ABSTRACT

Title of Dissertation: *LEUNIG*, *LEUNIG HOMOLOG*, AND *SEUSS* ARE TRANSCRIPTIONAL CO-REPRESSORS THAT REGULATE FLOWER DEVELOPMENT, MUCILAGE SECRETION, AND PATHOGEN RESISTANCE

Minh Bui, Doctor of Philosophy, 2009

Directed By: Associate Professor William J. Higgins, Department of Biology

Transcriptional repression is an important regulatory mechanism for development. My thesis focuses on dissecting the function of Groucho (Gro)/Transducin-Like Enhancer of split (TLE) family of transcriptional repressors in plant development. My work characterizes two *Arabidopsis thaliana* genes, *LEUNIG* (*LUG*), first discovered to repress transcription of the floral homeotic gene *AGAMOUS* (*AG*), and *LEUNIG_HOMOLOG* (*LUH*), a gene with the highest sequence similarity to *LUG*. To investigate the functional redundancy between *LUG* and *LUH*, I constructed and analyzed *lug*; *luh* double mutants, and concluded that both *LUG* and *LUH* repress *AG* expression in the flower, with *LUG* playing a more prominent role than *LUH*. The double mutant also revealed a previously unknown function of *LUG* and *LUH* in embryogenesis because *lug-3*; *luh-1* double mutants are embryo lethal, while the single mutants develop normal embryos. During the course of this study, I developed a new genotyping method called Simple Allele-

discriminating PCR (SAP), which is cost-effective, quick, and easy to perform. This method has greatly facilitated my research as well as others in the lab.

A second part of my thesis addresses the role of *LUG* and *LUH* in other developmental processes besides flower development. My data indicate that these two genes, like their counter parts in fungi and animals, act as "global co-repressors" in various developmental and physiological processes. My thesis work revealed that both co-repressors, together with its interacting protein SEUSS (SEU), repress the Salicylic Acid (SA) pathogen defense pathway. Although *lug-3*, *luh-1*, and *seu-1* mutants induced *PR1* expression at higher levels than wild-type, only *lug-3* and *seu-1* mutants were pathogen resistant. Furthermore, *LUH* functions as a positive regulator in seed mucilage secretion, a process important for proper seed germination, hydration, and dispersal. I propose a possible connection between the defect in mucilage secretion and pathogen defense in *luh-1* mutant plants and seeds, which places the foundation for further investigation and may uncover mucilage secretion as a major defense mechanism. My thesis has provided important insights into how transcriptional co-repressors regulate diverse developmental and physiological pathways in higher plants.

LEUNIG, *LEUNIG HOMOLOG*, AND *SEUSS* ARE TRANSCRIPTIONAL CO-REPRESSORS THAT REGULATE FLOWER DEVELOPMENT, MUCILAGE SECRETION, AND PATHOGEN RESISTANCE

By

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Dedication

This dissertation is dedicated to my wonderful and loving grandmother, Gai T. Nguyen (1916-April 28th, 2007). May she rest in peace. I miss you, I love you, will never forget you, and I hope you are proud of me.

Acknowledgements

My success would not be possible without the help and support from so many people throughout my academic career. I would like to thank my parents Quang Bui and Hoa Phan, sister Kathy Bui, and cousin Khanh Dinh, all who have provided a great deal of love, emotional, and financial support throughout the years.

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Other affiliations that played a key role in my success include members in the Biology Department such as Drs. Marco Colombini and Justicia Opoku, who provided valuable guidance and nurtured my love for teaching, and Lois Reid and Karen Speorl for their help and support throughout the years. Also, life partner Nathan Morris and dear friends including Dr. Leah Siskind, Amy Wayne, Boy and Khang Sharp, because without you guys, I would not have made it this far. Last but not least, past and present lab members including Channa Amarasekera, Boyana Grigorova, Fei Huang, Ranjani Ganesan, Paja Sijacic, Chloe Mara, and Courtney Hollender for your friendship, support, and thanks for making lab fun!

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Chapter 1: Introduction and Literature Review

1.1 Introduction

As scientific breakthroughs in the fields of medicine, mathematics, life and social sciences continue to make everyday life more livable and enjoyable, research becomes the forefront of all occupations to provide for these new beneficial technologies. There is no question that plants are at the bottom of the food chain, and their demise would also mean the end of human life as we know it. However, plant related research has faced many difficult challenges in times of financial crises. With growing emphasis on researching cures for human diseases, plant research has often been on the back-burners of political focus, but little do politicians and people know how important plants are to the world.

Plants are unlike most organisms in that they cannot move or escape from harsh environments or stresses. However, they are unique because they are highly adaptable, inhabiting almost every corner of the Earth, even in harsh climates that are uninhabitable for humans. However, like humans, plants are constantly under attack by various microbial pathogens such as bacteria, viruses, and parasites. These pathogens can be categorized as biotrophs which allow for co-survival between the host and pathogen, nectrotophs which induce death quickly upon their host, or hemi-biotrophs will allow for host survival in the beginning stages of infection but cause death later on.

To survive and co-habitate this world, the plant must fend off adversaries such as harsh environmental conditions including drought and pathogens to successfully live out its life. A plant's life cycle begins from a small seed, which will germinate, grow to reach adulthood, and then undergo a reproductive stage of development, and the process will begin anew. Hence, flower development and seed development are important aspects of plant biology to study, especially since it has direct human impact such as food and fuel production for an ever growing and demanding human population.

This literature review will attempt to educate and provide background information that will frame the research conducted within this dissertation. The literature review will first discuss the molecular aspects and physiology of flower development in plants, followed by the developmental aspects of seed production within the carpels of a flower. The third section will discuss the constant battle that exists between plants and their myriad pathogens, pathogens that take advantage of the plants' resources and aim to terminate or populate their host plants before the completion of host's life cycle. All these processes have a common theme, which is that they are all regulated by genes that encode transcriptional repressors. Therefore, the last part of this literature review will focus on the mechanisms of transcriptional repression that occur in organisms such as flies, yeast, and plants.

1.2 Flower Development

1.2.1 ABCE Model for Floral Organ Identity & Specification

Flowers are not only pleasing to the eye, but serve an important reproductive function and provide food for insects such as bees and humans alike. The intricate process of floral organ patterning and specification is largely determined by spatial and temporal regulation of floral homeotic genes. These floral organs are arranged in a series of four whorls including the sepals located in the outermost whorl, then petals in whorl two, stamens in whorl three, and fused carpels known as the gyneocium located in whorl four. Most angiosperm flowers consist of leaf-like sepals which protect the maturing flower, often colorful and extravagant petals that attract pollinators, stamens that carry pollen, and carpels that enclose ovules. Spatial organization of these floral organs requires highly regulated expression of A, B, C, and E class floral homeotic genes (**Fig. 1.1** and **Table 1.1**). When A class, A and B classes, B and C classes, and C class genes are expressed in whorls 1-4 of a flower, sepals, petals, stamens, and carpels develop, respectively (Liu, 2005).

Class	Gene(s)
A	*APETALA1* (*AP1*) and *APETALTA2* (*AP2*)
B	*APETALA3* (*AP3*) and *PISTILLATA* (*PI*)
C	*AGAMOUS* (*AG*)
E	*SEPALLATA1, 2, 3,* and *4* (*SEP1, 2, 3,* and *4*)

Table 1.1: The four classes of floral homeotic genes.

With the exception of *AP2*, the ABCE classes of genes encode MADS-box type II transcription factors (Nam et al., 2003). The name MADS is derived from the four founding members: *MCM1* in yeast, *AG* in *Arabidopsis*, *DEFICIENS* in

Antirrhinum, and *SRF* in human (Messenguy and Dubois, 2003). The MADs-box proteins possess a MADs-box domain which binds to the CArG-box (C AT-rich G) on the target promoter, and a K-box domain that is necessary for protein dimerization. In contrast, *AP2* encodes an *AP2/EREBP* (Ethylene Responsive Element Binding Protein) transcription factor with two AP2 domains (Okamuro et al., 1997). The single AP2 domain found in EREBP binds to the GCC-box on target promoters of the pathogenesis related (PR) genes expressed during pathogen infections (Buttner and Singh, 1997), while the two AP2 domains in *AP2* bind to the TTTGT sequence (Xuemei Chen, personal communication).

Loss-of-function A class mutants produce carpeloid sepals and stamanoid petals. Meanwhile, in loss-of-function B class mutants, petals and stamens are transformed to sepals and carpels, respectively. Finally, C class loss-of-function mutant flowers are indeterminate or produce additional flowers in the center, and only produce sepals and petals (Bowman et al., 1989). Because loss-of-function A class mutants develop flowers that ectopically express carpels in the outer whorls, and C class mutants ectopically expresse sepals in the innermost whorl, the A and C class genes negatively regulate each other (**Fig. 1.1**) (Lohmann and Weigel, 2002).

Although simultaneously knocking out the ABC genes resulted in flowers with all whorls of leaves instead of floral organs, ectopic over-expression of the ABC class genes in leaves was not sufficient to transform leaves into floral organs (Pelaz et al., 2001). This result led researchers to believe that there was another factor that is

necessary to transform leaves to floral organs. That factor was later found to be encoded by the E class genes. There are four highly redundant genes belonging to the E class including *SEPALLATA1 - 4* (*SEP1 - 4*) and they are expressed in all four whorls of the flower. *sep1 sep2 sep3* triple mutants develop flowers that have sepals in all four whorls (Pelaz et al., 2000). *sep4* single mutants are indistinguishable from wild-type plants, but quadruple *sep1-4* mutants develop leaf-like organs with branched stellate trichomes in all four whorls (Ditta et al., 2004). The knowledge that the E class genes are necessary for flower development led researchers to ectopically over-express the A, B, and E genes, resulting in plants that produce petals in lieu of roseatte leaves (Honma and Goto, 2001; Pelaz et al., 2001).

Current theories on flower development involve the formation of a tetrameric transcriptional regulating complex that encompasses all members of the A, B, C, and E class genes. Sepal development requires a transcriptional complex that consists of two A and two E-class proteins (AP1 and SEP4), petals require A, B, and the redundant E-class proteins (AP1, AP3, PI, SEP1-3), stamens require B, C, and E class proteins (AP3, PI, AG, SEP1-3), and carpels require two C and two E class proteins (AG, SEP1-3) (**Fig. 1.1B**). This tetrameric complex is conserved across different plant species (Kanno et al., 2003; Kramer and Hall, 2005).

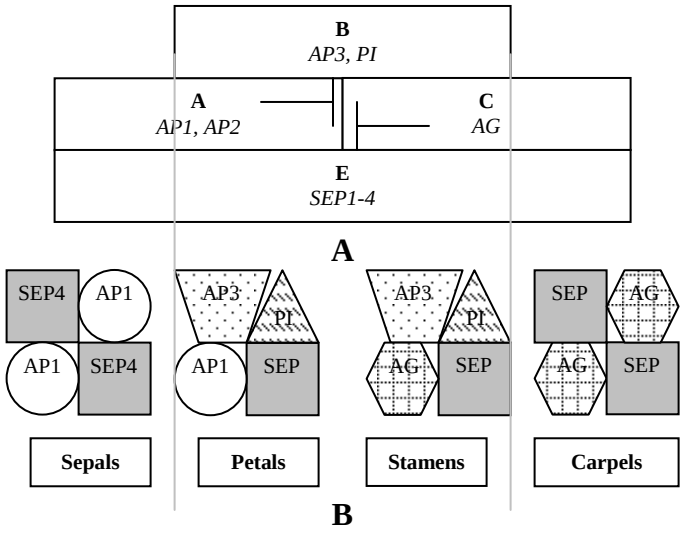


Fig. 1.1: The four floral whorls and the tetrameric complexes that determine floral organ identity. A) The ABCE model of genes that regulate expression of the four floral organs, sepals (A, E), petals (A, B, E), stamens (B, C, E), and carpels (C, E). B) Depiction of the different tetrameric complexes with different A, B, C, E class combinations that produce different floral organs.

1.2.2 Flower Development & Co-Repressors

lug loss-of-function mutants were originally isolated as an enhancer of the *ap2-1* mutant phenotype (Liu and Meyerowitz, 1995). *lug-3* is a strong allele due to a non-sense mutation in the LUFs domain at the N-terminus of LUG. *lug-3* mutants display floral organs that are predominantly carpelloid or staminoid in whorls 1-2. This phenotype revealed that the function of LUG is to repress the C class gene, *AGAMOUS* (*AG*). In *lug-3* mutants, *AG* is ectopically expressed in the outer two whorls of the flower and thus results in flowers with carpelloid and staminoid organs (Liu and Meyerowitz, 1995).

How does LUG normally repress *AG* in whorls 1 and 2 to allow for sepal and petal development? Both genetic and molecular studies revealed that LUG interacts with another protein called SEUSS (SEU) to form a co-repressor complex that

prevents *AGAMOUS* (*AG*) from being expressed in the outer two whorls of the flower (Franks et al., 2002; Sridhar et al., 2004). Because neither LUG nor SEU proteins possess DNA-binding domains, the LUG/SEU complex must interact with and be ferried to *AG*'s cis-element by a DNA-binding transcription factor. Sridhar *et a*l. (2006) report that SEU interacts with the A-class APETALA1 (AP1) and E-class SEPALLATA3 (SEP3) MADS-box proteins to form a multimeric complex, and AP1/SEP3 bring the LUG/SEU co-repressor complex to the *AG* cis-element to repress *AG* expression (**Fig. 1.2**).

How does LUG/SEU repress *AG* only in the outer two whorls? Sridhar *et al*. (2006) proposed three models. One possibility is that LUG/SEU interacts with the DNA-binding partners that are expressed in the outer two whorls, the second is LUG/SEU represses an activator of *AG*, and the third and most plausible model is LUG/SEU represses *AG* in all four whorls but factors present within the inner two whorls will antagonize the LUG/SEU repressor effect. The third model is supported by the fact that LUG/SEU interacts with both AP1 and SEP3, and likely with the other redundant SEP proteins that are expressed in all four whorls. The antagonizing factors within the inner two whorls include AG itself, which competes with LUG/SEU for SEP3 (Sridhar et al., 2006).

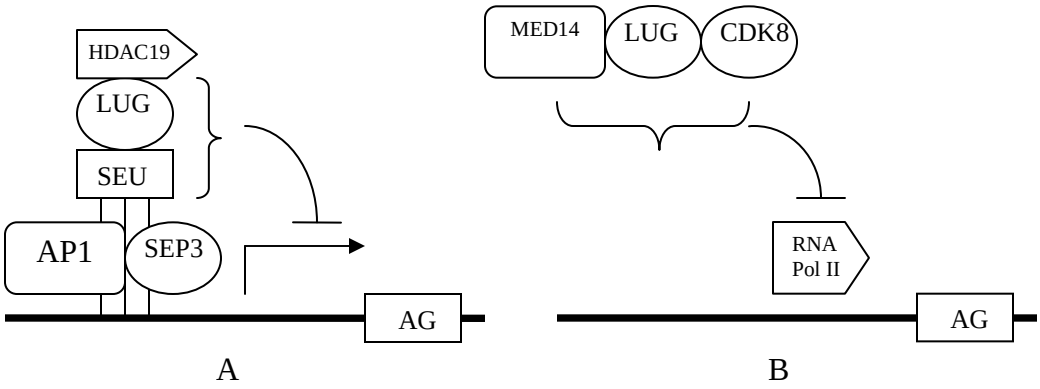


Fig. 1.2: Model depicting the transcriptional machinery that regulates *AG* expression. A) Putative model involving LUG and SEU as co-repressors in the transcriptional repression of *AG* with AP1 and SEP3 serving as the DNA-binding partners (Sridhar et al., 2004; Gonzalez et al., 2007). B) In the second model, LUG interacts with MED14 and CDK8 to inhibit the RNA Polymerase II transcriptional machinery (Gonzalez et al., 2007).

1.3 Seed Coat Development and Mucilage Production & Secretion

1.3.1 Seed Development

The development and formation of viable seeds preserves the plant species. The process involves fertilization (syngamy) of the egg by the pollen nuclei cell to create a diploid zygote. Meanwhile, a second sperm nuclei fuses with the two polar nuclei located within the embryo sac to form a nutritive triploid endosperm that nourishes the growing embryo (Taiz and Zeiger, 2006), which is the second product of double fertilization in flowering plants (Sorensen et al., 2001).

Once the egg is fertilized, the zygote undergoes cell divisions to resemble a multicellular plant, a process termed embryogenesis. Embryogenesis consists of five primary stages of development including the 1) zygotic stage that follows fusion of the sperm and egg; 2) globular stage which is due to a series of cellular divisions that

ultimately lead to the octant (eight-cell) embryo; 3) heart stage owing to rapid cell divisions on either side of the shoot apical meristem, producing protrusions that become the cotyledons; 4) torpedo stage which is the further development of cotyledonous protrusions and elongation of the axis; and finally 5) mature stage which occurs when the seed becomes desiccated and enters dormancy (Taiz and Zeiger, 2006). Dormancy is necessary for the seed to withstand long periods of environmental conditions that are unsuitable for growth such as cold winters or dry summers. When environmental conditions are favorable, the seed will continue on to the next stage which includes germination, vegetative development, and reproductive development. These favorable conditions include the right type and period of light the seed receives, and enough moisture to aid in biochemical reactions such as the synthesis of enzymes to break-down nutritive sources.

The seed germination process is antagonistically regulated by the plant hormones gibberellic acid (GA) and abscisic acid (ABA) (Piskurewicz et al., 2008). GA promotes seed germination by destroying RGA-LIKE2 (RGL2), a DELLA transcriptional repressor that inhibits germination, while ABA blocks germination by activating ABA-INSENSITIVE5 (ABI5), a basic leucine zipper that represses germination (Piskurewicz et al., 2008). Thus, the ratio between ABA and GA in the seed determines whether a seed germinates or not, depending on the suitability of the growth conditions that can facilitate growth (Taiz and Zeiger, 2006). In fact, the right environmental conditions will increase GA concentration while simultaneously

decreasing ABA levels, resulting in transcriptional changes between RGL2 and ABI5 (Gough, 2008).

Although the fertilization model for seed development is well-acknowledged, there is evidence of seed development that occurs independently of fertilization. *pistillata* (*pi*) mutants which lack the B-class floral homeotic gene, develop short siliques that have no seeds, but ethyl methanesulfonate(EMS) mutagenesis in the *pi* mutants resulted in suppressor mutations that produced long siliques that contained developing seeds. However, the flowers of these double-mutants were male sterile or lacked pollen because B-class mutants lack petals and stamens (Chaudhury et al., 1997). The group later isolated the suppressor gene by back-crossing to wild-type plants, mapped the three alleles to three different chromosomes, and named the genes *FERTILIZATION-INDEPENDENT SEED1, 2,* and *3* (*FIS1-3*). *fis1* and *fis2* mutants remain male sterile, but form proembryos that do not develop past the globular stage. When the *fis/+* mutants were fertilized with wild-type pollen, the siliques yielded 50% wild-type (+/+) seeds that were fully developed, and the other 50% mutant seeds (*fis/+*) that shriveled up, failed to germinate, and had embryos arrested at the torpedo stage.

FIS3 is allelic to *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) which encodes a WD40 protein, with mutants that lacked an embryo and endosperm (Ohad et al., 1996; Chaudhury et al., 1997; Ohad et al., 1999). *FIS1* encodes a protein that shares homology to *Drosophila Enhancer-of-zeste* and is identical to *MEDEA* in

Arabidopsis. *FIS2* encodes a zinc-finger domain that is capable of binding DNA and has three putative nuclear localization signals (Luo et al., 1999). *fis1* and *fis2* mutants produce seeds even in the absence of fertilization, lacked an embryo but develop normal endosperm. Thus, *FIS1* and *FIS2* genes repress endosperm development prior to fertilization (Sorensen et al., 2001).

1.3.2 Developmental Stages of the Seed Coat

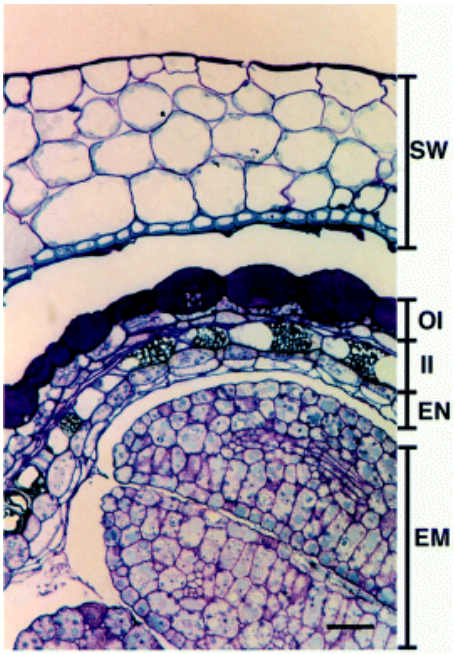


Fig. 1.3: The cellular structure of the seed coat (Windsor et al., 2000). Silique Walls (SW) surround the ovary. Two-cell layers of the seed coat form the Outer Integument (OI). Three cell-layers of the Inner Integument (II) and the cellular Endosperm (EN) protect and nourish the growing Embryo (EM) as indicated.

The outer integument undergoes a dynamic cellular and morphological rearrangement throughout the development of the growing embryo before it reaches maturity. The outer integument is the precursor to the seed coat with two cell-layers, while the inner integument has three cell layers that surround the cellular endosperm and protects the inner growing embryo (Windsor et al., 2000) (**Fig. 1.3**). During the

first stage of outer integument development, a large vacuole becomes apparent in both inner and outer layers. In the second globular stage, starch granules become visible toward the distal end of the outer layer but proximal end to the embryo in the inner layer. During the heart shaped embryo stage, mucilage is deposited in a ring around the lateral walls of the outer epidermal cell layer, which forces a protruding column to appear in the center of the epidermal cell and later becomes a columella (**Fig. 1.4**). During the fourth stage, the vacuole has disintegrated, mucilage production is complete, and columella is evident at the center of each epidermal cell. During the last stage, a thick primary cell wall (OW) is formed and positioned next to the columella and covers the radial walls (RW) located to the periphery of the columella (**Fig. 1.4**). During this final stage, starch granules are no longer present. When seeds are imbibed or hydrated, the mucilage stored in the epidermis will break through the secondary cell wall and be released into its surroundings.

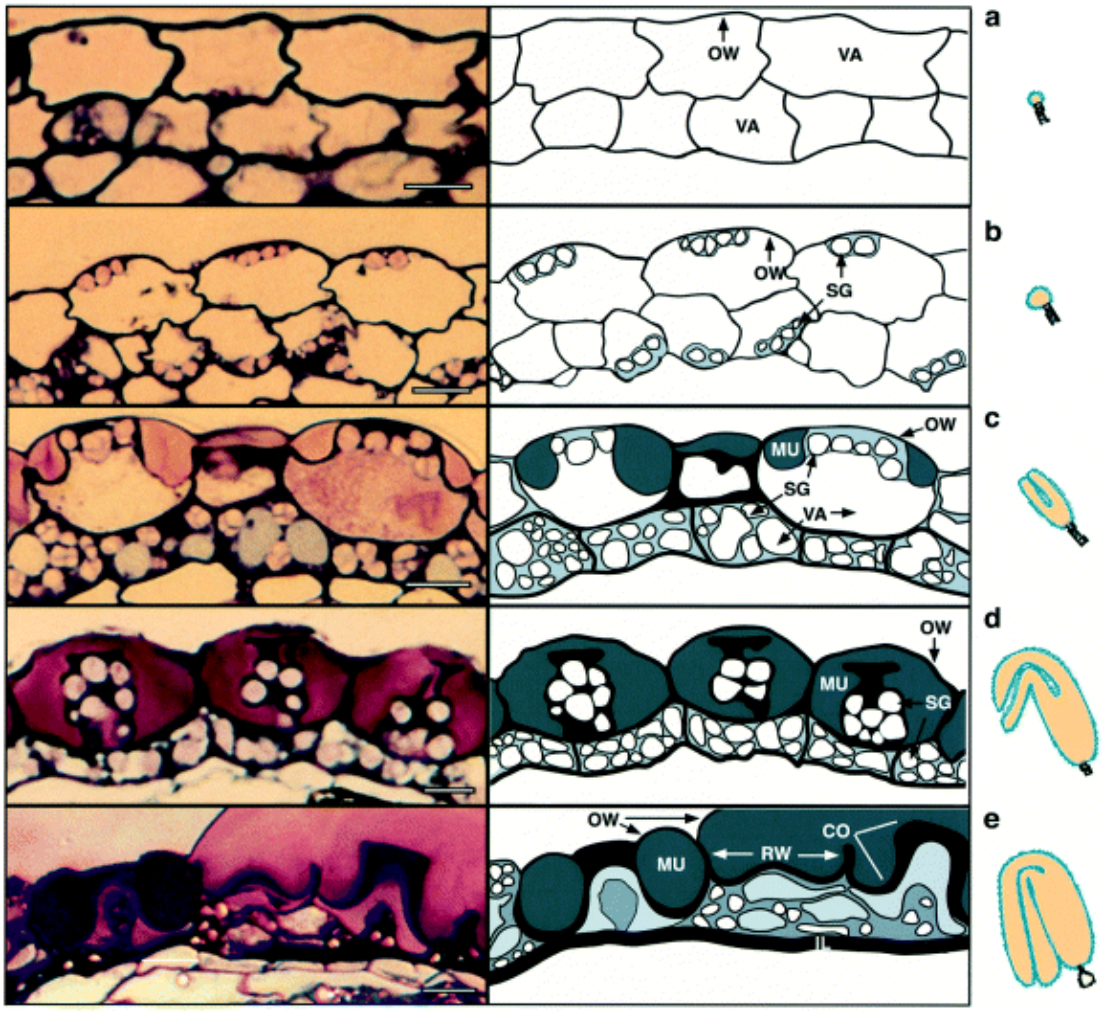


Fig. 1.4: Different developmental stages of the outer integument. A, B, C, D, and E represent stages 1-5 respectively from Windsor et al., 2000. OW=outer (primary) wall; VA=vacuole; SG=starch granules; MU=mucilage; CO=columella; IL=inner cell layer of outer integument; RW=radial wall. Bar=10 um.

1.3.3. Mucilage Composition and Synthesis

Mucilage is a gelatinous mixture that consists mostly of a pectinacious polysaccharide. Its production is known as myxospermy, a process that is common to the Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae plant families (Frey-Wyssling, 1976). The mucilage in the seed is important for protection of the growing embryo, seed germination, seed hydration and dispersal, and mucilage in the root cap

protects the growing root (Frey-Wyssling, 1976; Esau, 1977). Furthermore, mucilage is found in the transmitting tract of the gynoecium that extends from the stigmatic surface to the ovule micropyles. The hydrophilic mucilage, rich in arabinogalactan sugars facilitates the growth of pollen tube towards the ovule (Webb and Williams, 1988). Mucilage secretion accumulates large amounts between the plasma membrane and the outer cell wall in the seed coat, which is actin-directed vesicular transport and secretion of mucilage to a specific region on the membrane (Cai et al., 1997; Western et al., 2000).

Pectins, the primary component of mucilage that surrounds cell walls in dicotyledonous plants, consist of a heterogenous group of complex acidic polysaccharides such as unbranched polygalacturonic acid (PGA) and highly branched rhamnogalacturonan I (RGI). Both PGA and RGI are manufactured by the Golgi and transported extracellularly by vesicles (Brett and Waldron, 1990). RGI is the most abundant pectin component and isolated from plant cell walls. RGI has alternating oligo α-(1-3) arabinose and oligo β-(1-4) galactose branches (http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/carbohydrate-analysis/carbohydrate-analysis-iii.html#Pectin), which are later cleaved by β-galactosidase. The cleaved RGI has fewer branches, making pectin more permeable to water, and facilitates mucilage secretion to the outer surface of the seed upon imbibition (Dean et al., 2007) (**Fig. 1.5**).

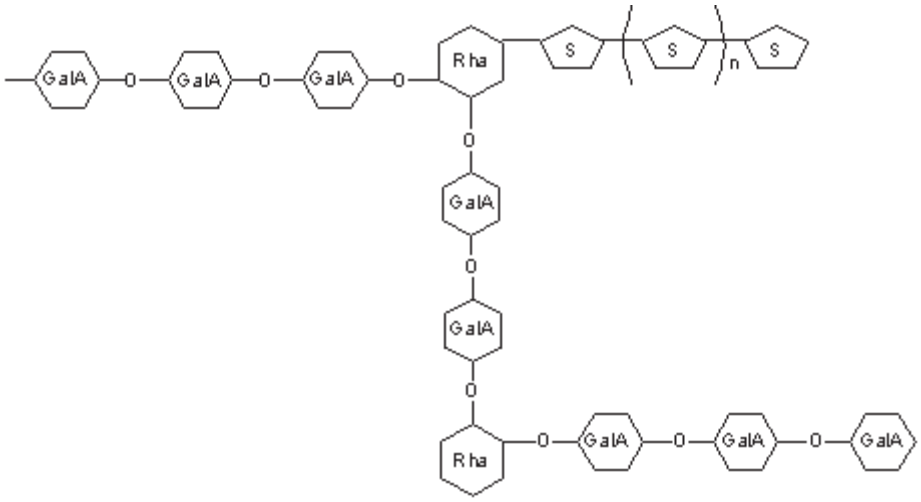


Fig. 1.5: Rhamnogalacturonan I (RGI) is one of the primary pectin components of mucilage that consists of GalA: Oligo-β-(1-4)-D-galactose, Rha: α-(1-2)-L-rhamnose, and neutral sugars such as S: Oligo-α-(1-3)-D-arabinose from Sriamornsak, 2003. Enzyme β-galactosidase targets and cleaves GalA residues.

Beyond the plasma membrane, plant cells have a primary and secondary cell wall, and a middle lamella that that acts as the glue that links adjacent cells together. The primary cell wall is made up of cellulose while the secondary cell wall is composed of lignin (Taiz and Zeiger, 2006). The middle lamella is largely composed of cellulose and pectins, pectins being the primary component of mucilage (**Fig. 1.6**).

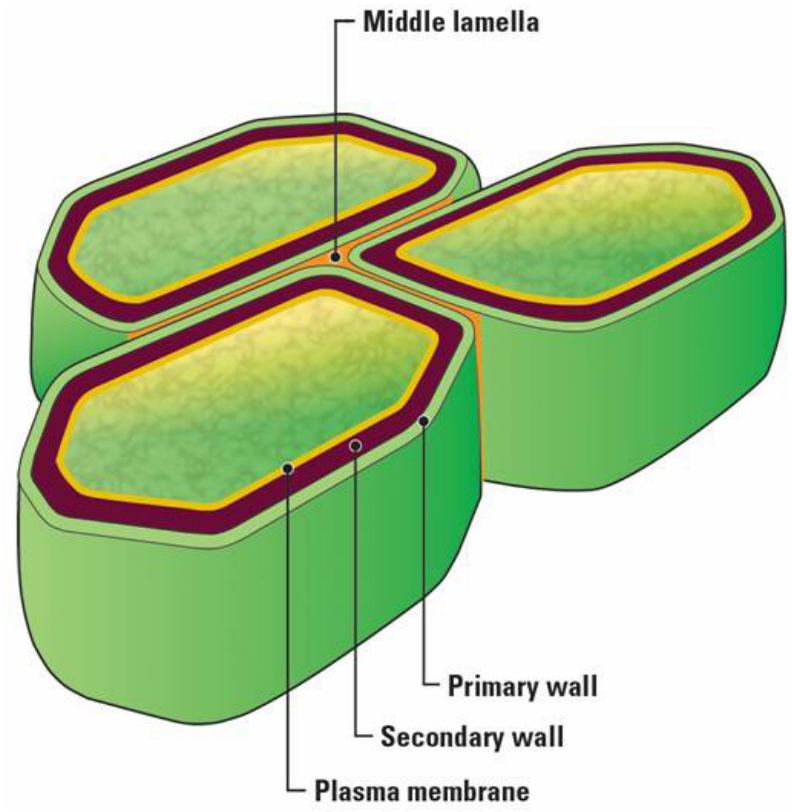


Fig. 1.6: An illustration of the plant cell membrane structures including the primary and secondary cell walls, and the middle lamella that "glues" the cells together from Taiz, 2006. The primary cell wall is composed of cellulose, one of the most abundant organic polymers found on Earth. The secondary cell wall is made up of lignin, an excellent source for ethanol fuels. The middle lamella contains pectins and cellulose, components that are used in the cotton fiber industry to make fabric and clothing.

1.3.4 Genetic Control of Epidermal Seed Development

The development and differentiation of the seed epidermis is important and necessary for the seed to maintain its dormancy until all growth conditions are met. One gene that is implicated in the epidermal seed coat development is *APETALA2* (*AP2*), which is an A class floral homeotic gene that is important for the production of sepals and petals (See earlier section and **Table 1.1**). *AP2* is expressed throughout the plant, including the flower, leaf, inflorescence, stem, and root (Okamuro et al., 1997),

and regulate different developmental pathways in different parts of the plant. *AP2* is transcriptionally and post-translationally regulated by microRNA 172 and post-translationally modified by phosphorylation (Aukerman and Sakai, 2003; Chen, 2003).

a*p2* mutant seeds were larger in size with larger embryo cells, and weighed more due to the increased total protein and fatty acid content (Jofuku et al., 2005). Most striking is the severely defective seed epidermis, which has thin walls and lack columella, volcano-like structures present on the surface of the seed epidermis (Western et al., 2001).

AINTEGUMENTA (*ANT*), a gene homologous to *AP2* has a role in both flower and ovule development. The *ant* mutants failed to initiate both inner and outer integuments during ovule development, did not have an embryo sac, and had a collasped nucellus region (Elliott et al., 1996). Therefore, both *AP2* and *ANT* regulate the earliest stages of epidermal seed development and formation, as both *ap2* and *ant* mutant seeds had abnormal epidermis (Elliott et al., 1996; Baker et al., 1997; Western et al., 2001).

In addition to the *AP2*-domain proteins, other transcription factors mediate proper epidermal cell differentiation and development within the seed. One of those genes is *TRANSPARENT TESTA GLABRA1* (*TTG1*), which encodes a WD40 domain and is required for development of two different epidermal cell types: trichome and seed coat development (Walker et al., 1999; Western et al., 2004; Zhao et al., 2008).

Additionally, the *ttg1* seed epidermis failed to synthesize mucilage and to produce columella (Western et al., 2004).

GLABRA2 (*GL2*) is another gene that regulates epidermal cell development. *gl2* mutants lack columellae within the seed coat, lack trichomes on adaxial leaf surfaces, and *GL2* is required for root hair development and controls seed oil accumulation (Rerie et al., 1994; Di Cristina et al., 1996; Shen et al., 2006). *GL2* encodes a homeodomain-leucine zipper transcription factor found only in plants, and. is activated by a transcriptional complex that consists of a R2R3 Myb protein, GLABRA1 (GL1), and a basic helix-loop-helix transcription factor, GLABRA3 (GL3) (Wang and Chen, 2008). In *Gossypium arboretum* (cotton), a functional homolog of *GL2* called *GaHOX1* successfully rescued the trichome developmental defect in *gl2-2* mutants when *GaHOX1* was expressed under the control of the *GL2* promoter. Northern analyses, Reverse transcriptase Polymerase Chain Reaction (RT-PCR), and *in situ* hybridization confirmed that *GaHOX1* is expressed in cotton fiber cells and plays an important role in cotton fiber formation (Guan et al., 2008).

Another R2R3 Myb transcription factor besides *GL1* is *AtMYB61*. Both GL1 and AtMYB61 are members of the largest class of Myb transcription factors that possess two bHLH repeat domains (R2R3) in plants and are capable of binding DNA. *myb61* mutants failed to secrete or extrude mucilage upon imbibement, and under reduced water conditions, *myb61* mutants has poor germination rates (Penfield et al.,

2001). Thus, providing insight into the function of mucilage, which is to aid in seed germination during dry environmental conditions.

A sixth gene that regulates trichome, root, and seed development is *TRANSPARENT TESTA GLABRA2* (*TTG2*), which encodes a zinc finger-like WRKY transcription factor. The protein contains two Myb binding sites and acts as direct targets for WEREWOLF (WER), GL1, and TRANSPARENT TESTA2. Gene expression analysis of the *ttg2* mutants revealed that *GL2* is significantly reduced in roots, and epistatic analysis firmly places *TTG1* and *GL3* upstream of *TTG2*, and *GL2* downstream of *TTG2* (Ishida et al., 2007) (**Fig. 1.7**). To date, the WRKY class of transcription factors has been characterized as a regulator of pathogen response, mechanical stress, and senescence (Eulgem et al., 1999; Eulgem et al., 2000). Although *TTG2* regulates trichome branching, seed coat development, and mucilage production, there has been no evidence that links *TTG2* to pathogen response (Johnson et al., 2002).

A pair of genes that regulate both trichome and seed coat development is *MYB5* and *MYB23*. *MYB5* is expressed in both trichomes and the seed coat, and *myb5* loss-of-function seeds develop epidermal cells that are irregularly shaped, have poorly developed columellae, and synthesize less mucilage than wild-type (Li et al., 2009). Single *myb5* mutants displayed minimal changes in trichome morphology, but double *myb5*; *myb23* mutants have a more severe trichome phenotype including small and two-branched (as opposed to three-branched) rosette trichomes (Li et al., 2009).

This study provides insight into the somewhat conserved yet unique operative genetic components between seed and trichome development (**Fig. 1.7**).

Another class of genes that regulate mucilage development in seeds, particularly the *MUCILAGE MODIFIED 1-5* (*MUM 1-5*) genes (Western et al., 2001). The mutants were identified through an ethane methyl sulfonate (EMS) mutagenesis screen using wild-type Columbia 2 seeds. The mutagenized seeds were screened using Toluidine blue stain, and seeds that did not secrete mucilage after imbibement were isolated, and studied for their mucilage defects during epidermal seed development.

Table 1.2 summarizes the ten genes and their respective mutant defects in seed epidermis and/or mucilage synthesis or secretion. *ap2* mutants had the most severe defects, including the lack of columella and failure to produce mucilage since *AP2* is the first gene to initiate epidermal development. Without proper development of a functional and morphologically sound epidermal cell structure, the outer integument cannot proceed with mucilage synthesis and deposition (Western et al., 2001). A second group consists of *gl2*, *ttg1*, and *mucilage-modified4* (*mum4*) mutants which produce less mucilage and develop reduced columella. The regulation of *MUM4* and where it fits into the mucilage synthesis pathways is depicted in **Fig. 1.7**, with *MUM4* being activated upstream by *GL2* (Western et al., 2001; Western et al., 2004). A third class encompasses *mum3* and *mum5* mutants, which develop normal epidermis and columella but secrete reduced mucilage that is of a different

biochemical composition from wild-type (Western et al., 2000; Western et al., 2001; Western et al., 2004; Dean et al., 2007). However, the molecular nature of *MUM3* and *MUM5* is unknown. It was proposed that the composition of RGI in *mum3* and *mum5* mutants is not sufficiently modified to allow for efficient mucilage secretion post-imbibition. *mum1* and *mum2* seeds develop normal columella and are able to synthesize mucilage, but fail to secrete it upon imbibition (Western et al., 2001; Dean et al., 2007). *MUM2* encodes a β-galactosidase, an enzyme capable of cleaving RGI as indicated in **Fig. 1.5**. In *mum2* mutants, RGI branches in mucilage is not cleaved, which prevents the pectin to become water soluble and the seed fails to extrude mucilage upon imbibition (Western et al., 2001; Dean et al., 2007). These different mutant classes have different functional roles during seed coat development and mucilage secretion as summarized in **Fig. 1.8**.

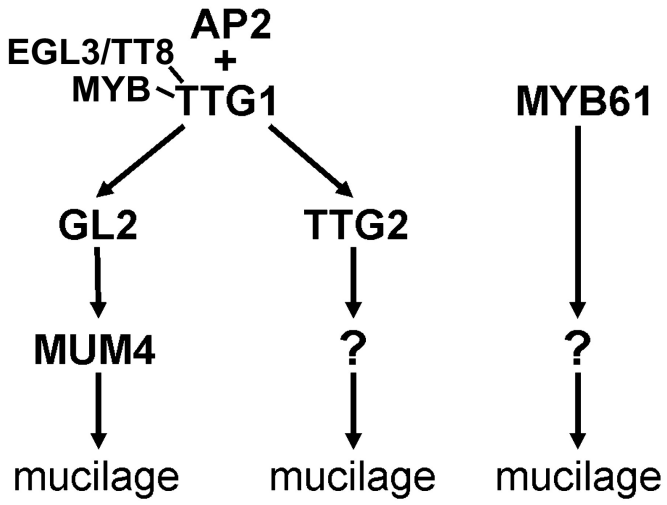


Fig. 1.7: Three distinct pathways for the synthesis of mucilage in *Arabidopsis* seed from (Western et al., 2004). In one pathway, *MUM4* (NDP-L-rhamnose synthase) is activated by *GL2* (homeodomain). The second pathway of mucilage synthesis involves *TTG2* (WRKY) and the third, *MYB61* (R2R3 Myb). The last two pathways have not identified downstream genes, however, it is hypothesis that they operate in different pathways because neither *TTG2* nor *MYB61* regulates the *MUM4* gene.

Gene Name	Acronym	*Arabidopsis* Gene ID	Encoding Protein	Phenotype	Reference
TRANSPARENT TESTA GLABRA1	*TTG1*	At4g24520	WD40	Reduced mucilage synthesis & columella	(Western et al., 2004)
TRANSPARENT TESTA GLABRA2	*TTG2*	At2g37260	WRKY	Reduced mucilage synthesis & columella	(Western et al., 2004)
GLABRA2	*GL2*	At1g79840	Homeodomain	Reduced mucilage synthesis & columella	(Western et al., 2001)
MYB61	*MYB61*	At1g09540	R2R3 Myb	Reduced mucilage synthesis & no mucilage secretion after imbibition	(Penfield et al., 2001)
APETALA2	*AP2*	At4g36920	AP2/EREBP	Seed epidermis that lack columella, thin walled & rectangular shaped cells	(Western et al., 2001)
MUCILAGE MODIFIED1	*MUM1*	*Not assigned*	Unknown	Failed to secrete mucilage upon imbibition	(Western et al., 2001)
MUCILAGE MODIFIED2	*MUM2*	At5g63800	Beta-galactosidase	Failed to secrete mucilage upon imbibition	(Western et al., 2001)
MUCILAGE MODIFIED3	*MUM3*	*Not assigned*	Unknown	Aberrant mucilage composition	(Western et al., 2001)
MUCILAGE MODOFIED4	*MUM4*	At1g53500	NDP-L-rhamnose synthase	Reduced mucilage synthesis & columella	(Western et al., 2000; Western et al., 2001)
MUCILAGE MODIFIED5	*MUM5*	*Not assigned*	Unknown	Aberrant mucilage composition	(Western et al., 2001)

Table 1.2: Genes that regulate seed coat and/or mucilage development, their molecular nature, and mutant phenotypes.

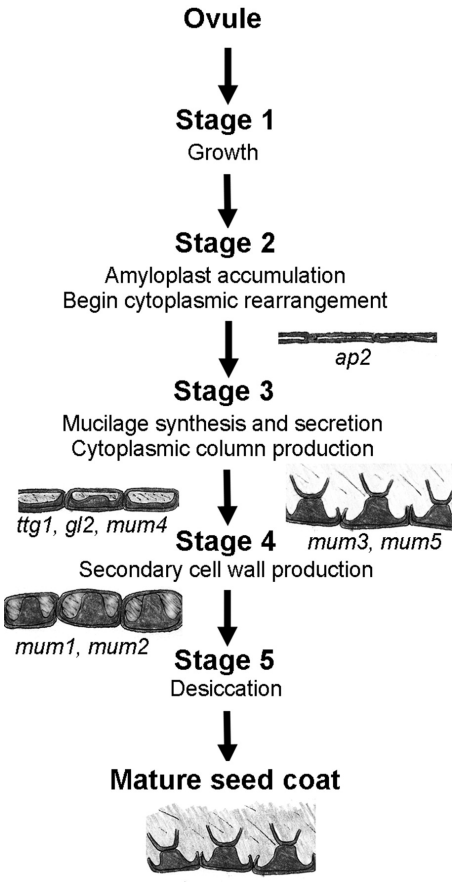


Fig. 1.8: Mutants affecting different stages of seed coat development (Western et al., 2004). Stage 1: seed growth; Stage 2: cellular morphogenesis; Stage 3: mucilage synthesis and secretion; Stage 4: secondary cell wall production; and Stage 5: seed desiccation. Note the normal columella in *mum3*, *mum5*, *mum1*, and *mum2* compare to the reduced columella in *ttg1*, *gl2*, and *mum4* mutants. Also note the trapped mucilage in *mum1* and *mum2* mutant seeds in contrast to the reduced mucilage in *ttg1*, *gl2*, and *mum4*.

1.4 Bacterial Pathogen Resistance

1.4.1 Plant Innate Immunity

Like humans and most multi-cellular organisms, plants are constantly under attack by foreign pathogens such as parasites, fungi, and bacterial prokaryotes. In the end, survival of the individual organism and ultimately the species relies on a defensive response to combat these foreign pathogens. In plants, the response is highly regulated and specific to the invading pathogens, and involves a series of gene

expression that result in molecular and physiological changes that eradicate or lessen the impact of invading pathogen.

Plants have developed a series of physical barriers including the production of leaf hairs or trichomes on the adaxial leaf surface (Calo et al., 2006; Brininstool et al., 2008), waxy cuticles which provide a layer of protection for maturing fruits (Burnett et al., 2000), and stomata that not only regulate gas exchanges between the plant and surrounding environment, but also prevent pathogen entry into plant tissues (Prats et al., 2006; Melotto et al., 2008).

Plant resistance is divided into two primary categories: 1) Non-host resistance and 2) Host-resistance. Non-host resistance is the most evolutionarily ancient, considered a major contribution to the safety and survival of plants, and is activated when the plant recognizes pathogen-associated molecular patterns (PAMPs) that are products of the invading pathogens. These PAMPs include lipopolysacharrides (LPS) found on the surface of the prokaryotes' cell walls which can induce the plant non-host resistance (Esposito et al., 2008), flagellin from bacteria which the plant can sense and induce apoptosis or cell death (Takai et al., 2008), and chitin and glucan that are found in fungi. There is also evidence that these fungi are capable of degrading pectins, primary components of mucilage on plant cell walls (Simon et al., 2005). Degradation of the pectinacious layer surrounding the plant cell walls suggests that mucilage plays an important and protective in plant defense, and that fungi and other pathogens have evolved mechanisms that target this layer for degradation to

increase accessibility to the cell. A component of non-host resistance is basal resistance, which remains intact even in plants that are pathogen susceptible to limit the growth of the pathogen and is not specific to any particular type of pathogen.

The second category is host-resistance, which acts at the subspecies level as different ecotypes or plant varieties will react differently to the same pathogen. Unlike non-host resistance which is induced by PAMPs, host-resistance relies upon the interaction of the plants' Resistance (R)-genes and pathogen's secreted Avirulence (Avr) factors. The plant responds appropriately by enacting apoptosis. This is commonly referred to as Hypersensitive Response (HR), consisting of local resistance that acts at the site of infection, and Systemic Acquired Resistance (SAR), which acts in tissues neighboring the infected site. The disadvantage of R-gene mediated host-resistance is that it is race-specific and cannot confer broad-spectrum resistance against multiple or similar strains of the same pathogen as in basal non-host resistance (Parlevliet, 1982; Joosten et al., 1994; Lin and Chen, 2008). A flowchart that summarizes the different levels of plant innate immunity is in **Fig. 1.9**.

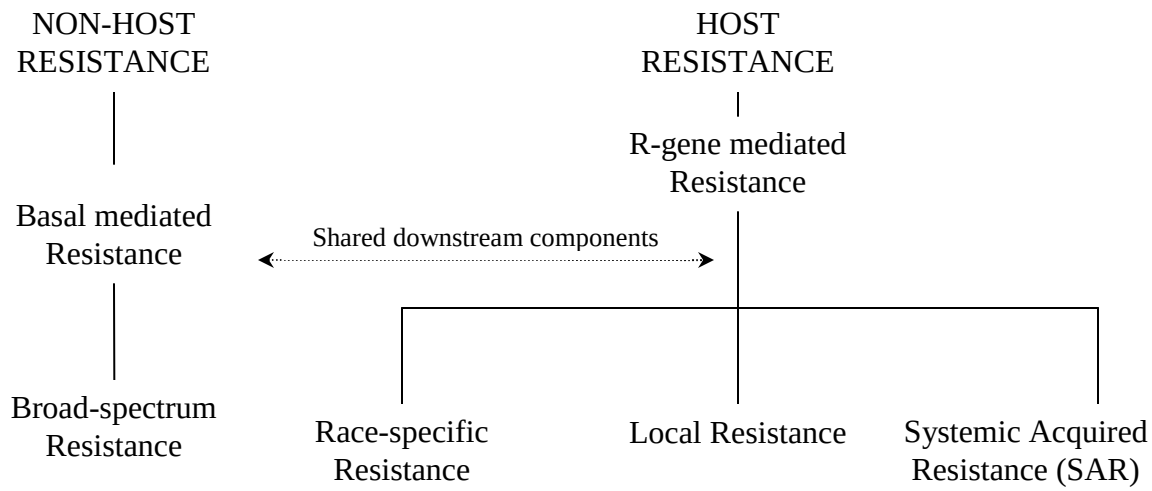


Fig. 1.9: Flowchart depicting the multi-layered defense mechanisms found in plants. Dotted arrow between Basal and R-gene mediated resistance means that the two pathways share common components. Adapted from Xiao, 2006.

1.4.2 Plant Resistant (R)-Genes

Resistance (R)-genes encode proteins that are the guardians to the pathogen resistance pathway. There are currently five classes of R-genes but the most abundant one is the nucleotide-binding-site leucine-rich-repeat (NBS-LRR) proteins (Rommens and Kishore, 2000). This class has a Toll/Interleukin1 receptor (TIR) domain, which is homologous to Toll-like receptors that trigger innate immune response in animals (Holt et al., 2003). In addition to pathogen response, TIR-NBS-LRR type proteins are also involved in shade-avoidance (Faigon-Soverna et al., 2006). Other classes include a) one with a transmembrane (TM) and an extracytoplasmic Leucine Rich Repeat (LRR) domain known as receptor-like proteins (RLPs); b) a cytoplasmic serine-threonine (Ser/Thr) receptor-like kinase (RLKs) with and without an LRR domain; c) a fourth member that is similar to TIR-NBS-LRR but has a coiled coil domain that

replaces the TIR domain (CC-NBS-LRR). The different types of R proteins are shown in **Fig. 1.10**.

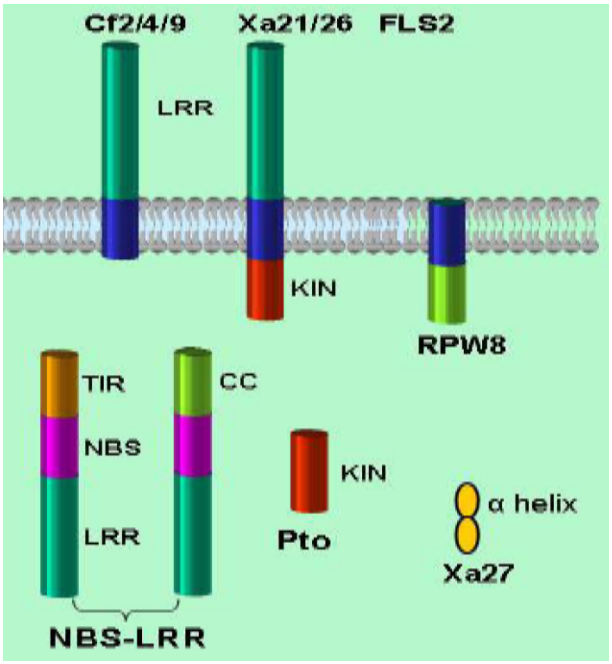


Fig. 1.10: The different types of plant Resistance (R) proteins from (Xiao, 2006). CF2/4/9 has a Transmembrane (TM) and Leucine Rich Repeat domains, and are thus known as Receptor-like Proteins (RLPs). Xa21/26 are similar to RLPs but have a cytoplasmic serine-threonine (Ser/Thr) kinase domain and are known as Receptor-like Kinase (RLKs). Two other similar R proteins with shared Nuclear Binding Site (NBS) and LRR domains, but have unique Toll/Interleukin1 receptor (TIR-NBS-LRR) and Coiled-coiled domains (CC-NBS-LRR). RPW8 and Xa27 are considered atypical R proteins while Pto is a protein kinase.

The TIR-NBS-LRR protein is well characterized because the TIR domain is homologous to the animal Toll/Interleukin 1 receptor. The NBS domain is important for ATP-binding and ATP-hydrolysis, which is triggered by the presence of a pathogen (Ellis and Jones, 1998; van der Biezen and Jones, 1998). Meanwhile, the LRR-domain is important for protein-protein interactions and likely responsible for recognizing the PAMPs or proteins elicited by the pathogen (Young, 2000; Fluhr, 2001). The TIR domain essentially functions as a receptor for the Avirulence (Avr) factors secreted by pathogens, and not only regulates immunity to pathogens, but also

plant development as well (Faigon-Soverna et al., 2006). Among the TIR-NBS-LRR proteins are most notably *RECOGNITION OF PERONOSPORA PARASITICA4* (*RPP4*), *RECOGNITION OF PERONOSPORA PARASITICA5* (*RPP5*), and *SUPPRESSOR OF NPR1 CONSTITUTIVE1* (*SNC1*). *Peronospora parasitica* is a fungus responsible for downy mildew disease, which incurs yellowing spots on the upper leaves due to sporulation and cotton-fiber like growth on the abaxial leaf surface (Koch and Slusarenko, 1990). RPP4 can operate in a WRKY70-dependent and -independent manner, which is a transcription factor that is implicated in both pathogen defense and plant senescence (Ulker et al., 2007). RPP4 also requires PAD4 and the phytohormone salicylic acid (SA) to mediate basal defenses against downy mildew (Knoth et al., 2007; Ulker et al., 2007). Landsberg *RPP5* is an ortholog Columbia *RPP4* and both confer resistance to downy mildew, and sequence comparisons revealed significant polymorphisms (Noel et al., 1999).

There are two models for pathogen recognition between the R protein and Avr factors, which occur before activation of the SA pathway (**Fig. 1.11**). One model is known as the “gene-for-gene”, where the Avr factor will interact with and be recognized by R proteins (Scofield et al., 1996; Jia et al., 2000; Deslandes et al., 2003). However, this model is often considered the exception and not the rule because there many more Avr factors than plant R genes, so there has to be another mechanism that can broadly recognize different Avr factors. Recently, the “guard hypothesis” was proposed, where the R protein will monitor several “guardee” proteins that are targeted by the pathogen Avr elicitors for phosphorylation or

degradation (Mackey et al., 2002; Axtell and Staskawicz, 2003). For example, both RPM1 and RPS2 R proteins monitor the RIN4 "guardee" proteins. After the "guardee" is chemically modified by the pathogen Avr ellicitor, the "guard" is activated and initiates the SA pathway to fend off pathogens.

1.4.3 The Salicylic Acid (SA) Pathway in Plant Defense

Following the release of the Avr factors by the pathogen and recognition of the Avr factors by the R proteins, the plant produces salicylic acid (SA). The level of SA is significantly induced by as much as 500-1000 fold, which subsequently induces plant defense genes and incur Systemic Acquired Resistance (SAR), a response that yields broad spectrum "whole plant" resistance to neighboring tissues (Verberne et al., 2000).

How is SA induced after R protein recognition? *ENHANCED DISEASE SUSCEPTIBLITY 1* (*EDS1*) is up-regulated following an infection and R protein activation. *EDS1* is responsible for increasing SA levels because *eds1* mutants produce significantly less SA (Falk et al., 1999). Genetic analyses determined that EDS1 acts downstream of several R proteins such as RPP1, RPP10, and RPP14-, (Aarts et al., 1998) and EDS1, PHYTOALEXIN DEFICIENT4 (PAD4), and SENESCENCE ASSOCIATED GENE101 (SAG101) interact to form a trimeric complex that activates the SA pathway (Wiermer et al., 2005). EDS1 encodes a protein with sequence homology to the catalytic site of eukaryotic lipases (Falk et al., 1999). Thus, EDS1 likely functions to hydrolyze lipids (Falk et al., 1999) and also regulates SA by activating *EDS5* and *SID2* (**Fig. 1.11**).

NONEXPRESSOR OF PR GENES 1 (NPR1) is activated by SA. NPR1 encodes a transcription factor that contains ankyrin repeats and confers systemic acquired resistance (SAR) to both *Pseudomonas syringae* and *Peronospora parasitica* (Ryals et al., 1997). The NPR1 protein shares homology to I-kappa B (IkB) which mediates innate immune response in wide range of organisms from *Drosophila* to mammals (Baldwin, 1996; Spoel et al., 2003). This SA signaling pathway in plants is representative of an ancient and highly conserved defense mechanism. Yeast-two-hybrid and electrophoretic mobility shift assays revealed that NPR1 increased the binding of a basic leucine zipper transcription factor to the promoter of *PATHOGENESIS-RELATED 1* (*PR1*) to activate *PR1* expression (Despres et al., 2000). NPR1 is an important modulator of SA/JA cross-talk by serving a novel function when in the cytosol. NPR1 exists as a multi-meric complex in the cytosol until it is activated by SA, resulting in dissociation of one NPR1 protein that will translocate into the nucleus and interacts with the TGA/OBF family of bZIP transcription factors to activate plant defense (Spoel et al., 2003). Activation of NPR1 occurs under the reducing conditions that follow and oxidative burst that occurs during defense responses (Dong, 2004).

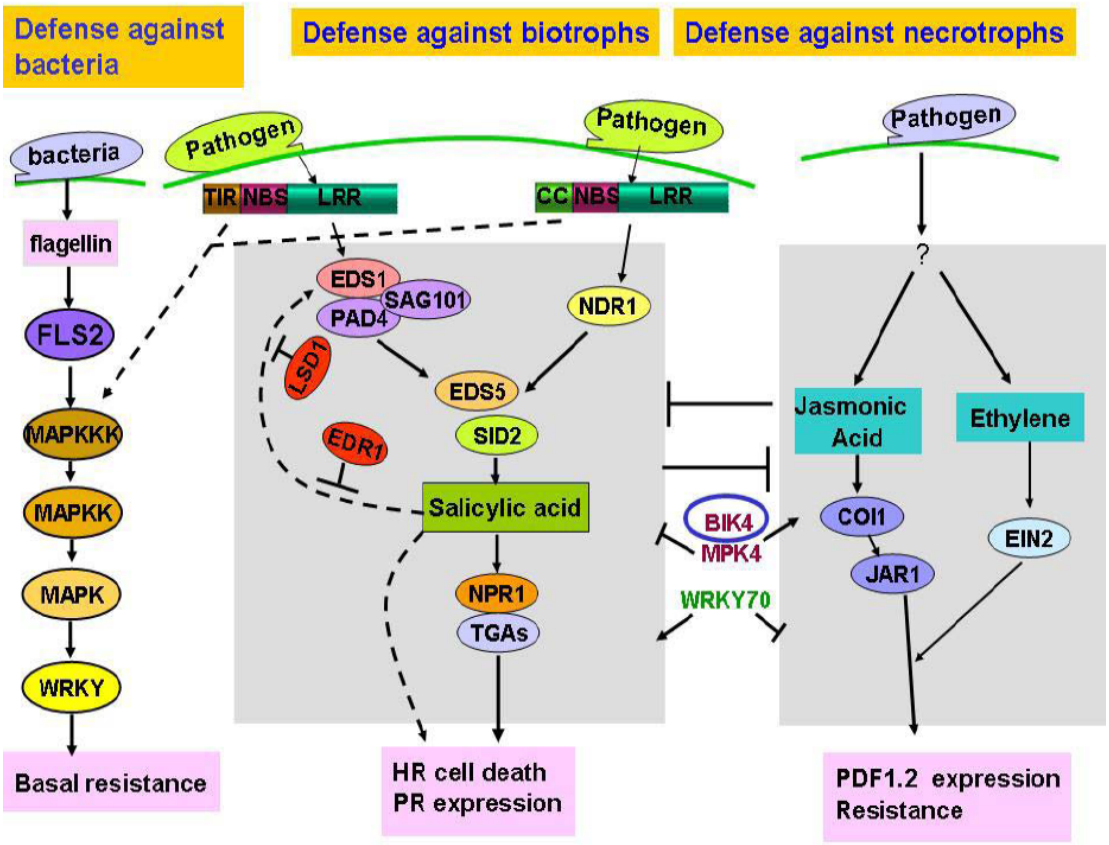


Fig. 1.11: The three primary pathogen defense pathways in plants. The salicylic acid (SA) pathway is the primary response to bacterial pathogens and is R-gene mediated, while the jasmonic acid (JA) and ethylene (ET) pathways mediate resistance to insect herbivory. Adapted from Xiao, 2006.

1.4.4 *PATHOGENESIS RELATED* (*PR*) *1-5* Genes

To quantify by molecular means how well plants respond to a pathogen through the SA pathway, researchers measure the expression of the *PATHOGENESIS RELATED* (*PR*) *1-5* genes, which are the targets of the SA/jasmonic acid (JA) defense pathways. There are 22 predicted *PR1* genes that encode basic and acidic proteins that respond to pathogens, but At2g14610 is the predominant gene that mediates systemic acquired resistance (SAR) (Mitsuhara et al., 2008). *PR2* encodes a B-1,3-glucanase important for destabilizing and degrading fungal cell walls (Stewart et al., 2001; Doxey et al., 2007), *PR3* encodes a chitinase enzyme capable of breaking

down fungal cell walls (Jung et al., 1993), and *PR5* encodes another membrane permeablizer that can destabilize yeast cell walls (Salzman et al., 2004). Therefore, *PR1-5* genes are effector genes with anti-bacterial and anti-fungal properties. Furthermore, they serve as convenient molecular markers for the SA pathway and SAR.

1.4.5 Hormonal Cross-talk Among Pathogen Resistance Pathways

Due to the many type of plant pathogens that exist and such as fungi, bacteria, and insects, plants have evolved different mechanisms to fend off specific intruders, with the key players being the salicylic acid (SA), jasmonic acid (JA), and ethylene pathways (ET). The role SA is apparent after plants are transformed with a transgene that constitutively expresses the bacterial NahG, an enzyme that degrades SA into inactive catechol (Delaney et al., 1994). These NahG plants exhibited enhanced susceptibility to several micro-organisms including oomycetes, fungal, bacterial, and viral pathogens. Genetic studies described earlier indicate that activation of SA by R proteins constitute a major defense pathway in plants.

Phytohormone JA responds to micro-organisms including *Alternaria brassicicola* and *Botrytis cinerea* fungi, and *Erwinia carotovora* bacteria (Thomma et al., 1999; Norman-Setterblad et al., 2000). *Arabidopsis jasomonate resistant 1* (*jar1*) mutants are insensitive to JA and have enhanced susceptibility to *Pseudomonas syringae* pv. *tomato* in (Pieterse et al., 1998). *fatty acid desaturase 3, 7,* and *8* (*fad3 fad7 fad 8*) triple mutants lack the ability to synthesize the JA precursor linolenic acid, had high mortality rates from fungal gnat larvae attacks (McConn et al., 1997),

demonstrating that JA plays an important role in herbivory. Certain pathogens induce the Jasmonic Acid (JA) /Ethylene (ET) pathway, which ultimately leads to increased expression of the *PLANT DEFENSIN 1.2* (*PDF1.2*) gene. *PDF1.2* is a pathogenesis-related protein that functions independently of SA, has anti-fungal properties, and is only regulated by JA/ET (Penninckx et al., 1998). Both phytohormones JA and ET will synergize to activate *PDF1.2* (**Fig. 1.11**) via AP2/EFR-domain transcription factors called ORA59 and ORCA3, which is an essential integrator of the JA and ET signaling pathways during plant defense (van der Fits and Memelink, 2001; Pre et al., 2008).

Different pathogens will induce different pathways, but to say that JA/ET is solely induced by herbivory while SA is strictly induced by bacterial pathogen is incorrect. One particular example is the bacterial pathogen *E. carotovora*, which when inoculated on *Arabidopsis* leaves induced JA/ET-induced molecular markers such as *PDF1.2* but did not induce SA response genes (Vidal et al., 1997). There are several studies that report the SA pathway is induced by insect herbivory. One example of herbivory-induced SA response is that by spider mites, which causes plants to emit methyl salicylate (MeSA), which leads to activation of SA-inducible defense genes and causes the release of SA and JA-induced blends of volatile compounds that attract carnivores to exterminate the herbivores (Dicke et al., 1999; Ozawa et al., 2000).

While JA/ET acts synergistically to fend off insect pathogens, both JA/ET pathways are known to antagonize the SA pathway. When SA is exogenously applied to plants, JA/ET marker gene *PDF1.2* was suppressed and SA-related *PR* genes were reduced when methyl jasmonate (MeJA) was exogenously applied to the plant (Koornneef et al., 2008). Transgenic tobacco plants with reduced SA levels exhibited reduced resistance to Tobacco Mosaic Virus (TMV) but had enhanced response to herbivorous *Heliothis virescens* larvae (Felton et al., 1999). The opposite is true for the application of BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester), which is a synthetic analog of SA, will increase resistance against *Pseudomonas syringae* but make the plant more vulnerable to leaf chewing by *Helicoverpa zea* (corn earworm) larva (Stout et al., 1999). Similarly, application of BTH to field-grown tomato plants reduced resistance to beet armyworm (*Spodoptera exigua*) (Thaler et al., 2002).

The significance of these multiple defense pathways is still very controversial. Most agree that the diversity of plant defense pathways will allow the plant to fine-tune their response to specific pathogens over-time, depending on the virulence strategy utilized by each particular pathogen. The antagonistic effect observed between JA/SA is likely to prevent inappropriate responses. For example, induction of programmed cell death by SA can be prevented especially when a necrotropic pathogen takes advantage of this process to induce massive plant cell death by secretion of toxins such as virulence factors. However, the plant-pathogen interaction is often described as an arms race as on-going adaptations in both the plant and

pathogen aim to terminate the other. Of course pathogens have also learned to adapt and regulate these plant defense pathways. One example is the report that *Pseudomonas* bacteria will utilize coronatine to activate the JA signaling pathway, and thereby antagonize the SA pathway indirectly through JA induction, delaying or inhibiting the plant's response and allow the pathogen a window of opportunity to colonize its host (Feys et al., 1994; Reymond and Farmer, 1998; Kloek et al., 2001).

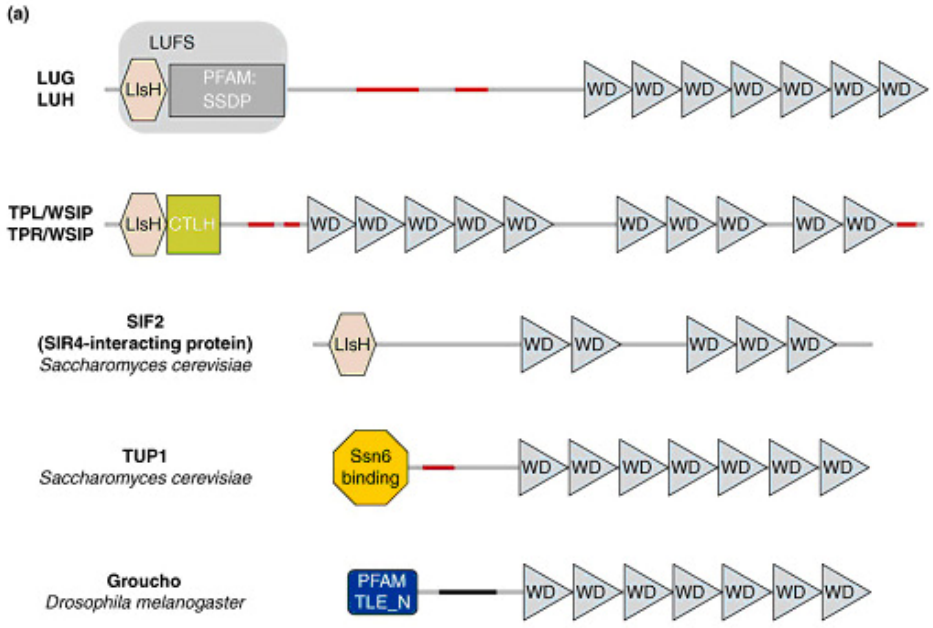
Although the purpose of having multiple and complex regulatory defense pathways is still very controversial, there is no doubt that these pathways give the plant more options and tools to co-evolve and co-adapt with its pathogen adversaries. These pathways may at first seem redundant or excessive, but a plant that is better equipped is likely to survive over-time. When the SA pathway is activated, it will repress transcription of JA-response genes, making transcriptional repression an important mechanism to study.

1.5 Transcriptional Co-repressors, Structure, & Function

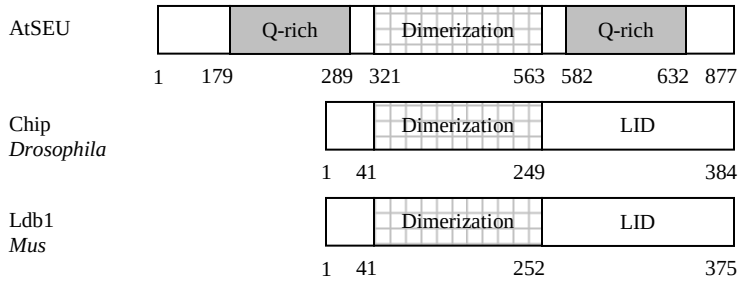
1.5.1 Groucho and TLE Co-repressors

Transcriptional co-repression is recognized as one of the key strategies utilized by both animnals and plant to regulate gene expression (Liu and Karmarkar, 2008). Transcription co-repressors have no DNA-binding motifs, but instead, they interact with various DNA-binding transcription factors that usher the co-repressor complex to the cis-acting elements of target genes. One example is Groucho (Gro) from *Drosophila melanogaster* and its mammalian homologs called Transducin-Like Enhancer of split (TLE). The Groucho/TLE proteins consist of an N-terminal

glutatmine (Q)-rich domain and a seven tryptophan-aspartic acid (WD)-repeat domain at the C-terminus (**Fig. 1.12**). Both Gro and TLE do not bind DNA, but are recruited by sequence-specific DNA-binding transcription factors to regulate genes in different developmental pathways (Chen and Courey, 2000). The Q-rich domain is responsible for homo-tetramerization, while the 7WD repeat domain forms a propeller-shaped structure that allows for protein-protein interactions with other transcription factors such as Engrailed and Dorsal to regulate embryo development (Tolkunova et al., 1998; Pickles et al., 2002). Groucho's interaction with Dorsal forms a "repressosome" complex that represses target gene expression via a mediator complex (**Fig. 1.13**) (Courey and Jia, 2001).



A



B

Fig. 1.12: Depiction of the similar and different domain structures of the Gro/Tup1 and SEU classes of transcriptional repressors. A) Comparison of the structural similarities between LUG/LUH and the transcriptional repressors Tup1 and Groucho from (Liu and Karmarkar, 2008). LUFS domain: LUG, LUH, Flo8, and Single-strand DNA binding; LisH: Lissencephaly Homology; CTLH: C-terminal to LisH; WD: tryptophan (W) aspartic acid (D) B) Domain structure alignment between SEU and two similar animal proteins, Chip and Lim Domain-binding 1 (Ldb1). All three contain the same conserved dimerization domain. LID: LIM Interaction Domain (adapted from Franks *et al*., 2002).

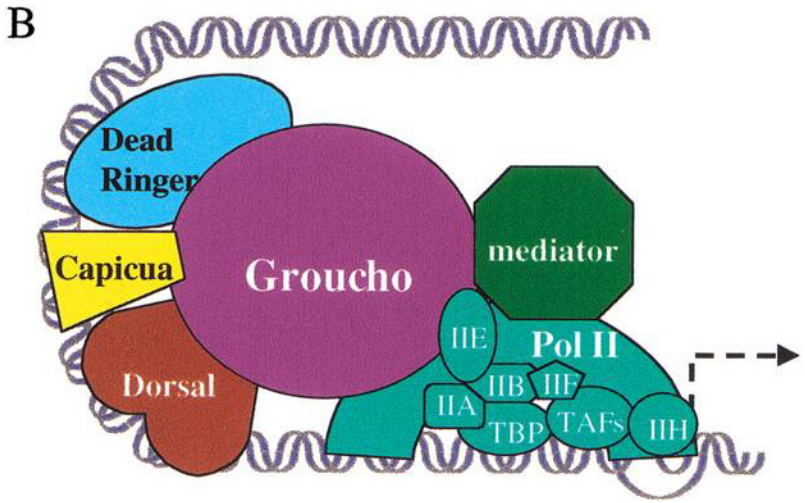


Fig. 1.13: Groucho "repressosome" complex. Adapted from (Courey and Jia, 2001). In this model, Groucho interacts with several key DNA-binding proteins including Dead Ringer, Capicua, and Dorsal to repress transcription. Transcriptional repression is accomplished by Groucho interacting with the basal transcription complex, specifically the TFIIE and mediator components, thereby blocking the function of the RNA-polymerase II transcriptional machinery.

Groucho regulates developmental processes including segmentation, sex determination, eye patterning, and dorsal-ventral and terminal patterning by interacting with different DNA-binding factors such as homeodomain proteins Engrailed, Rel-family Dorsal, and basic helix-loop-helix (bHLH) Hairy (Courey and Jia, 2001). The ability to regulate so many developmental processes is due to its interaction with so many DNA-binding partners. In mammals, TLE interacts with Acute myeloid leukemia 1 and 2 (AML1 and 2) to regulate hematopoiesis and osteoblast differentiation, and with lymphocyte enhancer factor 1 (LEF1) to determine cell fate (Levanon et al., 1998; Daniels and Weis, 2005).

1.5.2 TUP1 Functions as a Global Co-Repressor in Yeast

Saccharomyces cerevisiae has a Groucho-like protein named Tup1. Tup1 protein has a centrally located glutamine (Q)-rich domain and a seven WD-repeat domain at the C-terminus (**Fig. 1.12**). Unlike Groucho, Tup1 has a unique N-terminal domain that interacts with the tetratricopeptide repeat protein called Ssn6, forming a complex that consists of one Ssn6 and three Tup1 subunits (Varanasi et al., 1996; Redd et al., 1997; Liu and Karmarkar, 2008). The Tup1-Ssn6 co-repressor complex mediates repression of genes that are repressed by glucose (*SUC2*), respond to hypoxia (*ANB1*), or induced by DNA damage (*RNR2*) (Davie et al., 2003).

Neither Tup1 nor Ssn6 binds DNA directly. Instead, they are recruited to the target gene's promoter via interaction with pathway-specific DNA-binding transcription factors. In this case, Ssn6 acts solely as an adaptor protein that bridges

the interaction between Tup1 and pathway specific DNA-binding factors. Ssn6 cannot mediate transcriptional repression without Tup1. However, Tup1 can regulate transcription without Ssn6 if Tup1 is tethered to the target promoter by fusion to the DNA-binding domain (Keleher et al., 1992; Tzamarias and Struhl, 1994, 1995). The requirement of Ssn6 as an adaptor is in sharp contrast to Groucho in *Drosophila*, which directly binds to short Trp-Arg-Pro-Trp peptide motifs found on DNA-binding transcription factors such as Hairy, Dorsal, and Engrailed (Liu and Karmarkar, 2008), This explains why Tup1 has an additional N-terminal domain required to interact with its adaptor Ssn6.

There are at least three different mechanisms that Tup1 utilizes to repress transcription. One involves Tup1-Ssn6 physically interacting with several histone deacetylases (HDACs), in particular Reduced Potassium Dependency 3 (Rpd3), HAD One Similar (Hos1) and Hos2 (Davie et al., 2003). Recruitment of HDACs by Tup1-Ssn6 allows for histone deacytlation, thus stabilizing the interaction between the histone proteins and the Tup1 complex, resulting in a long-range repressive state.

Another mechanism involves Tup1-Ssn6 interacting with several components of the Mediator transcriptional machinery (**Fig. 1.13**). In fact, Ssn6-Tup1 require mediator components such as Sin4 (Jiang and Stillman, 1992), Srb10, Srb11 (Wahi and Johnson, 1995), Srb8 (Wahi et al., 1998), and Med3 (Papamichos-Chronakis et al., 2000), all of which are associated with the RNA polymerase II holoenzyme

(Carlson, 1997). Interaction with the Mediator complex causes competition between the Tup1 co-repressors and its co-activators.

1.5.3 Plant Co-Repressors

SEUSS (*SEU*) was originally identified as a mutant that had a similar phenotype to *lug* (Franks et al., 2002). *SEU* encodes a protein with a conserved central domain that is similar to the dimerization domain that is found in Chip in *Drosophila* and LIM domain-binding 1 (Ldb1) in mouse (**Fig 1.12**). The LID domain of Chip and Ldb1 interact with the LIM-homeodomain proteins to activate gene expression (Breen et al., 1998; Dawid et al., 1998). However, SEU in *Arabidopsis* acts as an adaptor for the LUG co-repressor, but can self-activate in yeast (Sridhar et al., 2004). Expressing *SEU* with the *35S::SEU-BD* construct alone did not repress reporter gene expression. In contrast, LUG-BD can repress reporter gene expression when tethered to SEU (Sridhar et al., 2004).

In addition to binding LIM proteins with the LID domain, Chip and Ldb1 directly interact with Single stranded DNA-binding protein (Ssdp) (Chen et al., 2002), via the N-terminal LUFS domain found in Ssdp, very similar to the SEU-LUG (LUFS) interaction in *Arabidopsis thaliana* (**Fig. 1.12**).

Both LUG and LUH belong to a thirteen member group of Gro/Tup1-like proteins in *Arabidopsis*. Other members in this family include TOPLESS (TPL), TOPLESS-RELATED (TPR), and WUSCHEL-INTERACTING PROTEINS (WSIP) (Liu and Karmarkar, 2008). All these *Arabidopsis* Gro/Tup1-like proteins have a

Lissencephaly homology (LisH) dimerization domain at the N-terminus similarly to Sucrase-isomaltase footprint protein (SIF2p) in yeast (**Fig. 1.12**). They are subdivided into two classes, based on the domain immediately following the LisH domain as well as the additional WD domains. One subclass that consists of TPL, TPR, and WSIP has both centrally and C-terminally located WD-repeat and an additional CTLH (C-terminal to LisH) domain. The other subclass with LUG and LUH as members, has a LUFS domain that combines the PFAM Ssdp and the N-terminally located LisH domains (Liu and Karmarkar, 2008) (**Fig. 1.12**). Both CTLH and LUFS domains are important for protein-protein interactions and are responsible for regulating many different developmental processes in *Arabidopsis*. Reminiscent to the model shown in **Fig. 1.2**, LUG and TPL are transcriptional repressors that interact with their respective adaptors, SEU and IAA12 to repress *AG* expression and auxin signaling, respectively.

tpl-1 mutants have a severe embryonic defect, resulting in shoot poles that are transformed into a root, in embryos which do not express shoot apical meristem markers such as *SHOOT MERISTEMLESS* and *UNUSUAL FLORAL ORGANS* (Long et al., 2002). *tpl-1* mutants are temperature sensitive and high temperatures can induce transformation of the cotyledons and shoot apical meristems into root fate. It was recently reported that TPL mediates the auxin-regulated transcriptional repression of root genes during embryogenesis (Szemenyei et al., 2008). Through its ETHYLENE RESPONSE FACTOR (ERF)-associated amphiphilic repression (EAR)

repressor domain, TPL physically interacts with repressor protein INDOLE-2-ACETIC ACID 12 (IAA12) to mediate repressor activity.

In *Arabidopsis*, the other class of Gro/TLE transcriptional repressors is LUG and LUH. LUG and LUH proteins are most similar in domain structure and in amino acid sequence with a 44% identity (Conner and Liu, 2000). Chapter 2 of this thesis is aimed at addressing the function of *LUH* and determines whether *LUG* and *LUH* are functionally redundant.

Yeast-two-hybrid and *in vitro* pull-down assays were conducted to understand the mechanism underlying LUG and SEU's ability to repress *AGAMOUS* (*AG*) transcription. Results revealed that DNA-binding proteins AP1 and SEP3 are recruited by LUG/SEU to the *AG* cis-elements (Sridhar et al., 2006). SEU acts as a bridging protein that links LUG to two other MADS-box transcription factors, AP1 and SEP3, forming the tetrameric transcriptional repressor complex that targets the second intron of *AG* (Sieburth and Meyerowitz, 1997; Sridhar et al., 2006).

1.5.4 Co-Repressor Mechanisms

Transcriptional regulation plays a vital role for developmental processes and underlies morphological changes that plants, and all living organisms for that matter, undergo to become functional and adaptive organisms. In humans, transcriptional silencing is not only important for development, but also for differentiation and oncogenesis, such as the case of SMRT, a potent co-repressor for retinoid and thyroid-hormone receptors (Chen and Evans, 1995; Choi et al., 2008). SMRT has also

been shown to interact with a class II histone deacetylase to promote nuclear retention of Bach2, a leucine zipper family protein that represses DNA transcription (Hoshino et al., 2007).

DNA is negatively charged due to its phosphate side groups, which attracts positively charged and repels negatively charged molecules. Nucleosomes are considered the basic units of the chromosomal structure in eukaryotes, and consist of an octameric histone core with eight histone proteins that tightly wrap around nuclear DNA. These eight histone proteins include two of each H2A, H2B, H3, and H4. A single nucleosome will contain approximately 200 nucleotide pairs of DNA that wraps around the octameric histone core 1.65 times. The nucleosomes are strung together to form a long and compact chromatin (Alberts et al., 2002).

The N-terminal tails of each of the eight histone proteins in a nucleosome are highly conserved in their sequence and are important for regulating the structure of chromatin. These histone tails are subjected to a number of chemical modifications, such as lysine acetylation via the enzyme acetyl transferase (HATs) and deacetylation via the enzyme histone deacetylase (HDACs). HATs and HDACs operate by adding or removing negatively charged acetyl groups on the lysine residue of the histone tails. Therefore, histone acetylation destabilizes the chromatin structure by removing the positive charge on the lysine residue of the histone tails, making it more challenging for the histones to neutralize the negatively charged DNA molecule. However, histone deacetyalation works in the opposite fashion, by exposing the

positive charges of the histone tails. This will subsequently neutralize the negatively charged DNA molecular, making it easier to form a compacted chromatin. Therefore, histone acetylation is associated with gene expression while histone deacetylation is associated with gene silencing (Alberts et al., 2002).

HDACs is studied for its immediate benefits in controlling and curing human diseases such as the study of HDAC inhibitors as a useful tool for controlling Huntington's, Alzheimer's, and Parkinson's diseases (Kazantsev and Thompson, 2008). Cancer research is also are targeting the production of HDAC inhibitors that induce cell-cycle arrest and apoptosis by altering the acetylation pattern of the histone tails and the structure of the chromatin fiber (Adcock, 2007). Additionally, HDAC inhibitors are also being studied for its therapeutic applications in central nervous system disorders such as Rubinstein-Taybi syndrome, Rett syndrome, Friedreich's ataxia, and multiple sclerosis (Kazantsev and Thompson, 2008). These direct benefits to humans from scientific studies strongly support and necessitate the need to study transcriptional regulation as a mechanism in treating and curing many diseases.

In plants, LUG interacts directly with HDA19, a potent class 1 histone deacetylase enzyme, giving insight into one mechanism of LUG-dependent transcriptional repression (Sridhar et al., 2004; Gonzalez et al., 2007). Gonzalez *et al*. (2007) reported a second mechanism of transcriptional repression that is HDAC-independent. This mechanism involves LUG interacting with AtMED14/SWP and AtCDK8/HEN3, both of which are members of the Mediator complex. This supports

the idea that LUG utilizes both HDAC-dependent and HDAC-independent mechanisms to repress transcription. These mechanisms highlight the importance and conservation of transcriptional repression processes in plants, fungi, and animals.

1.6 Significance

My thesis work is focused on understanding the mechanisms underlying how LEUNIG (LUG)/SEUSS (SEU) co-repressors regulate diverse developmental and physiological processes. In other organisms such as yeast, co-repressor *ScTUP1* has been called a "global repressor" because it regulates a myriad of processes including glucose repression, sporulation, sterility of alpha cells, and flocculence (Williams and Trumbly, 1990; Edmondson et al., 1996; Zhang et al., 2002). Because *ScTUP1* regulates so many different processes in yeast, it is likely *LUG* and its homolog *LEUNIG_HOMOLOG* (*LUH*) also regulates many developmental and physiological processes in plants. If so, what processes do *LUG* and *LUH* regulate? My thesis focuses on three areas *LUG* and *LUH* regulates, including flower development, seed mucilage synthesis and secretion, and pathogen defense.

Another focus of my research is to identify the functions of *LUH*. LUH shares similar protein domain structure and sequence identity to *LUG*. It is likely *LUG* and *LUH* possess similar or redundant functions. However, they may also exhibit functional divergence and may regulate completely different pathways. A third possibility is that some processes are shared by *LUG* and *LUH*, while others are exclusive to one or the other. My thesis work attempts to distinguish among these alternatives and provide important insights into *LUH* function and the conservation

and divergence between these two co-repressor proteins in plant growth and development.

Chapter 2: LEUNIG_HOMOLOG and LEUNIG Are Partially Redundant During *Arabidopsis* Embryo and Floral Development

2.1 Abstract

Transcription co-repressors play important roles in animal and plant development. In *Arabidopsis thaliana, LEUNIG (LUG)* and *LEUNIG_HOMOLOG (LUH)* encode two highly homologous genes that are similar to the animal and fungal *Gro/Tup1* type co-repressors. LUG was previously shown to form a putative co-repressor complex with another protein SEUSS (SEU) and repress the transcription of *AGAMOUS* in floral organ identity specification. However, the function of *LUH* is completely unknown. Here, we show that single *luh* loss-of-function mutations develop normal flowers, but *lug*; *luh* double mutants are embryo lethal, uncovering a previously unknown function of *LUG* and *LUH* in embryonic development. In addition, *luh/+* enhances the floral phenotype of *lug,* revealing a minor role of *LUH* in flower development. Functional diversification between *LUH* and *LUG* is evidenced by the inability of *35S::LUH* over-expression to rescue *lug* mutants and by the opposite expression trends of *LUG* and *LUH* in responses to biotic and abiotic stresses. *luh-1* mutation does not enhance the defect of *seu* in flower development but LUH could directly interact with SEU in yeast. We propose a model that explains the complex relationships among *LUH, LUG* and *SEU*. As most eukaryotes have

undergone at least one round of whole-genome duplication during evolution, gene duplication and functional diversification are important issues to consider in uncovering gene function. Our study provides important insights into the complexity in the relationship between two highly similar paralogous genes.

2.2 Introduction

Transcription repression plays a key regulatory role in cell fate specification, hormone signaling, and plant stress responses. *LEUNIG (LUG)* was first identified in *Arabidopsis thaliana* based on its role in regulating the stage- and domain-specific expression of the C class floral homeotic gene *AGAMOUS (AG)* in flower development (Liu and Meyerowitz, 1995). In *lug* mutants, ectopic *AG* expression in the outer two whorls of a flower leads to homeotic transformation of sepals into carpels and petals into stamens, as well as a reduction of floral organs. LUG protein is similar in domain structure and biochemical function to the Groucho (Gro), *Transducin-Like Enhancer of Split* (TLE), and Tup1 family of co-repressors in *Drosophila*, mammals and yeast, respectively (Liu and Karmarkar, 2008). These co-repressors do not possess a DNA-binding domain and are recruited to their regulatory targets by interacting with DNA-bound transcription factors.

The N-terminus of LUG possesses a conserved domain, the LUFS domain named after the four founding members LUG, LUH, yeast Flo8, and human Single Stranded DNA Binding Protein (SSDP). The LUFS domain of LUG is essential for the direct interaction with its cofactor SEUSS (SEU) (Sridhar et al., 2004). *SEU* encodes a Q-rich protein with a centrally positioned dimerization domain also present

in the LIM-Domain-Binding (Ldb) family of transcriptional co-regulators in mammals and *Drosophila* (Franks et al., 2002). Recruitment of the LUG/SEU co-repressor complex by the MADS-box proteins *APETALA1 (AP1)* and *SEPALLATA3 (SEP3)* was shown to target the LUG/SEU repressors to the *AG* cis-regulatory element leading to repressed chromatin at the *AG* locus (Sridhar et al., 2006). The repressor activity of *LUG* was shown to depend on a HDAC activity and LUG was shown to directly interact with histone deacetylase 19 (HDA19) (Sridhar et al., 2004; Gonzalez et al., 2007), suggesting that the plant Gro/Tup1 family co-repressors mediate transcription repression by histone modification and chromatin re-organization. Recently, *LUG* was shown to repress gene expression via a HDAC-independent but mediator-dependent mechanism (Gonzalez et al., 2007).

Like co-repressors in animals and fungi, *LUG/SEU* possesses functions outside flower development. *lug* mutants showed defects in gynoecium development, female and male fertility, leaf and floral organ shape, and vasculature (Liu and Meyerowitz, 1995; Chen et al., 2000; Liu et al., 2000; Cnops et al., 2004; Franks et al., 2006). *Antirrhinum* mutants *of STYLOSA*, a *LUG* ortholog, not only showed abnormal flower development but also exhibited hypersensitivity towards auxin and polar auxin inhibitors (Navarro et al., 2004). A transcriptome study identified *LUG*-regulated genes in abiotic and biotic stress response, meristem function, and transport (Gonzalez et al., 2007). Therefore, *LUG* likely encodes a global regulator for multiple developmental processes and signal pathways.

In *Arabidopsis*, *LUG* belongs to a small family of about 13 genes (http://smart.embl-heidelberg.de/), including *TOPLESS (TPL), TOPLESS-RELATED (TPR),* and *WUSCHEL-INTERACTING PROTEINS (WSIP)* (Kieffer et al., 2006; Long et al., 2006; Liu and Karmarkar, 2008). These genes are involved in regulating embryonic shoot-root axis determination and appear to repress auxin-mediated signaling events during embryogenesis (Long et al., 2006). The *TPL/WSIP* genes are also involved in mediating the effect of *WUS* in target gene repression to maintain stem cell pool at the shoot apical meristem (Kieffer et al., 2006). Therefore, the plant Gro/Tup1 family co-repressors are emerging as a fundamentally important class of regulators in plant development.

Among the 13 *Arabidopsis* Gro/Tup1 co-repressor-like proteins, *LUH* (AT2G32700) is most similar to *LUG* (Conner and Liu, 2000). Both proteins possess a N-terminal LUFS domain that is 80% identical (Fig. 2A). In addition, both proteins possess seven WD repeats at the C-terminus that show 58% identity to each other. A third domain that immediately precedes WD repeats (residue 369 to 500) also shows a high level of sequence similarity (57%). Both LUG and LUH have centrally located Glutamine (Q)-rich regions, but the Q-rich regions in LUH are less continuous and less extensive than those in LUG.

Despite the significant sequence similarity, almost nothing is known about *LUH* function or expression. This chapter presents data indicating that *LUH* possesses

both unique and overlapping functions with *LUG*, and that *LUH* activity is required for proper embryo and flower development. We propose a model that explains the complex relationship between *LUH* and *LUG*.

2.3 Materials and Methods

2.3.1 Plant Growth and Mutant Identification

Arabidopsis plants were grown on Sun Shine professional soil in controlled growth chambers at 20°C and 55% humidity under long day (16 hour light) conditions. Seeds used in germination and root elongation assays were sterilized with 70% ethanol and 0.6% hypochlorite (bleach), plated on 0.44% Murashige and Skoog Basal Medium (MS) plates, incubated in the dark for 3 days at 4ºC, and then grown for 5 days at 20^{o}C under long day before transferring to another MS plate for root analyses.

lug-3, *lug-16* and *seu-1* were generated in L*er* background and were previously described (Conner and Liu, 2000; Franks et al., 2002). *luh-1 (*luh_172H3; ABRC stock CS91893) and *luh-2* (luh_147A6; ABRC stock CS91036) were generated by the *Arabidopsis* TILLING Project by EMS mutagenesis in the Columbia *erecta-105* background (McCallum et al., 2000). *luh-3* (SALK_107245) was generated by T-DNA insertion (Alonso et al., 2003).

2.3.2 Double Mutant Construction and Genotyping

To generate double mutants, luh-1 pollen was used to pollinate lug-16, lug-3 or seu-1. F2 plants were analyzed by PCR-based genotyping methods. luh-1 dCAPS

marker uses primers 5'-GCACCTGGAGGGTTTCCATTTGAGTG-3' and 5'-CGCTTTACCTTGTTGTGCCTAAAATT-3' in 35 cycles of PCR at 94°C 30 seconds, 50° C 30 seconds, 72°C 30 seconds. 6µL PCR reactions were digested with BstX1 at 55°C and analyzed on 2.5% agarose gels. luh-1 PCR products were resistant to BstX1. seu-1 dCAPS primers (Franks et al., 2002) amplified genomic DNA at 94 oC 30 second, 50°C 30 second, 72°C 30 seconds for 35 cycles. The PCR products were digested with Rsa1. seu-1 PCR products are resistant to the RsaI digestion.

Since the dCAPS assay was not always reliable, an alternative fluorescent-based SNP assay (Amplifluor ® SNPs Genotyping) was adopted for luh-1 and lug-16. Individual leaf or single embryo was pressed onto FTA MicroCard (Whatman). A 0.2mm diameter disc was punctured out of the FTA MicroCard and served as templates for PCR following manufacturer's protocol. Primers for lug-16 (5'GTTAAGTAGGAAGTTAAGCCC3' and 5'-GAGAACACCATTCAACTGTAC-3') and luh-1 (5'-GTTTGGGCTTTTATTCAGGTT-3' and 5'-GCACTAGCATTAGACTGCCC-3') were first used in a conventional PCR. 25ng diluted PCR products then served as templates for Amplifluor ® SNPs Genotyping System (Assay Development kit from Chemicon International, a subsidiary of Serologicals Corp.) using Platinum Taq DNA Polymerase (Invitrogen). Amplifluor AssayArchitect program (Chemicon International) was used for primer design; allele-specific primer has a tail sequence complementary to either fluorescent FAM- or JOE-labeled primer. For lug-16 locus (tail sequence underlined), wild type specific primer is 5'-GAAGGTGACCAAGTTCATGCTTCACCAGGTGCGTCAATAGCT-

3’ and lug-16 specific primer is 5’-GAAGGTCGGAGTCAACGGATTTCCACCAGGTGCGTCAATAGT-3’. Both allele-specific primers pair with the same reverse primer 5’-CTGCAGTTGCTCTGTTTCCTAA-3’. All three primers were used in the same PCR genotyping reaction. For luh-1 locus (tails underlined), wild type specific primer is 5’-GAAGGTCGGAGTCAACGGATTTGTCCCAAAACACAGACCAC-3’ and luh-1 specific primer is 5’-GAAGGTGACCAAGTTCATGCTAATGTCCCAAAACACAGACCAT-3’. The reverse primer is 5’-GCACCTGGAGGGTTTCTTTTT-3’. PCR was run on conventional PCR machine programmed: 1) 96ºC for 4 minutes, 2) 96ºC for 12 seconds, 3) 57ºC for 5 seconds, 4) 72ºC for 10 seconds, 5) Repeat steps 2 to 4 for 15 cycles, 6) 96ºC for 12 seconds, 7) 55ºC for 20 seconds 8) 72ºC for 40” seconds, 9) Repeat steps 6-8 19 cycles, 10) 72ºC for 3 seconds, 11) Hold at 20ºC. Allelic discrimination was determined by reading FAM and JOE fluorophore signals using the BioRad iQ5 PCR machine.

2.3.3 Microscopy and Photography

Floral, silique, and seedling photographs were captured with a Nikon SMZ1000 microscope equipped with a NIKON digital camera. The green and white seeds were dissected from siliques and fixed in Hoyer's solution for 15 minutes (Liu and Meinke, 1998) and then examined and photographed with a Nikon ECL1PSE E600W microscope with Nomarski optics and equipped with a DXM1200 digital still camera. Images were processed with Adobe Photoshop version 7.0.

2.3.4 Molecular Analyses of *LUH*

LUH (At2g32700) has 17 exons. 5' RACE was performed to verify the *LUH* transcript using the GeneRacer Kit (Version F, Invitrogen) and total RNA from *Arabidopsis* flowers. 5' nested primer GGACACTGACATGGACTGAAGGAGTA was used, and the RACE products were cloned in pCRII TOPO (Invitrogen) and sequenced. *LUH* full-length cDNA (RAFL09-12-E08 (R12254)) was obtained from RIKEN Genomic Sciences Center and sequenced for confirmation.

To generate *35S::LUH,* the full length *LUH* cDNA from RIKEN was amplified by PCR with primers (35SLUH-F 5'-ATTACCCGGGGATGGCTCAGAGTAATTGGGAAG-3'; 35SLUH-R 5'-TCCCCCGGGCTACTTCCAAAT CTTTACGGA-3') containing engineered Xma1 sites with the high fidelity Taq polymerase (Roche). The PCR product was cloned in the PBI121 vector at Xma1 site and verified by sequencing. Plasmids were transformed into GV3101 *Agrobacterium* through electroporation. *luh-1* and *lug-16* plants were transformed by the floral dip method (Clough, 2005). Kanamycin resistant T1 seedlings were identified on MS plates containing 50µM Kan and transferred to soil.

For RT-PCR, reverse transcription was performed with oligo dT and Superscript RT II enzyme (Invitrogen). All RT-PCR reactions were carried out for 25 cycles and were repeated at least twice. Primers are *LUH* (5'-TGGCTCAGAGTAATTGGGAAG-3' and 5'-CCAGGCTTT GATTGCAGA-3') and

ACT2 (5'-GTTGGGATGAACCAG AAGGA-3' and 5'-CTTACAATTTCCCGCTCTTC-3'). The primers were designed to span introns to avoid amplification from contaminated genomic DNA. *ACT*2 was used as a loading control. The RT-PCR reactions were quantified using Image quant 1.1 (NIH) software, based on the intensity of the ethidium bromide staining.

2.3.5 Yeast-two-hybrid Assay and Repression Assay

Full length *LUH* cDNA was PCR amplified using high fidelity *Taq* polymerases (Roche) and the RIKEN (RAFL09) cDNA as a template with engineered primers. PCR products were cloned into the pCRII TOPO vector (Invitrogen). The clone was sequenced to verify amplification accuracy. The primers LUH-BD-f and LUH-AD-f 5'-ATTACCCGGGGATGGCTCAGAGTAATTGGGAAG-3' and LUH-BD-r 5'-ACGCGTCGACATCTACTTCCAAATCTTTACGGA-3' and LUH-AD-r 5'-ATTCTCGAGCTACTTCCAAATCTTTACGGA-3' contain Sal1 and Xma1 sites for the BD fusion and Xho1 and Xma1 sites for the AD fusion. The *LUH* fragments were excised from corresponding pCRII TOPO vectors and inserted into pGBKT7 and pGADT7 (Clontech), respectively, at corresponding enzyme sites. The yeast host (PJ69-4A), genotype *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), was used for transformation as described previously (Sridhar et al., 2004).

For the X-Gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside) overlay assay, 0.125 g agarose was dissolved in 25 ml sterilized Z-buffer (60 mM

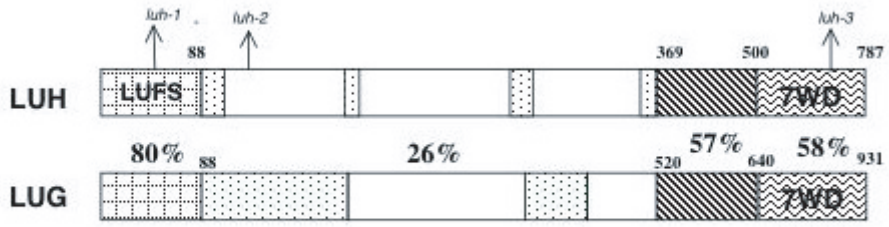
$Na_2HPO_4 \cdot 2H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, pH: 7.0) by heating in microwave oven. After cooling to 50ºC, 0.5 ml 10% SDS and X-Gal dissolved in DMF (final concentration of 2 mg/ml) were added. The molten agarose solution was poured over one to two plates containing yeast colonies. After 30-minute incubation at 37°C, the plates were photographed.

2.4 Results

2.4.1 *luh-1* Mutants Exhibit Vegetative and Development Defects

Through the Arabidopsis TILLING Project (McCallum et al., 2000), a former Ph.D. student Jayashree Sitaraman obtained several *luh* mutations (see Materials and Methods). *luh-1* (luh_172H3) is caused by G to A change resulting a conversion of tryptophan (W_{55}) to STOP codon (**Fig. 2.1B**), truncating the protein at the 55th residue. *luh-2* (luh_147A6) changes C to T, resulting in an amino acid substitution from serine (S_{123}) to Phenylalanine (F). *luh-3* (SALK_107245C) is caused by a T-DNA insertion in the third to the last exon (**Fig. 2.1B**), disrupting the last three WD repeats. We have focused on *luh-1* as it likely represents a null or a strong loss-of-function mutation.

A



B

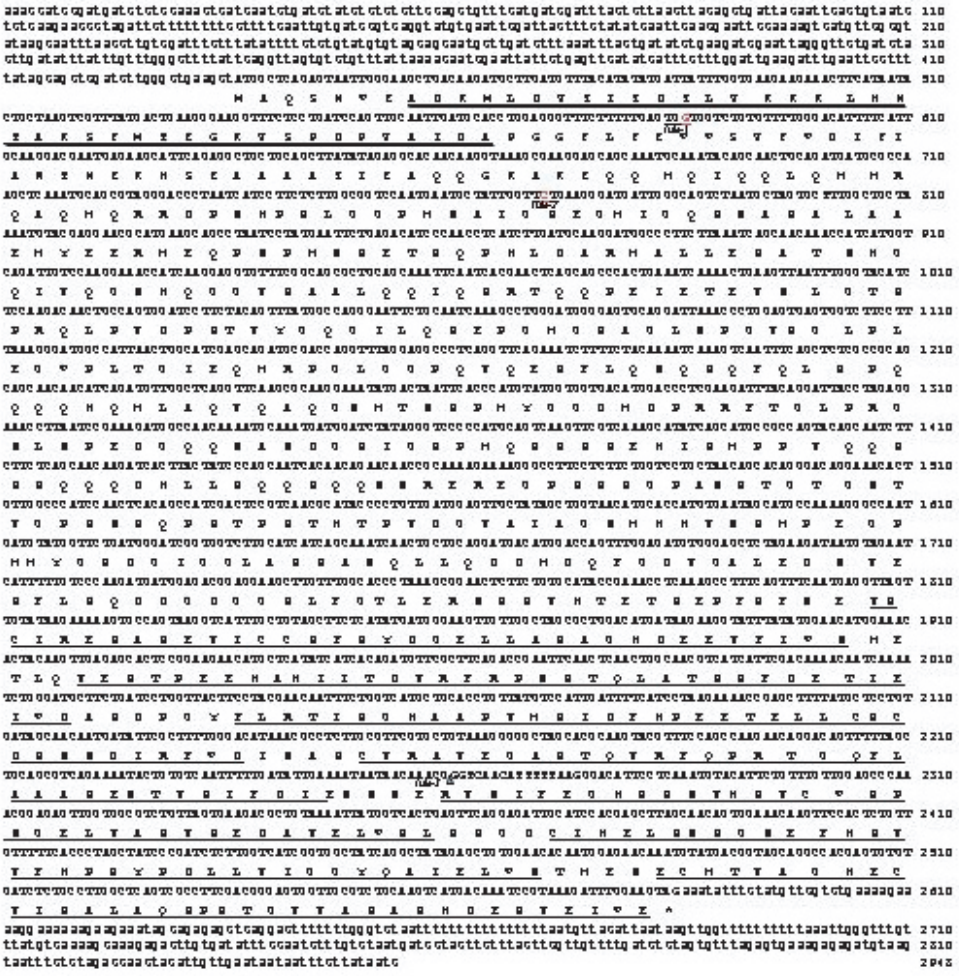


Fig. 2.1: LUH protein structure and gene sequence. A). A schematic diagram showing the protein domains of LUH and LUG. Numbers represent the amino acid, and the % numbers indicate % identity between LUH and LUG. The location of each luh allele is indicated by an arrow. (B). Sequence of LUH cDNA and deduced amino acid sequence. Changes in luh alleles are highlighted in red. 5' and 3' UTR are included. The amino acids in bold constitute

the LUFS domain. Within the LUFS domain, the LisH domain is doubly underlined. Seven WD repeats are also underlined.

Initial characterization by Jayashree Sitaraman indicated that *luh-1* single mutants did not exhibit any abnormality in flowers (**Fig. 2.2A**, **B**). Nevertheless, *luh-1* mutant seedlings showed slower and poorer germination on MS medium (**Fig. 2.2C**, **D**), with a germination rate of about 80% of WT (**Fig. 2.2H**). In addition, *luh-1* mutants grew slower compared to wild type at 3-week age (**Fig. 2.2E**), but eventually caught up. Finally, the roots of *luh-1* seedlings were significantly shorter than wild type (**Fig. 2.2F**, **G**). To test if these phenotypes are caused by the *luh-1* mutation, *35S::LUH* cDNA was transformed into *luh-1* mutants. Eight transgenic plants were obtained and two of these transgenic lines (#4 and #5) showed a higher level of *LUH* mRNA (**Fig. 2.2I**) and were further analyzed. The developmental defects of *luh-1* described above were rescued by the *35S::LUH* transgene. **Fig. 2.2G** and **2.2H** illustrate the rescue of root length and germination rate, respectively.

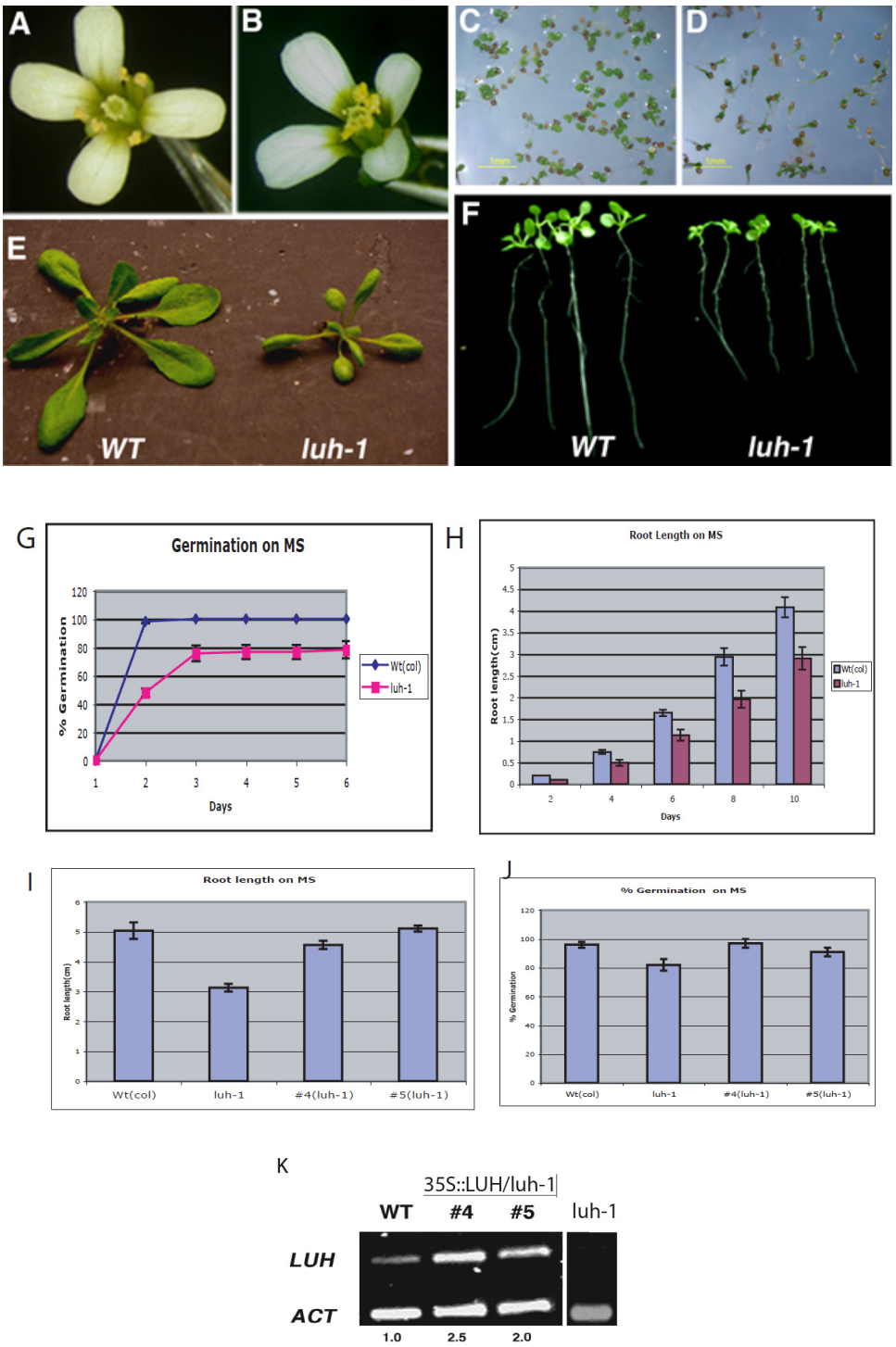


Fig. 2.2: luh-1 develops normal flowers but exhibits defects in vegetative growth. (A) A wild-type (Col-er) flower. (B) A luh-1 flower in Col-er background. (C) Germination of wild-type seeds on MS medium at 5 days. (D) Germination of *luh-1* seeds on MS medium at 5 days. (E) 3-week old wild type and *luh-1* plants. (F and H) Root elongation of wild type and *luh-1* seedlings on MS medium. Germinated seedlings were transferred to MS plates and grown vertically. Photos were taken after 7 Days in F. (G) Germination rate between wild-type and *luh-1* seeds. (I) *35S::LUH* complemented the *luh-1* defect in root elongation. Root length

measured after 7 days was expressed as the mean ± Standard Error (SE). (J) Germination phenotype of *luh-1* is complemented by *35S::LUH*. Germination is expressed as the mean ± SE. (K) Semi-quantitative RT-PCR showing the expression of LUH mRNA in wild type (Col-er), *luh-1*, and two different *35S::LUH* transgenic lines (#4 and #5) in *luh-1* background. The ratio between *LUH* band intensity and that of *ACTIN2* (*ACT*) control band was shown below each lane. (Results shown here were obtained by Jayashree Sitaraman).

2.4.2 *luh-1* Mutation Enhances *lug* Mutant Flower Phenotype

It is possible that the function of *LUH* in flowers is not necessary when *LUG* is intact, but becomes necessary when *LUG* is absent or reduced. If this is the case, *luh-1* may enhance the phenotype of *lug*. To test this, both the weak *lug-16* and the strong *lug-3* were crossed into *luh-1* to construct *lug-16; luh-1* and *lug-3; luh-1* double mutants. F2 progeny segregated *lug* single mutants as well as mutants with a more severe phenotype than *lug* single mutants. Allele-discriminating SNP assays (see Materials and Methods) were used to genotype those F2 plants with a more severe phenotype than *lug* single mutants, and they were found to be homozygous for *lug* and heterozygous for *luh-1*. Specifically, while *lug-16* single mutants developed elongated siliques (**Fig. 2.3A**), the *lug-16/lug-16; luh-1/+* plants did not show any silique elongation and were completely sterile (**Fig. 2.3B**). However, the floral phenotype of *lug-16/lug-16; luh-1/+* was similar to *lug-16* single mutants (**Fig. 2.3C**, **D**).

lug-3/lug-3; luh-1/+ flowers exhibited a more severe phenotype than *lug-3* single mutant flowers (**Fig. 2.3E**, **F**). *lug-3/lug-3; luh-1/+* mutant flowers consisted of only a few carpelloid sepals and sepal-like organs topped with horns, suggesting a more severe homeotic transformation possibly caused by more extensive ectopic expressions of *AG*. There were no petals, and stamens were either completely absent

or partially fused to first whorl organs (**Fig. 2.3F**, **G**). The *lug-3/lug-3; luh-1/+* flowers are completely sterile and they resemble *lug; seu* double mutant flowers (Franks et al., 2002).

Among the F2 progeny of *lug-3* and *luh-1* cross, *lug-3; luh-1* double mutants were never found, although they should occur at a frequency of 1 in 16. Only two *lug-16; luh-1* double homozygous mutants were found after screening several hundred F2 progeny of *lug-16* and *luh-1* cross. The *lug-16; luh-1* double mutants were extremely small in stature; the entire mature plant is smaller than a single rosette leaf (**Fig. 2.3G**). Inflorescence meristem only bears 3 to 5 flowers consisting of only carpels (**Fig. 2.3H**).

Since SEU acts as an adaptor for LUG and *seu* mutation enhances *lug* mutants (Franks et al., 2002; Sridhar et al., 2004), we tested the genetic interaction between *luh* and *seu*., *seu-1; luh-1* double mutants were identified by genotyping F2 as well as F3 plants of the *seu-1* and *luh-1* cross. The *seu-1; luh-1* double mutants are morphologically indistinguishable from *seu-1* single mutants (**Fig. 2.3I**, **J**).

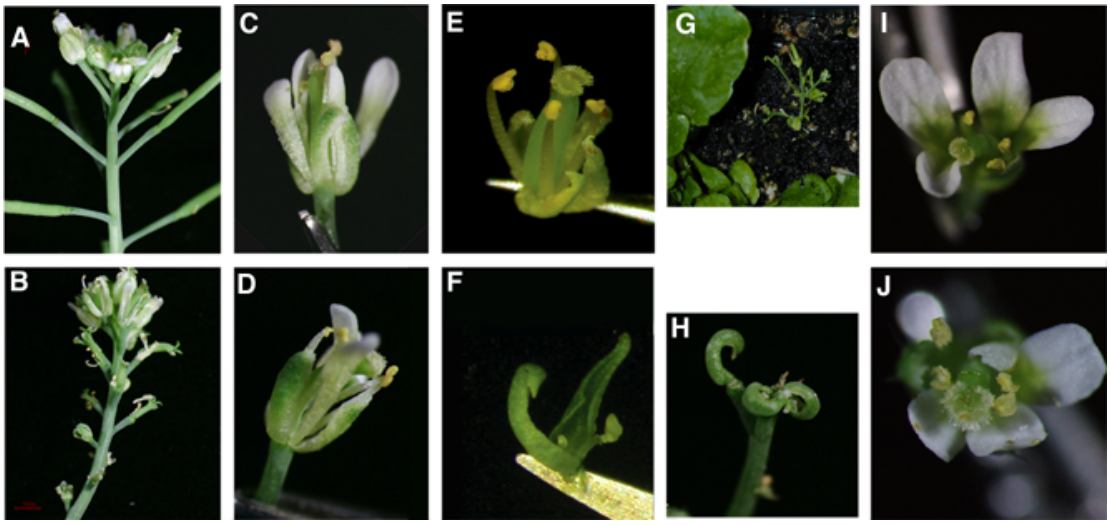


Fig. 2.3: *luh-1* enhances *lug-16* and *lug-3* during flower development. (A) An inflorescence shoot of lug-16. Note the elongating siliques. (B) An inflorescence shoot of *lug-16/lug-16*; *luh-1/+*. Note the absence of silique development. (C) A *lug-16* flower with narrow sepals and petals. (D) *A lug-16/lug-16; luh-1/+* flower. (E) A lug-3 flower. (F) A *lug-3/lug-3; luh-1/+* flower. (G) A *lug-16; luh-1* double mutant. Note the extreme small statue; the mature plant is smaller than a rosette leaf. (H) A close-up of an inflorescence shoot of *lug-16; luh-1* double mutant. (I) A *seu-1* flower. Note the reduced stamen number. (J) A *seu-1; luh-1* double mutant flower.

2.4.3 *lug; luh* Double Mutants Are Embryo Lethal

The absence of *lug-3; luh-1* double mutants and a significant reduction of *lug-16; luh-1* double mutants among the F2 progeny suggest most *lug-16; luh-1* double mutants die prematurely. The complete sterility of *lug-16/lug-16; luh-1/+* plants (**Fig. 2.3A**, **B**) makes it impossible to identify *lug-16; luh-1* double mutants in the next generation. Instead, we identified several *luh-1/luh-1; lug-16/+* plants through genotyping F2 progeny of the *lug-16* and *luh-1* cross. Surprisingly, these *luh-1/luh-1; lug-16/+* plants developed wild type-like flowers albeit at a slightly smaller size (**Fig. 2.4A**, **DC**). Thus, it appears that *LUG* is more critical for proper flower development than *LUH*, as *luh-1/luh-1; lug-16/+* plants with only one copy of wild type *LUG* are capable of normal floral development, but *lug-16/lug-16; luh-1/+* plants with only one copy of wild type *LUH* fail to develop normal flowers (**Fig. 2.3**).

When *luh-1/luh-1; lug-16/+* plants were self-fertilized and their siliques were examined, white and abnormal seed occurred at a frequency of about 36% in a silique (**Fig. 2.4E**; **Table 2.1**). This is in contrast to *luh-1* and *lug-16* single mutants, whose siliques contain only about 5% of white seeds and close to 95% green seeds (**Fig. 2.4B**, **C**; **Table 2.1**). To verify the genotype of these white and green seeds segregated by *luh-1/luh-1; lug-16/+* plants, 8 white seeds and 10 greens seeds (collected from several different siliques) were individually genotyped. All 8 white seeds were found to be *luh-1/luh-1; lug-16/+*. Among the 10 green seeds, 6 were *luh-1/luh-1; +/+* and 4 were *luh-1/luh-1; lug-16/+*. This suggests that *luh-1/luh-1; lug-16/+* seeds could either develop into normal green seeds or abnormal white seeds. An absence of *luh-1/luh-1; lug-16/lug-16* genotype among the 8 white and 10 green seeds indicated that the *luh-1/luh-1; lug-16/lug-16* embryos died early during embryogenesis before visible seeds were formed. Therefore, significant functional redundancy must exist between *LUH* and *LUG* during early embryo development.

To better pinpoint the stage at which embryo development is affected in the white seeds, we examined the white and green seeds dissected from the same siliques of *luh-1/luh-1; lug-16/+* plants. While the green embryos were already at the torpedo stage (**Fig. 2.4E**), the white embryos from the same silique were arrested at the late globular stage (**Fig. 2.4F**). In some of the white seeds, the globular embryos appeared disintegrated (Data not shown).

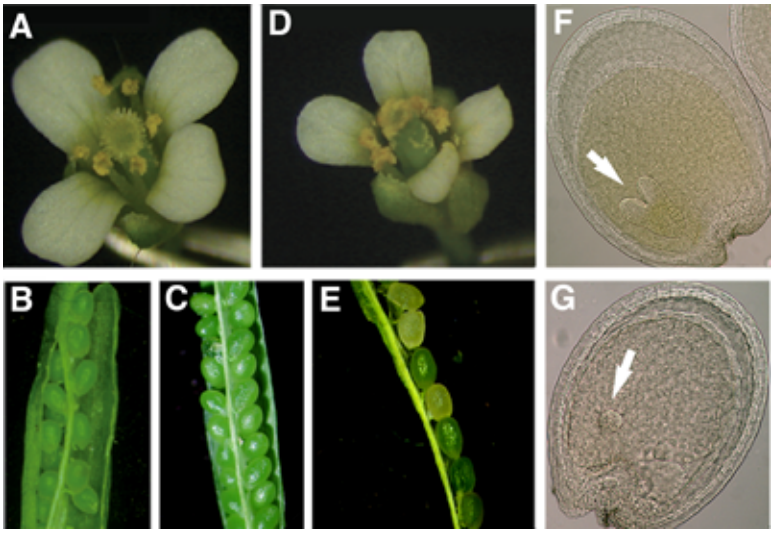


Fig. 2.4: luh-1; lug-16 double mutants are embryo lethal. (A) luh-1 flower. (B) An open silique of luh-1 showing green seeds inside. (C) An open silique of lug-16 showing green seeds inside. (D) A luh-1/luh-1; lug-16/+ flower. Note the smaller flower size. (E) An open luh-1/luh-1; lug-16/+ silique showing white seeds among green seeds. (F) Normaski image of a green seed in a silique derived from a luh-1/luh-1; lug-16/+ plant. Arrow indicates the embryo proper at the torpedo stage. (G) Normaski image of a white seed from the same silique as (F). Arrow indicates an embryo at the late globular stage.

2.4.4 *LUG* and *LUH* Have Divergent Functions and Expression Patterns

Functional diversification between *LUH* and *LUG* could result from their differences in expression or in protein coding sequences or both. To test this, we transformed *35S::LUH* into *lug-16* mutants. *lug-16* is the most fertile allele and can be easily transformed. If over-expressing *LUH* could rescue *lug-16*, LUH coding region may be equivalent to LUG. None of the 12 T1 transformants was able to rescue *lug-16*. On the contrary, five of these 12 lines showed an enhanced phenotype with more carpelloid sepals and a greatly reduced organ number (**Fig 2.5C**, **D**). We hypothesized that these five lines exhibited co-suppression and silencing of the endogenous *LUH*. Semi-quantitative RT-PCR was performed for three of the five

lines, revealing that that the *LUH* mRNA level in these lines was approximately half of that in wild type plants (**Fig 2.5E**) and supporting the co-suppression hypothesis.

The remaining seven *35S::LUH; lug-16* lines did not show a co-suppressed phenotype but the *35S::LUH* transgene did not rescue *lug-16* (data not shown). Either they did not express high enough levels of *LUH* or LUH protein is not equivalent to LUG in function. To distinguish these alternative explanations, *35S::LUH*; *luh-1* transgenic line *#5*, previously shown to rescue *luh-1* phenotype (**Fig. 2.2**), was crossed into *lug-16*. The F2 plants harboring *35S::LUH* #5 and carrying wild type *LUH* and *LUG* are wild type in phenotype (**Fig. 2.5F**, **G**). However, the F2 *35S::LUH* #5 plants carrying wild type *LUH* allele but *lug-16/lug-16* mutant allele exhibited phenotypes identical to *lug-16* single mutants (**Fig. 2.5H**, **I**), suggesting that increasing (and ectopic expressing) *LUH* transcripts could not substitute for *LUG*.

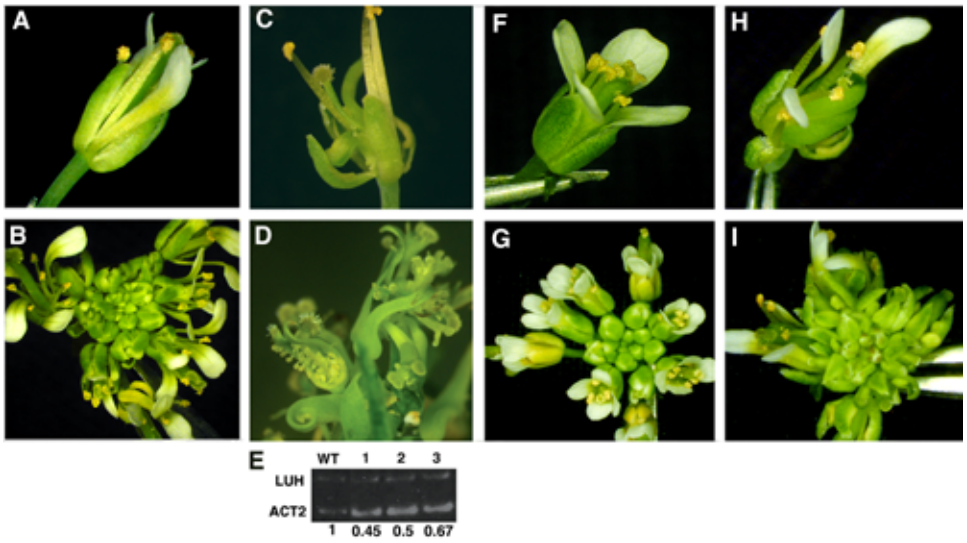


Fig. 2.5: *35S::LUH* failed to rescue *lug-16* mutants. (A) A *lug-16* mutant flower. (B) A *lug-16* inflorescence. (C). A *35S::LUH*; *lug-16* flower, where the 35S::LUH appears to enhance the defect of *lug-16* probably by silencing endogenous LUH. (D) An inflorescence of a

35S::LUH; lug-16 transgenic plant similar to (C). (E) RT-PCR result showing reduced *LUH* mRNA in the *35S::LUH; lug-16* transgenic lines 1, 2 and 3. The numbers below represent the relative mRNA level normalized to ACT2 and compared with wild type, which is taken as 1. (F) and (G) *35S:LUH* (#5) in wild type, causing no obvious phenotype. H) and (I) *35S::LUH* line #5 in lug-16, showing phenotypes identical to *lug-16* mutants shown in (A) and (B).

To compare the expression pattern of *LUG* and *LUH* during development, a former undergraduate student Parsa Hosseini utilized the AtGenExpress atlas data that compares the expression profiles of 22,746 probe sets on the Affymetrix ATH1 array using triplicate expression estimates from 79 diverse development samples ranging from embryogenesis to senescence and from roots to flowers (Schmid et al., 2005). *LUG* and *LUH* were shown expressed in all 79 samples in comparable levels (**Fig. 2.6A**). Interestingly, *SEU*, the partner of *LUG*, showed almost identical expression profile to *LUG*, supporting that proteins present in the same complex are likely expressed in similar profiles (Schmid et al., 2005). A comparison between LUH and SEU revealed highly similar but not identical profiles (**Fig. 2.6A**).

In addition, the expression profiles of *LUG*, *SEU* and *LUH* were compared using the AtGenExpress data using Arabidopsis samples challenged with biotic and abiotic stresses, hormones, lights, and nutrients (www.weigelworld.org/resources/microarray/AtGenExpress/) (Kilian et al., 2007). As shown in **Fig. 2.6B**, an increased expression upon a particular treatment is indicated by Magenta and a decreased expression upon a treatment is indicated by green. The clustergram showed that LUH and LUG exhibited almost opposite expression trends upon treatment with similar conditions. For example, *LUH* transcription is induced by exposures to biotic stress (nematode and *B. cinerea*) and abiotic stress (salt,

genotoxic, wounding, drought, oxidative), *LUG* transcription, on the contrary, is reduced or unchanged under these same conditions. Additionally, certain chemicals (cycloheximide, 2,4-dichlorophenoxyacetic acid, AgNO3, AVG), biotic stress (A. tumefaciens), and abiotic stress (hypoxia) caused an increased *LUG* expression but reduced *LUH* expression. *SEU* appears to exhibit an expression pattern more similar to *LUH*. This analysis suggests that *LUG* and *LUH* play opposite regulatory roles in different stress signaling pathways. How can *LUG* and *LUH* be expressed at opposing levels and both are localized within the nucleus? One possible explanation is that LUG and LUH regulate different targets, despite the fact they both require SEU as an adaptor. If LUG represses gene X which acts as a positive regulator of biotic stress and LUH represses gene Y which acts a negative regulator, during stress, LUG expression is reduce and LUH is induced to initiate stress response.

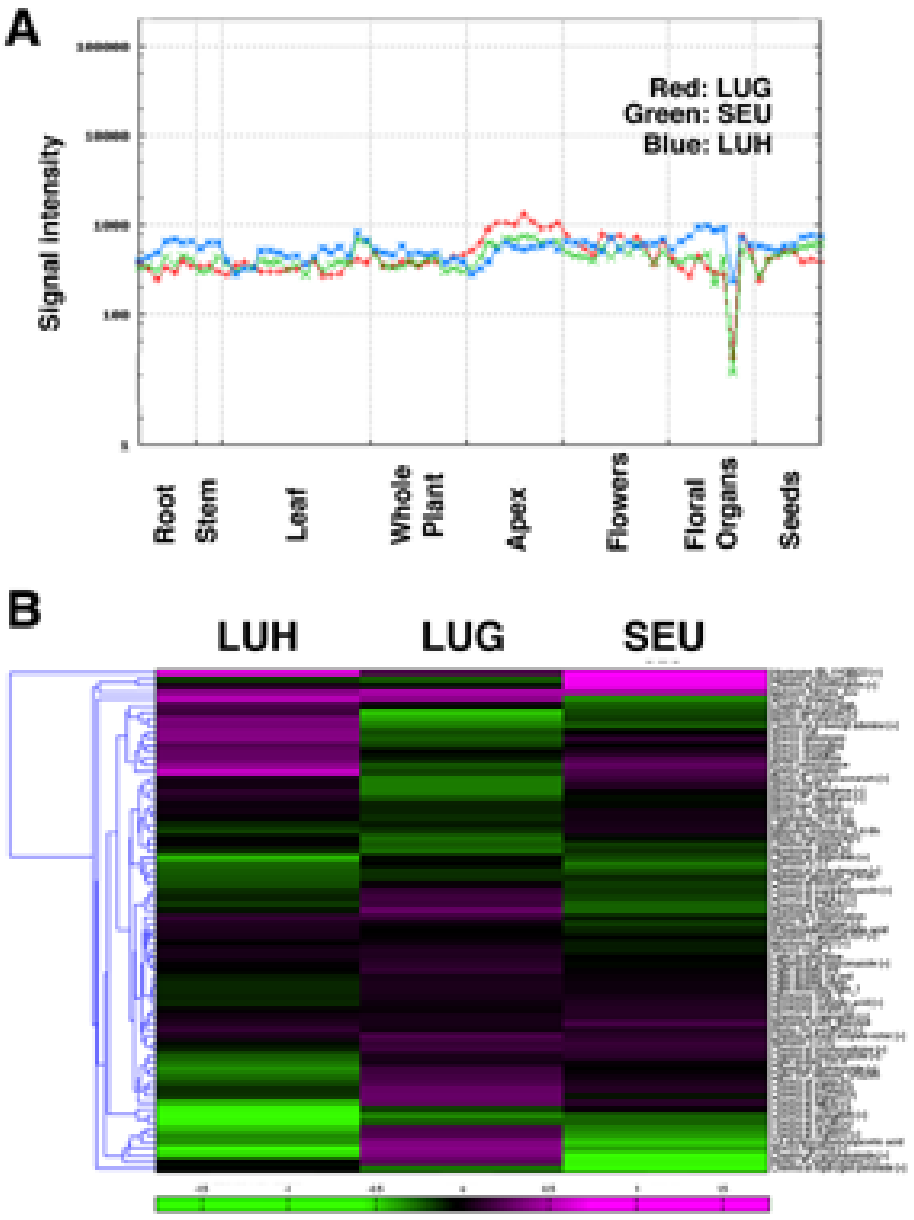


Fig. 2.6: *LUH* expression in comparison to *LUG* and *SEU*. (A) Expression profile of *LUH*, *LUG*, and *SEU* in different developmental tissues and stages. The data was generated by AtGenExpress (Development) and presented using the AtGen Expression Visualization Tool (Schmid et al., 2005). *LUG* (red) and *SEU* (green) showed almost complete co-expression in all tissues. *LUH* (blue) closely resembles but not identical to the *LUG/SEU* profile. (B) Hierarchal cluster analysis of environmental regulation of *LUH*, *LUG* and *SEU* expression using AtGenExpression (Abiotic, Light, Hormone, Pathogen) expression estimates by gcRMA (Kilian et al., 2007) (www.weigelworld.org/resources/microarray/AtGenExpress/). The clustergram was generated with the Matlab RC13 (Mathworks) Bioinformatics Toolbox. An increase in expression is indicated by Magenta and a decrease in expression is indicated

by green (see bar on the bottom). (This analysis was performed by Parsa Hosseini, a former undergraduate student in the Liu lab).

2.4.5 LUH Interacts Directly with SEU But Not with LUG

If *LUH* and *LUG* have both overlapping and unique functions, do their proteins directly interact with each other to form heterodimers? Yeast two-hybrid assays failed to detect an interaction between LUH-AD (full length LUH fused to the GAL4 Activation Domain) and LUG-BD (full length LUG fused to GAL4 DNA Binding Domain) (**Fig. 2.7A**), nor could LUG homodimerize (**Fig. 2.7B**). Thus LUG and LUH likely act independently or in parallel to regulate common as well as unique target genes.

Previously, LUG was reported to directly interact with SEU via the N-terminal LUFS domain (Sridhar et al., 2004); **Fig. 2.7A**, **B**). Because of the 80% sequence identity between LUG and LUH at the LUFS domain, we tested if LUH could also interact with SEU. A strong interaction was detected between full length LUH-AD and SEU (ND)-BD (Fig. 7B). SEU (ND) is a truncated SEU with its C-terminal domain (capable of self-activation) removed. Therefore, like LUG, LUH can also form a potential co-repressor complex with SEU.

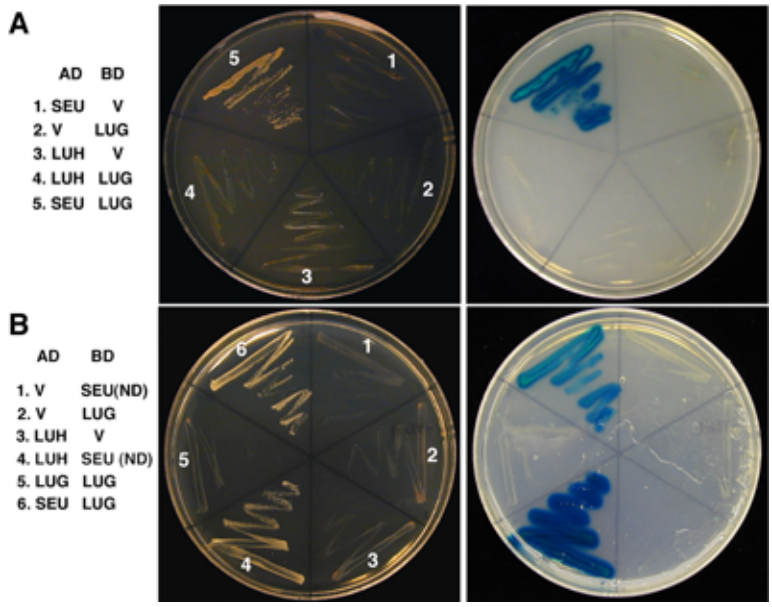


Fig. 2.7: LUH interacts with SEU but not LUG in yeast. (A) Yeast two-hybrid assay showing a lack of interaction between LUH and LUG. Positive interaction is indicated by the activation of HIS3 and ADE2 reporter genes allowing colony growth on -Trp, -Leu, -His, and -Ade plates containing 3mM 3-amino-1,2,4-triazole (left). The activity of a third reporter gene LacZ encoding β-galactosidase was tested by the X-gal overlay assay (right); blue color indicates a positive interaction. (B) Yeast two-hybrid assay showing a positive interaction between LUH and SEU(ND).

2.5 Discussion

2.5.1 LUG and LUH Exhibit Partially Redundant But Not Identical Functions

In this study, we investigated the function of *LUH*, the closest homolog of *LUG* in *Arabidopsis.* We discover that these two genes play redundant roles during embryo development, revealing a previously uncovered role of *LUG* during embryonic development. Second, we identified a relatively minor role of *LUH* compared with *LUG* in flower development as *luh-1* single mutation does not affect flower development, but *luh-1/+* can enhance the floral phenotype of *lug*. Third, since over-expressing *LUH* could not rescue the weak *lug-16* mutation, the

divergence in their coding sequences rather than their expression level or pattern likely contributes to their functional difference.

2.5.2 LUH and SEU Act in the Same Pathway to Regulate Flower Development

We showed that both LUG and LUH physically interact with SEU in yeast, suggesting the possibility of forming both LUG/SEU and LUH/SEU co-repressor complexes. Interestingly, *lug* and *luh* mutations exhibited drastically different genetic interactions with *seu*. Specifically, *lug*; *seu* double mutants exhibited a synergistic genetic interaction (Conner and Liu, 2000; Franks et al., 2002). In contrast, *seu-1* mutant flowers are indistinguishable from *seu-1; luh-1* double mutant flowers. Since *luh-1* does not exhibit any mutant floral phenotype, one likely interpretation is *LUH* plays only a minor role in flower development, so *luh-1* loss-of-function flowers look identical to wild-type. However, *seu-1; luh-1* mutants exhibit a phenotype that is identical to *seu-1* single mutants because it does not matter whether LUH is present or not, as LUG will compensate for the loss. An alternative interpretation is that *SEU* and *LUH* function independently, with *SEU* playing a more significant role. We favor the first interpretation because yeast-two-hybrid detected an interaction between LUH and SEU, so they are not likely to act independently (see discussion).

2.5.3 LUG and LUH Have Divergent Regulation During Environmental Stress

Recent genome-wide transcriptome studies comparing wild type and *lug-3* mutant tissues revealed dramatic changes in the expression of genes involved in abiotic and biotic stress response (Gonzalez et al., 2007). It is thus of particular interest to note the almost opposite expression trends between *LUG* and *LUH* under different biotic and abiotic challenges. This difference in gene expression pattern

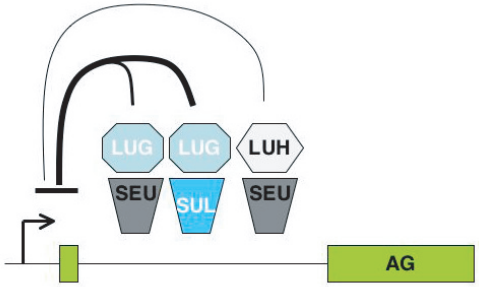
between *LUH* and *LUG* upon exposures to different environmental conditions (**Fig. 2.6B**) is in sharp contrast to the highly similar gene expression pattern between *LUH* and *LUG* in different tissues and developmental stages (**Fig. 2.6A**). This suggests that substantial differences may have occurred in the cis-regulatory elements of *LUH* and *LUG* involving responses to environmental signals.

Gene duplications are important evolutional strategies in facilitating species adaptation, buffering deleterious mutations, subdividing their function, or evolving new functions (Lynch and Force, 2000; Lynch et al., 2001). Based on analyses of 2022 recent duplicated gene pairs in *Arabidopsis,* duplicate genes with functions in developmental processes were found to be largely co-regulated while duplicate genes acting in abiotic or biotic stress responses were found to exhibit divergent expression profiles (Ha et al., 2007). This is consistent with our finding that *LUG* and *LUH* showed similar expression profile during development but exhibited almost opposite expression trends when challenged with various environmental stresses. Our observation suggests that *LUG* and *LUH* may have substantially divergent function when they act in stress response pathways.

2.5.4 Proposed Model for Transcriptional Repression of AGAMOUS

Previously, SEU was shown to function as an adaptor protein bridging the interaction between LUG and DNA-binding transcription factors such as *AP1* and *SEP3* to repress *AG* expression in flowers (Sridhar et al., 2004; Sridhar et al., 2006). Synergistic genetic interaction between *lug* and *seu* (Franks et al., 2002) suggests that SEU may function as an adaptor for LUG as well as for other co-repressors and that

LUG may utilize SEU as well as other adaptor proteins. Removing both *SEU* and *LUG* function in *seu; lug* double mutants thus has a more severe effect than removing either function. In contrast to synergistic interactions between *lug* and *seu*, *luh; seu* double mutants did not exhibit synergistic interactions in flowers, and their flowers resembled those of *seu* single mutants. Since *luh-1* does not have a flower phenotype, either the *luh-1; seu* double mutants exhibited an additive phenotype (and acting independent of each other) or *seu-1* is epistatic to *luh-1* for following reasons. First, LUH activity in flowers can be completely substituted for by LUG in *luh-1* mutants because of functional redundancy. Thus, it does not matter whether LUH is present or not in *seu* mutants, as LUG will compensate for the loss of LUH. A second possibility is that while LUG could form a co-repressor complex with SEU as well as SEUSS-like (SLK) proteins (Franks et al., 2002), LUH could only pair with SEU. When *seu* is mutated, LUH activity is then not needed and thus *luh: seu* double mutant flowers resemble *seu* single mutant flowers. This second explanation is illustrated in Fig. 8, where in *luh* mutants, the LUG/SEU and LUG/SLK complexes are sufficient to cover the loss of LUH/SEU. In *seu* single or *seu; luh* double mutants, the LUG/SLK complex can still provide most if not all of the function. In *lug* mutants, LUH-SEU can also perform most of the jobs. In *seu; lug*, or *luh; lug* double mutants, however, none of the LUH/SEU, LUG/SEU, or LUG/SLK complex is formed, leading to a much enhanced defect in the repression of *AG* and explaining the similar mutant floral phenotype between *seu-1; lug-3* and *luh-1/+; lug-3/lug-3* (**Fig. 2.3**; Franks et al., 2002).



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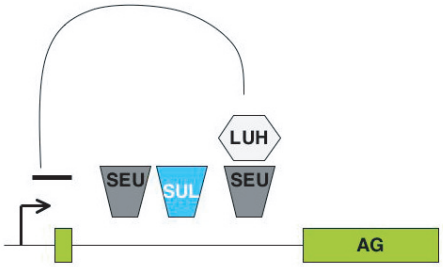
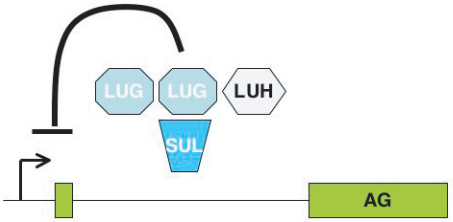
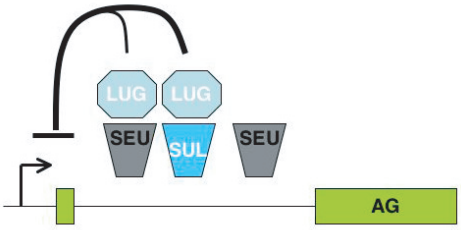


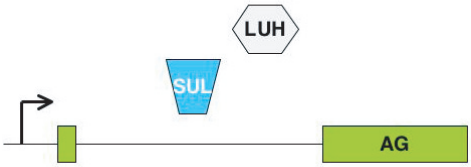
lug



seu

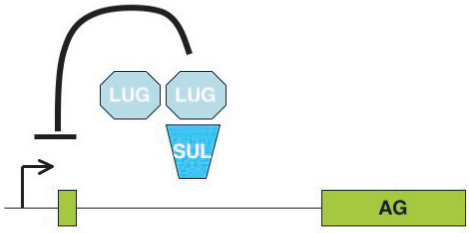


luh



lug; seu

lug; luh



seu; luh

Fig. 2.8: A model on the repression of AG by LUG, LUH, and SEU during flower development. The LUG/SEU, LUG/SLK, and LUH/SEU putative repressor complexes all act through the second intron of AG. The arrow indicates the transcription initiation from AG promoter. Bar indicates repressor activity. Thick lines connecting to the bar indicates stronger repressor activity than thin lines. The specific repressor complexes are indicated in single and double mutant combinations. SUL/SLK = SEUSS-LIKE.

2.6 Conclusion

Plant *Gro/Tup1* type co-repressors constitute an important class of regulatory molecules with roles in embryo shoot-root axis determination, stem cell pool maintenance, and floral homeotic gene regulation. Among the 13 Gro/Tup1 type co-repressors in *Arabidopsis*, *LUG* and *LUH* are most similar to each other. We show that *LUH* and *LUG* exhibit both redundant and divergent functions in embryonic development, floral homeotic gene regulation, and plant's biotic and abiotic stress responses. Gene duplication and functional diversification are important for species adaptation. Our study provides important insights into the complexity in the relationship between two paralogous genes.

2.7 Acknowledgements

I would like to thank former graduate student Jayashree Sitaraman for initiating this project and identifying and initially characterizing the *luh-1* mutant allele as shown in Figure 1-2. I would also like to thank former undergraduate student Parsa Hosseini for analyzing *LUG* and *LUH* expression data shown in Figure 6. My thanks also go to Arabidopsis Biological Resource Center and *Arabidopsis* Tilling Project for the LUH cDNA clones and *luh* mutant seeds, and Robert Franks, Paja Sijacic, and Courtney Hollender for critical comments on the manuscript. This work is supported by National Science Foundation Grant IOB0616096 to Z. L.

Chapter 3: *LUH* Regulates Mucilage Secretion and Both *LUG* and *LUH* Regulate Pathogen Resistance in *Arabidopsis*

3.1 Abstract

Both *LEUNIG* (*LUG*) and *LEUNIG_HOMOLOG* (*LUH*) encode proteins that belong to the Gro/TLE family of transcriptional co-repressors. Both LUG and LUH can physically interact with SEUSS (SEU) to repress *AGAMOUS* (*AG*) in flower development. Here we report the defect of *lug, luh* and *seu* mutants in seed coat development, seed mucilage synthesis and secretion, and in pathogen defense. We proposed and tested the hypothesis that seed mucilage secretion is related to general mucilage secretion in plants, and that mucilage secretion in xylem vasculature may be important for defense against pathogens and may underlie the defects of *luh-1* mutants in pathogen resistance and seed mucilage secretion. .

This work is the first of its kind to link the importance of mucilage synthesis or secretion to pathogen defense using molecular genetic approaches. Although the result presented here is far from conclusive, it provides important ground work for future investigation into this important link.

3.2 Introduction

Due to the constant adaptation and arms race that occurs between plants and their infectious pathogens, plants have responded accordingly by producing defense mechanisms such as physical barriers on the leaf, cellular barriers such as cell walls, and metabolites that will inhibit feeding and development of the herbivores (Walling, 2000). Additional adaptations include proteinase inhibitors such as polyphenol oxidases, arginases, and threonine deaminases that inhibit the herbivore's digestive enzymes and decrease the nutrition gained from plant tissues (Ryan, 2000; Ussuf et al., 2001; Chen et al., 2005). Plants also have the ability to secrete volatile chemicals that attract parasitoids and predators that eliminate the herbivorous pathogen (Baldwin et al., 2002; Dicke et al., 2003).

Because plants are incapable of moving or avoiding their antagonistic pathogens, they have evolved intricate molecular, cellular, and physiological components that share similar and differential aspects to that of animal systems. During a bacterial infection, plants are capable of sensing general features such as pathogen-associated molecular patterns (PAMPs) such as lipopolysacharides (LPS) and flagellin from bacteria, and chitin from fungi. This response, termed non-host resistance is the most ancient and contributes significantly to the well being of plants. A sub-category of non-host resistance is basal resistance, a type of defense that functions to minimize the growth of pathogens and remains intact even when all other defenses fail. For a pathogen to be successful, it must evade or deconstruct its host plant's basal defenses.

In contrast to non-host resistance, an evolutionarily more recent approach to plant defense involves resistant (R)-genes which encode proteins with conserved nucleotide-binding (NB) and C-terminal leucine-rich repeat (LRR) domains that act as pathogen sensors. The NB site shares homology among other plant R proteins and animal apoptotic effecter proteins including Apaf-1 and Ced4 (van der Biezen and Jones, 1998; Aravind et al., 1999). The NB-LRR class of R-genes is subdivided into two types with conserved structures but respond to a diverse set of pathogens. The CC-NB-LRR type has an N-terminal coiled-coil (CC) domain , while the TIR-NBS-LRR type shares structural homology to the *Drosophila* Toll and mammalian interleukin (IL)-1 receptor (TIR). Due to its considerable diversity, the TIR-NB-LRR type of R genes out-number the CC-NB-LRR class by 20%, and the majority of the TIR-NB-LRR R-genes cluster around the *RECOGNITION OF PERONOSPORA PARASITICA5* (*RPP5*) locus. This locus has *RPP4* and seven other *RPP5* homologues (Dangl and Jones, 2001). *RPP4* is a member of the *RPP5* multi-gene cluster and is found in the Columbia background, while *RPP5* is an ortholog found in Landsberg (van der Biezen et al., 2001). Ecotype differences are not only observed in pathogen defense, but also in plant development and growth (Beemster et al., 2002). These R proteins, upon recognizing specific pathogens, are responsible for activating the salicylic acid (SA) pathway, the primary defense pathway in plants. SA levels are significantly induced by 500-1000 fold by a broad range of pathogens, such as viruses and fungi. This in turn, will activate a number of plant defense genes, resulting in systemically acquired resistance (SAR) (Verberne et al., 2000).

ENHANCED DISEASES SUSCEPTIBILITY 1 (EDS1) acts downstream of several R-genes such as *RPP1*, *RPP10*, and *RPP14* (Aarts et al., 1998). PHYTOALEXIN DEFICIENT4 (PAD4) and SENESCENCE ASSOCIATED GENE101 (SAG101) interact to form a trimeric protein complex with EDS1 (Wiermer et al., 2005). *EDS1* has been shown to be up-regulated following a pathogen infection and that SA production is reduced in the *eds1* mutant (Falk et al., 1999). These studies indicate that *EDS1* is involved in transmitting the signal from R genes to SA induction.

To quantify by molecular means how well plants respond to a pathogen through the SA pathway, researchers measure the expression of the *PATHOGENESIS RELATED* (*PR*) *1-5* genes, which are induced by the SA pathway. *PR1* (At2g14610) encodes a basic protein that responds to pathogens (Mitsuhara et al., 2008). *PR2* encodes a β-1,3-glucanase important for destabilizing and degrading fungal cell walls (Stewart et al., 2001; Doxey et al., 2007). *PR3* encodes a chitinase enzyme capable of breaking down fungal cell walls (Jung et al., 1993). *PR5* belongs to an osmotin protein shown to bind β-1,3 glucans, which are cell surface components in fungi, thus allowing PR5 to recognize the fungal molecules (Salzman et al., 2004). Therefore, *PR1-5* genes are useful effector genes with anti-bacterial and anti-fungal properties. Furthermore, they serve as convenient molecular markers for the SA pathway and SAR response.

A microarray study compared gene expression between wild-type and the loss-of-function mutant *lug-3*, a null-allele of *LEUNIG* (*LUG*) in *Arabidopsis thaliana* (Gonzalez et al., 2007). The study revealed that *RPP4*, *SNC1*, and *RPP5*, members of the TIR-NB-LRR R-genes, were significantly up-regulated in the *lug-3* mutant compared to wild-type. *RPP4* is up-regulated 74-fold and 40-fold in *lug-3* leaves and flowers, respectively, thus LUG may repress R-gene transcription to allow for normal growth and development. This data implicates *LUG* as a repressor of the R-genes and thus the SA pathway.

Although the SA pathway has widely been studied as a key defense pathway, there are likely other pathways and mechanisms of deterring pathogen invasions. Besides the physical barriers and chemical deterrents previously mentioned, xylem occlusion by a gel-like substance has been reported to resist invading pathogens, thus preventing the systemic spread of pathogen. This gel-like substance in plants involves the formation and hyper-secretion of pectinacious mucilage, which has been reported in trees, bananas, peas, and corn (Beckman and Zaroogian, 1967; Vandermolen et al., 1983; Rioux et al., 1998; Crews et al., 2003; Perez-De-Luque et al., 2005).

However, xylem occlusion has not been vigorously tested as a defense mechanism in many plants. In addition to xylem occlusion, mucilage is well-studied as a functional aid in seed hydration and dispersal (Frey-Wyssling, 1976; Esau, 1977). The primary component of mucilage is carbohydrate-based pectin such as unbranched polygalacturonic acid (PGA) and highly branched rhamnogalacturonan I

(RG1), both of which are manufactured by the Golgi and transported extracellularly by vesicles (Brett and Waldron, 1990; Zhang and Staehelin, 1992; Dupree and Sherrier, 1998). Seeds secrete large amounts of mucilage when imbibed in a liquid environment. Previous studies from several labs have identified several transcription factors important for mucilage production such as *APETALA2 (AP2), TRANSPARENT TESTA GLABRA1 (TTG1), TTG2, and GLABRA2 (GL2)* (Western et al., 2001; Johnson et al., 2002). *ap2* mutants were first identified as a floral mutant that lacks sepals and petals, and later discovered to develop defects in the seed epidermis that affects mucilage synthesis and secretion (Western et al., 2001; Jofuku et al., 2005). Mutants of WD40-encoding *TTG1,* WRKY-encoding *TTG2,* and homeodomain protein *GL2* exhibited trichome developmental defects. Later, they were found to be necessary for mucilage and columella production in the seed (Bowman and Koornneef, 1994; Rerie et al., 1994; Walker et al., 1999; Western et al., 2001; Johnson et al., 2002). Both TTG1 and GL2 have been shown to interact with Myb transcription factors GLABRA1 (GL1) and WEREWOLF (WER) to regulate trichome and root development (Oppenheimer et al., 1991; Lee and Schiefelbein, 1999). These studies indicate that all these transcription factors regulate multiple cell fates including mucilage synthesis in seed, trichome development in leaf, and root hair growth in roots.

In addition to transcription factors, *MUCILAGE MODIFIED2* (*MUM2*) and *MUM4* genes encode mucilage modification and biosynthetic enzymes, respectively. *MUM2* encodes a beta-galactosidase and *MUM4* encodes an NDP-rhamnose synthase

necessary for rhamnogalacturonin I (RGI) synthesis, one of the primary components of pectin in mucilage (refer to **1.3.4** and **Fig.1.6**) (Usadel et al., 2004; Western et al., 2004; Dean et al., 2007). *mum4* mutants had reduced mucilage secretion and columella size (Western et al., 2001) and *MUM4* transcription is regulated by AP2, TTG1, and GL2 (Western et al., 2004). On the other hand, mutants of *MUM2/BETA GALACTOSIDASE6* produced mucilage in the seed epidermis, but failed to secrete it upon imbibition. (Dean et al., 2007). It was later found that *MUM2/BGAL6* is required for RG1 maturation by removing galactose/galactan branches to increasing the hydrophilic properties of mucilage, necessary for extrusion post-imbibition (Macquet et al., 2007).

A correlation between mucilage secretion and pathogen resistance in plants or seeds has not been demonstrated, especially in a genetic model system like *Arabidopsis thaliana*. In this study, we report novel defects in *luh-1* mutants, which fail to secrete seed mucilage and have increased susceptibility to pathogens. Assuming that mucilage syntheses in seed and vascular tissue are similar, we tested whether these two defects may be linked.

3.3 Materials and Methods

3.3.1 Plant Growth and DNA Extraction

For infection assays, *Arabidopsis thaliana* wild-type and mutant plants were grown under short day conditions (8 hr light, 16 hr dark) at 21ºC and 65% humidity for 4-6 weeks. Plants were infected before bolting and when roseatte leaves are fully expanded. For assays not requiring infection such as genotyping using genomic DNA,

plants were grown under 16 long day (16 hr light, 8 hr dark) and a single leaf was collected, DNA extracted using Edwards buffer (200 mM Tris, pH: 7.5; 250 mM NaCl; 25 mM EDTA, pH: 8.0; 0.5% SDS), precipitated with isopropanol, washed with 70% ethanol, and resuspended in distilled water.

Primers for genotyping e*ds1-2* mutants (F: 5'-ACACAAGGGTGATGCGAGACA-3', R1: 5'-GGCTTGTATTCATCTTCTATCC-3', R2: 5'-GTGGAAACCAAATTTGACATT-3') and SAP primers for *lug-3* mutants (Common F: 5'-ACTAAGCTGGAGTATTTCTATTT-3', wild-type R: 5'-TTGATGTTGTTGTTGCTGCCG-3', mutant R: 5'-TTGATGTTGTTGTTGCTGCCA-3').

3.3.2 Resin Embedding

Fixation and dehydration were adapted from (Weigel and Glazebrook, 2002). Samples were fixed overnight in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (1X PBS), pH: 7.0. Next day, samples were washed twice with 1X PBS and dehydrated at one hour intervals with ethanol series at 30%, 50%, 70%, 95%, and twice with 100%. Infiltration and embedding steps were done using JB-4 Embedding Kit according to manufacturer's protocol (Polysciences, Inc). (http://www.polysciences.com/SiteData/docs/123/204a24a728c8ffee6471ef33ede1e5d9/123.pdf). 1-2 um thick sections were done using a manual, Sorvall Porter-Blum JB-4 Microtome, then placed on water on superfrost/plus slides (Fisherbrand), and allowed to dry on a slide warmer at 42°C overnight.

3.3.3 Mucilage Staining

Plastic sections or whole seeds were stained according to previously described methods (Western et al., 2001). Briefly, seeds were stained in a tube for twenty minutes using 0.01% Toluidine blue dissolved in 0.01% Sodium Borate. They were gently transferred with a pipette to plates filled will water, and visualized under a dissecting microscope. Plastic sections were stained in using the same concentration of Toluidine blue as previously mentioned for five minutes, and then de-stained with distilled water for another five minutes.

3.3.4 Scanning Electron Microscopy

Seeds were dry mounted on aluminum stubs (Pella Cat. # 16111), coated with gold-palladium in a Denton DV 503 Vacuum Evaporator, and photographed with an Amray 1820D scanning electron microscope at 5.0 kV.

3.3.5 RNA/Northern Gene Expression Analysis

RNA extraction for leaves was carried out using TRIzol reagent according to manufactuer's protocol (Invitrogen Cat # 15596018). Total mRNA was converted to cDNA using Superscript III First Strand kit (Invitrogen Cat # 18080051) and then treated with RNase H (Invitrogen Cat # 18080044) to remove residual RNA contamination.

RNA extraction for siliques was done using a protocol from Dr. Tamara Western, located online at http://www.molecularstation.com/forum/arabidopsis-plant-biology/23161-rna-siliques.html. Briefly, 0.2 grams of silique tissues were placed into micro-centrifuge tubes, ground up, and using REB (25 mM Tris-HCL ph: 8.0, 25 mM

EDTA, 75 mM NaCl, 1% SDS in DEPC treated water), acid phenol (Sigma P4682), 24:1 chloroform:isoamy alcohol, 10 M LiCl (DEPC-treated), 3 M NaOAc pH: 5.2 (DEPC-treated), and DEPC water.

Primers were designed to span introns or have detectable size differences between genomic or cDNA amplification. Loading controls used were *ACTIN2* (F: 5'-GTTGGGATGAACCAGAAGGA-3'; R: 5'-CTTACAATTTCCCGCTCTTC-3') and *GAPC* (F: 5'-TCAATCACTGCTACTCAGAAG-3'; R: 5'-GATCAAGTCGACCACACGG-3'). Genes quantified included:

Gene	F/R Primer Sequence
BGAL4	F: 5'-CGATGAATACGGCTTACCAAG-3'
	R: 5'-CCCAAGTCATGCTTATTTGTTC-3'
MUM2/BGAL6	F: 5'-GTCTTTTAAGACAGCCTAAGTA-3'
	R: 5'-TCACATTCACTTTTGCAGTTTCA-3'
BGAL7	F: 5'-CCTAGCTCAGATCGAGAATG-3'
	R: 5'-ATGTAATAGTTTTGGAAAAGTTC-3'
BGAL11	F: 5'-ACATGGTACCAGACATACTTTG-3'
	R: 5'-CTCGATGACTTGCTTTGAAACA-3'
MUM4	F: 5'-TGGTTTAACTGGCAGACCCA-3'
	R: 5'-GAGCTCCTCAACCATGGCT-3'
PR1	F: 5'-GTAGGTGCTCTTGTTCTTCCC-3'
	R: 5'-CACATAATTCCCACGAGGATC-3'
PR5	F: 5' CCTCGTGTTCATCACAAGCG-3'
	R: 5'-GAAAATCCTCGAGTAGTCCG-3'
VSP1	F: 5'-GCTAAATATGGATATGGGACC-3'
	R: 5'-GTTACACCAACAGCCTTGAG-3'
VSP2	F: 5'-GCAAAATATGGATACGGAACAG-3'
	R: 5'-GTAGTAGAGTGGATTTGGGAG-3'
PDF1.2	F: 5'-TTCTCTTTGCTGCTTTCGAC-3'
	R: 5'-GTCATAAAGTTACTCATAGAGTGACAG-3'
RPP4	F: 5'-TAGTGACCGAGAAAACTGGG-3'
	R: 5'-CGAGGCCCTGAGGTAGATC-3'
RPP5	F: 5'-GTGGGATGGAACTCAGCC-3'
	R: 5'CGGTTGGAAAACTCTCTAGC-3'

Table 3.1: Primers used in RT-PCR during pathogen defense and mucilage studies. F=forward, R=reverse.

The PCR program for the alleles described here include 94ºC for 3 minutes, followed by 35-50 cycles of 94ºC for 20 seconds, 55-58ºC for 20 seconds, and 72ºC for 40 seconds, and ended by 72ºC for 3 minutes.

3.3.6 Pathogen Inoculation

Plants growing in short-day with fully expanded leaves were infected as previously described (Wang et al., 2007). Briefly, one leaf per plant was marked and infiltrated with ~100 uL of *Pseudomonas syringae* pv. *Maculicola* ES4326 (~10^4 CFU/mL or OD_{600}=0.0002 in 10 mM $MgCl_2$). After 3 days post infection (dpi), ~1 cm wide discs per leaf per plant were punched out at the site of infection, macerated with a grinding pestle, and serially diluted 5000X in 10 mM $MgCl_2$, of which, 100 uL was plated onto LB agar plates containing a final concentration of 100 ug/mL streptomycin. The plates were incubated at 30°C for two day, after which the number of bacterial colonies determined. This assay was repeated for ten different plants per genotype. The results presented as average +/- standard error of the mean.

For RT-PCR and Northern analyses, three leaves for three plants of the same genotype (9 leaves total) were locally inoculated as previously mentioned but using a *P. syringae* (OD_{600}=0.26). After incubating for 6 hours, locally and/or systemically infected leaves were pooled, respectively, before performing RNA extractions.

3.4 Results

3.4.1 *luh-1* and *lug-3* Exhibit Seed Mucilage Defects Upon Imbibition

Dry seeds of *luh-1*, *lug-3*, *seu-1*, *ap2-2*, and wild-type (Columbia and Landsberg *erecta*) were stained with 0.1% Toluidine blue for twenty minutes, and then observed under a dissecting scope. A halo of purple mucilage can be observed surrounding the wild-type seeds (**Fig. 3.1A** and **B**). *luh-1* mutant seeds exhibited the most striking defect with a complete absence of mucilage after being imbibed. The effect observed in *luh-1* mutant seeds was completely penetrant, with 100% lacking mucilage compared to 3% in wild-type (COL-er) (**Fig. 3.1A**). The two layers of mucilage can be separated by shaking (Western et al., 2001). While *luh-1* appears to lack both layers, strong loss-of-function *lug-3* mutants had a significant reduction in the outer mucilage layer and also had a very translucent or thin inner mucilage layer (**Fig. 3.1B**). After shaking to remove the outer mucilage layer, the inner mucilage layer of *lug-3* was often torn while the inner mucilage layer of wild-type seeds remained intact. This indicates the inner mucilage layer of *lug-3* seeds is very fragile. *ap2* mutant seeds was previously reported to exhibit seed coat defects and lacked mucilage (Western et al., 2001), and *ap2-2* seeds were used in our study as a control for seed coat and mucilage defects. *seu-1* mutants secreted mucilage but had a higher percentage of seeds that lacked mucilage (18%) compared to 1% in wild-type (Ler) (**Fig. 3.1B**).

Failure to secrete mucilage after imbibition may be due to a developmental defect within the outer integument layer of the seed, a defect in mucilage synthesis, or

failure to secrete mucilage even after imbibition. To distinguish among these possibilities, we conducted Scanning Electron Microscopy (SEM) to examine epidermal cell morphology of the seed. Both *lug-3* and *luh-1* seeds develop normal epidermal cells, with hexagonal shaped cells and a protruding central collumella (**Fig. 3.1A** and **B**). However, *ap2-2* seeds had reduced columella and elongated rectangular epidermal cells. *seu-1* seeds also had abnormal epidermal morphology with larger rectangular cells, but the defect was weaker than *ap2-2* seeds.

A transgene containing a strong constitutive *35S* promoter that drives *LUH* full-length cDNA was introduced into *luh-1* (Sitaraman et al., 2008). The transgenic seeds were examined for mucilage secretion after imbibement. These seeds secrete more mucilage than wild-type, which caused the seeds to stick to each other due to excessive amounts of mucilage (**Fig. 3.1A**). SEM photos indicate normal epidermal morphology in these transgenic seeds. Thus, *LUH* expression level appears to regulate the amount of seed mucilage secretion after imbibement.

3.4.2 Genetic Interaction Between *LUH* and *SEU*

Previously, SEU and LUH were shown to directly interact through the yeast-two-hybrid assay (Sitaraman et al., 2008). We tested the genetic interaction between *seu* and *luh* in seed coat development and mucilage secretion. *seu-1*; *luh-1* double mutants were generated and the seed epidermis examined for any defects. Similar to the *luh-1* single mutant seeds, the *seu-1*; *luh-1* double mutant seeds lacked mucilage (**Fig. 3.1A**). However, *luh-1* suppresses the *seu-1* seed coat morphogenesis defect, indicating that *luh-1* may be epistatic to *seu-1* in seed coat morphology.

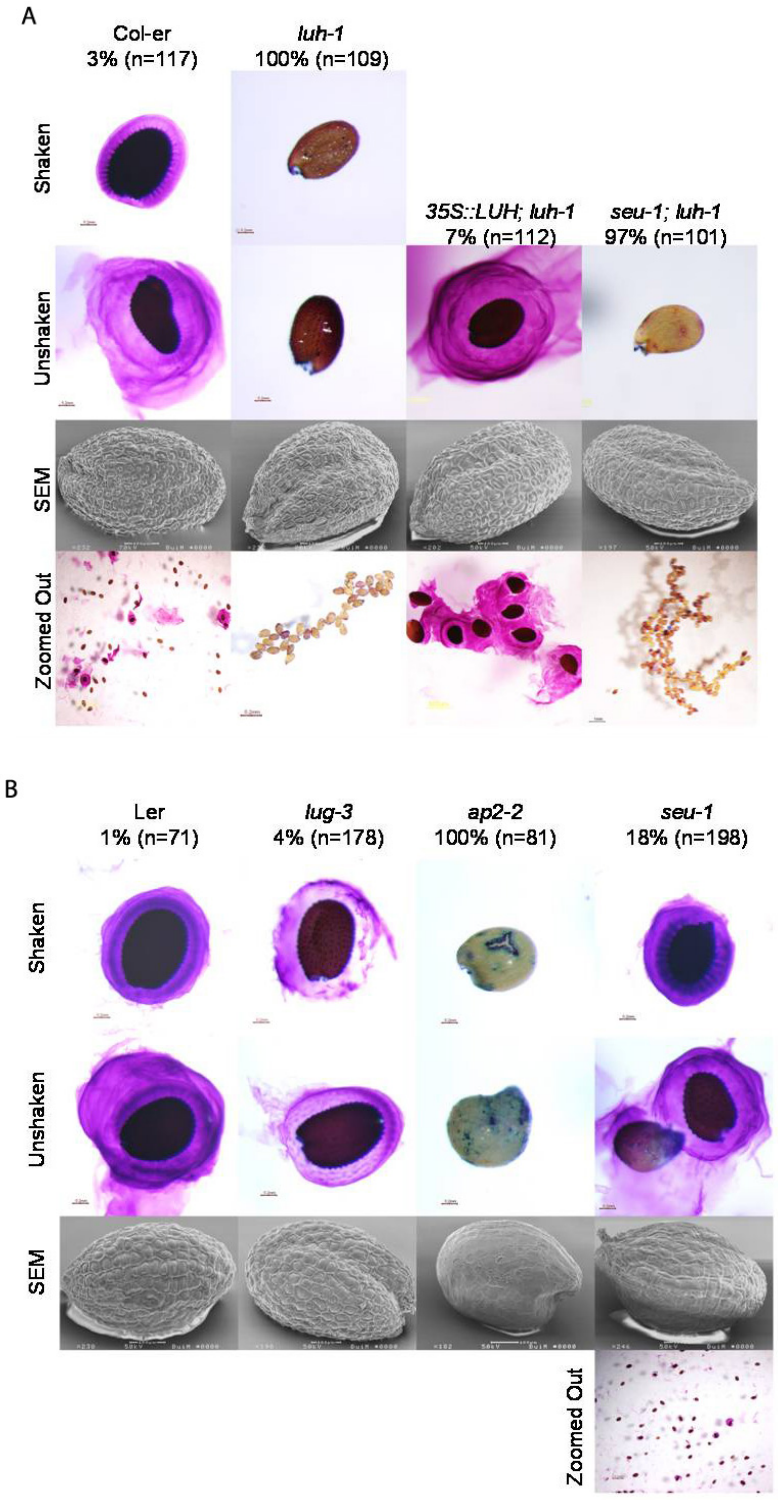


Fig. 3.1: Mucilage secretion after imbibement across different genotypes. Toluidine blue staining of shaken and unshaken seeds (top two rows), SEM photo of seed coat morphology (third row), and zoomed out photos. A) Wild-type (Col-er), *luh-1*, *35S::LUH; luh-1*, and *seu-1; luh-1* double mutant seeds. B) Wild-type (Ler), *lug-3*, *ap2-2*, and *seu-1* seeds. % indicates the percentage that completely lack mucilage, and n = number of seeds counted. Zoomed out

pictures for Ler and *seu-1* were 2X to show wild-type looking seed dispersal, *luh-1* and *seu-1; luh-1* were 4X to show the characteristic clumping, and *35S::LUH; luh-1* at 8X to show release of mucilage.

3.4.3 *luh-1* Mutant Seeds Fail to Secrete Mucilage

The absence of seed mucilage can be attributed to defects in several dependent or independent processes. First, the epidermis of the seed coat does not develop properly or is completely absent like in *ap2-2* mutants (Western et al., 2001). Second, the epidermal cells of the seed fail to synthesize or produce less mucilage, as in the case of *mum4* mutant seeds (Western et al., 2001; Western et al., 2004). *MUM4* encodes an NDP-L-rhamnose synthase, a mucilage biosynthetic enzyme. The third possibility is that the seeds fail to secrete mucilage post-imbibition even though they synthesize and store mucilage, as in the case of *mum2* mutants (Western et al., 2001; Dean et al., 2007). *MUM2* encodes the enzyme β-galactosidase. *mum2* mutants cannot cleave the pectin branches, a process required for mucilage hydration, expansion, and primary cell wall rupture, leading to mucilage secretion upon imbibition (Western et al., 2001; Dean et al., 2007; Macquet et al., 2007).

Since *luh-1* seed coat is morphologically similar to wild-type, *luh-1* seed coat development is normal. Therefore, the mucilage defect must either be due to a failure to synthesize or properly modify the pectin in mucilage. To distinguish between these two alternative possibilities, plastic sections and histological staining was performed for wild-type and *luh-1* seeds. *luh-1* mutants produce and accumulate mucilage that is kept in between the two cell wall layers before imbibition. As shown in **Fig. 3.2**, epidermal cells of the seed coat in *luh-1* are similar to wild-type. Each cell has a

centrally raised columella that is flanked by mucilage. Therefore, *luh-1* is capable of synthesizing mucilage but fails to secrete it upon imbibition.

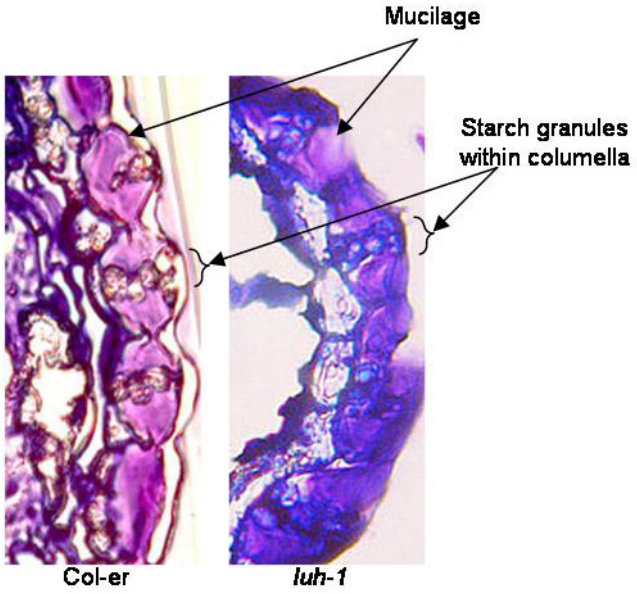


Fig. 3.2: Plastic section of seed epidermal cells, comparing wild-type (Col-er) to *luh-1*. Mucilage, starch granules, and columella are indicated.

Since both *luh-1* and *mum2-1* mutants can synthesize mucilage but fail to secrete it, one wonders if LUH is involved in the transcriptional regulation of *MUM2*. Preliminary Reverse Transcription Polymerase Chain Reaction (RT-PCR) was conducted to look at *MUM2/BGAL6*, *BGAL11*, and *MUM4* expression in wild-type and *luh-1* seeds. *BGAL11* was tested because it encodes another β-galactosidase enzyme similarly to *MUM2/BGAL6*. A slight reduction in *MUM2* expression is detected in *luh-1* and no significant difference in *BGAL11* is detected. Unexpectedly, *MUM4* is expressed at higher levels in *luh-1* than wild-type (**Fig. 3.3**).

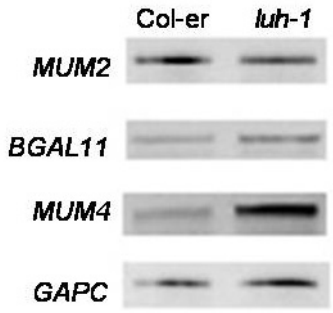


Fig. 3.3: RT-PCR gene expression analysis of cDNA from siliques/seeds. Shown are the results of *MUM2/BGAL6, BGAL11,* and *MUM4* RT-PCR in wild-type (Col-er) and *luh-1*. *GAPC* (*GLYCERALDEHYDE-3-PHOSTPHATE DEHYDROGENASE C SUBUNIT*) is used as a loading control.

3.4.4 *luh-1* Mutant Plants Are Susceptible While *lug-3* and *seu-1* Mutants Are Resistant to Bacterial Pathogen

A microarray study revealed a dramatic increase in the expression of R genes of the TIR-NBS-LRR class (up to 70 fold) in *lug-3* mutants (Gonzalez et al., 2007). Members included in the study are *RPP4*, *RPP5*, and *SNC1*. This led us to suspect that *LUG* represses R genes which are important in pathogen defense, so we tested the susceptibility of *lug-3*, *luh-1*, and *seu-1* mutants to the bacterial pathogen, *Pseudomonas syringae* ES4326, and quantified the number of bacterial colonies present in the leaves after three days. If a plant is more susceptible than wild-type, a higher titer of colonies will be observed. We observed that *lug-3* mutant plants were slightly and *seu-1* mutant plants were significantly resistant to bacterial pathogen infections when compared to wild-type (Ler) (**Fig. 3.4**). In contrast, *luh-1* was highly susceptible to pathogen infections when compared to wild-type (Col-er). We also tested *seu-1; luh-1* double mutant plants and found that the double mutants were resistant similarly to *seu-1* single mutant, indicating that *seu-1* is epistatic to *luh-1* in the defense pathway (**Fig. 3.4**).

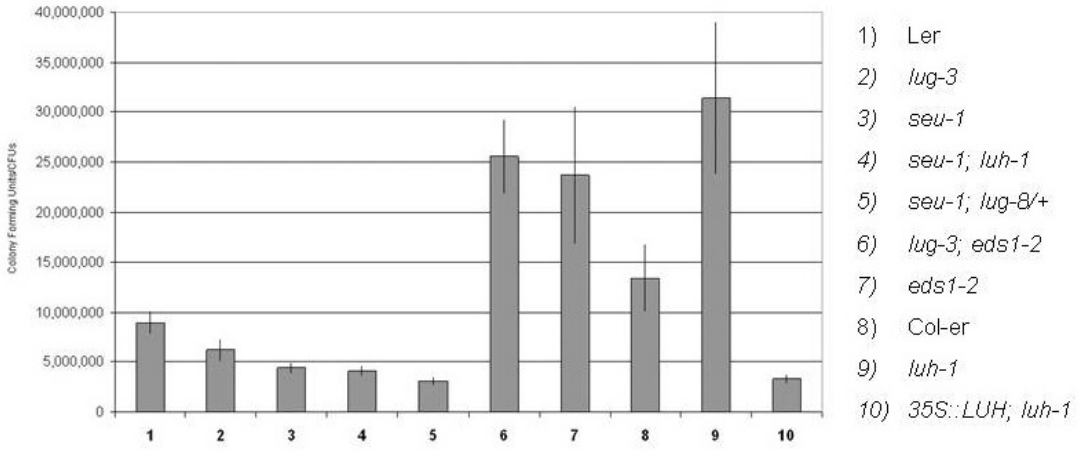


Fig. 3.4: *lug-3*, *seu-1*, and *35S::LUH*; *luh-1* plants are resistant, while *luh-1* is susceptible to bacterial pathogen. Bacterial titer 3 days after infection in *lug-3*, *seu-1*, *eds1-2*, *luh-1*, *35S::LUH*, and wild-type (Ler and Col-er) leaves. Graph indicates x= +/- SEM, n=10. One-tailed p-values vs wild-type (Ler): *lug-3*=0.052, *seu-1*=0.007, *seu-1; luh-1*: 0.048, *seu-1; lug-8/+*=0.051, *eds1-2*: 0.167. One-tailed p-values vs wild-type (Col-er): *luh-1*: 0.147, *35S::LUH; luh-1*: 0.101. One-tailed p-value between *eds1-2* and *lug-3; eds1-2*: 0.224.

We tested *35S::LUH; luh-1* transgenic plants and showed that they were extremely resistant to the bacteria compared to wild-type (Col-er) and is the most resistant one among all the genotypes in our study (**Fig. 3.4**).

If the pathogen resistance phenotype of *lug-3* is due to the mutant having increased R gene expression (including *RPP4*, *RPP5*, and *SNC1*), mutations in genes that act in the SA pathway should block this increased resistance phenotype observed in *lug-3* (Gonzalez et al., 2007). *ENHANCED DISEASE SUSCEPTIBLE 1* (*EDS1*) is a gene that acts immediately downstream of the TIR-NBS-LRR R gene, and the *eds1-2* mutant is more susceptible to bacterial pathogens than wild-type (Aarts et al., 1998; Falk et al., 1999; Wiermer et al., 2005). We generated a double mutant between *lug-3* and *eds1-2* to determine their genetic epistasis in pathogen defense. *lug-3; eds1-2*

double mutant plants resemble *eds1-2* single mutants (**Fig. 3.4**), revealing that *eds1-2* is epistatic to *lug-3*. This result supports the hypothesis that *lug-3* acts upstream of *eds1-2*, and *lug-3*'s effect on pathogen resistance is likely mediated by its mis-expression of the R genes.

3.4.5 *lug-3, luh-1,* and *seu-1* Exhibit a Constitutively Active, Hypersensitive Response to Pathogen Infections

The expression of *PATHOGENENESIS-RELATED 1* (*PR1*), a marker for the SA defense pathway was examined in *lug-3, seu-1*, and *luh-1* mutants. *PR1* is normally not expressed but is induced upon activation of the SA pathway. Since the SA and jasmonic acid (JA) pathways are antagonistic, JA-induced genes such as *VSP1*, *VSP2*, and *PDF1.2* are also tested and should show opposite expression trends from *PR1*. If local leaf infections lead to a Hypersensitive Response (HR), it will lead to Systemic Acquired Resistance (SAR) in untreated leaves of the same plant, and can be monitored using molecular markers such as *PR1* (Kiefer and Slusarenko, 2003; Mishina and Zeier, 2007).

RT-PCR data indicate that *PR1* is significantly induced in the *lug-3*, *seu-1*, and *luh-1* mutant plants after infection (**Fig. 3.5**). Our study shows a significant increase in *PR5* expression during local and/or systemic infections with *Pseudomonas* bacteria. *VSP1* and *VSP2* expression was significantly reduced in *lug-3* and *seu-1* mutants. Thus, the increase in *PR* gene expression and decrease in *VSP* gene expression in *lug-3* and *seu-1* is consistent with the antagonistic effect between SA and JA pathways. *PDF1.2* is a marker that is expressed in the JA/ET response

pathways, and our study shows that *PDF1.2* expression is also increased in *lug-3* and *seu-1* mutants.

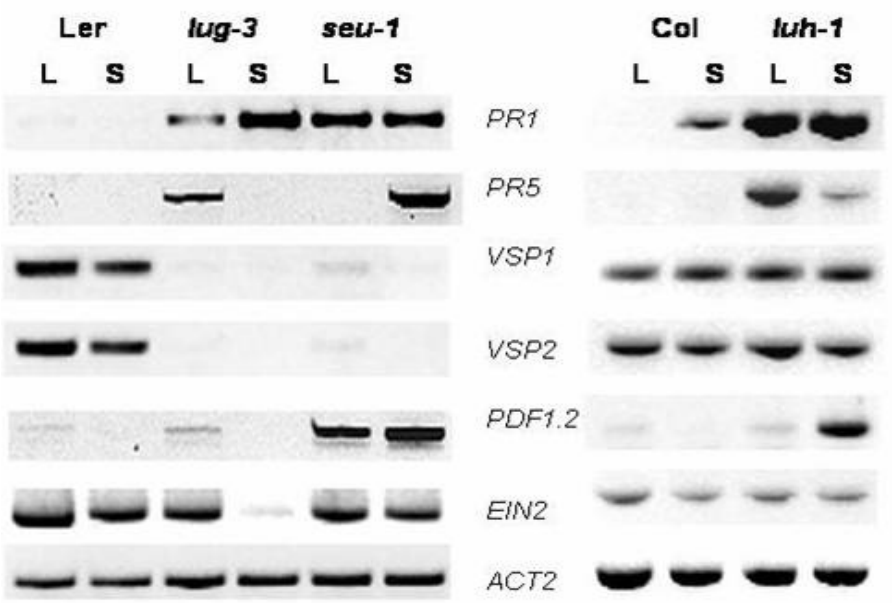


Fig. 3.5: RT-PCR analysis using molecular markers in the SA and JA/ET pathogen defense pathways. Leaves were infected with *Pseudomonas syringae* for six hours and RNA extracted from locally (L) and systemically (S) infected leaves.

In the case of *luh-1*, markers for SA and JA pathways including *PR1* and *PDF1.2*, respectively, are all increased compared to wild-type (**Fig. 3.5**). This data indicates that *LUH* plays a more complicated role than *lug-3* and *seu-1* in regulating pathogen defense.

3.4.6 Most *mum* Mutants Are More Susceptible to Bacterial Pathogens

The results on pathogen response (**Fig. 3.4**) revealed that *LUH*'s expression level correlates with its susceptibility to pathogen, as increased *LUH* expression in *35S::LUH* transgenic plants correlate with higher pathogen resistance. Could this increased resistance be attributed to higher mucilage secretion shown earlier in the chapter? If so, we would expect other *mum* mutants with decreased mucilage to be

susceptible to bacterial pathogens as well. We performed a similar inoculation assay that was described earlier, and quantified the number of colony forming units in the *mum* mutants, including *mum1-1*, *mum2-1*, *mum3-1*, and *mum5-1*. *mum2-1* plants are not significantly different than wild-type, but the other *mum1-1*, *mum3-1*, and *mum5-1* mutants are more susceptible to *Pseudomonas syringae* than wild-type (**Fig. 3.6**).

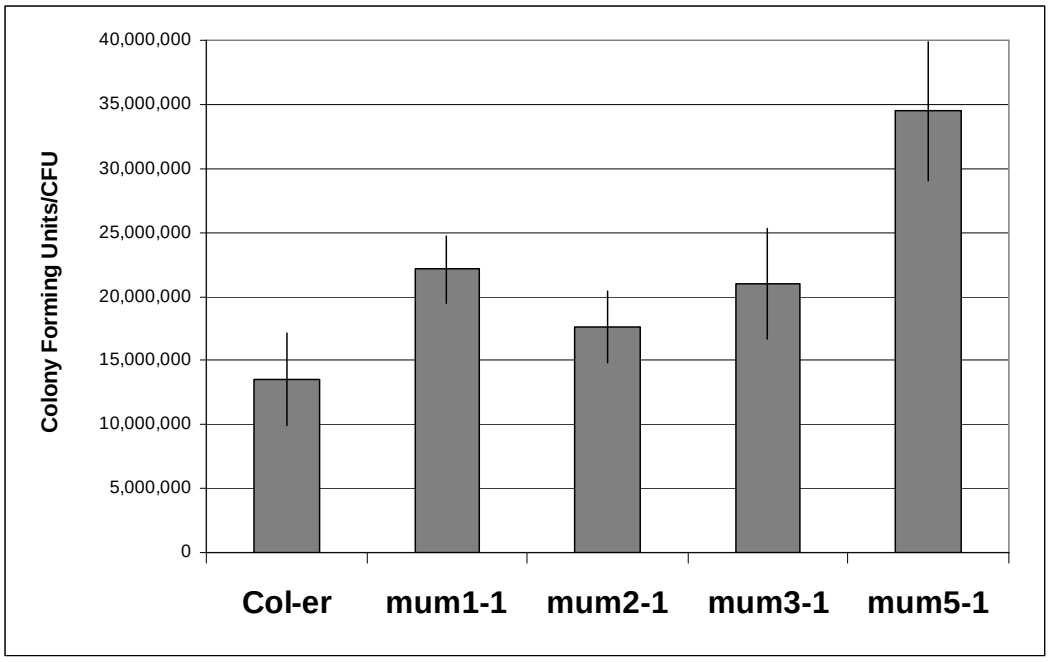


Fig. 3.6: Bacterial titer of infected *mum* mutants. Graph indicates x=+/- SEM, n=5. One-tailed p-values vs wild-type (Col-er): *mum1-1*=0.068, *mum2-2*=0.426, *mum3-1*=0.083, *mum5-1*=0.048.

mum2-1 is defective in *β-GALACTOSIDASE 6* (*BGAL6*) expression, but there are a total of 17 β-galactosidase genes in *Arabidopsis*, and they may be functionally redundant in other parts or pathways in the plant. We mined the microarray expression database on these 17 *BGAL* genes after *Pseudomonas syringae* DC3000 infection. Of all 17 (*BGAL*) genes, only *BGAL4* was significantly induced by the *Pseudomonas syringae* DC3000 (**Fig. 3.7**). All other *BGAL* genes are repressed after infection. Since *MUM2* is allelic to *BGAL6* and its expression is not induced upon

pathogen infection, *MUM2* may not play a role in mucilage secretion upon pathogen infection. *MUM2*'s role is likely specific to seed mucilage secretion. The molecular nature of *MUM1*, *MUM3*, and *MUM5* is not known.

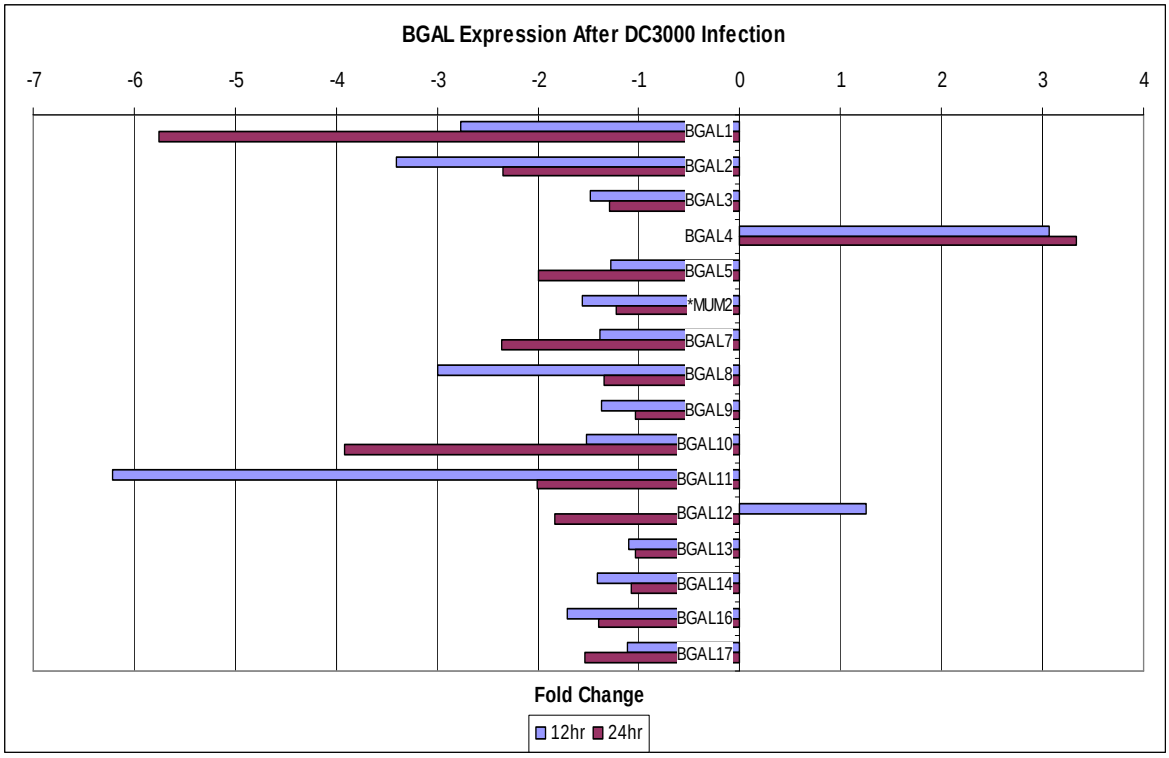


Fig. 3.7: Microarray data indicating the expression fold changes of the *β-GALACTOSIDASES* (*BGAL*) genes from De Vos, *et al*. (2005) (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=330). Data represents untreated versus 12 hr and untreated versus 24 hr post-innoculations with *Pseudomonas syringae* DC3000.

To test for genes that regulate mucilage secretion in plants, we measured *MUM2*/*BGAL6*, *BGAL4*, and *BGAL11* levels in uninfected leaves. *luh-1* mutants had a reduction in *MUM2*/*BGAL6*, *BGAL4* and *BGAL11* expression compared to wild-type, while *lug-3* had increased levels of corresponding genes compared to wild-type. The *RPP4* R gene is increased in *lug-3*, but *RPP4*'s effect in *luh-1* or *RPP5* in *lug-3*

cannot be determined due to PCR saturation. *MUM*4 expression is unchanged (**Fig. 3.8A**).

Northern blot is one assay one can utilize to measure and compare steady-state transcript levels between wild-type and mutant plants. We conducted a northern analysis of infected and uninfected leaves, measuring the amount of *MUM2*/*BGAL6* expression in *lug-3* and *luh-1* mutants. *MUM2* expression was reduced in the uninfected and infected *luh-1* leaf tissue compared to wild-type. In contrast, *MUM2* remained unchanged in *lug-3* (**Fig. 3.8B**). Thus, the susceptibility in *luh-1* could be partially attributed to a reduction in *MUM2* expression.

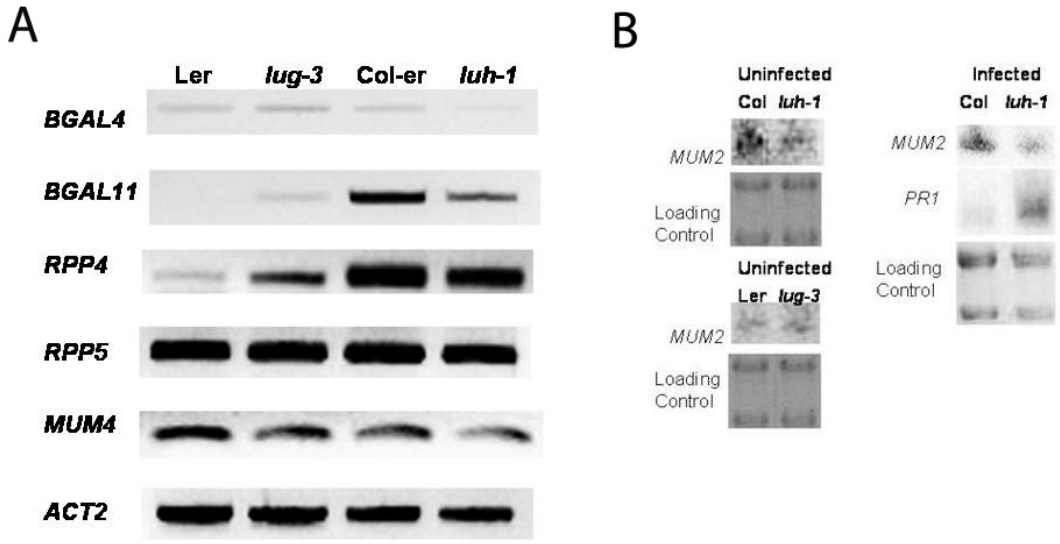


Fig. 3.8: RT-PCR analysis using putative SA pathway genes and genes involved in mucilage synthesis and secretion. A) Gene expression changes in uninfected leaves. B) Northern analysis comparing *MUM2* and *PR1* expression between *luh-1* and wild-type (Col-er) in both infected and uninfected leaves.

3.5 Discussion

Here, we report that mutants of *LEUNIG* (*LUG*) and *LEUNIG_HOMOLOG* (*LUH*) have defects in seed mucilage secretion. *luh-1* mutant seeds had the most

severe defect with complete absence of mucilage after imbibition, while *lug-3* seeds appear to secrete reduced levels of mucilage (**Fig. 3.1**). LUG and LUH are 44% identical in the amino acid sequence, and are among the most similar pairs of genes among the twelve Gro/TLE type co-repressors (Liu and Karmarkar, 2008). Our study indicates *LUH* plays a more significant role than *LUG* in seed mucilage secretion, while *LUG* plays a more significant role in flower development (Sitaraman et al., 2008). Previously, we revealed that *luh-1* seeds had a germination defect (Sitaraman et al., 2008), which may be attributed to the finding in our current study that *luh-1* fails to secrete mucilage, as mucilage functions as an aid for seed germination (Western et al., 2000).

Our result shows that *luh-1* mutant seeds produce mucilage normally but fail to secrete it. One important enzyme for the modification of mucilage is β-galactosidase, which is encoded by 17 genes in *Arabidopsis*. Among the 17 genes, mutants of *MUM2*/*BGAL6* were reported to have a mucilage secretion defect due to failure to modify one of the primary components of mucilage, rhamnogalacturonin I (RGI). Such modification allows for correct hydration properties of mucilage and leads to mucilage release (Western et al., 2001; Dean et al., 2007; Macquet et al., 2007). This led to the hypothesis that *LUH* may positively regulate *MUM2*. RT-PCR indicated reduced *MUM2* expression, although not sufficient to explain the complete absence of mucilage. It is likely *LUH* regulates other genes involved in mucilage modification and secretion. Future work includes the rescue of the *luh-1* defect by over-expressing *MUM2*.

Although the salicylic acid pathway has been widely studied as a key regulatory pathway for the control of pathogens, xylem occlusion by pectinacious mucilage has been reported to resist invading pathogens such as bacteria, fungi, and viruses from accessing nearby tissues in trees, bananas, peas, and corn (Beckman and Zaroogian, 1967; Vandermolen et al., 1983; Rioux et al., 1998; Crews et al., 2003; Perez-De-Luque et al., 2005). However, the molecular connection between mucilage synthesis and secretion, along with its significance in controlling infection in plants has not been investigated. In this study, we discovered defects of *luh-1* in both mucilage secretion and pathogen resistance. We tested potential connections between the two defects. Specifically, lack of mucilage secretion in *luh-1* xylem and other *mum* mutants may aid in the spread of pathogens upon infection. Consistent with this hypothesis is our data shows increased susceptibility to pathogens in *luh-1* and three of the four *mum* mutants, and reduced susceptibility in *35S::LUH* transgenic plants that hypersecretes mucilage. Future work includes the examination of xylem mucilage in *luh-1*, *35S::LUH*, and *mum* mutants is needed to support this hypothesis.

It has been reported that both LUG and LUH proteins directly interact with SEU (Sridhar et al., 2004; Sitaraman et al., 2008). We show that both *lug* and *seu* mutants are more resistant to pathogens (**Fig. 3.4**), have increased *PR* gene expression, and *LUG* acts upstream of *EDS1* and likely represses R gene expression by interacting with a DNA-binding transcription factor. However, our data does not eliminate the possibility that LUH too, may also interact with SEU to repress R gene

expression. However, the additional defects of *luh-1* in mucilage secretion may mask the effect of *LUH* in R gene regulation, leading to a more pathogen susceptible rather than resistant response. If this explanation were confirmed, it would indicate a more important role of mucilage in pathogen defense that operates independently of the SA pathway.

3.6 Acknowledgements

I would like to thank Graduate Student Boyana Grigorova for the Northerns generated in this study, Nathan Lim from Montgomery Blair High School who helped with the pathogen infection assays, and co-advisor Dr. William Higgins for his support and guidance.

Chapter 4: Conclusions

4.1 Summary

Plant research has gained widespread media attention particularly with transgenic crop foods aimed to reduce human hunger and diseases in plants. *Arabidopsis thaliana*, a model organism for plants has been widely studied since its genome was completely sequenced in 2000. Due to genetic and functional homology shared between *Arabidopsis thaliana* and other eukaryotes including *Homo sapiens*, results attained from studying *Arabidopsis* have led to a myriad of discoveries that greatly impacted agriculture as well as human health. The discoveries include the understanding of how the human innate immune system operates because plants and humans possess defense proteins with NBS-LRR domains, the discovery of COP1 and COP9 signalosome protein complexes and their association with cancer, the RNA silencing machinery, which is linked to certain auto-immune diseases, and the DNA methylation enzymes important for X chromosome inactivation and transposon silencing in humans (Jones et al., 2008). As the human population grows exponentially and plants being at the bottom of the food chain, it becomes more evident that plant research will gain the urgency and recognition that has been long over due. In anticipation of the rising demand for plants that can adapt to the changing environment, scientists have dissected, analyzed, and generated crop plants such as canola that are resistant to drought (Wang et al., 2005), wheat that is impervious to insect herbivory (Arzani A, 2004), and soybean that is resistant to

bacterial pathogen (Huynh et al., 1989). To address the problem of not having enough food to support the growing human population, many researchers have targeted their research to flower development. Plant crops such as rice provide sustenance to over half the world's population (Tyagi et al., 2004). It is the rice seed that is hulled and consumed, understanding flower development is essential to improving plant fertility and productivity that will help solve the global food crisis.

The Liu lab is particularly interested in understanding flower development in *Arabidopsis*. The lab has extensively studied the molecular, genetic, and physiological aspects of the male stamen and the seed-producing female carpel in the flower, which is largely determined and regulated by the gene *AGAMOUS* (*AG*). A repressor of *AG* was identified and named *LEUNIG* (*LUG)* that belongs to the Gro/TUP1 family of co-repressors. *lug* mutants produce flowers that have no petals and develop sepals that resemble carpels (Liu and Meyerowitz, 1995). The Gro/TUP1 family proteins regulate transcriptions of many genes in diverse pathways by interacting with different DNA-binding transcription factors, which give the repressors their pathway specificity.

In the overall grand scheme of the research, several key conclusions can be made. One, homologous genes due to gene duplications will have both redundant and unique or exclusive functions, such as the case of *LUG* and *LUH*. The work in Chapter 2 shows that *LUG* and *LUH* possess partially redundant functions during flower development but completely redundant functions during embryogenesis. The

work described in Chapter 4 shows that both *LUG* and *LUH* repress the SA pathway while *LUH* has a predominant role in seed mucilage secretion. My thesis work also reveals that like other Gro/TUP1 family members in other systems, *LUG* and *LUH* act as "global co-repressors" by regulating diverse developmental processes including embryo development, root elongation, seed germination, mucilage secretion in the seed, and bacterial pathogen resistance.

Several interesting discoveries derived from my research may have potential agronomic value, including the *35S::LUH* transgenic line that hyper-secretes mucilage and is more resistant to bacterial pathogen. The idea that mucilage secretion is linked to pathogen resistance has placed groundwork for future investigations that may lead to novel strategies that enhance resistance to pathogens in crop plants.

Cotton fibers consist of pectin cell wall components and are secreted from cotton seeds. A recent study indicates that *LUG/LUH* is induced in cotton seeds during fiber initiation (Samuel Yang et al., 2006). The textile industry can benefit immensely from plants that produce larger amounts of cotton fibers used for clothing, as cotton is one of world's most renewable resource and most widely used by humans (http://www.organicexchange.org/). Also, because sales of organically grown cotton have more than doubled from $245 million in 2001 to $583 million in 2005, this makes cotton an important global commodity. Another important reason for studying mucilage and plant cell wall components is that it is important for ethanol production, a bio-fuel that will one day replace human dependence on non-renewable resources

such as oil and coal, which have horrible environmental implications. The production of plants that make large amounts of lignin, the primary component of the ethanol fermentation process will be the aim for scientists and automobile makers as the world oil reserve continues to diminish.

One of the limiting factors of food crop production is the continued onslaught of plant pathogens and herbivores. Researchers are actively searching for ways to produce plants that are resistant to pathogens and herbivores. Thus, the research gained from my thesis work will have molecular genetics, plant physiological, and agricultural/agronomical impacts, and will potentially benefit humans as well.

4.2 Future Direction

4.2.1 Linking Mucilage Synthesis in Seed to Pathogen Defense

Although we conclude that *LUH* is important for mucilage secretion in the seed, we still do not know the molecular mechanism that underlies the defects, nor do we know the identity of the DNA-binding transcription factor that recruits LUH to regulate this process. Candidate interacting partners include AtMYB61 and MYB5. However, I have obtained negative result in a yeast-two-hybrid (Y2H) analysis between AtMYB61 and LUG, LUH, and SEU (*data not shown*). MYB5 may be a good candidate to test for Y2H interactions with the transcriptional co-repressors.

Also, over-expressing *MUM2* into the *luh-1* mutant background is a good way to test whether *MUM2* will rescue the *luh-1* seed mucilage defect. If it does, it will support the hypothesis that *LUH* regulates *MUM2* expression to regulate mucilage secretion.

Although we previously reported that *luh-1; lug-16* double mutant plants are mostly embryo lethal (Chapter 2 and Sitaraman et al., 2008), we could still test *luh-1/luh-1*; *lug-16/+* seeds or even double mutants, which exist in very small numbers, for mucilage defects. Thus far, our study has not looked into the potential functional redundancy in regulating mucilage synthesis/secretion between *LUG* and *LUH*.

Is it a coincidence that *luh-1* and *mum5-1* mutant plants are equally susceptible to pathogens? Is there a direct link between defects in mucilage secretion in seeds and pathogen susceptibility? Since the molecular nature of *MUM5* is not known, we currently can not test whether *LUH* regulates *MUM5* both in seed mucilage and xylem mucilage found in leaves and stems. Another crucial assay is to detect defects of mucilage secretion in *luh-1* root xylem upon pathogen infection. I have attempted this by looking at root mucilage secretion without success. Perhaps mucilage can also be found in the phloem of leaf tissue, which is involved in transporting sugars. Finally, chromatin immuno-precipitation (ChIP) would be able to identify targets of the *LUH* co-repressor complex. This will determine the genes that are responsible for mediating the *luh-1* mucilage defect in seeds and disease resistance in plants.

Additionally, utilization of biochemical methods such as GC/MS to quantify seed mucilage in different genotypes will allow us to detect subtle differences in mucilage levels that are not easily observed with plastic sections and staining. Also, seed mucilage development has common genetic components with trichome development. It is worthwhile to look into potential trichome defects that may exist in *luh-1* leaves, and compare that to the *35S::LUH* over-expression line and the wild-type. Preliminary data indicates that both *FASCIATA 1* (*FAS1*) and *FASCIATA 2* (*FAS2*) genes are reduced in both *lug-3* and *luh-1* mutant plants (**Fig. 4.1**). Both *FAS1* and *FAS2* encode subunits of the Chromatin Assembly Factor-1 (CAF-1) complex, which facilitate formation of nucleosomes on newly replicated DNA (Exner et al., 2006). *fas* mutants have defects in shoot apical meristem and develop highly branched trichomes in leaves.

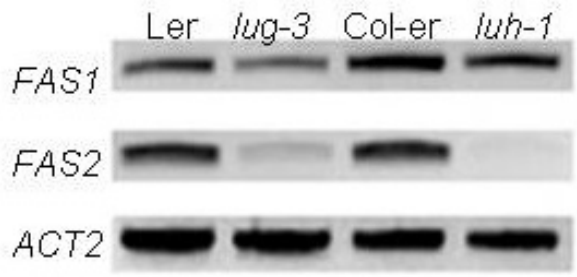


Fig. 4.1: RT-PCR analysis semi-quantitatively measuring *FAS1* and *FAS2* expression in *lug-3* and *luh-1* mutant leaves compared to wild-type, Ler and Col-er, respectively.

4.2.2 Pathogen Defense

Using semi-quantitative RT-PCR, our study focuses on gene expression changes 6 hours after infection, but the incubation period after infection is three days before the bacteria is extracted, and the CFUs determined. Perhaps monitoring the expression of the defense marker genes in a longer duration will provide a more

accurate and comprehensive observation as to what is occurring at the molecular level.

seu-1 and *lug-3* mutant plants exhibit symptoms of more severe reactive oxygen-species (ROS)-mediated stress upon growth on high osmotic media (**Fig. 4.2**). *luh-1* mutant plants exhibited no ROS-mediated stress, suggesting that *luh-1* mutants may be defective in activating ROS, giving insight into another possible explanation as to why *luh-1* is susceptible to bacteria pathogens. This result is consistent with the pathogen infection assay (**Fig. 3.4**), which shows that *seu-1*and *lug-3* had the greatest resistance to bacteria. This observation is consistent with the hypothesis that *LUG* and *SEU* repress the SA pathway, which induces ROS and ROS-mediated cell death to fend off pathogens.

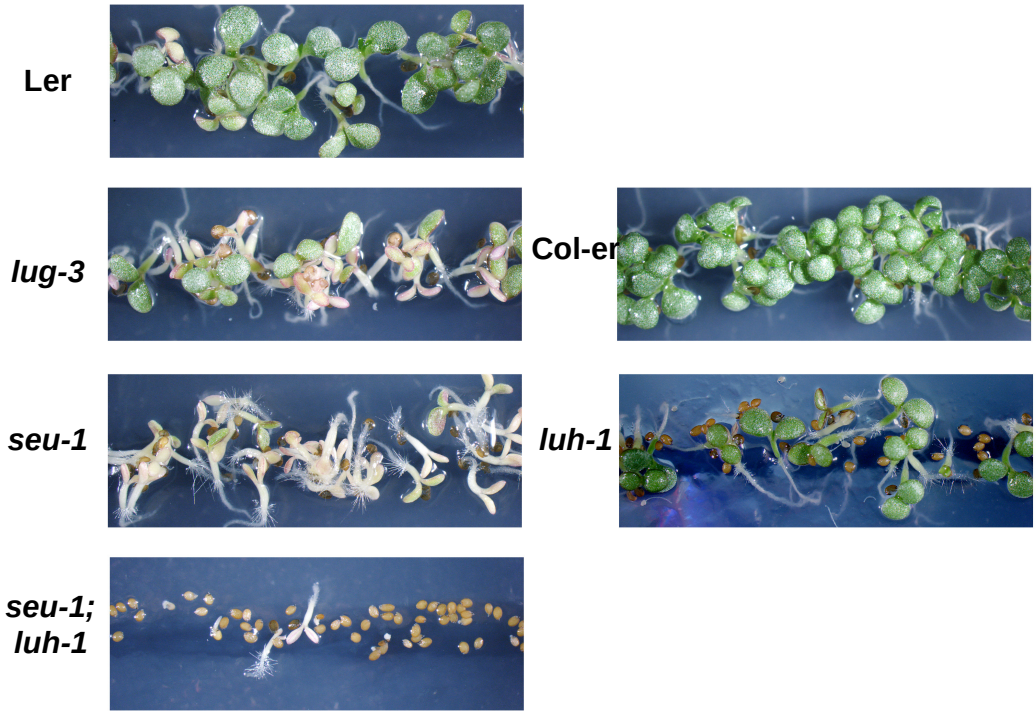


Fig. 4.2: Different genotypes grown on MS media supplemented with 270 mM mannitol for 21 days to induce osmotic stress, resulting in increased ROS levels and leaf chlorosis.

Because *seu-1*, *lug-3*, and *35S::LUH* mutant plants are resistant to bacteria, we can potentially generate super pathogen resistant plants by obtaining double or tripe mutants between these mutations or transgenes. Additionally, since *eds1-2* and *luh-1* plants are susceptible to bacteria while *35S::LUH* is pathogen resistant, *35S::LUH* should be introduced into *eds1-2* to test whether *EDS1* and *LUH* act in the same or parallel pathways.

Appendix I: Simple allele-discriminating PCR for cost-effective and rapid genotyping and mapping (SAP)

A1.1 Abstract

A1.1.1 Background

Single nucleotide polymorphisms (SNPs) are widely observed between individuals, ecotypes, and species, serving as an invaluable molecular marker for genetic, genomic, ecological and evolutionary studies. Although a large number of SNP-discriminating methods are currently available, few are suited for low-throughput and low-cost applications. Here, we describe a genotyping method named Simple Allele-discriminating PCR (SAP), which is ideally suited for the small-scale genotyping and gene mapping routinely performed in small to medium research or teaching laboratories.

A1.1.2 Results

We demonstrate the feasibility and application of SAP to discriminate wild type alleles from their respective mutant alleles in *Arabidopsis thaliana*. Although the design principle was previously described (Ferrie et al., 1992; Little, 1995), it is unclear if the method is technically robust, reliable, and applicable. Three primers were designed for each individual SNP or allele with two allele-discriminating forward primers (one for wild type and one for the mutant allele) and a common reverse primer. The two allele-discriminating forward primers are designed so that

each incorporates one additional mismatch at the adjacent (penultimate) site from the SNP, resulting in two mismatches between the primer and its non-target template and one mismatch between the primer and its target template. The presence or absence of the wild type or the mutant allele correlates with the presence or absence of respective PCR product. The presence of both wild type-specific and mutant-specific PCR products would indicate heterozygosity. SAP is shown here to discriminate three mutant alleles (*lug-3*, *lug-16*, and *luh-1*) from their respective wild type alleles. In addition, the SAP principle is shown to work in conjunction with fluorophore-labeled primers, demonstrating the feasibility of applying SAP to high throughput SNP analyses.

A1.1.3 Conclusions

SAP offers an excellent alternative to existing SNP-discrimination methods such as Cleaved Amplified Polymorphic Sequence (CAPS), or derived CAPS (dCAPS) method. It can also be adapted for high throughput SNP analyses by incorporating fluorophore-labeled primers. SAP is reliable, cost-effective, fast, and simple, and can be applied to all organisms not limited to *Arabidopsis thaliana*.

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A1.2 Introduction

Genetic and genomic research has entered a new era with the ever-improving and novel sequencing technologies (Service, 2006). Researchers, now more than ever, are taking advantage of the available genomic information for research, teaching, and applications. Single Nucleotide Polymorphism (SNP), the most abundant form of DNA polymorphisms, serves as the most valuable molecular marker for research and application, including the detection of risk associated alleles linked to human diseases (Eberle et al., 2007), the study of evolutionary conservations between different species and building recombination maps (Hillier et al., 2007), gene mapping and cloning (Wang and Liu, 2006), and crop breeding (Rafalski, 2002). In many small to medium size academic laboratories as well as teaching laboratories around the world that utilize *Arabidopsis thaliana* or other genetic model systems, SNPs have become indispensable for genotyping progeny of genetic crosses, discriminating between mutant alleles from wild type alleles or isolating genes using the map-based approach. Efficient and robust genotyping assays are also essential for the identification of individuals carrying suppressor or enhancer mutations that manifest no visible phenotypes of their own (Resnick et al., 2006). Therefore, robust, reliable, inexpensive, and fast SNP-discriminating methods are needed.

Currently, a large variety of techniques for high throughput SNP genotyping are available (Gut, 2001; Kwok, 2001). They can be grouped into four main classes: allele-specific hybridization, allele-specific nucleotide incorporation, allele-specific

oligonuleotide ligation, and allele-specific invasive cleavage. For example, the TaqMan genotyping method (Livak et al., 1995) and the Amplifluor SNP HT genotyping System (Myakishev et al., 2001) are PCR-based, suitable for large scale high throughput applications. Both methods, however, require expensive instrumentation and reagents such as synthetic oligonucleotides labeled with different fluorescent dyes. Various genome resequencing methods are also extremely powerful for large scale SNP-discrimination (Huang et al., 2004; Shapero et al., 2004), yet are impractical for assaying a selected set of SNPs in specific genomic regions, and are usually beyond the reach of small to medium size laboratories with limited resources.

To date, a widely utilized SNP detection method for low-throughput applications in plant research is the Cleaved Amplified Polymorphic Sequence (CAPS), which requires locus-or gene-specific primers to amplify the region of interest, followed by restriction enzyme digestion, and electrophoresis (Konieczny and Ausubel, 1993). In a modified CAPS method called "derived CAPS" (dCAPS) (Neff et al., 2002), an engineered primer creates a restriction enzyme recognition site that can be used to distinguish the targeted SNP. dCAPS is more widely applicable than CAPS because it does not require the SNP to create or destroy a restriction enzyme site. While CAPS and dCAPS are suitable for small to medium scale genotyping, both methods require enzymatic digestion, increasing the cost as well as experimental time. One serious limitation of CAPS and dCAPS is that the restriction enzyme required could be inefficient and costly and that incomplete enzyme digestion

hinders one's ability to distinguish heterozygocity from homozygocity of the tested SNP.

During the course of genetic research in construction of double mutants between *leunig (lug)* and *leunig-homolog* (*luh*) (Sitaraman et al., 2008), we encountered situations in which the dCAPS markers for *lug* and *luh* mutations yielded ambiguous results. We first turned to direct sequencing and subsequently to the Amplifluor SNP HT genotyping System (Myakishev et al., 2001). These methods, while reliable, tend to have a high cost when the number of mutants requiring genotyping increases. In addition, Amplifluor SNP HT requires the access to a real time PCR machine not readily available to us. We searched for alternative genotyping methods and came across with the "amplification refractory mutation system (ARMS)" (Ferrie et al., 1992; Little, 1995) developed more than 10 years ago in mammalian systems.

The ARMS technique is based on the extension of primer only when its 3'-end is a perfect complement to the allele present in the input sample. However, when terminal mismatching has only weak-destabilizing effect, single mismatch at the terminal base may not discriminate between wild type and mutant templates. Therefore, an additional deliberate mismatch is introduced at the penultimate (second to the terminal) base of the primer to increase the specificity of the PCR reaction. As different mismatches have different destabilizing effects (Ferrie et al., 1992; Little, 1995), both the terminal and the penultimate mismatches are considered together. If

the terminal and natural mismatch is highly unstable, a weak additional mismatch will be introduced at the penultimate site, and vice versa. This principle is further elaborated recently in a graphic dial (Wangkumhang et al., 2007) and can now be designed through a website (http://bioinfo.biotec.or.th/WASP).

Based on the principle of ARMS, we designed allele-specific primers by introducing additional mismatch at the penultimate site aimed at destabilizing base pairing between the primers and corresponding non-target templates. We demonstrate that this method offers an excellent alternative to CAPS or dCAPS because of its simplicity, low cost, robustness, speed, and reliability. We named this method SAP (Simple Allele-discriminating PCR) instead of ARMS (amplified refractory mutation system) as SAP more readily explains its application and thus may help popularize its utility. We describe primer design rules and show the successful application of the SAP principle to fluorescent-labeled universal primers in allele-discrimination PCR, allowing high throughput applications. The SAP method provides a practical and useful alternative to existing genotyping methods and will greatly facilitate plant research and teaching.

A1.3 Materials and Methods

A1.3.1 Plant Growth and DNA Extraction

Arabidopsis thaliana wild type and mutant plants were grown under 16-hour long day conditions at 20ºC and 65% humidity for 4 weeks. One to two leaves were collected from individual *Arabidopsis* plants, and DNA was extracted using Edwards buffer (200 mM Tris, pH: 7.5; 250 mM NaCl; 25 mM EDTA, pH: 8.0; 0.5% SDS),

precipitated with isopropanol, washed with 70% ethanol, and resuspended in 50 to 100 μL distilled water, 2 μL of which (roughly about 10 ng genomic DNA) was used in 20 μL PCR reactions.

DNA template was sometimes obtained through the FTA card (Whatman) following manufacturer's instructions. One single leaf was pressed onto the FTA card and allowed to dry. 1.2 mm diameter discs were punched out of the DNA-containing FTA cards using the 1.2 mm micro punch. The discs were first washed with 20 μL FTA Purification Reagent (Whatman) and washed again with 20 μL 1X TE buffer. Each DNA disc was used directly in individual PCR reactions.

A1.3.2 Primers and PCR

Primers were designed as described in the Result section. PCR program for all alleles described here was the same, beginning with 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 20 seconds, 55-57ºC for 20 seconds, and 72ºC for 40 seconds, and ended by 72ºC for 3 minutes. WT and MT primer pairs were designed to have similar annealing temperatures to allow simultaneous PCR. Primer sequences are provided in **Table A1.3**. Standard PCR reaction was used with the final primer concentration at 0.5 μM and the final dNTP concentration at 0.2 μM in a 20 μL PCR reaction. Taq DNA Polymerase was purchased from GeneScript Corporation (Cat# E00007). 1% agarose gels were made with Invitrogen's UltraPure Agarose. 5 μL PCR reaction was loaded in each lane of the 1% agarose gel

A1.3.3 High-throughput Application

The Amplifluor SNPs Genotyping System for Assay Development kit was purchased from Chemicon International (Millipore Cat# S7907). AS primers for WT LUG and MT *lug-16* were designed with a 5' tail sequence identical to the 3' region of the FAM or JOE universal primers, respectively (**Table A1.3**). PCR reaction mixture and PCR program were set up following the manufacturer's instruction and using the Platinum Taq DNA Polymerase (Invitrogen). End-point fluorescence detection was carried out using BioRad's iQ5 Multicolor Real-Time PCR Detection System and software.

A1.4 Results

A1.4.1 SAP Primer Design for Genotyping three mutant alleles in *Arabidopsis thaliana*

To discriminate single base change between wild type and the mutant allele, a forward primer that exclusively anneals to WT and another forward primer that exclusively anneals to the mutant allele are designed. These two allele-specific (AS) primers are paired with a common reverse primer for standard PCR reactions. The AS primers are designed based on the principle that if the existing SNP mismatch results in a weak destabilization between the AS primer and its non-template target, a strong destabilizing mismatch will be introduced at the penultimate site. Conversely, if the SNP mismatch already has a strong destabilizing effect, a weak destabilizing mismatch should be introduced at the penultimate site. If a medium destabilizing effect exists at the SNP mismatch, a weak or medium mismatch will be created at the penultimate site.

Table A1.1 indicates the weak, medium, strong, or maximum destabilization effect of each mismatched pair, based on Little (1995). In general, the purine-pyrimidine mispairing (G-T and A-C) are more stable and exhibit a weaker destabilization effect than the purine-purine or pyrimidine-pyrimidine mismatches. Purine-pyrimidine mismatches still form two hydrogen bonds in a geometry similar to G-C and A-T, and they don't require contracting or expanding the double helix. Pyrimidine-pyrimidine or purine-purine mispairings, in contrast, are more unstable because of the altered geometry in the double helix as well as reduced hydrogen bonding. For more detailed analyses of thermodynamics of mismatches, one can consult Peyret et al. (1999).

When designing the AS primers, the specific type of nucleotide introduced at the penultimate site should be determined by consulting **Table A1.1** or **Table A1.2**. A step-by-step illustration of AS primer design for the *seuss (seu)-1* mutant (Franks et al., 2002) is shown in **Fig. A1.1A**. The terminal mismatches (GT, AC) in this case are weak destabilizing, thus a strong destabilizing mismatch (GA) is introduced at the penultimate site. A second example shows a C (WT) to A (MT) mutation, which resulted in strong destabilizing mismatches at the terminal site between the WT and MT primers and their corresponding non-target templates, respectively (**Fig. A1.1B**). As a result, a weak destabilizing mismatch is introduced at the penultimate site. A web-based computational design tool using this principle can be found at http://bioinfo.biotec.or.th/WASP (Wangkumhang et al., 2007).

	A	T	G	C
A	A/A	A/T	A/G	A/C
T	T/A	T/T	T/G	T/C
G	G/A	G/T	G/G	G/C
C	C/A	C/T	C/G	C/C

Yellow: Weak Destabilizing Effect
Blue: Medium Destabilizing Effect
Red: Strong Destabilizing Effect
Green: Normal Base Pairing

Table A1.1: The strength of destabilization for all combinations of nucleotide pairing.

Template			Primer		
Penultimate (2)	Ultimate (1)		Penultimate (2)'	Ultimate (1)'	
	SNP_1	SNP_2	$Base_1$<-->$Base_2$ or Common Base	SNP_1	SNP_2
A	A <-->	T	C<-->A	T <-->	A
		G	G		C
		C	C		G
	T <-->	C	G	A <-->	G
	G <-->	T	C	C <-->	A
	C <-->	G	A<-->C	G <-->	C
T	A <-->	T	G	T <-->	A
		G	C<-->T		C
		C	G		G
	T <-->	C	T<-->C	A <-->	G
	G <-->	T	G	C <-->	A
	C <-->	G	G	G <-->	C
G	A <-->	T	T<-->G	T <-->	A
		G	A		C
		C	T		G
	T <-->	C	A	A <-->	G
	G <-->	T	T	C <-->	A
	C <-->	G	G<-->T	G <-->	C
C	A <-->	T	A	T <-->	A
		G	T<-->C		C
		C	A		G
	T <-->	C	C<-->T	A <-->	G
	G <-->	T	A	C <-->	A
	C <-->	G	A	G <-->	C

Table A1.2: An alternative comprehensive table for the design of SAP primers.

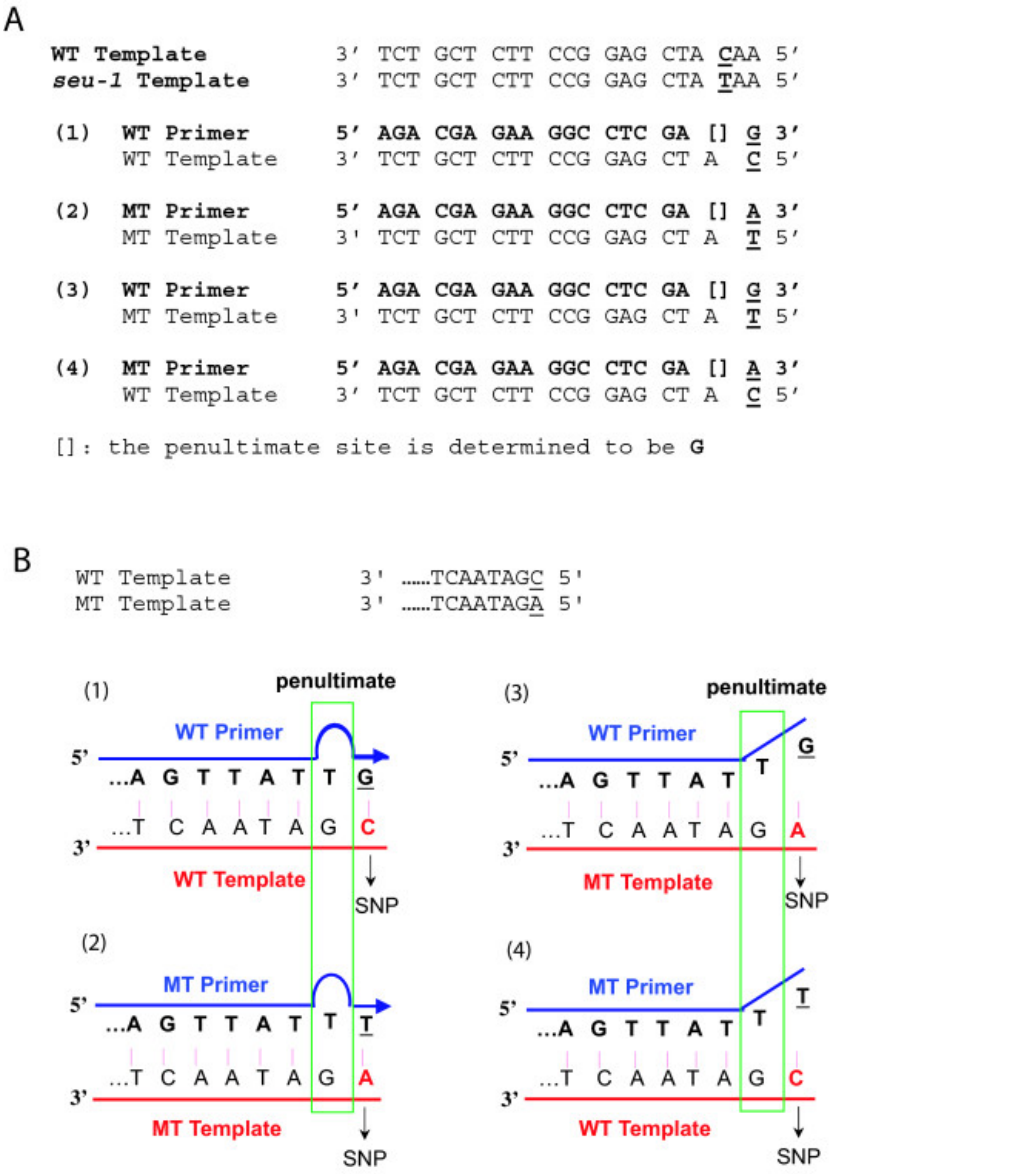


Fig. A1.1: Illustration of the SAP principle. (A) A step-by-step illustration of the AS primer design for the *Arabidopsis seu-1* mutant. The WT (SEU) sequence and the *seu-1* mutant sequence are shown on top. The mutated base is underlined. The WT (SEU)-specific primer is first designed based on its complementarity to WT template sequence shown in (1); the MT (*seu-1*)-specific primer sequence is designed based on its complementarity to the MT template sequence shown in (2). The primer sequence is always from 5' (left) to 3' (right). The penultimate base in the AS primers is indicated by a bracket. Subsequently, the WT primer is paired against the MT template (3) to determine the terminal mismatch (GT). Similarly, MT primer sequence is paired against WT template sequence (4) to determine the terminal mismatch (AC). By referring to Table 1, the GT and AC terminal mismatches identified above both exhibit weak destabilization effect. Thus, the penultimate mismatch should exhibit a strong destabilization. By referring to Table 1, the strongest destabilization mismatch that involves "A" is "GA". Therefore, G is chosen at the penultimate site of both WT and MT AS primers. (B) Four possible annealing scenarios for a hypothetical C to A mutation, which is underlined. Because the terminal mismatches (GA and TC) are strong

destabilizing, the penultimate site thus selects a weak destabilizing mismatch (TG), which is indicated within the green rectangle. (1) Proper annealing of a WT primer to the WT template, which will lead to successful PCR amplification. (2) Stable annealing of the MT primer to the MT template, leading to successful PCR amplification. (3) Unstable pairing of the WT primer to the MT template due to two consecutive mismatches. No PCR product is expected. (4) Unstable pairing of the MT primer to the WT template. No PCR amplification is expected.

The initial application of the SAP assay to genotyping three mutant alleles, *lug-16, luh-1* and *lug-3*, is shown (**Fig. A1.2A, B; Table A1.3).** Subsequently, several other mutations were genotyped by the SAP (data not shown). In all cases, the SAP assay was successful. For example, **Fig. A1.2A** shows the PCR amplification of WT template with the WT (LUG) primer and the amplification of *lug-16* MT template by the *lug-16* MT primer. It also shows the failure of PCR amplification of WT template by the *lug-16* MT primer, and failure of PCR amplification of *lug-16* MT template by the WT (LUG) primer, suggesting that the WT (LUG) and MT (*lug-16*) primers are highly specific to their target templates. Similar genotyping result was obtained for *luh-1* (**Fig. A1.2A**). Additionally, **Fig. A1.2B** illustrates the utility of SAP in identifying an F1 progeny (heterozygote) of a genetic cross between wild type and *lug-3* mutants.

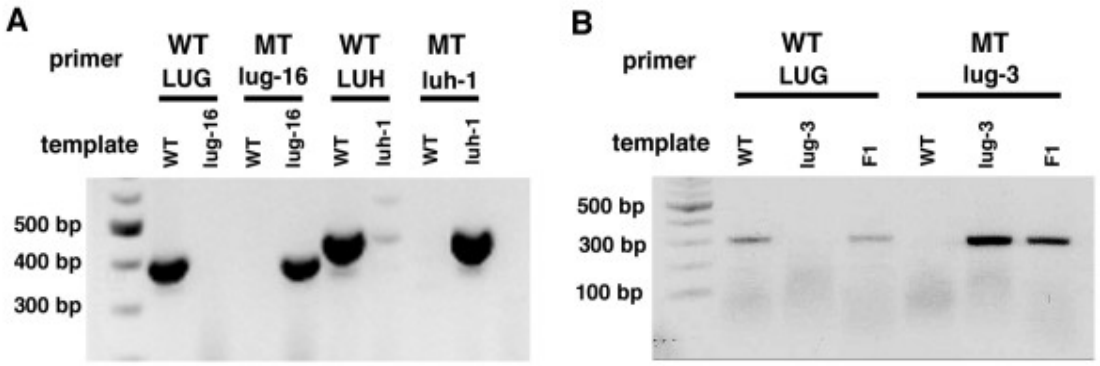


Fig. A1.2: SAP-based genotyping of three different mutant alleles. (A) WT LUG and MT lug-16 genotypes were identified by the positive amplification of a 401 bp band when the WT (LUG) primer and the lug-16 MT primer amplify their WT and MT target template DNA, respectively. Similarly, WT (LUH) and MT luh-1 genotypes were identified when a 456 bp PCR fragment was amplified with respective primers. (B) Presence of WT (LUG) and MT lug-3 template DNA correlates with the amplification of a 301 bp PCR band using respective WT (LUG) and MT lug-3 primers. A heterozygote (F1 progeny of a cross between wild type and lug-3) correlates with the positive PCR amplifications with both WT (LUG) or MT lug-3 primers.

SNP	Primer Type	Direction	Sequence (5'-3')
lug-16	WT-Specific	Reverse	CCACCAGGTGCGTCAATATC
	Mutant-Specific	Reverse	CCACCAGGTGCGTCAATATT
	Common	Forward	TTGTATGCAAGTATGTGACTTTA
*lug-16** (Amplifluor)	WT-FAM	Reverse	**GAAGGTGACCAAGTTCATGCT**TCCACCAGGTGC GTCAATATC
	Mutant-JOE	Reverse	**GAAGGTCGGAGTCAACGGATTT**CCACCAGGTGC GTCAATATT
	Common HT	Forward	CTGCAGTTGCTCTGTTTCCTAA
luh-1	WT-Specific	Forward	GGAGGGTTTCTTTTTGAGTTG
	Mutant-Specific	Forward	TGGAGGGTTTCTTTTTGAGTTA
	Common	Reverse	CCATGATGGTTTGTTGCTGAT
lug-3	WT-Specific	Reverse	TTGATGTTGTTGTTGCTGCGG
	Mutant-Specific	Reverse	TTGATGTTGTTGTTGCTGCCA
	Common	Forward	ACTAAGCTGGAGTATTTCTATTT

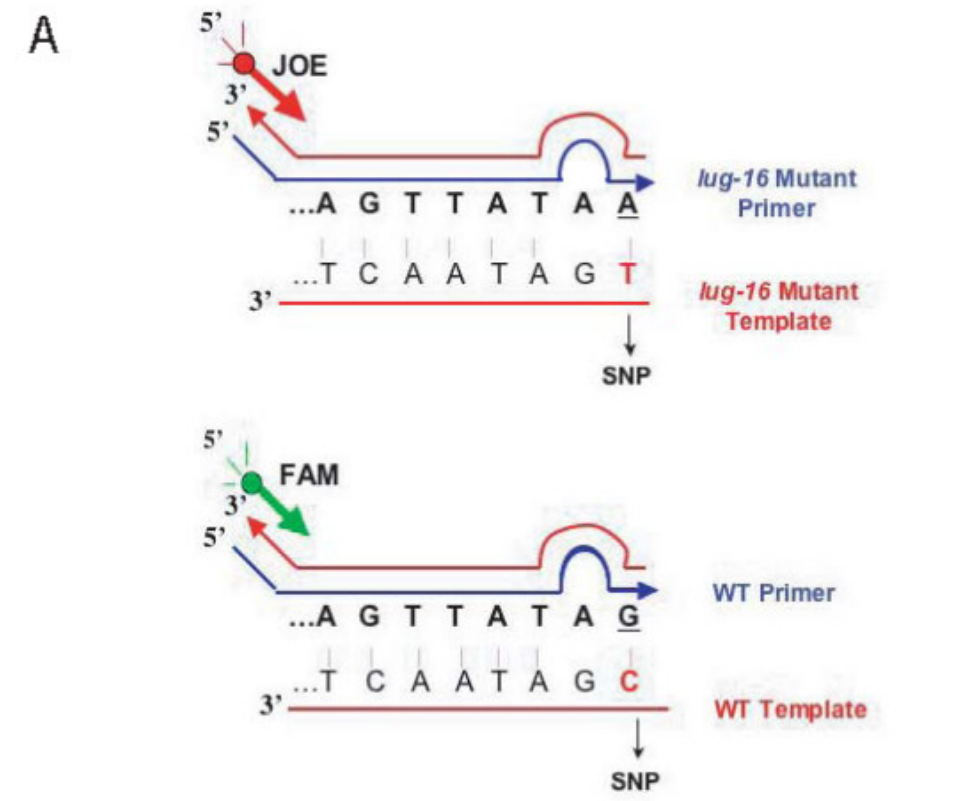
Table A1.3: Primer sequences for three different alleles. *: The bold and underlined sequences are 5' extended primer sequences that specifically pair with the 3' region of the FAM or JOE universal fluorescent primers, respectively, from the Amplifluor SNPs Genotyping System.

The SAP assay is normally set up in two parallel PCR reactions. One set of PCR reaction combines the WT-specific primer with the common reverse primer. The second set of PCR reaction combines the MT-specific primer with the same common reverse primer. When the SAP assay is first developed for a specific SNP, different annealing temperatures should be tested using WT and MT DNA templates to identify the optimal annealing temperature that allows positive amplification of AS primers with respective target templates and negative amplification with non-target templates. Ideally, the optimal anneal temperature for the WT-specific amplification is the same as that of the MT-specific amplification, allowing for single PCR runs. However, this is sometimes difficult to achieve, and separate PCR runs using different annealing temperatures for WT and MT-specific PCR reactions are necessary.

A1.4.2 Feasibility in High-throughput Applications

In certain instances, when large-scale analyses are required or when there is a small amount of genetic material, SAP can be applied in a high-throughput and highly sensitive manner. To demonstrate such an application, the AS primer design principle was utilized and adapted to the Amplifluor SNPs Genotyping System (Chemicon) (Myakishev, Khripin et al. 2001). This technology uses energy transfer (ET) universal primers that generate fluorescent PCR products (**Fig. A1.3A**). While the allele-discriminating principle is the same as SAP, the detection of the PCR products requires a machine capable of reading fluorescence signal such as a fluorescence plate reader or a qRT-PCR machine.

In our experiment, the WT primer is annealed to the Amplifluor SNPs Genotyping primer FAM, while the MT *lug-16* primer is annealed to the Amplifluor SNPs Genotyping primer JOE. After PCR, the data were transferred to Microsoft Excel, and scatter plots were generated (**Fig. A1.3B**). Homozygous wild type (+/+) showed a high FAM signal and some background JOE signal. In contrast, homozygous mutant (*lug-16/lug-16*) showed a high level of JOE signal and some background FAM signal. Heterozygote (*lug-16/+)* showed significant signal from both JOE and FAM. The successful discrimination between *lug-16/lug-16* homozygotes, wild type (+/+), and *lug-16/+* heterozygotes indicates that the SAP-based principle can be applied to high-throughput and highly sensitive applications. In addition, this method is highly sensitive, requiring only 0.4 ng template DNA in 10 microliter PCR reactions.


A
5'
JOE
3'
5'
...A G T T A T A A
lug-16 Mutant Primer
...T C A A T A G T
3'
lug-16 Mutant Template
SNP
5'
FAM
3'
5'
...A G T T A T A G
WT Primer
...T C A A T A G C
3'
WT Template
SNP

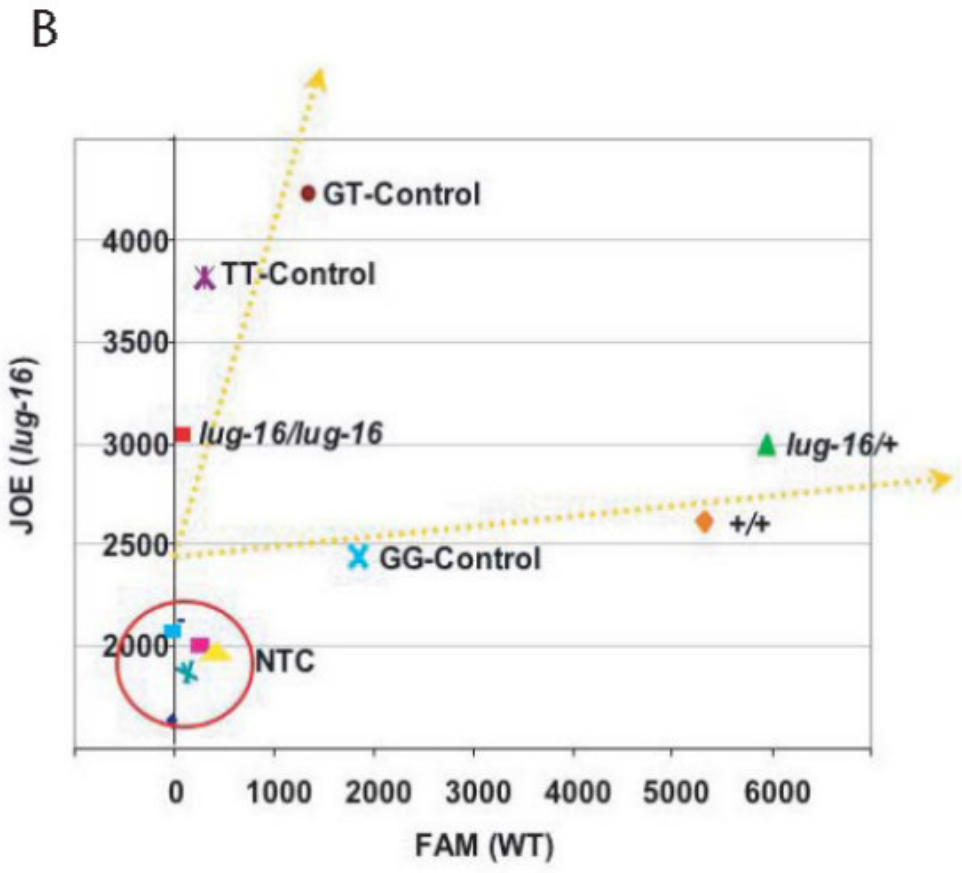

B
GT-Control
TT-Control
lug-16/lug-16
lug-16/+
+/+
GG-Control
NTC
JOE (lug-16)
FAM (WT)
4000
3500
3000
2500
2000
0
1000
2000
3000
4000
5000
6000

Fig. A1.3: Adaptation of SAP for high throughput applications. (A) Diagram illustrating the Amplifluor SNP genotyping assay system. The allele-specific primer each has a unique 5' tail sequence that is identical to the 3' region of one of the Amplifluor SNP Universal Primers (FAM or JOE indicated by green and red arrows respectively). When combined with the common reverse primer, PCR amplification results in the synthesis of the tail sequence complement (thin red line). The Amplifluor® SNPs Universal Primer then anneals specifically to the tail of reverse complement and is elongated by Taq Polymerase. Subsequent PCR cycles unfold the hairpin structure (indicated by filled circle) of the Amplifluor® SNPs Universal Primers, which results in fluorescent signals. (B) A scatter plot showing results of a SAP-based Amplifluor SNP assay. X-axis represents the FAM signal measuring the amplification of WT LUG (+), and the Y-axis indicates the JOE signal that measures lug-16-specific PCR amplification. Two types of controls were used. First, the manufacturer's template controls (GG, GT, TT) utilize FAM/JOE SNP primers and the control templates (GG, TT, and GT), both of which are provided by the manufacturer's kit. Second, the non-target control (NTC) uses water instead of DNA template. Results of three experimental samples (lug-16/lug-16, lug-16/+, and +/+) are shown. DNA template was from known genotype. The experiment has been performed twice with similar results. The result from one such an experiment is shown.

Unlike the low throughput examples discussed earlier, both the WT and the MT AS primers are added into the same PCR mix and used to amplify their target templates using the same PCR program. If the two AS primers do not amplify their target DNA with equal efficiency, one fluorescent signal (such as FAM shown in Fig. 3B) could be significantly higher than that of the other fluorescent signal (such as JOE in Fig. 3B). Therefore, it is important to always include wild type and mutant control templates in the same experiment.

A1.5 Discussion

We describe a simple SNP-discriminating method and demonstrate its utility for plant research. Although the design principle was previously described (Little 1995; Wangkumhang, Chaichoompu et al. 2007), it is has not been shown to be utilized in plants, nor is it known if the method is technically robust, reliable, and applicable.

Several important lessons were learned in the course of developing the SAP assay. First, a primer that is too stable will not distinguish between the target and the non-target templates. In contrast, an unstable primer will not effectively amplify its target template. To weaken undesirable stability between the primer and its non-template target, either the primer length is reduced, or the PCR annealing temperature is increased. The general rule of thumb is to maintain primer G/C contents at 36% to 66%, primer length between 18 and 22 bases, the amplicon size around 200-600 bases, and the annealing temperature between 55^{o}C to 60^{o}C. WT and MT allele-specific primers are best kept at similar length to allow for same PCR conditions. When the last nucleotide at the 3' end of the AS-primer is a G or C, there is often an increased likelihood of a faint, non-specific background PCR band. Accordingly, an increase in annealing temperature or a shortening of primer length may be necessary. Finally, PCR conditions have to be first optimized using the wild type and mutant DNA template controls. PCR optimizing runs on a temperature gradient are highly recommended when one develops the SAP assay. It is also possible to further optimize the assay by adjusting appropriate primer and dNTP concentrations.

A1.6 Conclusion

The aforementioned SAP method described is a cost-effective, time-efficient, robust and reliable method for the identification and discrimination of different alleles. SAP offers several advantages over existing CAPS and dCAPS genotyping assays and can be adapted for high-throughput applications. SAP may be broadly applied to a wide range of research in any organisms.

A1.7 List of Abbreviations

AS: Allele Specific; PCR: Polymerase Chain Reaction; SAP: Simple Allele-discriminating PCR; SNP: Single Nucleotide Polymorphism; WT: Wild Type; MT: Mutant; WASP: Web-based Allele-Specific PCR.

Appendix II: Ethane Methyl Sulfonate (EMS) Induced Mutagenesis Screen for *LUH* Suppressors

To identify repressors of *LUH*, we conducted EMS mutagenesis (0.15% EMS with 10,000 seeds) in *luh-1* mutant seeds. Because *luh-1* seeds fail to secrete mucilage, Toluidine blue staining will identify mutagenized *luh-1* seeds that are now capable of secreting mucilage again. This study will help in the identification of genes that act downstream of *LUH* (see **Fig. A2.1**). Preliminary screens of how many seeds 1,200 have successfully identified about twenty mutagenized *luh-1* seeds that secrete mucilage. These seeds are picked out and grown on MS media, transferred to soil, where it is grown to maturity, and the seeds collected and stored for the next generation. Eventually, the suppressor gene will be identified through map-based cloning, and further studied for mucilage and developmental seed defects. The genetic concept and pictures of mutagenized *luh-1* seeds that secrete mucilage are shown in **Fig. A2.1**.

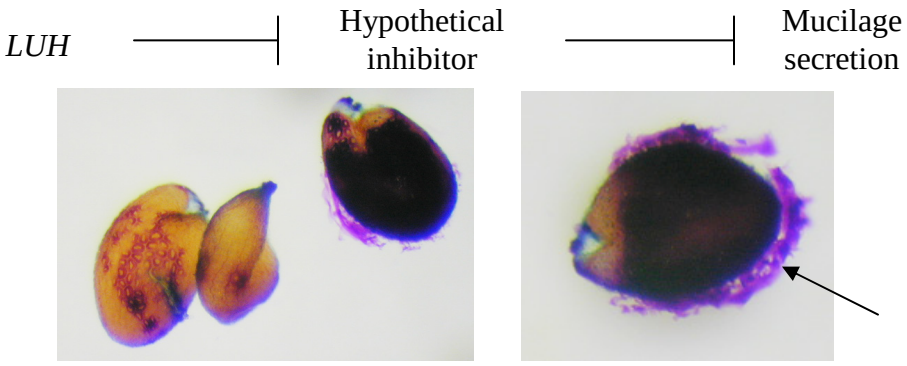


Fig A2.1: Photograph of *luh-1* seed (left) and a potential *luh-1* suppressor mutant seed. Mucilage is secreted in a mutagenized seed (arrow).

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