but double mutant causes embryonic lethality revealing, for the first time, the importance of *LUG* and *LUH* in embryo development.

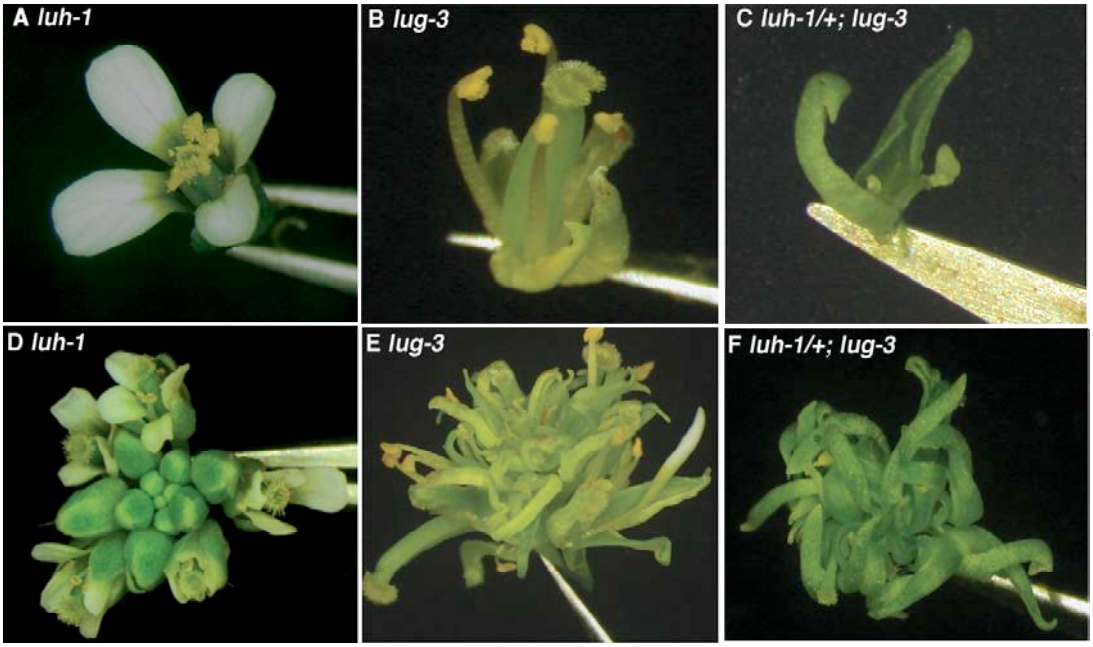


FIG 2-2. *luh-1* enhances *lug-3* phenotype A) *luh-1* single flower B) *lug-3* single flower C) *luh-1/+, lug-3* single flower D) *luh-1* inflorescence E) *lug-3* inflorescence F) *luh-1/+, lug-3* inflorescenceIn C, the absence of gynoecium is obvious. Horn-like projections on tip of sepals indicate their carpelloid identity.

Occasionally, some seeds in the siliques of *luh-1/luh-1; lug-16/+* plants showed precocious germination (vivipary) (Fig 2-3E), i.e., seeds start to germinate even when residing in the siliques. Such viviparous phenotypes have been observed

in maize *vp1* mutants and occasionally in *Arabidopsis abi3* mutants (McCarty et al., 1991; Finkelstein et al., 1994). *VP1* and *ABI3* genes are homologous to each other and are involved in ABA signaling. This vivipary is due to a reduced level of ABA biosynthesis or insensitivity to ABA mediated inhibition of seed germination. ABA plays a major role in controlling embryo dormancy in the seed and in suppressing precocious germination.

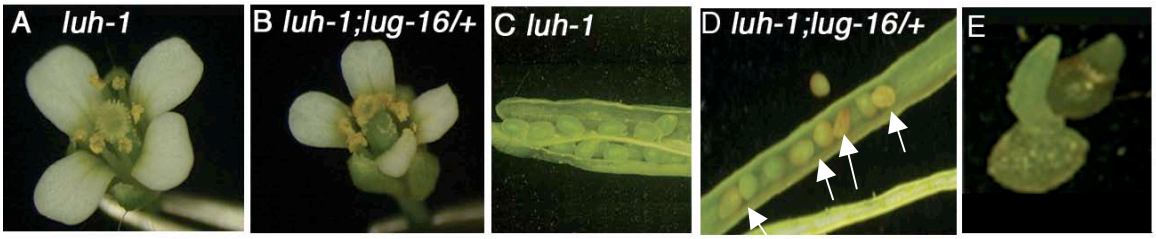
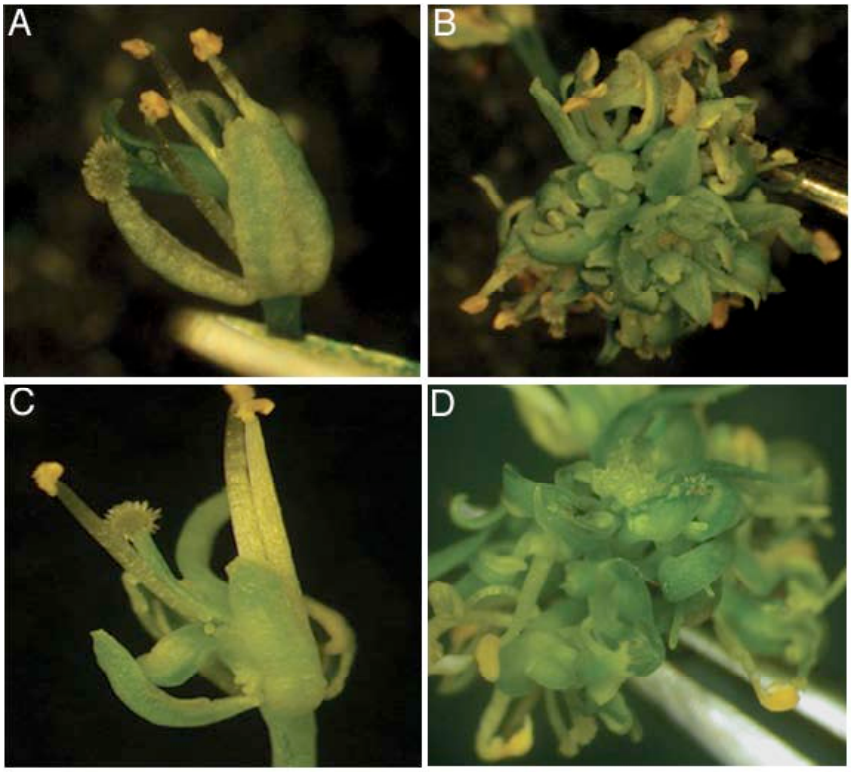


FIG 2-3. A) *luh-1* flower B) *luh-1; lug-16/+* flower C) *luh-1* silique D) *luh-1;lug-16/+* silique showing dead embryos (arrows) E) *luh-1; lug-16/+* seeds showing precocious germination.

***luh-1* shows dominant genetic effect in *lug* background**

Genetic analyses showed that the presence of one mutant copy of *LUH* can enhance *lug-16 or lug-3*, leading to the formation of more severely affected flowers. This was further confirmed by a separate experiment, where *lug-16* mutant plants were transformed with 35S:: *LUH*. The T1 transformants not only failed to show a rescue of *lug-16* phenotype, on the contrary, they showed an enhanced floral phenotype;

flowers developed more carpelloid sepals and a greatly reduced number of organs (Fig 2-4C, D). These flowers are similar to the *lug-16/lug-16; luh-1/+* flowers (Fig 2-4 A, B). One explanation is that the 35S::*LUH* did not over express *LUH* but exhibited co-suppression effect, reducing the mRNA level of endogenous *LUH*. To test this hypothesis, RT-PCR of these 35S::*LUH/lug-16* inflorescences was performed. The results indicated a reduced *LUH* mRNA level (Fig 2-4E). RT-PCR results showed that the *LUH* mRNA level in these plants was approximately half of that in wild type plants (Fig 2-4E). Instead of over expressing *LUH*, 35S::*LUH* suppresses the *LUH* expression in these *lug-16* plants. This suggests that the reduction of functional *LUH* by half results in an enhancement of *lug-16* phenotype indicating that *LUH* dosage exhibits dominant genetic effect in *lug-16* background. In the T2 generation, only those plants that showed enhanced flower phenotype had the transgene as showed by genomic PCR (data not shown). This indicates that the presence of 35S::*LUH* transgene causes enhanced phenotype.



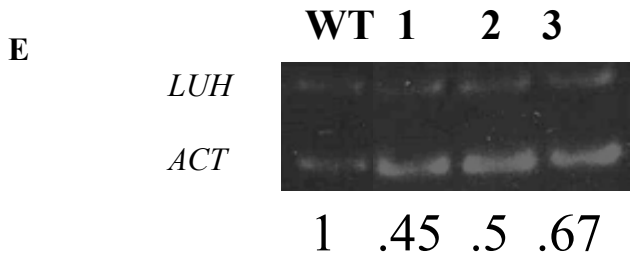


FIG 2-4. Phenotype of 35S::*LUH/lug-16* plants. A) *lug-16/+, luh-1* single flower B) *lug-16/+, luh-1* inflorescence C) 35s*LUH/lug-16* single flower D) 35s*LUH/lug-16* inflorescence E) RT-PCR showing the reduced *LUH* mRNA in 35s*LUH/lug-16* transgenic lines 1, 2 and 3. *ACT* is the loading control. The numbers represent the relative mRNA level normalized to *ACT* and compared with WT, which is taken as 1.

LUH and SEU physically interact in yeast two- hybrid assay

Previous work in our lab demonstrated that LUG physically interacts with SEU through the LUFS domain. Since the LUFS domain is highly conserved between LUG and LUH with 80% identity LUH might also interact with SEU through its LUFS domain. To test this, yeast- two hybrid assay was performed with LUH fused to GAL4 BD (Binding domain) and SEU fused to GAL4 AD (Activation domain). Three reporters ADE2, HIS3 and ß- galactosidase were integrated in the yeast strain.

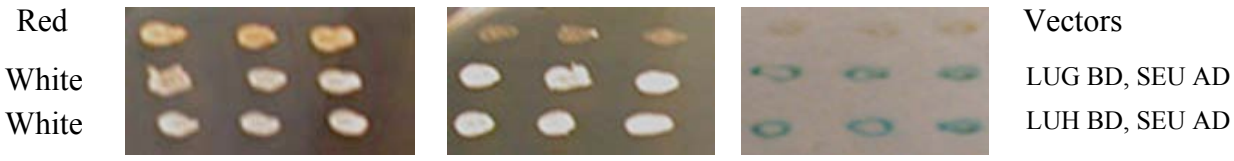


FIG 2-5. Yeast two- hybrid assay for LUH and SEU.
Physical interaction between LUH and SEU as indicated by the growth of yeast on plates lacking and histidine (HIS), blue color colonies by lacZ filter lift assay and white rather than red color colonies in -ADE plates indicate positive reporter expression. The three spots are three different colonies for each combination; Vector alone (upper); LUG, SEU positive control (middle) and LUH and SEU (Lower).

Only if there is a physical interaction between LUH and SEU, can yeast grow on the selective plates (Fig 2-5). The growth of yeast on plates lacking histidine plates, the white colonies on ADE plate, and the blue color in lacZ filter assay suggested an interaction between LUH and SEU. Yeast two hybrid assay between LUG and LUH did not reveal a physical interaction between LUG and LUH (data not shown).

LUH enhances *SEU* in *luh*, *seu* double mutant

Since LUH interacts with SEU physically, they might also interact genetically. In order to test this, *luh-1 seu-1* double mutants were generated. Seeds from F1 wild type plants were collected and analyzed for segregation in the F2 generation. In the F2 generation, *seu* mutants segregated along with two (out of 34) plants that exhibited a more severe phenotype than *seu-1* plants (Fig 2-6). *seu-1* is a weak allele and *seu-1* plants exhibited narrow sepals and petals. However, the *luh-1 seu-1* double mutant plants are very short (one-fourth the size of *seu-1* plants) with extremely small leaves and small flowers (Fig 2-6C). There is loss of second and third whorl organs and the flowers are sterile (Fig 2-6). Genotyping indicated that these plants are homozygous for *seu-1* and heterozygous for *luh-1*.

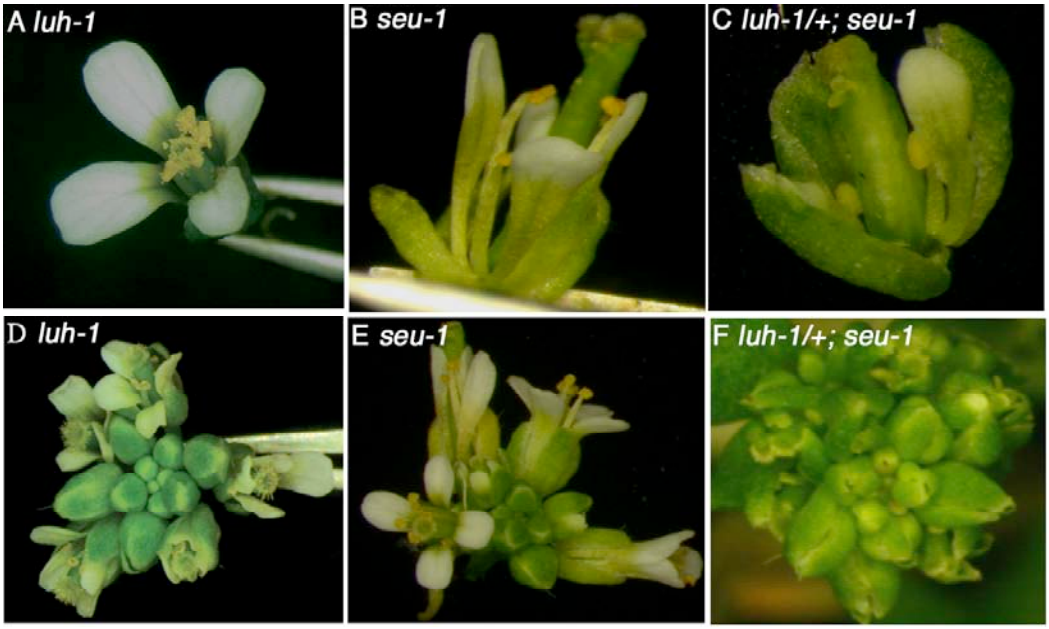


FIG 2-6. *luh-1* enhances *seu-1* phenotype A) *luh-1* single flower B) *seu-1* single flower C) *luh-1/+; seu-1* flower at twice the magnification as others D) *luh-1* inflorescence E) *seu-1* inflorescence F) *luh-1/+ seu-1* inflorescence

DISCUSSION

AG expression is negatively regulated by many repressors, indicating that it is a complex process. *AG* repression is essential not only in maintaining the spatial specification of the floral organs but also in maintaining the floral meristem determinacy. In the absence of *AG* this determinacy is lost, leading to the formation of reiterated flowers. Thus, the proper expression of *AG* is required for the formation of reproductive parts of the flower and hence plant propagation. This calls for a much robust mechanism involving concerted action of several transcription factors.

Although all the players involved in the complex formation or the exact mechanism of action of these factors is not yet known, a picture is emerging as to the possible roles of participating factors. LUG, LUH and SEU may all be part of a multi-protein complex. *lug* or *seu* single mutants do not show a dramatic phenotype as compared to *lug seu* double mutants. Similarly, *luh* single mutants do not show any phenotype at all. This may be due to the fact that the functions of *LUG* and *LUH* are partially redundant. In other words, while *LUG* alone is able to completely compensate for *LUH*, *LUH* alone cannot completely (but only partially) substitute for *LUG* activity as evidenced by *lug* single mutant phenotypes. However, the absence of both *LUG* and *LUH* results in embryo lethality revealing a previously unknown function of these two proteins. Moreover, the production of seeds with precocious germination in *luh-1, lug-16/+* mutants (Fig 2-3E) suggests that although *LUH* might have a prominent role in ABA signaling, the absence of *LUG* will enhance the ABA phenotype of *LUH*, indicating *LUG* plays a role in ABA as well. Since, *luh lug* double mutants are embryo lethal, it is not possible to test the double mutant for ABA phenotype. However, the enhanced floral phenotype of *lug; luh-1/+* suggests that *LUH* too has a role in flower development (Table 2-2).

The presence of viviparous seeds in the siliques of *luh-1/luh-1; lug-16/+* plants indicate that both *LUH* and *LUG* might be involved in ABA signaling. Such viviparous seeds are not seen in *luh-1* single mutant siliques. ABA is a plant hormone

involved in seed dormancy, seed maturation and desiccation. The presence of ABA during seed dormancy ensures that the seeds do not germinate precociously. Either a block in ABA biosynthesis or a reduced sensitivity to ABA can lead to precocious germination. Maize *viviparous 5* (*vp5*) and *viviparous 7* (*vp7*) mutants are blocked in ABA synthesis (Neill et al., 1996) whereas *vp1* mutant is defective in its response to ABA (Robichaud et al., 1980). Since *LUH* is involved in ABA signaling (chapter 3), loss of *LUH* might result in reduced response to ABA mediated seed dormancy. This effect is more pronounced when both *LUH* and *LUG* are absent (Table 2-2).

TABLE 2-2 The role of *LUG* and *LUH* in different developmental processes

Phenotype	*luh*	*luh*	*luh+lug*
Embryo lethal	-	-	++
Flower	-	++	+++
ABA signaling	++	-	+++ (Vivipary)

+ indicates the severity of the phenotype with ++ and +++ indicating more severe phenotype; - indicates there is no phenotype.

Since both LUG and LUH interact with SEU physically via their LUFS, it is possible that in flowers, LUG, LUH and SEU form a repression complex. The *in vivo* repression activity of *LUG* has been demonstrated already (Sridhar et al., 2004). However, *SEU*, by itself, does not show repression activity and has been proposed to be an adaptor protein for LUG (Sridhar et al., 2004). Since LUH and LUG have similar structural motifs, it is possible that *LUH*, like *LUG*, may confer repression activity to the complex as well. This is yet to be tested. The repression activity of *LUG* was shown to be reduced by inhibitors to Histone deacetylase (HDAC), such as Trichostatin A (Sridhar et al., 2004). It would be interesting to see if *LUH* is involved in chromatin modification as well.

It would be interesting to see what other proteins LUH may interact with. Future studies would be aimed at identifying specific DNA binding factors that mediate the effect of *LUH/SEU/LUG* in ABA response and flower development.

Chapter Three

LUH PLAYS A POSITIVE REGULATORY ROLE IN ABA SIGNALING

ABSTRACT

ABSCISIC ACID (ABA) is an important plant hormone that mediates seed maturation and stress response in higher plants. I show here that regulators of flower development also function in ABA signaling. The function of *LEUNIG HOMOLOG (LUH)*, the only *Arabidopsis* gene with a high degree of sequence similarity to *LUG*, was characterized. In addition to enhancing the floral phenotype of *lug* mutants, *luh-1*, a putative null mutant, exhibited decreased sensitivity to ABA, reduced expression of ABA-inducible genes *KIN2* and *RD29A,* and increased rate of water loss. These *luh* loss-of-function phenotypes indicate a positive role of *LUH* in ABA signaling. The similarity of LUH to the transcription co-repressor LUG suggests that *LUH* may act to repress the expression of negative regulators to exert a positive effect on ABA signaling. I showed one putative target of *LUH* is *ERA1*. Taken together, these data indicate a novel function of the flower development gene *LUH* in promoting ABA hormone signaling.

INTRODUCTION

Plants, being sessile, need to have a robust defense response in case of adverse external conditions. The plant hormone ABSCISIC ACID (ABA) orchestrates many important physiological responses, including seed maturation and dormancy, desiccation tolerance and water stress under drought conditions (Leung and Giraudat, 1998; Finkelstein et al., 2002). Under severe water deficiency, plants respond by reducing water loss through transpiration by the induction of ABA, which induces the closure of stomata present in the guard cells located in the abaxial side of the leaves. The guard cells are responsible for water and gas exchanges. Since crop loss due to drought conditions is a major problem in agriculture, it is of fundamental importance to understand the mechanism of ABA signal transduction in order to better manage and reduce crop loss by drought.

However, many genes that play either positive or negative roles in ABA signaling have been identified based on the altered sensitivity of mutant seedlings to the inhibition of germination by exogenous ABA (Koornneef et al., 1984), or altered reporter gene expression to different stresses such as ABA, low temperatures (Xiong et al., 2001). Recent isolation and cloning of many genes in ABA signaling suggests that ABA signal transduction is a complicated process involving a plethora of proteins with different biochemical functions.

Five ABA- INSENSITIVE (ABI) genes have been identified and isolated. *ABI* (*ABA INSENSITIVE*) genes, *ABI1* and *ABI2* encode serine/threonine phosphatase 2C (Leung et al., 1994; Meyer et al., 1994; Leung et al, 1997); *ABI3*, *ABI4* and *ABI5* encode transcription factors (Finkelstein et al., 1998; Finkelstein et al., 2000; Giraudat et al., 1992) with roles specific for seed development. Of particular importance to this study is *abi1-1*, which shows reduced reponsiveness to ABA. As *abi1-1* is semi-dominant, it was unclear whether *ABI1* is a positive or a negative regulator of the ABA response. Gosti et al., (1999) identified seven intragenic revertants of the *abi1-1* mutant. These revertants were more sensitive to ABA in the inhibition of seed germination and root growth and had reduced PP2C activity. This led to the conclusion that the lack of the ABI1 enzyme activity resulted in enhanced ABA responsiveness. Thus *ABI1* and later *ABI2* were proposed to encode negative regulators of ABA signaling. However, Wu et al., (2003) have shown that an over expression of *ABI1* does not block the ABA signaling pathway.

An increasing number of mutants that are hypersensitive to ABA have also been identified. *ERA1* (Enhanced Response to ABA) encodes the β subunit of a Farnesyl transferase (Cutler et al., 1996; Pei et al., 1998). A farnesyl transferase transfers a farnesyl group to a protein and may activate or inactivate a protein through farnesylation. Farnesylation has been implicated in signal transduction. Interestingly *ERA1* has been shown to be involved in meristem development as well as in ABA

signaling (Bonetta et al., 2000). *era1* mutants were shown to have increased sensitivity to ABA mediated inhibition of germination and were shown to lack farnesyl transferase (Cutler et al., 1996). This suggested that farnesylation is required to negatively regulate ABA signaling. *ABH1* (ABA Hypersensitive1) encodes an mRNA Cap binding protein (Hugouvieux et al., 2001). *abh1* mutants have been shown to be drought resistant by inducing stomatal closure and exhibit hypersensitivity to ABA- mediated inhibition of seed germination. *SAD1* (Supersensitive to ABA and Drought) encodes a protein involved in RNA splicing and export (Xiong et al., 2001). Based on their hypersensitive response to ABA *ERA1*, *ABH1* and *SAD1* have been suggested to encode negative regulators of ABA signaling.

An emerging theme in several model systems is that, a single regulatory gene could function in multiple developmental processes. For instance, Tup1, a global co-repressor in yeast, represses genes in cell type specification, in glucose and oxygen metabolism, in DNA damage repair and in other cellular stress signaling (Redd et al., 1997). *LEUNIG* (*LUG*), a co-repressor in *Arabidopsis* with sequence similarities to Tup1 (Conner and Liu, 2000) was initially identified due to its function in repressing floral homeotic gene *AGAMOUS* (*AG*) (Liu and Meyerowitz, 1995). LUG contains Q-rich and WD domains which is similar to Tup1. *lug* mutants exhibit pleiotropic defects not restricted to flower organ identity specification including abnormally

narrow leaves, split gynoecium and possibly other unidentified defects. Recently, functional similarity between *LUG* and Tup1 was demonstrated as *LUG* can repress gene expression in yeast as well as in plants (Sridhar et al., 2004).

Co-repressors do not contain a DNA-binding domain but are recruited to the promoters of target genes by interacting with DNA–binding transcription factors. Recently, it was shown that *LUG* does not function alone but forms a complex with *SEUSS* (*SEU*), another repressor of *AG* expression in flowers (Franks et al., 2002; Sridhar et al., 2004) and SEU interacts specifically with the N-terminal LUFS domain of LUG. The LUG / SEU complex is responsible for the repression of *AGAMOUS* (*AG*) in the first two whorls of *Arabidopsis* flowers. Like *lug* mutants, *seu* also exhibits pleiotropic defects not restricted to flowers, including reduced plant height, narrow leaves and floral organs and insensitivity to auxin (Pfluger et al., 2004). *SEU* is a novel plant transcription factor and encodes a conserved domain similar to the dimerization domain of Ldb family of transcriptional co-regulators in mammals. However, SEU does not have a DNA-binding domain (Franks et al., 2002). SEU was proposed to act as an adaptor protein between LUG and DNA-binding transcription factors. The pleiotropic defects of *lug* and *seu* mutants suggests that the *LUG* /*SEU* complex may interact with different DNA-binding transcription factors to repress different target genes in different developmental processes. However, the role of LUG/SEU outside the flowers is not characterized yet.

Previously, we identified a homolog of *LUG* in Arabidopsis, known as *LUG HOMOLOG* (*LUH*), that shows 44% identity to *LUG*. Although *luh-1* single mutants do not show a flower phenotype, *luh-1* can enhance *lug* and *seu* floral phenotypes even as a heterozygote, suggesting that *LUH* functions in the flower, but this function is partially redundant to that of *LUG* (see chapter 2). More surprisingly, the abnormally high level of *LUH* expression in *abi1-1* mutants (Schroeder J. et al., Stanford Microarray Database, EXPT ID 11895 and Fig 2-5A) led to the identification of a role for *LUH* in ABA signaling.

Flower development responds to both internal (hormone, developmental clock) and external cues (day light, cold). Although many genes have been identified that function in flower development and patterning or in hormone signaling, most studies are limited to either flower development only or hormone signaling only. Only recently, it was increasingly realized that similar components could function in both processes. In this chapter of my thesis, I have shown that *LUH* functions in ABA hormone signaling. I propose that *LUH* positively regulates ABA signaling by repressing the expression of *ERA1*, the negative regulator of ABA. The common regulatory proteins shared between hormone signaling and floral development suggest that these two processes may cross-talk to each other. For instance, ABA may affect *LUG*, *LUH* and *SEU* expression and, as a result, affect *AG* expression and flower development.

Materials and methods

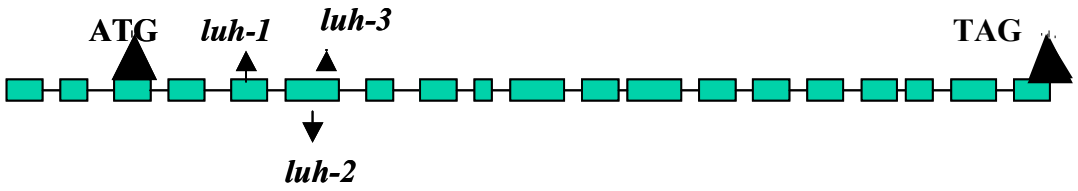
(See Chapter five)

RESULTS

LUH structure and sequence

LUH is the only *Arabidopsis* gene (At2g32700) with significant sequence similarities to *LUG* throughout the entire gene. *LUH* has 17 exons and is located on chromosome 2 in the *Arabidopsis* genome. 5' RACE was performed to verify the *LUH* transcript. The longest 5' RACE product has 5 nucleotides more than the predicted sequence in the Genbank database (FIG. 3-1B). Three ESTs [187H14T7, H7B3T7 (Arabidopsis Biological Resource center) and RAFL09-12-E08 (RIKEN)] were obtained and RAFL09-12-E08 is the full length EST. Sequencing of these ESTs confirmed the intron/exon boundaries by the TIGR annotation. The intron-exon structure of LUH and the alleles of LUH obtained through TILLING are shown in FIG 3-1B.

A



B

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 Q  I  V  Q  G  N  H  Q  G  G  V  S  A  A  L  Q  Q  I  Q  S  R  T  Q  Q  P  E  I  K  T  E  V  N  L  G  T  S
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 P  R  Q  L  P  V  D  P  S  T  V  Y  G  Q  G  I  L  Q  S  K  P  G  M  G  S  A  G  L  N  P  G  V  S  G  L  P  L
TAAAGGGATGGCCATTAACTGGCATCGAGCAGATGCGACCAGGTTTAGGAGGCCCTCAGGTTCAGAAATCTTTTCTACAAAATCAAAGTCAATTTCAGCTCTCGCCGCAG 1210
 K  G  W  P  L  T  G  I  E  Q  M  R  P  G  L  G  G  P  Q  V  Q  K  S  F  L  Q  N  Q  S  Q  F  Q  L  S  P  Q
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 Q  Q  Q  H  Q  M  L  A  Q  V  Q  A  Q  G  N  M  T  N  S  P  M  Y  G  G  D  M  D  P  R  R  F  T  G  L  P  R  G
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 N  L  N  P  K  D  G  Q  Q  N  A  N  D  G  S  I  G  S  P  M  Q  S  S  S  S  K  H  I  S  M  P  P  V  Q  Q  S
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 M  M  Y  G  S  D  G  I  G  G  L  A  S  S  A  N  Q  L  L  Q  D  D  M  D  Q  F  G  D  V  G  A  L  E  D  N  V  E
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 S  F  L  S  Q  D  D  G  D  G  G  S  L  F  G  T  L  K  R  N  S  S  V  H  T  E  T  S  K  P  F  S  F  N  E  V  S
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 N  G  E  L  V  A  S  V  S  E  D  A  V  K  L  W  S  L  S  S  G  D  C  I  H  E  L  S  N  S  G  N  K  F  H  S  V
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 V  I  S  A  L  A  Q  S  P  S  T  G  V  V  A  S  A  S  H  D  K  S  V  K  I  W  K  *
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taatttctctagaccaactacattgttgaataataatttcttataatc                                                              2948
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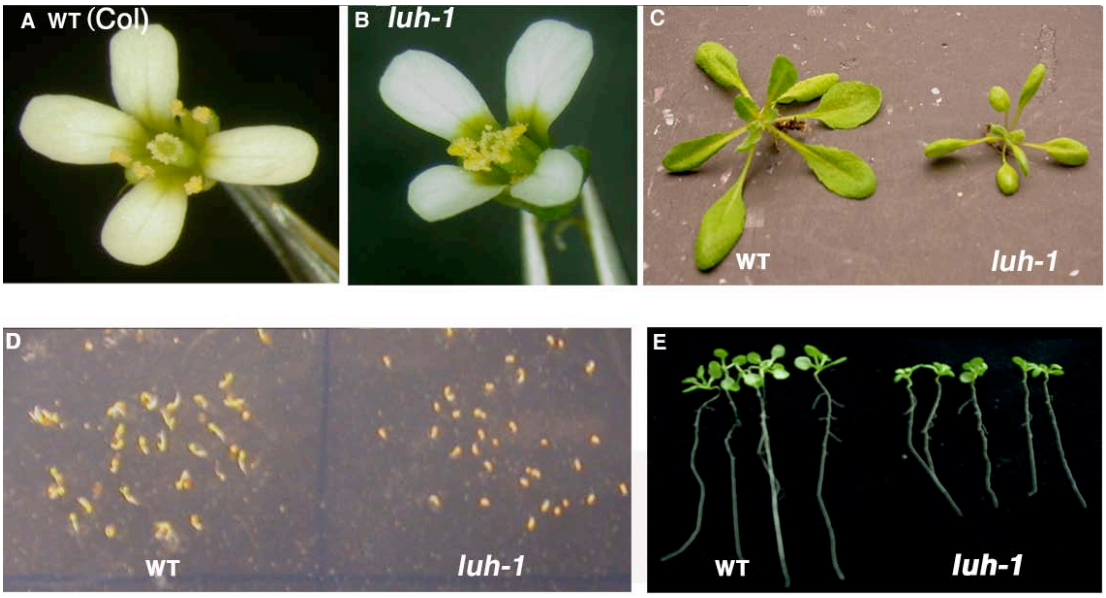
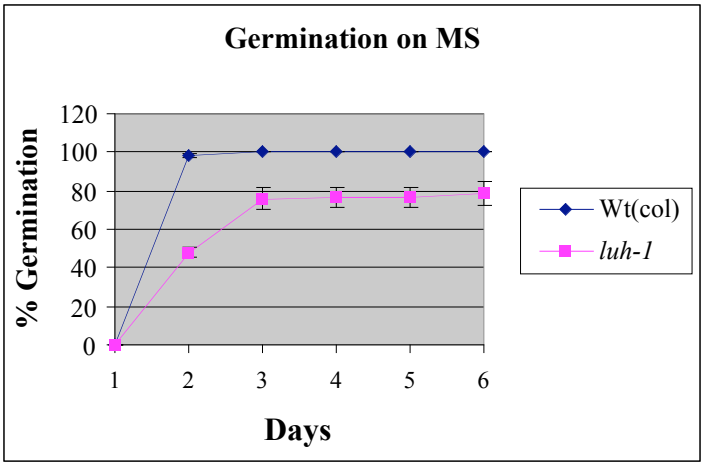
FIG 3-1. A) A schematic diagram showing showing LUH gene structure and the position of different alleles on the gene. Closed boxes represent exons and lines represent introns B) Sequence of *LUH* cDNA and deduced amino acids. 5' and 3' UTR are included. The nucleotides in bold in the 5' UTR are the extra nucleotides obtained through 5' RACE. The 7 WD repeats are underlined and the 88 amino acids of the LUFS domain are shown in bold.

luh-1 loss of function mutants exhibit vegetative defects

In order to identify the function of *LUH*, we sought to isolate mutations in *LUH* via a reverse genetic approach. Through the TILLING (McCallum et al, 2000) facility, we identified several *luh* mutations including *luh-1*, a putative null. *luh-1* mutant protein is truncated by a stop codon at the 55 amino acid position. Two other missense alleles, *luh-2* and *luh-3* change a single amino acid from L to F at 114 and from S to F at 123, respectively (FIG 3-1A). A fourth allele identified from SALK line (SALK _ 043396.56.00.x) has a T-DNA inserted in the 5' UTR. RT-PCR using primers downstream of the T-DNA insertion detected *LUH* mRNA in this T-DNA line. Therefore, we have chosen to analyze *luh-1*. Unlike *lug*, *luh-1* single mutants did not show any abnormality in flowers (FIG 3-2A, B). Nevertheless, *luh-1* mutant seedlings showed poor germination on MS medium, with germination rate only about 80% of WT (FIG 3-2 D, F). In addition, the root length of *luh-1* mutant seedlings is only about 75% of WT (FIG.3-2 E, G). The growth of *luh-1* mutant plants is slower compared to WT plants as shown by the size of the rosette plants at 3-week stage (FIG 3-2C). *luh-2* mutant did not show any developmental defect (data not shown). All the developmental defects in *luh-1* could be complemented by the over expression of the *LUH* cDNA in *luh-1* plants (FIG 3-3), indicating that these phenotypes are indeed due to a lack of *LUH* activity. Specifically, two different transgenic lines #4 and #5 that harbored 35S::*LUH* and showed higher levels of *LUH* mRNA (FIG 3-





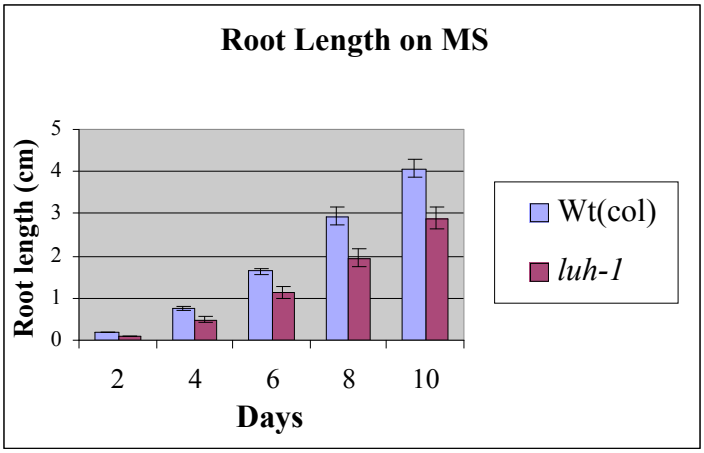


FIG 3-2. *luh-1* develops normal flowers but exhibits defects in vegetative growth. A) A WT flower B) A *luh-1* flower C) 3-week old WT and *luh-1* plants D) Germination of WT and *luh-1* seeds on MS medium (picture taken 4 days after germination) E) Root growth of WT (Col) and *luh-1* seedlings on MS medium (one week old seedlings). F and G are quantitative measurements of germination and root growth respectively.

3A), were analyzed. Both lines showed germination rate and root growth similar to WT (FIG 3-3B, C). The sensitivity to ABA in these lines was similar to that of wild type (data not shown).

LUH is expressed in all tissues and is induced by ABA

Consistent with their pleiotropic effects, *LUG* and *SEU* have been shown to be expressed in all organs and tissues of plants (Conner et al., 2000; Franks et al., 2002). To examine *LUH* expression, RT-PCR was performed on RNA extracted from flowers, leaves, stem, root and seedlings. *LUH* mRNA is expressed in all tissues tested (FIG 3-4), with a 4-fold higher expression level in flowers than in other tissues. RT-PCR also showed that *LUH* mRNA is expressed in the seedlings starting as early as 2 days after germination, suggesting that *LUH* may play a role at the very beginning of development.

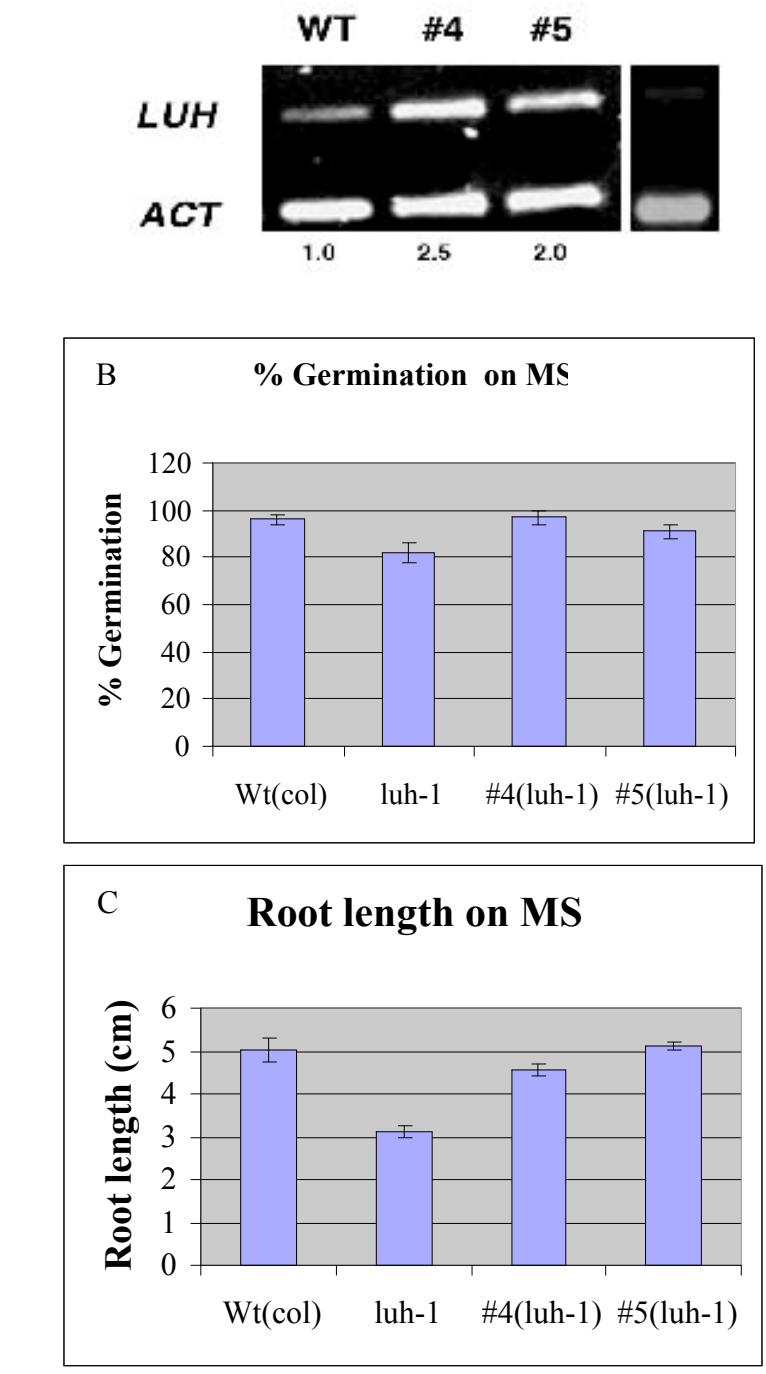


FIG 3-3. A) RT-PCR showing the expression level of *LUH* mRNA in WT (Col) and two different transgenic lines (#4 and #5) of *luh-1*plants harboring 35S::*LUH*. *ACT2* is used as the loading control. *LUH* mRNA is absent in *luh-1* mutant, indicating it is a RNA null. Numbers indicate relative *LUH* level after normalization with *ACT*. B) Germination phenotype of *luh-1* is complemented by 35S::*LUH*. Germination is expressed as the mean ± of 2 duplicates with ~ 50 seeds each. C) Over expression of *LUH* from 35S::*LUH* complements the *luh-1* defects in root elongation. Root length is the average length of 2 duplicates measured after 7 days.

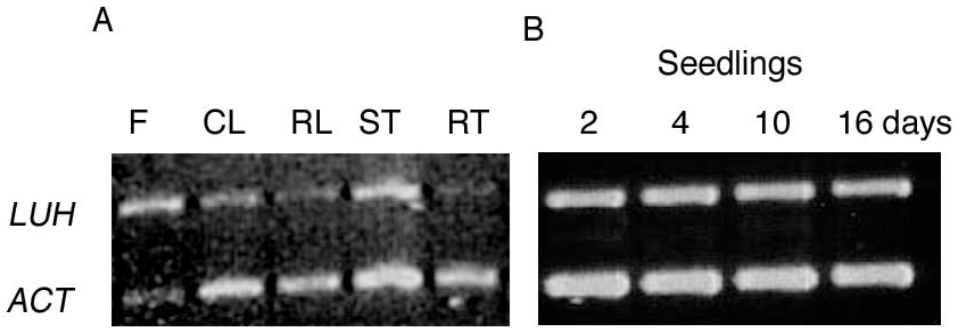


FIG 3-4. *LUH* mRNA is expressed in all tissues tested. Semi quantitative RT-PCR showing the relative amounts of *LUH* mRNA present in different tissues of WT (Col) plants. F-Flower, CL-Cauline leaf, RL-Rosette leaf, ST-Stem, RT-Root. *ACT* is the loading control. (B) RT-PCR showing the relative amount of *LUH* mRNA in WT (Col) seedlings 2, 4, 10 and 16 days after germination.

Since *luh-1* single mutants did not show any floral phenotype, Stanford microarray database was searched, in order to see if *LUH* had any function in other processes of plant development. A search of Stanford micro array database (http://genome.www5.stanford.edu/cgi-bin/SMD) revealed that *LUH* mRNA level is increased about 10-fold in *abi1-1* mutants (Schroeder et al., EXPT ID 11895). However, we could not find any micro array data for either *LUG* or *SEU*. To verify *LUH* mRNA expression in *abi1-1*, an RT-PCR was performed to examine *LUH* mRNA expression in WT and *abi1-1* mutant seedlings. *LUH* mRNA is increased by about 3-fold in the *abi1-1* mutants (FIG 3-5A). Further, when exposed to 100μM ABA, *LUH* mRNA is induced by about 2-fold (FIG 3-5B). To test if *LUG* and *SEU* expression are also regulated by ABA, RT-PCR was performed. *SEU* and *LUG*

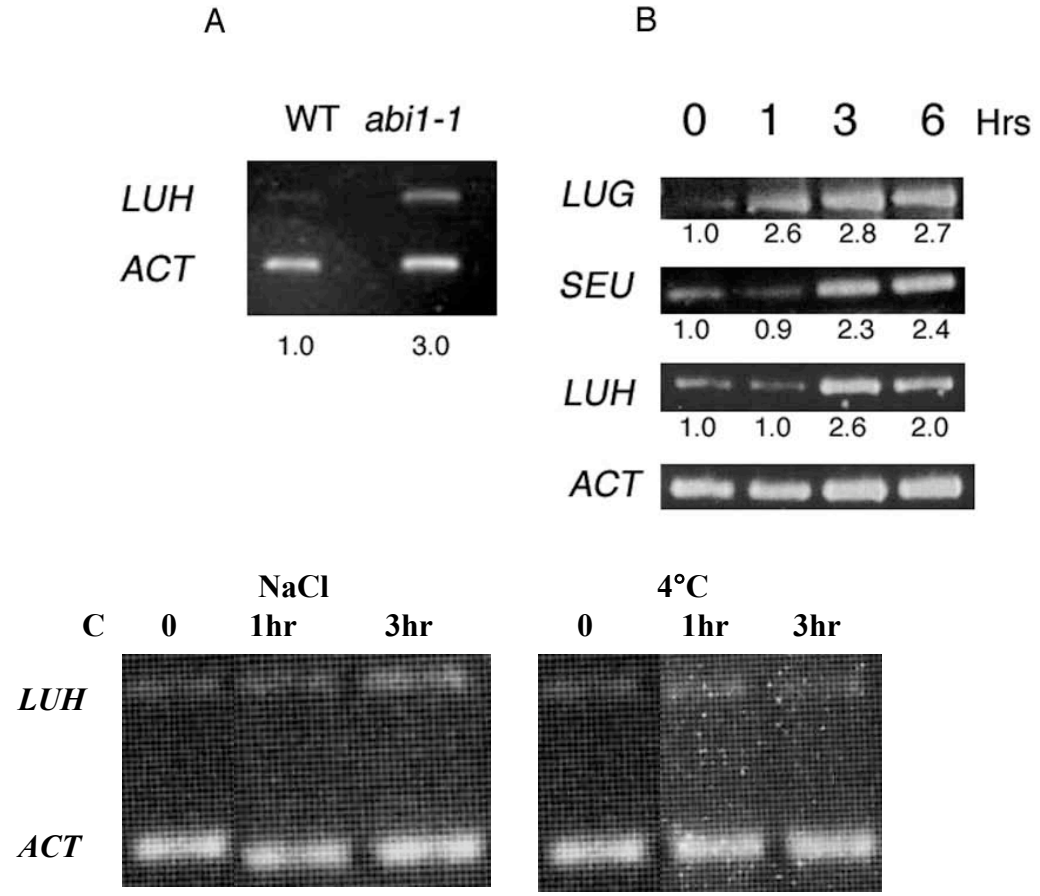


FIG 3-5. Expression of *LUH*, *LUG* and *SEU* mRNA are induced by ABA. A) RT-PCR showing the expression of *LUH* mRNA in WT (Ler) and *abi1-1* (Ler) seedlings grown on 1X MS. *ACT* is the loading control. The numbers represent the relative mRNA level normalized to *ACT* and compared with WT, which is taken as 1.0. B) RT-PCR of 10-day old seedlings after exposure to 100μM ABA. 0, 1, 3, 6 hours after ABA treatment. C) *LUH* mRNA expression is not affected after exposure to 1, 3 hours of 300mM Nacl or low temperature (4°C). D) RT-PCR showing the increase in *LUH* mRNA expression in 3-week old leaves after treatment with 100μM ABA.

expression are also induced by ABA. Interestingly, *LUG* is induced one hour earlier than *SEU* and *LUH* (FIG 3-5B). As shown in FIG 3-5C, *LUH* mRNA levels are not affected when seedlings are exposed to NaCl or low temperature, suggesting that *LUH* is specifically induced by ABA. *LUH* mRNA expression is also increased in leaves treated with ABA (FIG 3-5D), indicating that *LUH* might have a role in stomatal regulation.

luh-1 exhibits defects in ABA signaling

To test if *LUH*, *LUG* or *SEU* have a role in ABA signaling, *luh-1*, *lug-3* and *seu-1* seedlings were tested for their sensitivity to exogenous ABA (FIG 3-6). The germination of *lug-3* and *seu-1* seedlings (in Ler background) on ABA is similar to that of WT (Ler). However, the germination of *luh-1* (Col-*er105*) seedlings on ABA containing plates was less sensitive compared to that of WT (both Col and Col-*er105* background) seedlings (FIG 3-6A). A similar effect is observed on inhibition of root elongation by ABA. *luh-1* mutant seedlings show reduced sensitivity to inhibition of root elongation by ABA (FIG. 3-6B). A control experiment in which WT and *luh-1* seedlings were grown on medium containing IAA (auxin) shows similar inhibition of root elongation for both the genotypes (data not shown). This indicates that the defects in *luh-1,* with the inhibition of germination and root elongation are specifically due to ABA.

Mutants of ABA signaling tend to show altered stomatal regulation. Failure in stomatal closure leads to faster rate of water loss. This can be studied by measuring the time course of water loss in detached rosette leaves. FIG. 3-6C shows that the amount of water loss for *luh-1* plants, is more than WT (Col-*er*) indicating that loss of *LUH* leads to disruption in ABA-mediated stomatal closure. FIG 3-6D shows the water loss in Ler, *lug-3, seu-1* and *abi1-1* rosette leaves. Although *luh-1* plants show greater water loss than WT, it is not drastic as *abi1-1*. However, *lug-3* and *seu-1* do not show any change in water loss compared with WT.

LUH **may promote ABA response by repressing a repressor in the ABA signaling pathway**

Since ABA induces the expression of downstream response genes such as *KIN2* and *RD29B*, we tested if the expression of *KIN2* and *RD29B* was affected in *luh-1*, *lug-3* or *seu-1* mutants. The expression of both *RD29B* and *KIN2* mRNA is significantly reduced in *luh-1* mutants (FIG 3-7A), one hour after treatment with ABA. This suggests that *LUH* activity is required to positively mediate the ABA response. However, the expression of *KIN2* and *RD29B* seems relatively unchanged in *lug-3* and *seu-1* mutants (FIG 3-7A).

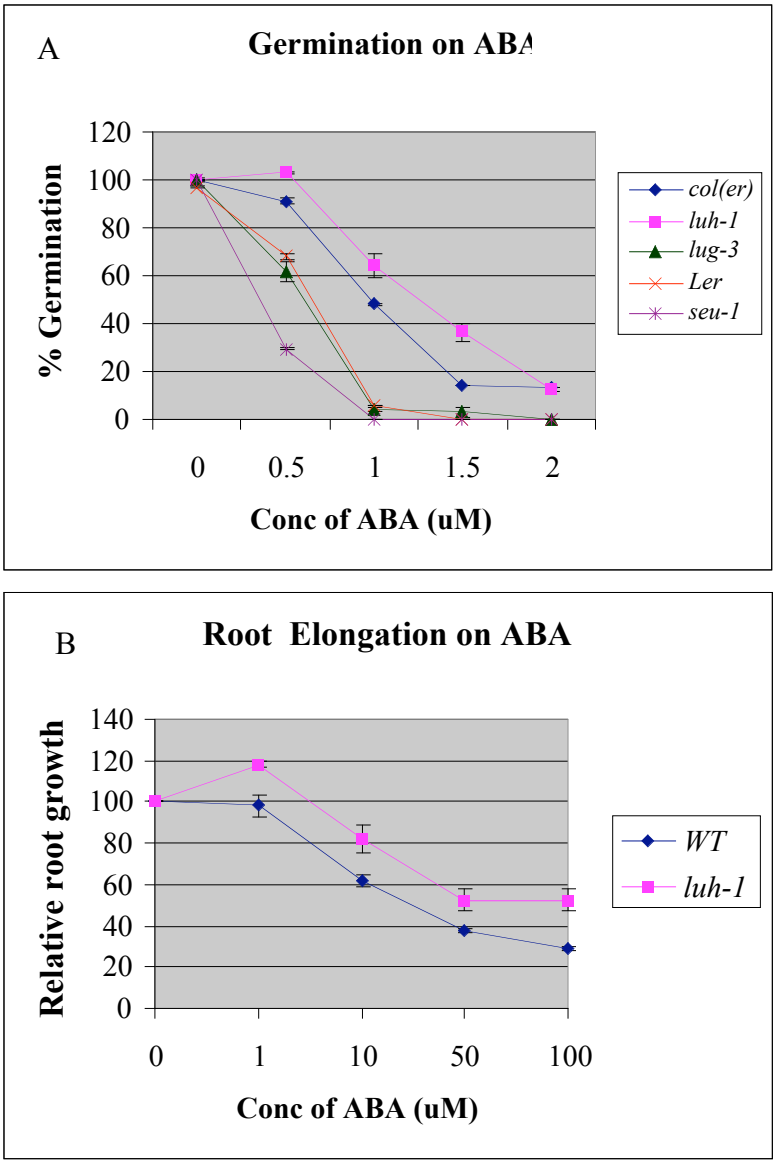
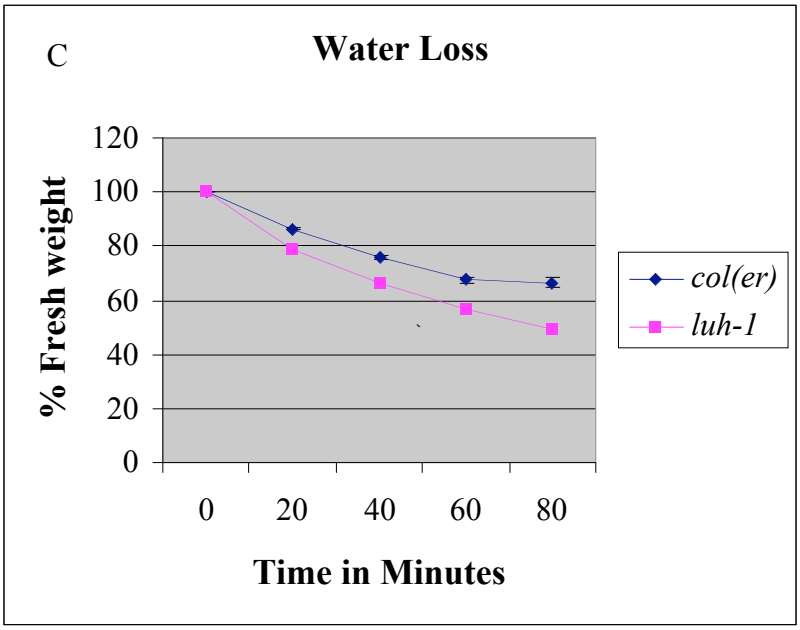


FIG 3-6. A) Germination of WT, *luh-1*, *lug-3* and *seu-1* seedlings on MS medium containing different concentrations of ABA. MS plates containing the seeds were kept at 4° C for 2 days and germination was scored 3 days after the plates were transferred to the growth chamber. For *luh-1*, % germination on MS was corrected to 100%. Data represent the mean of 2 duplicates ± SE , with 50 seeds each. *luh-1* is in Col -er background and is compared with Col-er. *lug-3*, *seu-1* are in Ler background and should be compared with Ler.

B) Relative root growth of WT (Col-er), *luh-1* on ABA containing plates. 4 days old seedlings grown on MS plates were transferred to ABA containing plates and grown vertically. Root elongation was measured 7 days after the transfer. Relative growth was measured as a % of elongation of roots grown on ABA containing plates relative to those grown on ABA free plates for all the genotypes. Data represent the mean of 15 to 20 seedlings ± SE.



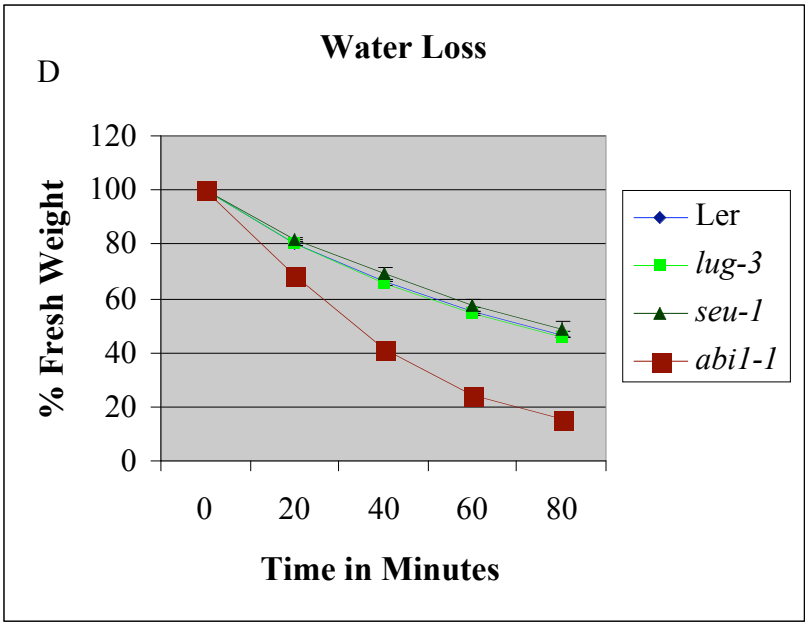


FIG 3-6. C) Rate of water loss in detached 3-week old rosette leaves of WT (Col-er) and *luh-1*. Water loss is measured as the percentage of initial fresh weight, in duplicates (n=7). Data shows mean ± SE.

D) Similar to C, water loss in *lug-3* and *seu-1* and *abi1-1* (all in Ler background) compared to Ler

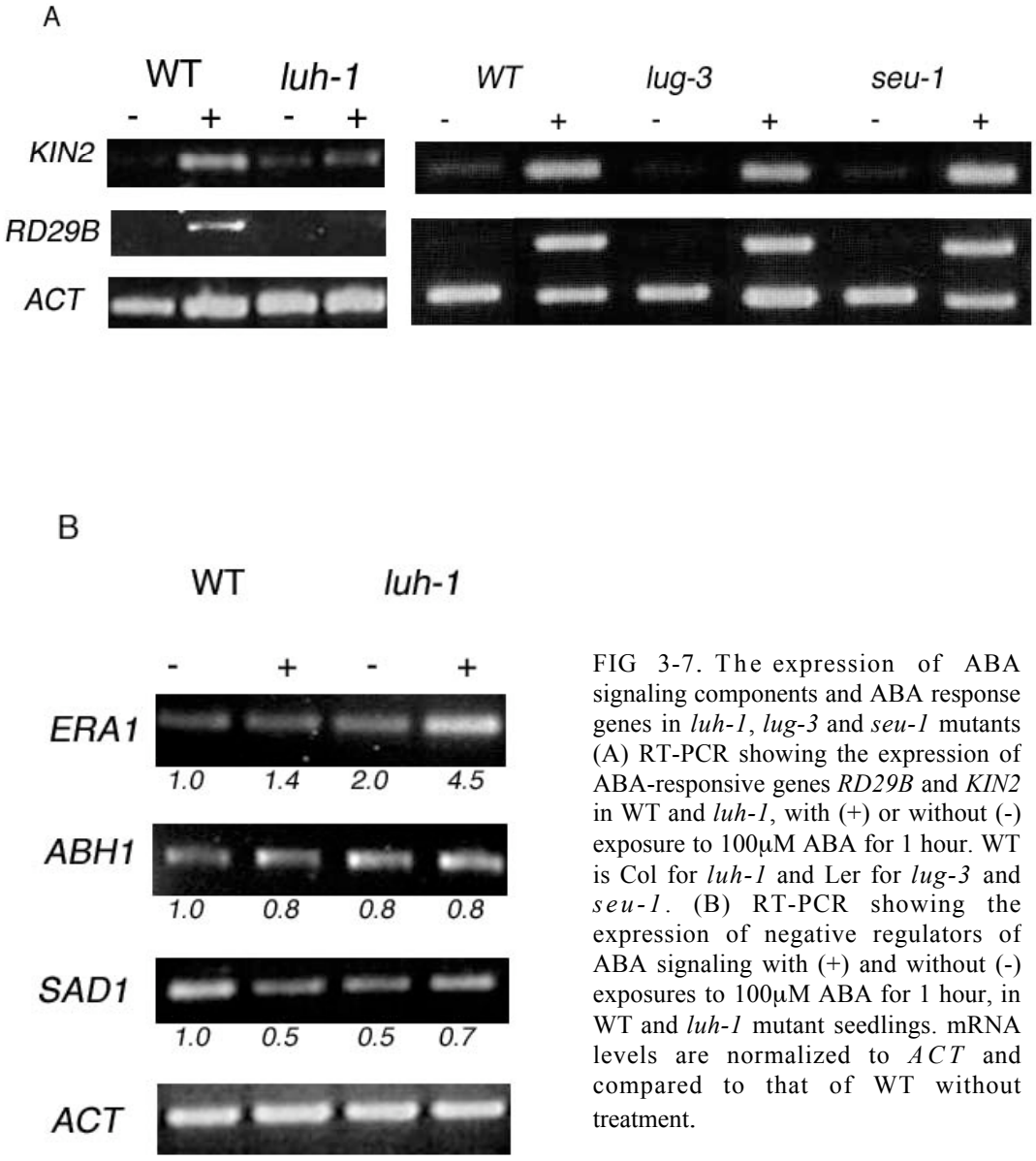


FIG 3-7. The expression of ABA signaling components and ABA response genes in *luh-1*, *lug-3* and *seu-1* mutants (A) RT-PCR showing the expression of ABA-responsive genes *RD29B* and *KIN2* in WT and *luh-1*, with (+) or without (-) exposure to 100μM ABA for 1 hour. WT is Col for *luh-1* and Ler for *lug-3* and *seu-1*. (B) RT-PCR showing the expression of negative regulators of ABA signaling with (+) and without (-) exposures to 100μM ABA for 1 hour, in WT and *luh-1* mutant seedlings. mRNA levels are normalized to *ACT* and compared to that of WT without treatment.

Brady et al., (2002), have shown that *era1* is epistatic to *abi1*. That is *ERA1* acts downstream of *ABI1* in the signal transduction cascade. Due to the structural similarity between LUG and LUH, it is possible that LUH might also function as a repressor in ABA signaling pathway. However, the phenotypes of *luh-1* mutant seedlings shown thus indicate a positive role for *LUH* in ABA response. Therefore, *LUH* may exert its positive effect by repressing the expression of a negative regulatorof ABA signaling. In order to see if the expression of any of the negative regulators of ABA response is altered in *luh-1* mutants, the expression of *ERA1*, *ABH1* and *SAD1* in WT and *luh-1* seedlings was examined with and without exposures to ABA. As seen in FIG. 3-7B, the basal level of *ERA1* mRNA in the absence of ABA is increased 2-fold in *luh-1* mutants. This increased expression of *ERA1* is further enhanced by the treatment with ABA. In contrast, the expression of *ABH1* or *SAD1* is not significantly altered in *luh-1* mutants. Thus, it is possible that *LUH* might function to relay the signal from *ABI1* down to *ERA1*. Further confirmation of this requires constructing double mutants between *abi1* (null) and *luh-1* and between *era1* and *luh-1*.

DISCUSSION

LUH, a positive regulator of ABA response

The ubiquitous expression of *LUH*, *LUG* and *SEU* (FIG. 3-4, Conner & Liu, 2000; Franks et al, 2002) together with the pleiotropic defects in these three mutants suggests that these three genes might function outside flower development, providing co-repressor activities to a variety of target genes in different developmental processes by interacting with different DNA-binding factors. We showed that *luh-1* seedlings are less sensitive than WT to ABA mediated inhibition of germination and root elongation (FIG 3-6). Combined with the observations that ABA induces *LUH* expression and the expression of ABA response genes *KIN2* and *RD29B* are reduced in *luh-1* mutant background, *LUH* is likely a positive regulator of the ABA response. *LUH* might therefore function as a repressor of a negative regulator of ABA signaling pathway to exhibit a positive regulatory effect.

To test if *LUH* is a repressor of a negative regulator of ABA signaling, we examined the expression levels of known negative regulators of ABA signaling, including *ERA1*, *ABH1* and *SAD1*. Only the expression of *ERA1* is increased in *luh-1* mutants both in the basal level in the absence of ABA and in the induced level upon exogenous ABA application, suggesting that *LUH* normally represses *ERA1* expression. Epistatic analyses between *luh-1* and *era1* will further establish the regulatory relationship between *LUH* and *ERA1*.

The fact that *LUG* and *SEU* are induced in response to ABA (Fig 3-5B) suggests a possible role of these genes in promoting ABA responses. However, an absence of altered sensitivity in ABA responses suggests that *LUG* and *SEU* might be functionally redundant with *LUH* in ABA signaling. One emerging model is that while *LUH* plays a prominent role in ABA signaling and a minor role in flower development, *LUG* plays a more prominent role in flower development but a minor role in ABA signaling. Therefore, a role of *LUG* in ABA signaling may be revealed only in *luh-1* background.

The siliques of double mutant plants *lug-16/+; luh-1/luh-1* contain seeds that germinate precociously in the siliques (chapter 2). Such precocious germination or vivipary is due to a lack of seed dormancy. ABA is required for seed maturation and dormancy thus ensuring that seeds do not germinate precociously. When ABA synthesis is reduced or ABA signaling is defective, seeds do not mature and dessicate and germinate precociously. I have shown that *LUH* is a positive regulator of ABA signaling and hence could facilitate ABA mediated seed dormancy. *luh-1* mutants, however, do not contain such viviparous seeds. The presence of these viviparous seeds in *luh-1/luh-1; lug-16/+* suggests that *LUG* plays a redundant role in ABA signaling. It is possible that *lug* might enhance *luh* phenotype in ABA signaling in a *luh lug* double mutant. However, the *luh lug* and *luh seu* double mutants are sterile,

making it impossible to test the response of the *luh lug* mutant seedlings to exogenous ABA. The water loss in *lug-16/+; luh-1/luh-1* plants is currently being studied.

ATHB6, a homeobox protein involved in ABA signaling, has been shown to be the substrate of ABI1, which removes phosphate group from ATHB6 (Himmelbach et al., 2002). *ATHB6* is suggested to function downstream of *ABI1*, as a negative regulator of ABA signaling pathway. *LUH* could be downstream of *ABI1* and *ATHB6*. Analysis of *LUH* promoter sequences shows a putative *ATHB6* binding site (CAATTATTA). Experiments testing direct binding of *ATHB6* to *LUH* promoter will verify this. Ectopic expression of *ATHB6* results in a reduced sensitivity of the seeds to ABA mediated inhibition of germination, thus making a negative regulator of ABA signaling (Himmelbach et al., 2002).

ABA normally inhibits germination. If *LUH* is a positive regulator of ABA signaling, *luh-1* mutants should show increased germination. However, in MS medium without ABA, *luh-1* seedlings show reduced germination compared to WT seedlings (FIG. 3-2 E, G). Since germination is a complex process involving many different factors such as hormones and light, it is possible that *LUH* might be a factor involved in regulating multiple processes including ABA response or in the cross-talk between ABA and other hormones involved in germination.

Gibberellic acid (GA) and brassinosteroids promote seed germination while ABA and ethylene inhibit seed germination. However, the defect in *luh-1* could not be rescued by germinating *luh-1* seeds on GA or brassinosteroid medium (data not shown). The level of sugar and salt in the medium also affects germination. High sugar and salt inhibit germination. Therefore, a defect in glucose or salt signaling could also lead to altered germination response.

Similarly, the reduced root growth in *luh-1* might be due to either reduced number of cells or decrease in size of the cells. Microscopic analysis of roots of *luh-1* mutants in comparison with roots of WT seedlings would indicate if there is any change in the cell number or size of root cells in *luh-1* mutants. Alternatively, the reduced root growth might also reflect an altered nutritional status of the cells. Since glucose is also involved in germination of seeds, a defect in glucose signaling might affect both germination and root growth. Germination and root elongation of *luh-1* seedlings on sugar containing media could indicate if these two processes are affected in *luh-1* mutants in the presence of sugar.

ABI3 and *ABI5* were shown to be involved in a developmental checkpoint (Lopez-Molina et al., 2002). This ABA-mediated growth arrest of germinating seedling ensures that the embryos germinate only when proper nutritional and environmental conditions are met. This developmental checkpoint requires *ABI3*,

which has been shown to function upstream of *ABI5* in executing this checkpoint. This indicates that the germination and further development of the embryo is regulated at several stages.

In conclusion, I have shown a novel function for the floral repressor *LUH*. Further, I have demonstrated that *LUH* plays a unique function in ABA signaling. Fig 3-8 shows the schematic model of *LUH* in the ABA signaling pathway. Based on the repressor action of *LUH*, *LUH* is placed upstream of *ERA1*. Since *SEU* has been shown to be involved in auxin hormone response as well, it would be interesting to see if *LUG* and *LUH* have any role to play in other hormone signaling pathways. Since hormone cross talks are very common, *LUH*, *LUG* and *SEU* might be shared components of different hormone signaling pathways.

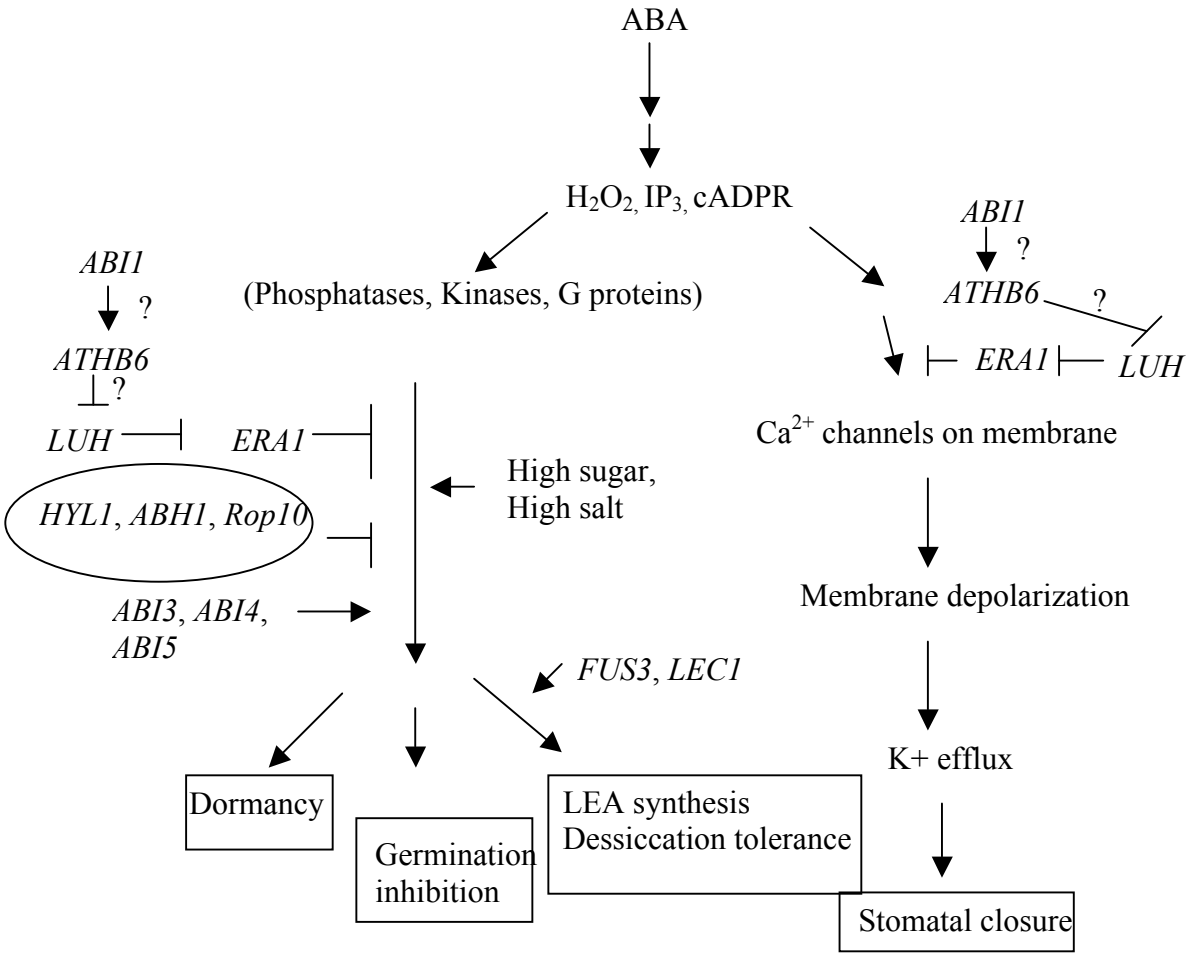


FIG 3-8. A schematic model of ABA signaling pathway indicating the roles of various genes in different ABA mediated processes. Arrows represent activation and bars represent inhibition. Question marks indicate that the relationship is not defined. CADPR-cyclic ADP Ribose; IP_3-Inositol triphosphate; H_2O_2-Hydrogen peroxide

Chapter Four

CONCLUSION

Flower development and floral patterning have been researched in-depth in the past 20 years. The transition of a vegetative plant to a flowering plant is an important and irreversible event in the plant life cycle. The transition to flowering is regulated by both internal and external stimuli/signals. In order to ensure proper development and reproduction, proper coordinations between different components in various signaling pathways must occur. The components involved in one pathway may be shared with other pathways, a way of coordination and cross-talk among different signaling pathways. The major finding of my thesis that the flower development regulators specifically *LUH*, and to some extent *LUG* and *SEU* also function in ABA hormone signaling pathway suggests that floral organ patterning and development may be coupled with or influenced by plant hormones.

Transcriptional repression is a complex process involving the interaction of multiple molecules and the formation of multimeric complexes. Tup1 is a global repressor in *Saccharomyces cerevisiae*, which represses genes involved in different processes including glucose metabolism, hypoxia, DNA-damage and cell type switching (Smith and Johnson, 2000). Groucho, a Drosophila co-repressor with sequence similarity to Tup1, similarly represses genes involved in many

developmental processes including lateral inhibition, segmentation, sex determination, dorsal/ventral pattern formation, terminal pattern formation, and eye development (Chen and Courey, 2000). Mut11 in Chlamydomonas, a uni cellular green alga, a TUP1 homolog functions as a repressor in transposon mobilization, cellular growth and sensitivity to DNA-damaging agents (Zhang et al., 2002). A *C.elegans* homolog of Groucho, UNC-37 has been shown to function as a repressor in pattern formation along the L/R axis in the nervous system and in motor neuron identity (Zhang and Emmons, 2002; Chang et al., 2003). The co-repressors form co-repressor complex, such as Tup1/Ssn6 and the complex recruits Rpd3 type Histone deacetylase to cause repressive chromatin or interact with RNA polymerase to inhibit transcription Davie et al., 2003).

LUH and LUG are partially redundant in flower development

The study of transcriptional repression in plant development is gaining grounds rapidly. The identification of many negative regulators of *AG* indicates that transcriptional repression in plants requires the concerted action of many components. Although *luh-1* mutants do not show any single flower phenotype, *luh-1* is able to genetically enhance the floral phenotypes of *lug-16* and *lug-3*. This suggests that *LUH* has a role in flower development but its function is redundant to that of *LUG*. Although yeast two- hybrid assay between LUG and LUH did not reveal any physical

interaction between LUG and LUH (data not shown), it is possible that both LUG and LUH function in the same repression complex by interacting independently with SEU. Alternatively, both LUH and LUG are shown to interact with SEU indicating the possibility of two types of SEU/LUG and SEU/LUH co-repressors (Fig 4-1). As shown in Fig 4-1, LUG can complex with either SEU or SEU-like. Similarly LUH can form a complex with SEU or SEU-like. However, in flowers, SEU/LUG might be the predominant complex in *AG* repression while in ABA signaling LUH/SEU-like may be the more prominent complex that mediates the repression of *ERA1*. This might explain why *lug* single mutant has a flower phenotype while *luh* single mutant shows ABA phenotype.

LUG was shown to interact with SEU via the LUFS domain in LUG. LUH may also interact with SEU through the LUFS domain in LUH. Each of the co-repressor complexes might be recruited by different DNA-binding proteins targeted to different target promoters. For example, AP1, a MADS box protein implicated in binding to *AG* cis-regulatory element, has been found to interact with SEU (VV Sridhar and Liu, unpublished). Therefore, AP1 might recruit the LUG/SEU co-repressor complex to the *AG* cis-regulatory element (Fig 4-1). *AP1* mRNA is expressed in the outer two whorls of the flower thereby imparting spatial specificity to the co-repressor complex and repressing *AG* expression only in the outer two whorls. It is likely that a different transcription factor with roles in ABA signaling can

recruit the LUH/SEU co-repressor comples to the cis-regulatory element of genes in ABA signaling such as *ERA1*.

The identification of specific DNA-binding factors that interact with LUH might shed some light as to the protein partners that are involved in the formation of a repression complex. One approach may be to isolate genetic enhancers of *luh*. Similar to *lug* and *seu*, it will be interesting to test if *luh* can genetically interact with *seu-like* mutants in flowers. Yeast-two hybrid screens using guard cell cDNA library as prey and LUH- BD as bait will likely identify the protein partners that function with *LUH* in ABA signaling.

Chromatin immunoprecipitation (ChIP) is a powerful technique that has been used to identify target gene sequences. ChIP can be used to identify the targets of *LUH*. Since *ERA1* has been shown to be downstream of *LUH*, ChIP can be used to analyze if *LUH* is bound to *ERA1* promoter. Precipitation of proteins involved in a complex, by an antibody specific to one of the proteins, could be used to pull down the complex and then probe with antibodies to putative partners in the complex. This technique could be used to identify protein partners of LUH, by immuno precipitating LUH with antibodies to LUH. Similarly, transgenic plants containing epitope-tagged LUH can be used to determine protein partners present in a complex in vivo.

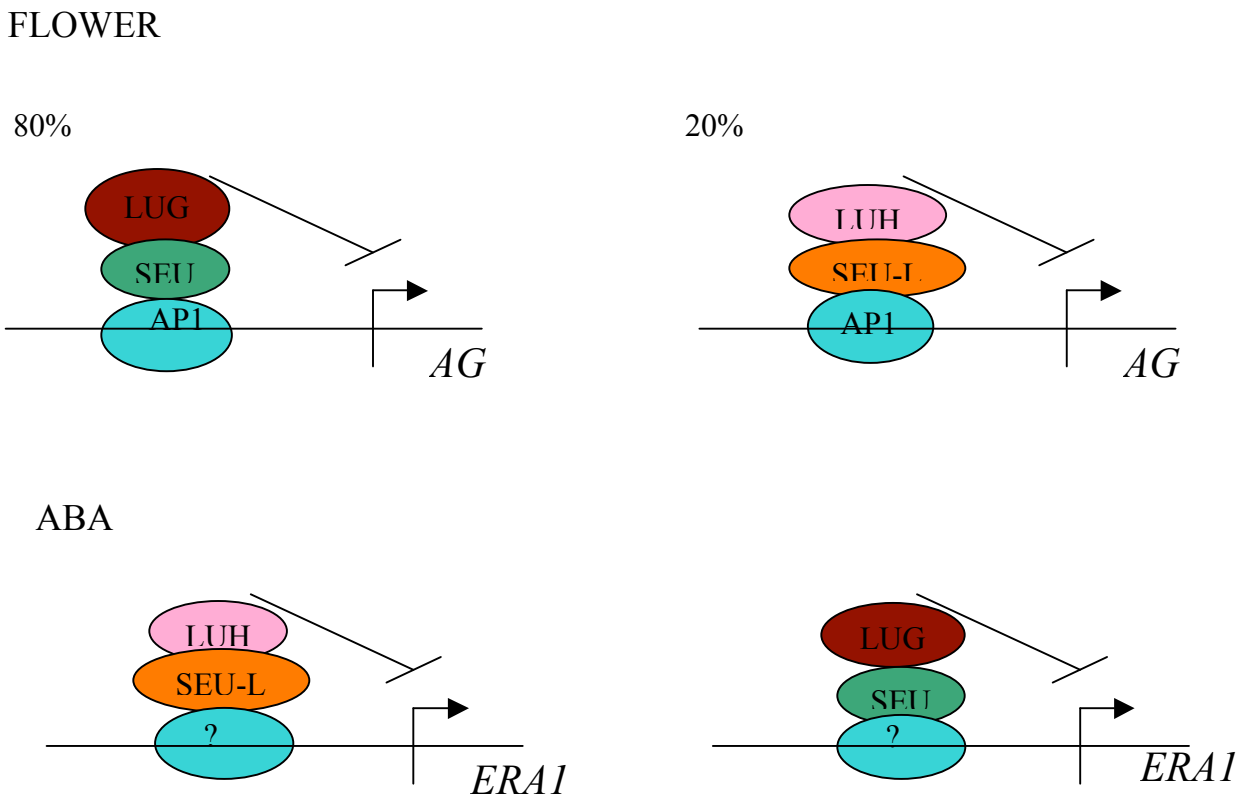


Fig 4-1. Schematic figure showing a model of action of *LUH*, *LUG* and *SEU* in *AG* repression and ABA signaling. LUH, LUG, SEU and SEU-like may form co-repressor complexes. The co-repressor is recruited by AP1 to repress *AG*, and recruited by an as yet unknown transcription factor to repress *ERA1*. The LUG/SEU co-repressor complex might be more in flower while the LUH/SEU-like complex might be more in ABA signaling. *SEU-L* is a *SEU* homolog in *Arabidopsis.*

LUH has a prominent role while _LUG_ has a minor role in ABA signaling

Like the co-repressors Tup1 and Gro mentioned above, *LUG*, *SEU* and *LUH* might also function as global co-repressors in plant development. They may function in different tissues by being recruited by different DNA-binding proteins. The fact that all three genes, *LUG*, *LUH* and *SEU,* are expressed ubiquitously in *Arabidopsis* supports this idea. I have shown in chapter three of the thesis that *LUH* functions in ABA signaling based on the phenotype of *luh-1* mutant seedlings in the presence of exogenous ABA. *LUH* may act as a repressor of *ERA1*, in the ABA signaling pathway (Fig 4-1). The lack of a single mutant phenotype for either *lug* or *seu* mutant seedlings in the presence of exogenous ABA suggests that *LUH* has a prominent role in ABA signaling while *LUG* and *SEU* may have a more prominent role in *AG* repression in flower development. The viviparous phenotype in the seeds of *lug-16/+; luh-1/luh-1* but not in *luh-1* single suggests that L*UG* too plays a role in ABA signaling, but its effect is only revealed in *luh-1* mutant background.

This thesis shows evidence for the role of *LUG* and *LUH* in embryo development. The lack of both *LUG* and *LUH* results in embryo lethality although the single mutants do not show any abnormality in embryo development. This novel function for *LUG* has not been reported so far. This suggests that *LUH* and *LUG* are

not only important for floral organ specification but also for the proper development of embryos.

Although the role of ABA in flower development or floral organ formation is not known, since it induces the expression of the floral regulators *LUG*, *LUH* and *SEU*, it is possible that ABA might have a role in floral organ development by regulating the expression of these genes. It would be interesting to see if the expression of *LUH*, *LUG* or *SEU* mRNA in flower is affected by exogenous ABA. ABA might alter the transcriptional activity of *LUH*, *SEU*, and *LUG* targets in flower development and hence might function indirectly in floral organ formation. These targets could be identified by Chromatin immuno precipitation and analysed to see if they have any function in flower development. Once the targets are identified, they could also be tested to see if their expression levels are affected in the presence of ABA.

Since *lug, luh/+* double mutants show severe flower phenotype and *luh, lug-16/+* mutants show viviparous phenotype, it provides a clue as to the link between ABA signaling and floral organ development. It remains to be seen if this effect is direct. This thesis provides some insights in that direction.

Chapter Five

Materials and Methods

Plant growth

Arabidopsis plants were grown on metromix 360 soil in controlled growth chambers at 20°C under long day conditions (16 hour light and 8 hour dark) and 50-60% humidity. Seeds used in germination and root elongation assays were harvested from WT and mutant plants grown at the same time.

luh-1 mutant identification

luh mutants were obtained through the TILLING (Targeting induced Local Lesions IN Genomes) facility (ABRC stock # CS91893). These plants were generated by EMS mutagenesis in Col *er-105* background (McCallum et al, 2000). *luh-1* mutant plants grown on soil were identified using dCAPS marker (see Table 5-1). Genomic DNA was amplified by dCAPS primers by PCR at 94°C 2 mins, 94°C 30 secs, 50° C 30 secs, 72°C 30 secs for 35 cycles. 6μL PCR reactions were digested with restriction enzyme BstX1 overnight at 55°C and were run on 2.5% agarose gels. Mutant PCR products were not cut and wild type PCR products were cut by BstX1 to

produce a 178 bp DNA fragment. *luh-2* and *luh-3* mutants were also grown for phenotypic analysis.

Molecular analyses of *LUH*

Total RNA was isolated from flowers (for RACE) and from 10 day old seedlings grown on MS plates (for ABA-response assays) with Tri-reagent (Sigma). WT and mutant seedlings grown on the same plates were sprayed with 100μM ABA (Sigma) for the indicated time periods. Control plates were sprayed with water. For the tissue specific expression of *LUH*, flowers, leaves and stems were harvested from 5 week old Col WT plants. Roots were harvested by removing them from seedlings grown vertically on MS plates for 2 weeks. Reverse transcription was performed with oligo dT and Superscript RT II enzyme (Invitrogen). All RT-PCR reactions were carried out for 25 cycles. All RT-PCR reactions were repeated at least twice. Primers were designed spanning introns so as to distinguish cDNA from genomic DNA contamination. *ACTIN* 2 primers were used as control Primer sequences (Table5-1). The RT-PCR reactions were quantified using Image quant 1.1 (NIH) software, based on the intensity of ethidium bromide staining. 5' RACE for *LUH* was performed with generacer (Version F, Invitrogen) kit with total RNA from *Arabidopsis* flowers. 5' nested primer GGACACTGACATGGACTGAAGGAGTA (Table 5-1) was used and the RACE products were cloned in pCR II TOPO (Invitrogen) and sequenced.

ABSCISIC ACID response assays

For germination, seeds harvested at the same time from WT (Col-er), *lug-3*, *luh-1*, *seu-1* mutant plants were surface sterilized and transferred to 0.5X MS plates containing various concentrations of ABA (Sigma) and stratified at 4°C for 2 days and transferred to growth chamber. Seeds were counted as germinated when the radicles completely penetrated the seed coat. For root elongation, surface-sterilised seeds were grown on 0.5X MS plates for 4 days in growth chamber after stratification for 2 days in cold room. After 4 days, seedlings were transferred to 0.5X MS plates containing various concentrations of ABA for 7 days and root elongation was measured with a ruler. Water loss was measured in detached rosette leaves from 3-week old plants. 7 leaves from two plants per genotype were left on laboratory bench abaxial side up, and water loss was estimated by weighing the leaves after regular periods of time.

Generation of Transgenic plants

LUH full length cDNA was obtained from RIKEN (RAFL09) and sequenced to confirm correct sequence. This full length *LUH* cDNA was amplified by PCR with primers (Primer sequence in Table 5-1) containing engineered Xma1 sites and high fidelity Taq polymerase (Roche). The PCR product was cloned in the PBI121 vector

at Xma1 restriction site and verified by sequencing. Plasmids were transformed into GV3101 *Agrobacterium* cells through electroporation. The primary shoots of newly bolted *luh-1* and *lug-16* plants were removed to stimulate growth of secondary shoots. A few days after the clipping, *luh-1* plants were transformed by the floral dip method (Clough et al., 1998). The inflorescences of the plants were dipped in an infiltration medium (0.5XMS salt, 5% sucrose and 0.03% silwet L-77) containing *Agrobacterium* cells. Each pot was immersed in the medium for about 10 minutes and the pots were placed on their side and covered with saran wrap. The next day, the plants were kept upright and grown until maturity. Kanamycin resistant T1 seedlings were identified on MS plates containing 50µM Kan and transferred to soil. Seeds were first washed with 70% ethanol followed by 0.05% tween. Seeds were then rinsed with sterile water and plated on MS Kan plates after mixing with 0.1% agarose. Plates were kept at 4°C for two days and transferred to growth chamber. Ten day old kanamycin resistant seedlings were transferred to soil. Out of 12 35S::*LUH/lug-16* transgenic plants that were found to have transgene by PCR analysis, 5 plants that showed an enhanced phenotype were further analyzed. For 35S::*LUH/luh-1* transgenic plants, 8 plants were found to have transgene in them and 2 of these lines that showed higher *LUH* mRNA expression were used for further analyses.

Leaf DNA extraction for PCR analyses

Two to three medium sized leaves were removed by forceps and placed in an Eppendorf tube containing 400 µL of Edwards extraction buffer (200 mM Tris pH 7.5; 250 mM NaCl; 25 mm EDTA; 0.5% SDS). Tissues were ground with pestles and the tubes were centrifuged at 14,000 rpm for 5 minutes at room temperature and 300 µL of the supernatant was transferred to a clean eppendorf tube. Equal volume of isopropanol was added and incubated for 5 minutes at room temperature. Tubes were centrifuged at 14,000 rpm for 5 minutes at room temperature. Pellets were washed with 300 µL of 70% ethanol three times and then air-dried. Pellets were resuspended in 30 µL of distilled water and stored at 4° C.

Double mutants and genotyping

For generation of double mutants between *luh-1* and *lug-16* (a weak allele of lug), and *lug-3* (a strong allele), *luh-1* pollen was used to pollinate *lug-16* and *lug-3* and the seeds from F1 WT plants were collected. F2 plants were analyzed for segregation of double mutant plants. Similarly, *luh-1 seu-1* double mutants were generated by crossing *luh-1* pollen to *seu-1* stigma. For genotyping, *luh-1* mutants were identified by *LUH* dCAPS marker (Table 5-1). The genotype at *LUG* locus (*lug-16, lug-3*) was determined by PCR amplification with primers (Table 5- 1). PCR products were purified with PCR purification kit (Qiagen) and sent for sequencing. For *lug-16* and *lug-3* mutants, sequencing was done to verify the sequence. *seu-1* dCAPS marker

(Table 5-1) was used to amplify the genomic DNA at 50°C for 35 cycles and digested with the restriction enzyme Rsa1 for 3 hours at 37°C.

Photography and microscopy

Whole mount floral photographs were taken with a Zeiss Stemi SV6 dissecting microscope with a NIKON digital camera and images were processed with Adobe photoshop version 7.0.

Yeast two- hybrid assay

Full length *LUH* cDNA (obtained from RIKEN) was PCR amplified as described for 35S::*LUH* and sequenced to confirm there were no amplification errors. The primers (Table 5-1) designed for PCR amplification were engineered to have enzyme sites (Sal1 and Xma1 for Binding domain-BD vector) and Xho1 and Xma1 for (Activation domain-AD vector). The amplified cDNA were cloned in-frame into pGBKT7 (BD vector) and pGADT7 (AD vector) (clontech) respectively at the restriction enzyme sites. The yeast host strain used for transformation was PJ69A whose genotype is *MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4delta gal80delta GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ* (James et al., 1996). Three reporter genes *HIS3*, *ADE2* and *lacZ* are responsive to GAL4 activation in the yeast strain PJ69-4A.

Transformation was done according to Agatep et al., (1998). Briefly, a small overnight culture of yeast (2-5mls) grown at 30° C was used to inoculate a 50 ml YPAD medium (Clontech) the next day and grown for about 4 hours. Cells were centrifuged at 5000 rpm for 5 minutes and resuspended in 25 ml sterile water and centrifuged again. Cells were resuspended in 1.0 ml 100 mM Lithium acetate (LiAc) and transferred to a 1.5 ml microfuge tube. The cells were centrifuged again and LiAc was removed. The cells were resuspended in 500 µl of 100mM LiAc which is sufficient for 10 transformations. Aliquots were then transferred to individual microfuge tubes and the transformation mix containing PEG (50% w/v), 2 mg/ml salmon sperm DNA, 1.0 M LiAc and plasmid DNA. The mix was incubated at 30°C for 30 minutes and heat shocked at 42°C for 30 minutes, centrifuged at 6000 rpm for 15 seconds to remove the transformation mix and resuspended in 100 µl sterile water. The resuspended cells were plated on selective plates (-leu-trp-ade or –leu-trp-his) and incubated at 30°C for 2-4 days to recover transformants.

X-Gal colony filter lift assay

Nitrocellulose filters were laid on the plate of yeast colonies and allowed to wet completely. Filter paper was lifted off the plate and placed in liquid nitrogen for a couple of minutes. Filter paper was then transferred to a Petri plate containing X-Gal solution (10% triton-X-100, 50 mg/ml X-Gal and in phosphate buffer), with cell side

up. Plates were incubated at 37°C and checked periodically for the formation of blue color.

Production of LUG-His tag proteins in bacteria

LUG cDNA corresponding to the amino acid region shown in Fig A-1, was amplified by PCR with primers engineered to have BamH1 and Xho1 restriction enzyme sites (Primer F 5' ccg ctc gag ggc ctt ggg agt tct gta ggg 3'; R 5' cgg gat cca tga aat gct acc atc cat cgt 3'). The amplified cDNA was cloned in pCRII TOPO vector (Invitrogen) and the insert was verified by sequencing. The insert was excised from pCR II TOPO and ligated with the vector pet14b at BamH1-Xho1 site (Novagen). This pet14b-LUG plasmid was transformed into BL21 host and selected on chloramphenicol (34 μg/μL)), ampicillin plates (50 μg/μL). The transformants were grown at 37° C overnight in a small volume of LB (5 mL). This culture was used to inoculate a 250 mL LB broth the next day. When the culture reached an OD_{600} of 0.5, the culture was induced with 1.0 mM isopropylthio-ß-D-galactoside (IPTG) and the cells harvested 3.5 hours post-induction. Cells were pelleted and the pellets were used for protein extraction.

Protein purification and Thrombin digestion

Proteins were isolated by the denaturing protocol (according to the Novagen His-bind kit literature) with 1X imidazole buffer (5mM imidazole, 0.5 M NaCl, 20mM Tris-Hcl pH 7.9) containing 6M urea. The proteins were purified by passing them through a pre-charged, single-use Nickel column (Novagen). The protein fractions were eluted with 1X elution buffer containing 20 mM imidazole, 0.5 M NaCl, 20mM Tris-Hcl pH 7.9. The purified protein was quantified by the Bradford method (Ausubel et al., 1991), using Bovine Serum Albumin (BSA) as the standard. Briefly, LUG-His tag protein diluted to 1:5, 1: 50 and 1: 100 were mixed with 5 ml Bio-Rad protein assay reagent, allowed to sit at room temperature for 2 minutes and spectrophotometrically measured at 595 nm (Beckman DU 600 Spectrophotometer). To cleave His-tag, 100 µg of the purified protein was digested with 0.1U of biotinylated-Thrombin (Novagen) at 20° for 16 hours, in 500µl total volume. After digestion, the mixture was passed through Streptavidin-agarose spin column (Novagen) to remove the thrombin.

Production of LUG antibodies

500 µg of purified LUG was sent to Cocalico biologicals to induce antibodies against LUG in rabbits. One rabbit was immunized with 100 µg of the LUG antigen for initial inoculation and with 50µg of the antigen for three booster doses. Three test

bleeds (6 to 8 mL each) were obtained and the final bleed was used to detect LUG in the Western blot shown in appendix Fig A-4.

Extraction of proteins from Arabidopsis flowers

Proteins were extracted from WT and mutant (*lug-3*) *Arabidopsis* flowers using EZ buffer according to a protocol described in Martinez-Garcia et al., (1999). Briefly, *Arabidopsis* materials were collected with forceps and placed in 1.5 mL eppendorf tubes containing buffer E (125 mM Tris-Hcl pH 8.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50mM sodium bisulphite), centrifuged at 14,000 rpm for 10 minutes, and the supernatant was saved. 100 µL of the supernatant was mixed with 10µL of Z buffer (125mM Tris-Hcl pH 6.8, 12% (w/v) SDS, 10% (v/v glycerol, 22% (v/v ß-mercaptoethanol, 0.001% (w/v) bromophenol blue). The extract obtained (EZ extract) was loaded in a pre-cast NuPage 4%-12% bis-tris poly-acrylamide gel (Invitrogen) and the proteins were separated after running the gel at 200 volts for one hour. Protein transfer was performed in XCell sure lock Mini Cell blot module (Invitrogen) according to manufacturer's protocol using PVDF (Millipore) membrane. Transfer was done for 1 hour at 30 volts. Western blot was performed according to methods described in Ausubel et al., (1991). Anti-LUG anti serum was diluted 1: 3000 and Goat-anti Rabbit antibodies conjugated to AP (Promega) was diluted 1:4000 to detect LUG, with western Blue (Promega), as a substrate.

Extraction of nuclei

Nuclei was extracted from *Arabidopsis* leaves by the procedure described by Kinkema (2002). Leaves were homogenized and the homogenate filtered through 62µm (pore size) nylon mesh (Small parts, inc). Triton-X-100 (0.5%) was added to the filtrate and incubated on ice for 15 minutes, centrifuged and washed with Honda buffer [(2.5% (w/v) Ficoll, 5% dextran T40, 0.4 M sucrose, 25mM Tris-Hcl pH 7.4, 10mM $MgCl_2$, 10mM ß- mercaptoethanol]. The pellet containing the nuclei was resuspended in 1 mL Honda buffer; centrifuged again for 5 minutes and the supernatant was retained. The supernatant was centrifuged again at 5000 rpm, to pellet the nuclei.

Table 5-1: Primers used for RT-PCR and genotyping

Gene name	Forward	Reverse
	Primer sequence (5' to 3')	
ACT2	gtt ggg atg aac cag aag ga	ctt aca att tcc cgc tct tc
ABH1	ggt tat aga cta gta tct aat cag	aga gct ctg tta aga aga gcc tct
ERA1	tct act aca aag att ata ttc aac	cag taa cat gtg tgg tag aag tca
LUG	tga gtg gtg gtc tgt ctt ctg ctg g	agc agc ctc atc taa gga ctc c
LUH	tgg ctc aga gta att ggg aag	cca ggc ttt gat tgc aga at
luh-1 (dCAPS)	gca cct gga ggg ttt cca ttt gag tg	cgc ttt acc ttg ttg tgc cta aaa tt
KIN2	atg tca gag acc aac aag aat gcc	cta ctt gtt cag gcc ggt ctt gtc
RD29B	gtg aag atg act atc tcg gtg gtc	gcc taa ctc tcc ggt gta acc tag
SAD1	atg gcg aac aat cct tca cag ctt	tca ttc tcc atc ttc ggg aga ccc
SEU	tag tat gaa gga cct gat aga tta	gtt tgg agg att gta agc agc att
seu-1 dCAPS	aca aca gat tct gct ctt ccg gag gta	tta cct gca aac acc gaa ca

pet14b-*LUG*	ccg ctc gag ggc ctt ggg agt tct gta ggg	cgg gat cca tga aat gct acc atc cat cgt
LUH (RACE)	gga cac tga cat gga ctg aag gag ta	gag cca tag ctt cag ccc aag atcg
LUH-AD	att acc cgg gga tgg ctc aga gta att ggg aag	att ctc gag cta ctt cca aat ctt tac gga
LUH-BD	att acc cgg gga tgg ctc aga gta att ggg aag	acg cgt cga cat cta ctt cca aat ctt tac gga
35S::*LUH*	att acc cgg gga tgg ctc aga gta att ggg aag	tcc ccc ggg cta ctt cca aat ctt tac gga
lug-16	att ttc tta ttg cat tgt ttc tta	gca gtt ata taa tca cta gta tcc
lug-3	caa ctc ttg ttg caa cgt gca cag	cgt ctc ttc tct tct gag gct gct gct

Table 5-1. (continued)

APPENDIX 1

Raising Antibodies Against LEUNIG

LEUNIG (LUG) is shown to negatively regulate *AGAMOUS* expression in the outer two whorls of *Arabidopsis* flowers. The mechanism of the outer two whorl-specific function of *LUG* is not known. Either *LUG* is only expressed in the outer two whorls or *LUG* activity is regulated post-transcriptionally or post-translationally. In situ hybridization showed that *LUG* mRNA is expressed in all four whorls in wild type flowers (Conner and Liu, 2000), indicating that the outer whorl-specific *LUG* function must be at the translational or post-translational levels. To test if LUG protein is only present in outer two whorls, antibody against LUG will be an essential tool. Further, anti-LUG antibody may facilitate future biochemical analyses of LUG.

Examining the expression of LUG protein in wild type *Arabidopsis* plants

To raise the anti-LUG antibodies, a portion of the *LUG* gene (Fig A-1), which is not conserved between *LUG* and *LUH*, was cloned in the vector pet14b (Novagen) containing His-Tag and transformed into BL21 bacterial hosts. The cells were then induced with 1 mM IPTG and harvested 3.5 hours post-induction. The cells were

lysed and the protein was isolated by the denaturing protocol (according to Novagen protocol).

MSQTNWEADKMLDVYIHDYLVKRDLKATAQAFQAEGKVSSDPVAIDAPGG

FLFEWWSVFWDIFIARTNEKHSEVAASYIETQMIKAREQQLQQSQHPQVSQQ

**lug-12* stop **lug-3*

QQQQQQQQIQM**Q**QLLLQRAQQQQQQQQQHHHHQQQQQQQQQQQQQ**Q**

QQQQQHQNQPPSQQQQQQSTPQHQQQPTPQQQPQRRDGSHLANGSANGLV

GNNSEPVMRQNPGSGSSLASKAYEERVKMPTQRESLDEAAMKRFGDNVGQ

LLDPSHASILKSAAASGQPAGQVLHSTSGGMSPQVQTRNQQLPGSAVDIKSE

INPVLTPRTAVPEGSLIGIPGSNQGSNNLTLKGWPLTGFDQLRSGLLQQQKPF

MQSQSFHQLNMLTPQHQQQLMLAQQNLNSQSVSEENRRLKMLLNNRSMSL

GKDGLGSSVGDVLPNVGSSLQPGGSLLPRGDTDMLLKLKMALLQQQQQHQ

QQGGGNPPQPQPQPQPLNQLALTNPQPQSSNHSIHQQEKLGGGGS ITMDGSI

SNSFRGNEQVLKNQSGRKRKQPVSSSGPANSSGTANTAGPSPSSAPSTPSTHT

PGDVISMPNLPHSGGSSKSMMMFGTEGTGTLTSPSNQLADMDRFVEDGSLD

DNVESFLSQEDGDQRDAVTRCMDVSKGFTFTEVNSVRASTTKVTCCHFSSD

GKMLASAGHDKKAVLWYTDTMKPKTTLEEHTAMITDIRFSPSQLRLATSSF

DKTVRVWDADNKGYSLRTFMGHSSMVTSLDFHPIKDDLICSCDNDNEIRYW

SINNGSCTRVYKGGSTQIRFQPRVGKYLAASSANLVNVLDVETQAIRHSLQG

HANPINSVCWDPSGDFLASVSEDMVKVWTLGTGSEGECVHELSCNGNKFQS

CVFHPAYPSLLVIGCYQSLELWNMSENKTMTLPAHEGLITSLAVSTATGLVA

SASHDKLVKLWK

FIG A-1. Amino acid sequence of LUG. The portion of LUG selected for raising antibody is underlined. The positions of *lug-3* and *lug-12,* are also indicated, both change a Q (bold) into a stop.

The protein was then purified through a Nickel column by virtue of its His-Tag (Fig A-2), digested with thrombin to cleave the His-Tag (Fig A-3). 500µg purified antigen was sent to Cocalico biologicals for raising antibodies in rabbit. The anti-serum obtained after 3 bleeds was used as primary antibodies to detect the LUG protein using protein extracts from Arabidopsis flowers. Goat anti-rabbit antibodies conjugated to the Alkaline Phosphatase (AP) enzyme were used as secondary antibodies in the western blots with western blue as a substrate (Promega). The detailed methods are described in the Materials and Methods section.

Protein extracts from *lug-3* and *lug-12* flowers served as the negative controls. These two nonsense mutants do not produce the peptide used for raising antibodies (Fig A-1). Although the antibodies recognized the purified bacterial protein (Fig A-4), they failed to detect a specific band expected to be present in WT but absent in *lug-3* on the western blot (Fig A- 4). One possibility is that LUG protein is not abundant and is difficult to detect. To enrich LUG protein, which was previously shown to localize to nucleus (Conner and Liu, 2000), nuclear extract was made and the western blot repeated. However, the antibodies still failed to detect LUG (data not shown).

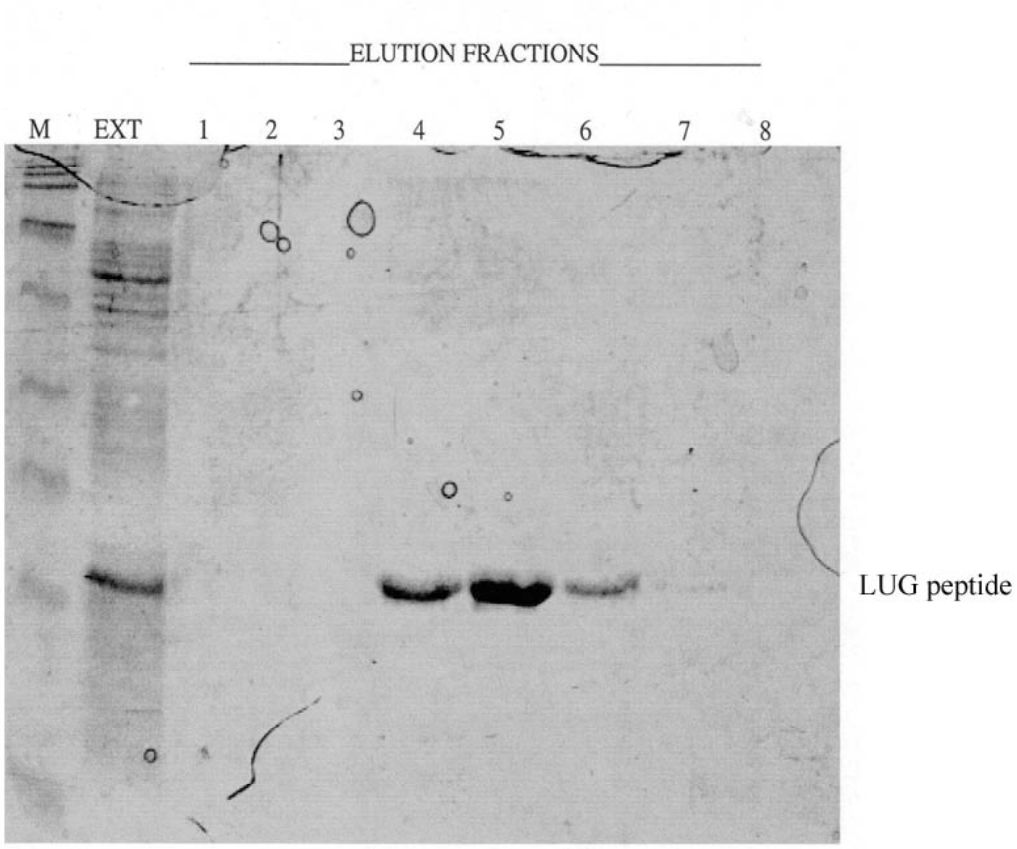


FIG A-2. Purification of LUG His-tag protein from E.coli. Numbers 1 to 8 represent the different elution fractions from the Nickel column. M- protein molecular weight marker; E-Extract before purification.

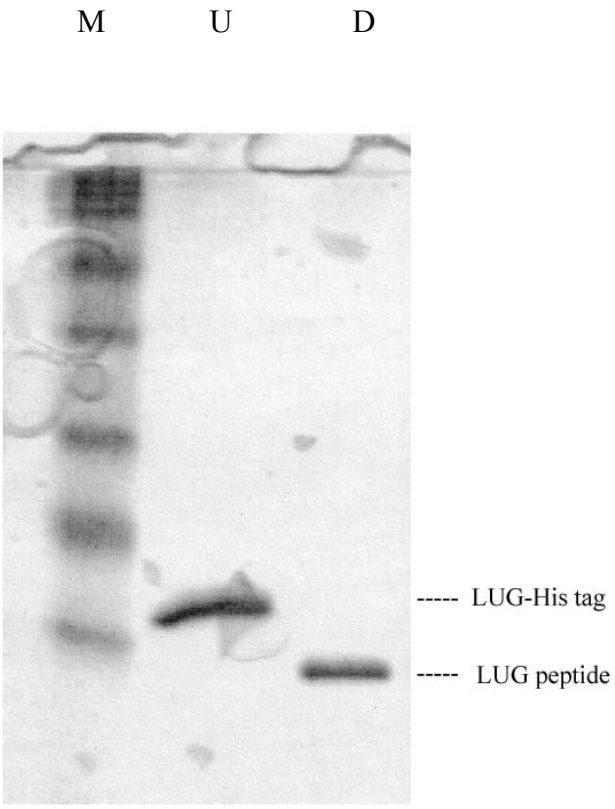


FIG A-3. Thrombin digestion of LUG His-tag proteinLUG His-tag protein cut with 0.1U Thrombin for 16 hours at 20°C. M: Protein molecular weight marker; U: Uncut LUG-His tag protein; D: Thrombin digested LUG protein

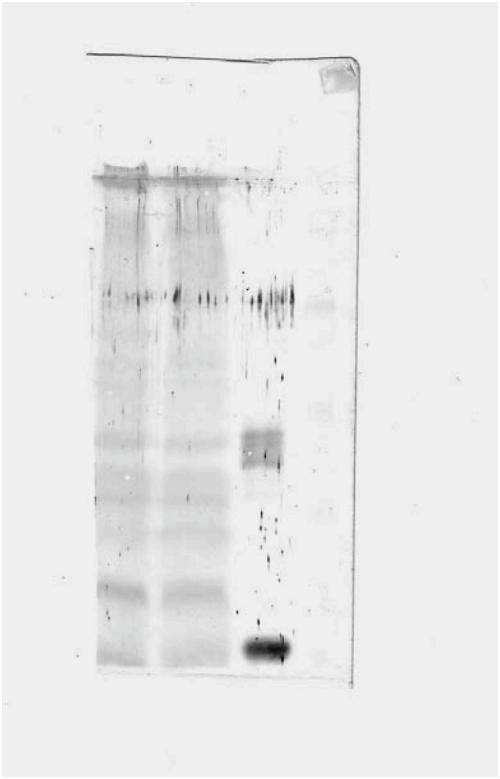


FIG A-4. A Western blot with Anti-LUG antibodies using protein extracts from *Arabidopsis* flowers Lane 1: *lug-3* flower; Lane 2: Wt *Arabidopsis* flower; Lane3: Purified epitope from bacteria. Primary antibody (Anti LUG antiserum) was diluted 3000 times; secondary antibody (Goat anti-rabbit antibody conjugated to Alkaline Phosphatase) was diluted 4000 times.

The failure to detect LUG in Western blot can be due to several possibilities. First, LUG is a high molecular weight protein (103 kD) (Fig A-4) and is likely expressed at a low level. Second, the primary antibodies were obtained only after three booster doses and may be very low in amount in the antiserum. Since the antibodies can detect LUG from bacterial extract, the antibody could be useful for in *vitro* experiments such as *in vitro* immuno- precipitation experiment. Furthermore, lower

dilution of the antibody (such as 1:100) should be tested on Western blots and *in situ* immuno localization of LUG could be tried, as antibodies that failed in Western blot were shown to work in immunolocalization.

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