# GIBBERELLIN REGULATES *ARABIDOPSIS* FLORAL DEVELOPMENT VIA SUPPRESSION OF DELLA PROTEIN FUNCTION

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**Summary**

Floral organ development, especially petals and stamens is impaired in severe *Arabidopsis* GA-deficient mutant *ga1-3*, suggesting that GA is a general regulator of floral development. However, the mechanism via which GA regulates petal and stamen development remains unclear. Although previous analysis have shown that GA promotes the elongation of plant's organs by opposing the function of the DELLA proteins, a family of nuclear growth repressors, it was not clear if the DELLA proteins are involved in the GA-regulation of petal and stamen development.

*Arabidopsis* genome encodes five distinct DELLA proteins (GAI, RGA, RGL1, RGL2 and RGL3). Previous genetic studies have shown that GAI and RGA have overlapping functions in the repression of plant stem growth, while RGL2 controls the seed germination. RGL1 may play a role both in stem elongation and seed germination. Although DELLA proteins GAI, RGA, RGL2 and RGL1 are all expressed in inflorescences, no obvious suppression of *ga1-3* floral phenotype was observed in *ga1-3* mutants lacking GAI, RGA, GAI and RGA, or RGL2. Using novel combinations of loss-of-function mutations of DELLA proteins, we determined that RGA, RGL1 and RGL2 act synergistically to repress stamen filament cell elongation and microsporogenesis. GA promotes stamen filament cell elongation and pollen development by opposing the function of DELLA proteins RGA, RGL1 and RGL2.

DELLAs act as negative regulators of GA response. However, as a group of putative transcription regulators, the molecular mechanism of DELLAs repressing floral development is largely unknown. Comparing the global gene expression patterns in unopened flower buds of the *ga1-3* mutant with that of the wild type and the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant, we found that about half of GA-regulated genes are regulated in a DELLA-dependent fashion. This data also

suggested that there might be a DELLA-independent or –partially-dependent component of GA-dependent gene regulation.

*MYB21*, *MYB24*, and *MYB 57* are flower-specific genes. DELLA proteins RGA or RGL2 repress expression of these genes in *ga1-3* flower buds. Genetic study showed that *MYB21*, *MYB24*, and *MYB 57* are necessary for normal stamen development. Absence of four DELLAs (GAI, RGA, RGL1 and RGL2) cannot suppress the short stamen /phenotype conferred by the loss-of-functions of *MYB21* and *MYB24*, suggesting that these *MYB* genes might act downstream of DELLA proteins in controlling the floral development.

Jasmonic acid (JA) is a lipid-derived signaling molecule that is required for normal stamen development. Recently, *MYB21* and *MYB24* were identified to be down-regulated in JA deficient mutant *opr3*, suggesting that JA might regulate stamen development via promoting the expression of *MYB21* and *MYB24*. It is intriguing to know if there is a cross-talk between GA and JA pathways in controlling stamen development. We found that JA was able to induce the expression of *AtMYB21* and *AtMYB24* in the absence of GA, but GA could not induce the expression of *AtMYB21* and *AtMYB24* in the absence of JA. These data suggested that JA might act downstream of GA in promoting the expression of *AtMYB21* and *AtMYB24*. Further study indicated that GA might regulate *AtMYB21* and *AtMYB24* through modulation of JA biosynthesis. However, JA induced expression of *MYB21* and *MYB24* in *ga1-3 gai-t6 rgl1-1 rgl2-1* mutant is necessary but not sufficient enough to induce the normal elongation growth of stamen filament in *Arabidopsis*, suggesting that *AtMYB21* and *AtMYB24* are not the master check-point for GA functions in regulating stamen development.

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**Cao DN, Cheng H (co-first author), Wu W, Soo HM, Peng J**
Gibberellin mobilizes distinct DELLA-dependent transcriptomes to regulate seed germination and floral development in *Arabidopsis*. *Plant Physiology* 142: 509-525, 2006.

**Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP**
Integration of plant responses to environmentally activated phytohormonal signals. *Science* 311(5757):91-4, 2006.

**Cao DN, Hussain A, Cheng H, Peng J**
Loss of function of four DELLA genes leads to light- and gibberellin-independent seed germination in *Arabidopsis*. *Planta* 223: 105-113, 2005.

**Hussain A, Cao DN, Cheng H, Wen ZL, Peng J**
Identification of conserved Ser/Thr residues important for gibberellin-sensitivity of Arabidopsis RGL2 protein. *Plant J.* 44:88-99, 2005.

**Cheng H, Qin L, Lee S, Fu X, Richards D, Cao DN, Luo D, Harberd NP, Peng J**
Gibberellin regulates *Arabidopsis* floral development via suppression of DELLA protein function. Development 131: 1055-1064, 2004.

**Cheng H, Soo HM, Peng J**
DELLAs repress flower-specific genes *AtMYB21*, *AtMYB24* and *AtMYB57* through modulation of JA pathway in *Arabidopsis*. In preparation

# Chapter 1

# Literature Review

## 1.1 Gibberellins

Gibberellins (GAs) are important plant hormone. They are classified on the basis of structure as well as function. All gibberellins are derived from the *ent*-gibberellane skeleton (Fig. 1.1). There are currently 136 GAs identified from plants, fungi and bacteria (http://www.plant-hormones.info/gibberellins.htm). Only a few of them (for example, GA1, GA3, GA4, GA5 and GA6) are bioactive. GA4 acts as an active GA in regulating stem elongation and flowering in *Arabidopsis* (Xu et al., 1997; Eriksson et al., 2006). In monocot *Lolium temulentum*, GA5 and GA6 are the active GAs in the induction of flowering, but have little effects on stem elongation (King et al., 2001b; King et al., 2003). GAs are mainly present in actively growing tissues such as shoot apices, young leaves and flowers, indicating that GAs are primarily synthesized at the sites of their action (Kaneko et al., 2003). Comparison of expression pattern of genes involved in GA biosynthesis or GA signaling revealed that the sites where bioactive GAs synthesized almost overlap with the sites where GA signaling occurred, with the exception in aleurone and anthers (Kaneko et al., 2003). On the other hand, the presence of long-distance transport of GA was also reported (Hoad, 1995).

Gibberellins (GAs) act throughout the life cycle of plants regulating vegetative growth (including stem, hypocotyl and root elongation), seed germination, as well as reproductive development (including floral induction, floral organ development, embryo development and pollen tube growth) (Swain and Singh, 2005; Fleet and Sun, 2005). They play important role in agriculture. Commercially, Gibberellins are widely used to increase malting of barley during beer production and to increase fruit size of

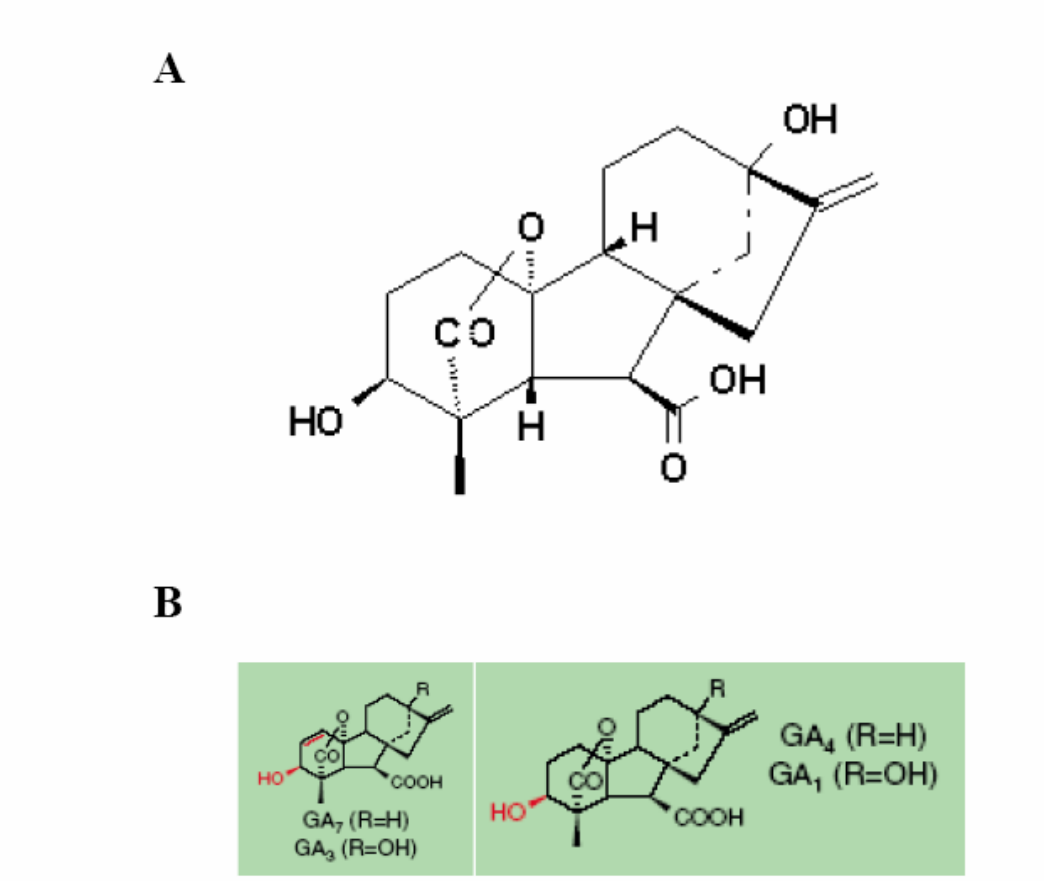


**Fig. 1.1. Gibberellins.** (A) Structure of *ent*-gibberellane skeleton. (B) Examples of structure of GAs derived from *ent*-gibberellane skeleton (Hedden and Phillips, 2000).

seedless grapes. The substantial increases in world wheat and rice yields during the "Green Revolution" were resulted from the introduction of dwarfing traits into the plants. Identification of these "Green Revolution" genes revealed that the interference with the action or production of GA resulted in dwarfing traits. Semi-dwarf wheat varieties carried a semi-dominant mutation in *Rht* genes which turned out to be the orthologues of *Arabidopsis* DELLA-domain genes *RGA* and *GAI* (GA-signaling components) (Peng et al., 1999; Silverstone and Sun, 2000). In contrast to wheat, dwarfing rice alleles contained a recessive mutation in *SD1* (*SEMIDWARF 1*) gene which is a GA biosynthesis gene encoding GA 20-oxidase (GA 20ox) (Hedden, 2003).

Insight into the mechanisms of GA-regulated plant growth and development has been gained from researches on both GA biosynthesis and signaling pathways. Majority of genes encoding enzymes involved in GA biosynthesis and catabolism pathways have been cloned and well characterized (Hedden and Phillips, 2000; Olszewski et al., 2002). Examination of the expression pattern of these genes by using reporter genes or *in situ* hybridization techniques led to the revelation of the sites of the GA metabolism during development and the homeostasis of bioactive GAs controlled by developmental and environmental cues.

Several factors have been identified to influence GA metabolism. These factors include type of tissue, development stages, light and responses to GA (hedden and Phillips, 2000). A set of 2-oxoglutarate-dependent dioxygenases, GA 20-oxdases (GA20ox) and GA 3-oxdases (GA3ox) which catalyze the later steps in the production of biologically active GAs, are the major targets for light regulation of GA metabolism (Kamiya and Garcia-Martinez, 1999). Distinct tissue and cell specific expression pattern of *GA3oxs* in *Arabidopsis* also suggested that individual *AtGA3ox* members played distinct developmental roles (Mitchum et al., 2006). Both *GA3oxs*

and *GA20oxs* are under feedback regulation by GA signaling (Sun and Gubler, 2004; Hedden and Phillips, 2000). In addition, it was reported that other endogenous signals such as auxin promote the expression of *GA20ox* and *GA3ox* (Garcia-Martinez et al., 1997; Van Huizen et al., 1997) (Fig. 1.2).

The signal transduction pathway transmits the GA signal from outside into cellular to regulate the gene expression and plant morphology. Significant progress has been made in understanding the molecular and biochemical basis of how plant response to GAs. These include the identification and characterization of the upstream GA signaling components, *trans*- and *cis*- acting factors that regulate downstream GA-response genes as well as the newly discovered molecular mechanism of GA-induced proteolysis of GA signaling repressors (Pimenta Lange and Lange, 2006).

## 1.2 The GA signaling components

Mutants with altered response to bioactive GA have been isolated through genetic screens. These GA response mutants fall into two phenotypic categories: with constitutively active GA responses (GA oversensitive) or impaired GA responses (GA insensitive). GA oversensitive mutants have a slender and paler-green phenotype which mimics wild-type plants that are treated with excessive GA. GA insensitive mutants display a dwarfed and dark-green phenotype which resembles the GA deficient mutants, but their dwarfing phenotype cannot be rescued by exogenous GA. Cloning of genes that are affected in these GA response mutants led to identify a number of negative and positive regulators of GA signal pathway (Sun and Gubler, 2004).

### 1.2.1 Negative regulators

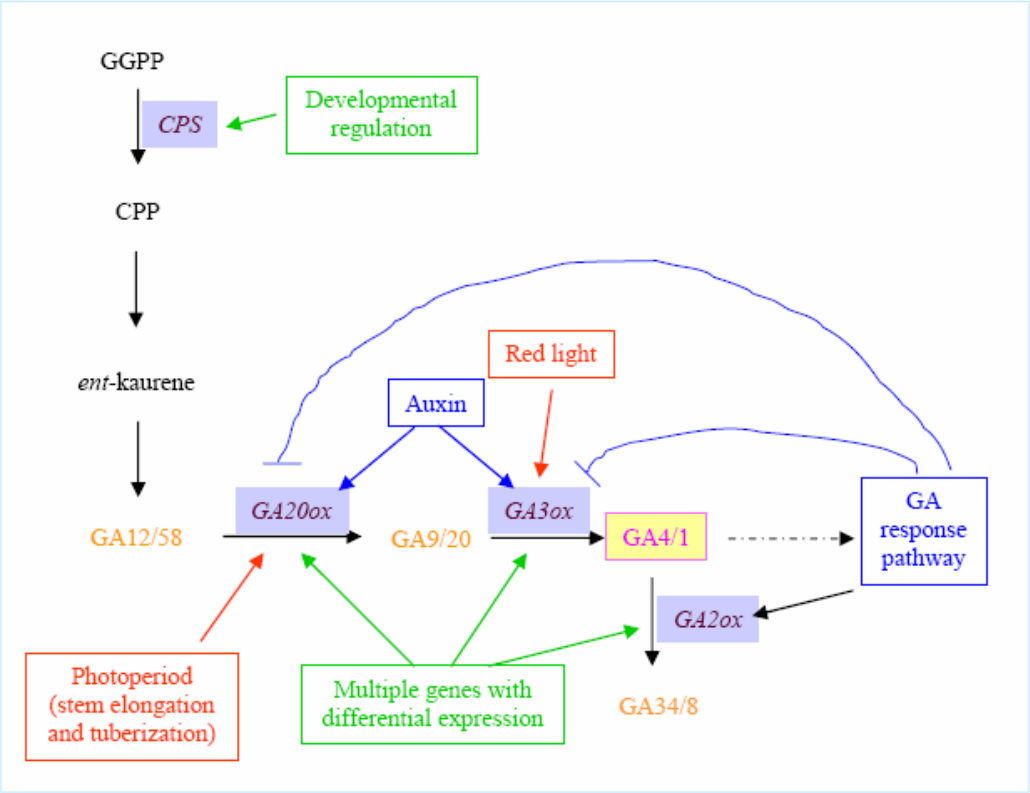


**Fig. 1.2. Regulatory mechanisms known to affect expression of the genes encoding enzymes for gibberellin (GA) metabolism (Hedden and Phillips, 2000).** Hormone and light regulation are indicated in blue and red, respectively, with arrow heads denoting enhanced gene expression and bars denoting suppressed expression. The green arrows indicate genes that have been shown to exhibit tissue-specific patterns of expression. The biologically active GAs are highlighted in yellow. Abbreviations: CPP, *ent*-copalyl diphosphate; *CPS*, CPP synthase; *GA2ox*, gibberellin 2-oxidase; *GA3ox*, gibberellin 3-hydroxylase; *GA20ox*, gibberellin 20-oxidase; GGPP, *trans*-geranylgeranyl diphosphate.

Several negative regulators of GA signaling including DELLA proteins, SPINDLY (SPY) and SHORT INTERNODE (SHI), have been identified by characterization of the recessive (loss-of-function) elongated GA-oversensitive mutants and the semi-dominant (gain-of-function) GA-insensitive mutants.

#### 1.2.1.1 DELLA proteins

DELLA proteins form the largest group of negative regulators of GA response. They are highly conserved in *Arabidopsis* (RGA, GAI, RGL1, RGL2, and RGL3) and several crop plants, including maize (d8), wheat (Rht), rice (SLR1), barley (SLN1), and grape (VvGAI) (Fig. 1.3) (Boss and Thomas, 2002; Olszewski et al., 2002). DELLA proteins belong to plant specific GRAS (GAI, RGA, SCARECROW) family of putative transcriptional regulators (Pysh et al., 1999). The *Arabidopsis* genome contains over 30 GRAS family members, all of which contain a number of characteristic features in C-terminal region, including 1) two leucine heptad repeats (LHR) which may mediate protein-protein interaction, 2) putative nuclear localization signals (NLS) which could localize the protein into nucleus (Itoh et al., 2002; Silverstone et al., 2001), and 3) a putative SH2 phosphotyrosine binding domain. Their N-termini are more divergent. DELLA proeins are named after their unique and conserved DELLA domain near the N terminus of the DELLA proteins. DELLA domain confers the GA response specificity of DELLA proteins. The polymeric Ser/Thr motif (poly S/T) could serve as the targets of phosphorylation or glycosylation (Fig. 1.4) (Richards et al., 2001).

##### 1.2.1.1.1 DELLA proteins in *Arabidopsis*

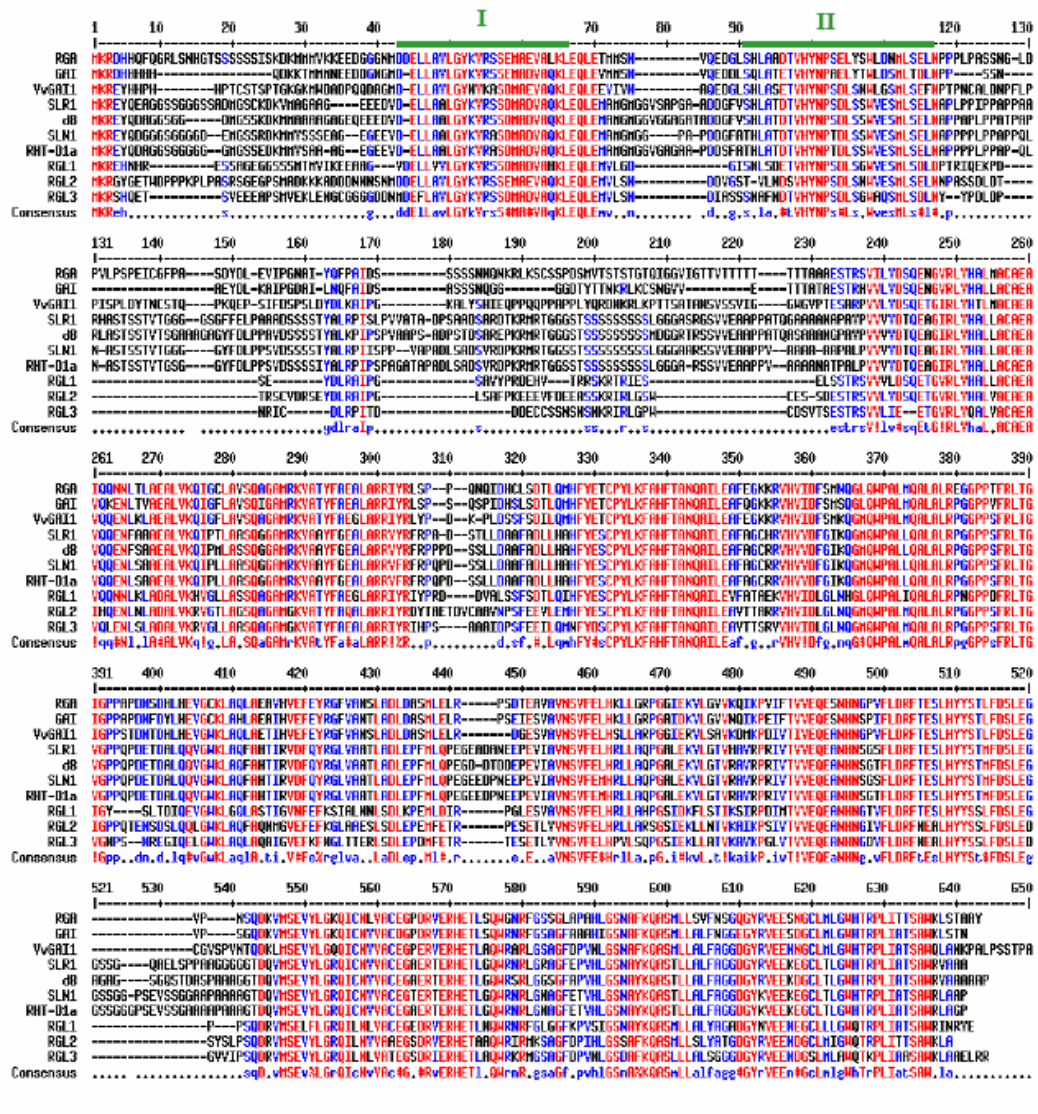


**Fig. 1.3. Alignment of DELLA protein sequences from *Arabidopsis* (GAI, RGA, RGL1-3), rice (SLR1), barley (SLN1), wheat (RHT1-D1a), maize (d8) and grape (VvGAI).** The highly conserved region I and II at N terminus are shown in green (Peng et al., 1999).

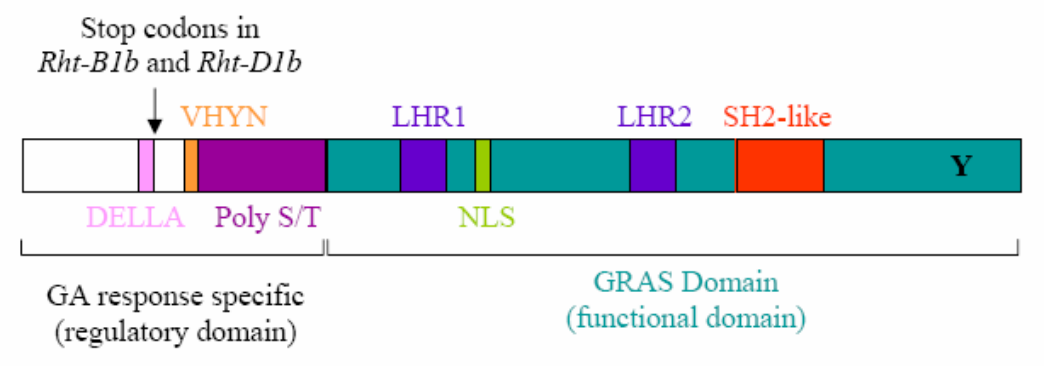


**Fig. 1.4. Schematic domain structure of DELLA protein (Sun and Gubler, 2004)**. The C-terminus (dark green) is highly conserved in all GRAS proteins and contains the repressor activity. Functional domains identified in this region include two leucine heptad repeats (LHR) (purple), the first of which mediates dimerization, a nuclear localization signal (NLS) and a SH2-like domain (red), which could indicate the involvement of phosphotyrosine signaling. The N-terminus (white) contains the GA-signaling domain. It is more variable, but includes two highly conserved motifs (named DELLA and VHYNP) that are required for GA-induced degradation, and a Poly S/T region. The arrow indicates position of stop codons in *Rht-B1b* and *Rht-D1b* (Hedden, 2003).

The first two DELLAs identified in *Arabidopsis* were *GAI* (*gibberellic acid insensitive*) and *RGA* (*REPRESSOR OF GA1-3 MUTANT* or *RESTORATION OF GROWTH ON AMMONIA*). The *Arabidopsis gai* mutants are dwarfed, dark green and flowering late in short day (Wilson et al., 1992; Koornneef et al., 1985). This phenotype cannot be rescued by GA treatment. *gai* mutants accumulate bioactive GAs to higher levels than wild type controls. *gai* is a semi-dominant mutation and was cloned by insertional mutagenesis (Peng et al., 1997). Wild type *GAI* encodes a protein that displays extensive homology with *SCARECROW* (*SCR*) at C-terminus (Sabatini et al., 2003; Di Laurenzio et al., 1996). The mutant *gai* allele contains a 51-base pair (in-frame) deletion in the sequence of wild type *GAI*, resulting in a mutant protein gai that lacks 17-amino acid residues in DELLA domain. This in-frame deletion confers a dominant dwarf, reduced GA responses phenotype (Peng et al., 1997).

*Arabidopsis RGA* gene was initially identified in a screen for mutations that suppressed the phenotype conferred by *ga1-3* (Silverstone et al., 1998). These recessive *rga* alleles partially suppress the defects conferred by *ga1-3* such as reduced stem elongation, delayed flowering as well as apical dominance. Like *ga1-3*, *rga ga1-3* plants are non-germinating and sterile. It contains low level of bioactive GA and application of GA can restore their fertility and other defects. Once cloned, *RGA* was found to be a homologue of *GAI* with 82% identity, and both of them belong to the plant specific GRAS family with a unique N terminus (Silverstone et al., 1998).

Although null alleles of *GAI* confer a no visible phenotype from wild type, they have increased PAC resistance, indicating that loss-of-function of *GAI* may partially suppress the effects of GA-deficiency (Peng and Harberd, 1993; Wilson and Somerville, 1995). Knock-out of both *RGA* and *GAI* allows for a GA-independent

rosette leaf expansion, flowering, and stem elongation (Dill and sun, 2001; King et al., 2001a). Therefore, *GAI* and *RGA* encode negative regulators of GA signaling in *Arabidopsis*.

Three other DELLAs, *RGL1*, *RGL2*, and *RGL3*, are present in *Arabidopsis*. Their specific roles in GA signaling were uncovered through reverse genetic studies. Screens of a *Ds*-transposant collection (Parinov et al., 1999; Sundaresan et al., 1995) for *Ds-GUS* within the *RGL1*and *RGL2* ORFs led to the isolation of recess mutants for *RGL1* (*rgl1-1*) and *RGL2* (*rgl2-1, rgl2-5, and rgl2-12*) (Lee et al., 2002). T-DNA insertion mutant alleles for *RGL1*, *RGL2*, and *RGL3* were also isolated (Tyler et al., 2004). *rgl2* mutants were strongly resistant to PAC in seed germination and loss-of-function of *RGL2* was able to suppress the non-germination phenotype of *ga1-3*, indicating that RGL2 may be the key suppressor in seed germination (Lee et al., 2002; Tyler et al., 2004). Further study showed that this function was enhanced by GAI and RGA (Cao et al., 2005; Penfield et al., 2006). None of the single mutation in *Arabidopsis* DELLA proteins shows any visible phenotype in floral development. RGA and GAI function together in controlling the stem elongation and flowering transition, but the floral organ development is still arrested in the *ga1-3rga-t2gai-t6* mutant (King et al., 2001a). Detailed analysis of different mutation combinations of DELLAs suggested that RGA, RGL1 and RGL2 act synergistically in repressing flower development (this thesis; (Cheng et al., 2004). Absence of RGA, RGL2, RGL1 and GAI leads to GA-independent plant growth (this thesis; Cheng et al., 2004; Tyler et al., 2004; Cao et al., 2005). These data indicated that four out of five DELLAs in *Arabidopsis* may play distinct and overlapping roles in *Arabidopsis* life cycle (Fig. 1.5).

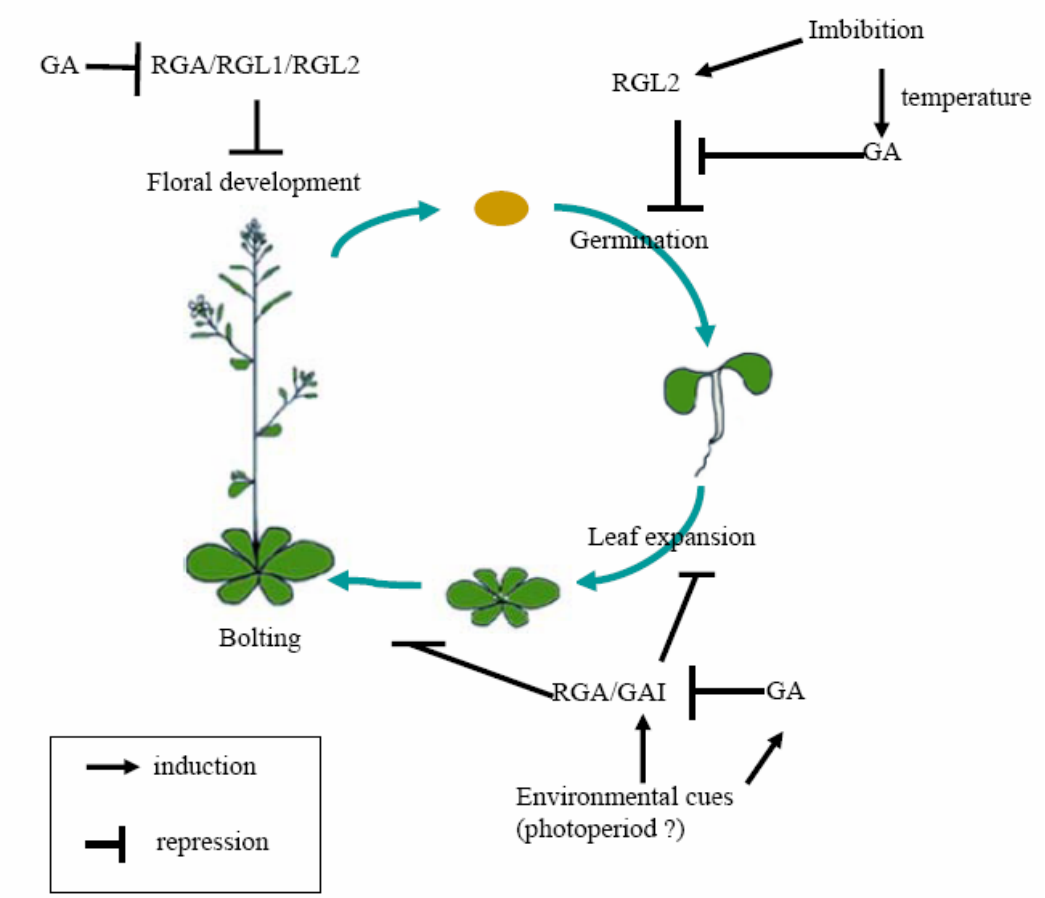


**Fig. 1.5. DELLA proteins function in *Arabidopsis* life cycle (Lee et al., 2002).** RGL2 links the environmental cue (moisture) with the GA-signaling pathway during the regulation of seed germination. Signaling through GAI and RGA mediates GA-promoted stem elongation, leaf expansion and flowering (peng et al., 1997; 1999, silverstone et al., 1998; Dill and Sun, 2001; King et al., 2001; Lee et al., 2002). Signaling through RGA, RGL1 and RGL2 mediates GA-promoted floral development (Cheng et al, 2004; Tyler et al., 2004).

#### 1.2.1.1.2 DELLA proteins in other species

Unlike in *Arabidopsis*, only one GAI/RGA functional ortholog is present in rice (*SLENDER RICE 1*(*SLR1*)) and barley (*SLENDER1* (*SLN1*)). Null alleles of *SLR1* and SLN1 displayed a constitutive GA response slender phenotype. Elongation was not affected by GA inhibitor in these mutants. Endogenous GA levels in mutants were lower than that in wild type. In contrast, over-expression of *SLR1* with a truncated DELLA domain showed a dominant GA-insensitive dwarf phenotype (Ikeda et al., 2001; Itoh et al., 2002). These data indicated that the function of DELLA proteins in repressing GA signaling may be highly conserved in various species.

In wheat and maize, *REDUCED HEIGHT-1* (*RHT-1*) and *dwarf-8* (*d8*) were identified as *GAI*/*RGA* functional orthologs, respectively. In particular, the introduction of wheat *Rht-B1b/Rht-D1b* semi-dwarf mutation alleles confer wheat semi-dwarf phenotype with an impressive increase in grain yields in 1960s, which was termed as "Green Revolution" (Peng et al., 1999). Molecular analysis revealed that *Rht-B1b/Rht-D1b* and *D8* alleles contained mutations that altered the N-terminal region of the protein. Genetic analysis indicated that *Rht-B1b/Rht-D1b* and *D8* made active products. All three *D8* alleles either have an in frame deletion within highly conserved region I (*D8-1*), or region II (*D8-2023*), or have a deletion that made an N-terminally truncated product that lacks region I and most of the region II (*D8-Mpl*) (Fig. 1.3, Fig. 1.4). The *Rht-B1b/Rht-D1b* mutations were both nucleotides substitutions that create stop codons to make N-trminally truncated products that lack region I (Peng et al., 1999).

Dwarfism associated "Green Revolution" mutation was also identified in grapevine. Genetic evidence showed that GAs inhibited flowering in grapevine (Boss et al., 2003). Characterization of a grapevine dwarf mutant revealed that the mutated

gene (called *VvGAI*) associated with the dwarf phenotype was a homologue of the wheat "green revolution" gene *RHT-1* and the *Arabidopsis* gene *GAI*. Sequence comparison of wild type *VvGAI* and its mutant allele *Vvgai1* indicated that mutant Vvgai1 contained a point mutation resulting in an amino acid change in DELLA region. The conversion of tendrils to inflorescences in the dwarf grapevine demonstrated that the grape tendril was a modified inflorescence inhibited from completing floral development by GAs (Boss and Thomas, 2002).

### 1.2. 1.2 SPINDLY (SPY) and SECRET AGENT (SEC)

Recessive mutations at the *SPY* locus of *Arabidopsis* conferred resistance to GA biosynthesis inhibitor PAC (Jacobsen and Olszewski, 1993). *spy* mutant plants were slender with constitutive GA response. Mutations in *SPY* partially rescued all the phenotypes of *ga1-3* including non-germination, dwarfing, dark green leaves, late flowering in long days and non-flowering in short days (Filardo and Swain, 2003); indicating that SPY might act as negative regulator of GA response. Over-expression of *SPY* in petunia phenotypically resembled PAC treated petunia wild type plants, further supporting the role of SPY as a negative regulator of GA action (Izhaki et al., 2001).

*SPY* encodes a putative OGT (O-linked N-acetyl-glucosamine transferase) and SPY was detected both in cytoplasm and nucleus in plant cells (Swain et al., 2002). In animal, OGT catalyze the transfer of O-linked N-acetylglucosamin (GlcNAc) from UDP-GlcNAc to Ser/Thr residues of proteins. GlcNAc modification may interfere or compete with kinases or phosphorylation sites and is implicated in regulating many signaling pathways (Roos and Hanover, 2000; Comer and Hart, 2000; Wells et al.,

2001). Like animal OGTs, purified recombinant SPY protein had OGT activity *in vitro* (Thornton et al., 1999).

SPY contains 10 copies of a tetratricopeptide repeats (TPR motif) at N-terminus and a catalytic domain at C-terminal (Jacobsen et al., 1996); Izhaki et al., 2001). The TPR of SPY interacts with SPY both in vitro and in yeast-hybrid assays, indicating that SPY may function as a homodimer by protein-protein interaction via TPR motif. Ectopic expression of TPR in *Arabidopsis* wild type caused a phenotype similar to loss-of-function *spy* mutants including resistant to GA biosynthesis inhibitors, short hypocotyl length and early flowering (Tseng et al., 2001). Over-expression of *SPY*'s TPR in petunia generated a dominant negative mutant and conferred resistance to PAC in seed germination (Izhaki et al., 2001). These data demonstrated that elevated TPRs alone may block the SPY functions by forming inactive hereodimers with SPY and/or by interacting with the target proteins of SPY, suggesting that the TPR domain could participate in protein-protein interactions and that these interactions were important for the proper function of SPY.

*spy* alleles were epistatic to *gai* and enhanced the *rga* phenotype, suggesting that SPY may act downstream of GAI (Jacobsen et al., 1996). However, if SPY is an OGT, it may modify GAI or RGA via addition of an O-GlcNAc moiety, rather than being a downstream signaling component (Swain and Olszewski, 1996; Harberd et al., 1998). It was reported that the function of OsSPY in GA signaling was not via changes in the amount or stability of SLR1, but by controlling the suppressive function of DELLA protein SLR1(Silverstone et al., 2006; Shimada et al., 2006).

There are two OGTs in *Arabidopsis*: SPINDLY (SPY) and SECRET AGENT (SEC). T-DNA insertion mutants of *SEC* did not exhibit obvious phenotypes. *sec* and *spy* mutations had a synthetic lethal interaction. SPY and SEC had overlapping

functions necessary for gamete and seed development (Hartweck et al., 2006). More recently, SEC has been shown to have unique role in the infection of *Arabidopsis* by *Plum pox virus* (Chen et al., 2005). More detailed analysis of the relationship between SPY and SEC revealed that unlike SPY, SEC had a limited role in GA signaling but it functioned in a partially redundant manner with SPY to regulate reproductive development (Hartweck et al., 2006).

*spy* mutants exhibited various phenotypic alterations that was not found in GA-treated plants. A detailed investigation of *spy* mutant phenotype suggested that SPY might play a role beyond in GA signaling (Swain et al., 2001). *spy* mutants were resistant to exogenously applied cytokinins, demonstrating that SPY acted as both a repressor in GA signaling and a positive regulator of cytokinin signaling (Greenboim-Wainberg et al., 2005). Study of HvSPY in barley aleurone showed that HvSPY played a negatively role for GA-induced promoter and a positively role for an ABA-induced promoter (Robertson et al., 1998). It was also reported that SPY and GIGANTEA (GI) interacted and acted in *Arabidopsis* pathways involved in light response, flowering, and rhythms in cotyledon development (Tseng et al., 2004). Therefore, the function of SPY in planta is more complicated than thought at first.

#### 1.2.1.3 SHORT INTERNODES (SHI)

*shi* (*short internodes*) mutant in *Arabidopsis*, caused by a transposon insertion, displayed a typical semi-dominant dwarf phenotype similar to GA deficient mutant. However this dwarfing phenotype could not be rescued by GA application, indicating *SHI* was involved in GA signal pathway. It contained elevated endogenous bioactive GA indicating that the feedback control of GA biosynthesis may be defective in this mutant. Cloning of the *SHI* gene revealed that suppression of GA response in *shi*

mutant was a result of over-expression of *SHI* (Fridborg et al., 1999). SHI contains a zinc finger domain, suggesting its role in transcriptional regulation. Transient expression of *SHI* in barley aleurone was able to suppress GA induction of barley α-amylase expression, supporting that SHI acts as a suppressor of GA response. SHI belongs to a gene family consisting nine members in *Arabidopsis*. Loss-of-function insertion alleles of *SHI* showed no phenotype. It was possible that SHI and SHI-related genes were functionally redundant (Fridborg et al., 2001).

### 1.2.2 Positive regulators

Several positive regulators of GA response including GA receptors and F-box proteins were identified by characterization of loss-of-function (recessive), GA unresponsive dwarf mutants. On the other hand, several other signaling components including U-box proteins and GAMYBs were also identified to function as positive regulators in GA signaling.

#### 1.2.2.1 GA receptor: GA INSENSITIVE DWARF 1 (GID1)

GAs are soluble in the inter- and intra-cellular compartment of plant tissues. It may cross the membrane by passive diffusion. Therefore, receptors on the protoplast surface may not be required for the perception of GA. It has been proposed that soluble GA receptors rather than membrane bound receptors may be involved in cell elongation (Hooley et al., 1992). The soluble 50 kDa GA-binding protein observed in aleurone by GA4 photoaffinity labelling may be a good candidate for a soluble GA receptor (Hooley et al., 1992). However, based on the induction of α-amylase gene expression in isolated aleurone protoplasts of *Avena fatua L.* by Sepharose beads-immobilized GA4, it was indicated that GA receptors might be located at, or near, the

external face of the aleurone plasma membrane (Hooley et al., 1990). Comparison of the effects of microinjected GA and extracellular GA on α-amylase expression in barley aleurone cells showed that injected GA did not elicit GA response, including induction of a-amylase expression, while extracellular GA did, further indicating that the perception of GA might occur at the external face of the plasma membrane of barley aleurone protoplasts (Gilroy and Jones, 1994). Therefore, it was proposed that plants might have both soluble and membrane-bound GA receptors. Two proteins (6 kDa and 18 kDa) were identified through gibberellin-photoaffinity labeling experiment in plant plasma membranes (Lovegrove et al., 1998). In contrast, *in vitro* binding and purification of radiolabeled GA4 have identified soluble GA-binding proteins in cucumber and *Azukia angularis* (Keith et al., 1982; Nakajima et al., 1997). Although both membrane-bound and soluble GA-binding proteins have been reported, their roles in GA perception or action await to be elucidated by the cloning and characterization of these genes.

Recently, rice GID1 was shown to possess the expected properties of the long-sought GA receptor. The rice *gid1* mutant appeared to be completely unresponsive to GA. SLR1 was epistatic to GID1 and was not degraded in the *gid1* mutant. The *GID1* encodes an unknown protein with similarity to the hormone-sensitive lipases and preferentially nuclear localized. The affinity between GID1 and bioactive GA were consistent with GID1 as a functional receptor. Most importantly, in a yeast two-hybrid assay, GID1 interacted with the rice DELLA protein SLR1 in a GA-dependent manner. Overexpression of GID1 resulted in a GA-hypersensitive phenotype. These data supported that GID1 was a soluble GA receptor. It was believed that on binding GA, AtGID1 binds to DELLA proteins to stimulate the ubiquitination of DELLA

proteins by promoting binding of DELLA to $SCF^{SLY1/GID2}$ E3 ubiquitin ligase (Ueguchi-Tanaka et al., 2005; Hartweck and Olszewski, 2006).

*Arabidopsis* contains three GID1 orthologs (AtGID1a, AtGID1b, and AtGID1c). The expression of *AtGID1*s in rice *gid1-1* mutant rescued the GA-insensitive dwarf phenotype of *gid1-1*, indicating that all three AtGID1s may function as GA receptors in *Arabidopsis*. Early genetic screens for GA response mutants did not identify any of these receptors, indicating there are significantly functional overlapping among these genes. Characterization of the knock-out mutants of *AtGID1s* suggested that they may function redundantly but specifically as well (Nakajima et al., 2006; Griffiths et al., 2006). *gid1a gid1b gid1c* triple mutant displayed a dwarf phenotype that was more severe than GA deficient mutant *ga1-3* and was completely insensitive to GA application. RGA accumulated in the *gid1a gid1b gid1c* triple mutant and loss of RGA function could partially rescue the phenotype of the triple mutant. Biochemical analyses revealed that all three recombinant proteins showed higher affinity to GA4 than to other GAs. Yeast two-hybrid and *in vitro* pull-down assays supported that AtGID1s interact with AtDELLAs in both GA4 and DELLA domain dependent manner. Furthermore, the GA-GID1 complex promotes the interaction between RGA and the F-box protein SLY1. All these results demonstrated that resembling rice GID1, AtGID1a, b and c also functioned as GA receptor in *Arabidopsis* (Nakajima et al., 2006; Griffiths et al., 2006).

#### 1.2.2.2 E3 ubiquitin ligases

The ubiquitin-proteasome pathway is very important for the hormone regulated cellular processes in plant. The general function of the ubiquitin/26S

pathway is to conjugate ubiquitin to Lys residues within the substrate proteins, thus targeting the degradation of these substrate proteins by the 26S proteasome. In the early steps of this pathway, three enzymes: the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) are involved. The E3 enzyme, containing either a HECT domain or RING/U-box domain, specifies the substrate (Moon et al., 2004).

#### 1.2.2.2.1 F-box proteins: GA-INSENSITIVE DWARF 2 (GID2), SLEEPY 1 (SLY1) and SNEEZY (SNE)

The SCF class of E3 ligases, which belongs to multiple subunit RING domain E3s, are the most thoroughly studied E3 in plant. The name is derived from their three subunits: SKP1 (ASK in plants for *Arabidopsis* SKP1), CDC53 (or Cullin), and the F-box protein. F-box proteins were named after the conserved 60-amino acid motif (F-box) at N terminus which is responsible for binding to ASK/SKP. F-box proteins represent the largest superfamily in *Arabidopsis*. The role of SCFs in plant development is extensive. They are involved in diverse processes including hormone response, phtotomorphogenesis, circadian rhythms, floral development and senescence (Moon et al., 2004).

Recently, characterization of the recessive GA-insensitive mutants identified several F-box genes involved in GA signaling. A severe GA-insensitive dwarf mutant, GA-*insensitive dwarf* 2 (*gid2*), was isolated in rice. *gid2-1 slr1-1* double mutant showed a slender phenotype identical to *slr1-1* single mutant, indicating that *GID2* functions upstream of *SLR1*. Positional cloning of *GID2* indicated that it encodes an F-box protein. Yeast two-hybrid analysis showed that GID2 interacted with OsSKP15 protein. GA-dependent degradation of SLR1 did not occur in *gid2* mutant. On the

other hand, an accumulation of phosphorylated SLR1 was observed in the GA-treated *gid2* mutant. These data suggested that GID2 may be a positive regulator of GA signaling and target the degradation of SLR1 which is initiated by GA-dependent phosphorylation ((Sasaki et al., 2003). Yeast two-hybrid assay and immuno-precipitation experiments demonstrated that GID2 formed a component of an SCF complex, specifically interacted with phosphorylated SLR1 proteins and triggered the GA-dependent degradation of SLR1 in rice (Gomi et al., 2004).

*sleepy 1* (*sly1*) was first isolated in a screen for suppressors of the ABA-insensitive mutant *abi1-1* in *Arabidopsis*. The *sly1* alleles were the first recessive GA-insensitive dwarfing mutants identified. They showed the full spectrum of phenotypes associated with severe GA deficient mutant, including the failure of germination in the absence of the *abi1-1* lesion (Steber et al., 1998). *rga* null allele partially suppressed the *sly1* mutant phenotype (McGinnis et al., 2003). Positional cloning of the *SLY1* genes revealed that it encodes a putative F-box protein. DELLA domain protein RGA was accumulated in *sly1* mutant even after GA treatment. These data suggested that $SCF^{SLY1}$ may mediate the degradation of RGA through 26S proteasome pathway (McGinnis et al., 2003). By yeast two-hybrid and in vitro pull-down assay, it was demonstrated that SLY1 directly interacted with RGA and GAI via their c-terminal GRAS domain (Dill et al., 2004; Fu et al., 2004).

Over-expression of *SLY1* in *sly1-2* and *sly1-10* mutant plants rescued the recessive GA-insensitive phenotype of these mutants. Surprisingly, antisense expression of *SLY1* also suppressed the phenotype of these mutants (Strader et al., 2004). These data led to the hypothesis that the *SLY1* homologue *SNE* could functionally replace *SLY1* in the absence of the recessive interfering *sly1-2* or *sly1-10* genes. This hypothesis was verified by the result that over-expression of *SNE* in *sly1-*

*10* plants restored normal RGA level and rescued the dwarf phenotype of *sly1-10* plants (Strader et al., 2004).

#### 1.2.2.2.2 U-box arm-repeat protein: PHOTOPERIOD REGULATED 1 (PHOR1)

U-box domain, designated after a 70 amino acid motif of yeast ubiquitination factor UFD2, is related to the RING finger motif of E3 ubiquitin ligase. U-box domain is similar in structure to RING finger domain, but does not require Zinc ions to stabilize the motif. The first U-box proteins identified, UFD2, CHIP and NOSA, were implicated to function as ubiquitin ligases in ubiquitin-dependent protein degradation (Hatakeyama et al., 2001; Jiang et al., 2001).

PHOR1 was identified to be a novel component of the GA signaling pathway in potato. Resembling the GA-deficient mutants, antisense inhibition of *PHOR1* produced semi-dwarf phenotype. The antisense lines showed a reduced response to GA application and accumulated higher GA levels than wild type, indicating that PHOR1 may function as GA signaling components. *PHOR1* encodes an arm repeat containing protein similar to the *Drosophila* segment polarity gene *Armadillo* and contains a U-box domain in its N-terminal (Amador et al., 2001). Three *PHOR1* homologs have been identified in *Arabidopsis*, suggesting that the function of U-box domain proteins might be conserved in different species (Monte et al., 2003). Further studies are required to demonstrate if U-box domain protein PHOR1 really functions as a ubiquitin E3 ligase to ubiquitinate its potential targets, DELLA proteins, for proteasome degradation.

### 1.2.2.3 GAMYB transcription factors

GA activates the expression of α-amylase gene expression in aleurone cells. Functional analysis of α-amylase gene promoters led to identify a 21bp GA response element (GARE) containing a conserved sequence TAACAA/GA. A number of other *cis*-acting elements including Box1/O2S-like element, pyrimidine box (C/TCTTTT) and TATCCAC box in α-amylase gene promoters have also been shown to act as enhancers within GA response complex (GARC) (Gubler and Jacobsen, 1992; Lanahan et al., 1992; Tregear et al., 1995; Woodger et al., 2003; Sun and Gubler, 2004).

The similarity of TAACAA/GA sequence to plant and animal MYB binding sites led to the isolation of a GA-regulated transcription factor HvGAMYB in barley aleurone cells. HvGAMYB contains a typical R2/R3-MYB DNA binding domain at N terminus and two transcriptional activation domains at C terminus. It bound specifically to the TAACAA/GA sequence of GARE (Gubler et al., 1995). Transiently expressed HvGAMYB strongly activated the α-amylase promoter, indicating that HvGAMYB functioned as a transcriptional activator of α-amylase (Gubler et al., 1999; Gubler et al., 1995). Transient silencing of *HvGAMYB* in aleurone cells caused a dramatic reduction of α-amylase promoter activity and constitutive expression of *HvGAMYB* triggered α-amylase promoter activity. These data indicated that *HvGAMYB* expression was necessary and sufficient for the GA induction of α-amylase gene expression (Zentella et al., 2002).

The involvement of GAMYB as a trans-activator of GA signaling was not restricted in the cereal aleurone system. In addition to aleurone, HvGAMYB was found to be strongly expressed in anthers. Transgenic barley plants with over-expression of *HvGAMYB* failed to dehisce and were male sterile, suggesting that GAMYB may function in anther development (Murray et al., 2003).

Long-day promotes the expression of *LtGAMYB* in grass *Lolium temulentum* in the apex, suggesting that *GAMYB* may play a role in flowering (Gocal et al., 1999). A role of *GAMYB* in flowering was also investigated in *Arabidopsis*. Based on the sequence similarity, *GAMYB*-like genes, *MYB33*, *MYB65*, *MYB97*, *MYB101* and *MYB120* have been identified in *Arabidopsis* (Gocal et al., 2001; Stracke et al., 2001). Transient expression assays indicated that MYB33, MYB65 and MYB101 were able to functionally substitute for HvGAMYB in transactivation of the α-amylase promoter in barley aleurone (Gocal et al., 2001). Like *LtGAMYB*, an increase of expression of *MYB33* at shoot apex coincided with the onset of flowering. In addition, expression of *MYB33* overlapped with the expression of floral meristem identity gene *LEAFY* (Gocal et al., 2001). Furthermore, MYB33 specifically bound to a MYB-binding site within *LEAFY* promoter, an element that was known to be essential for GA activation of *LEAFY* promoter (Blazquez and Weigel, 2000). Therefore, GA may regulate flowering through inducing the expression of *GAMYB* in the apex.

Recently, characterization of *GAMYB* knock-out mutants in both rice and *Arabidopsis* has led to define the roles of *GAMYB* in GA-regulated processes outside cereal aleurone. The induction of α-amylase expression by GA in the endoderm was blocked in the rice *gamyb* mutant alleles. No obvious phenotype was observed in the mutants at vegetative stage. After phase transition to the reproductive stage, the internodes of mutants were shortened and floral development, especially pollen development was affected. These results demonstrated that, in addition to its role in the induction of α-amylase in aleurone, *OsGAMYB* was also important for pollen development (Kaneko et al., 2004). Knockout mutants of *Arabidopsis MYB33* and *MYB65* were also isolated. Characterization of the mutant alleles revealed that *MYB33* and *MYB65* functioned redundantly in controlling anther development. Double mutant

*myb33myb65* was conditionally male sterile due to the premeiotically blocking of pollen development (Millar and Gubler, 2005).

Time-course studies have shown that GA induced a rapid increase in *HvGAMYB* gene expression prior to α-amylase gene expression in barley aleurone layers. The increase in *HvGAMYB* might due to an increase in the rate of transcription or at postranscription level (Gubler et al., 2002). DELLA protein SLN1 acted as a repressor in *HvGAMYB* expression in aleurone cells. There is a lag time of 1h between SLN1 degradation and the expression of *HvGAMYB,* indicating that SLN1 is not a direct repressor of *HvGAMYB*. Like many other GA response genes in cereal aleurone, *HvGAMYB* was also repressed by ABA which acts downstream of SLN1 (Gomez-Cadenas et al., 2001; Penson et al., 1996). The facts, that there were multiple isoforms of *HvGAMYB* in aleurone and *HvGAMYB* was detectable in the non-GA treated aleurone without the accumulation of α-amylase, indicated that *HvGAMYB* might be regulated at posttranscriptional level as well. In addition, MYB transcription factors may also be modulated by phosphorylation and acylation (Vorbrueggen et al., 1996; Tomita et al., 2000).

MYB transcription factors may operate as part of large transcriptional complex. HvGAMYB-binding proteins have been identified in barley aleurone. These factors could be involved in the posttranscriptional regulation of HvGAMYB. DOF (DNA-binding with one finger) transcription factors bind to pyrimidine boxes in hydrolase GARC and regulate hydrolase expression in aleurone cells (Washio, 2003). BPBF (Barley Prolamine Box-binding Factor), a barley DOF transcription factor, was found to interact with HvGAMYB and repress HvGAMYB-mediated trans-activation of the promoter of the GA-response protease gene, *AL21*, in barley aleurone (Mena et al., 2002; Diaz et al., 2002). On the other hand, SAD (Scutellum and Aleurone-

expressed DOF), another DOF transcription factor, interacted with HvGAMYB to positively regulate the function of HvGAMYB (Isabel-LaMoneda et al., 2003). In addition, KGM (Kinase associated with GAMYB), another GAMYB-binding protein, was isolated in barley aleurone and identified to be a repressor of GAMYB in barley aleurone (Woodger et al., 2003).

Recently, regulation of *GAMYB* by miRNA159 was reported. MiRNA159 is a microRNA of 21 nucleotides sequence which is near-perfect /closely complementary to an internal sequence of the mRNA of *AtGAMYBs*, *HvGAMYB* and *OsGAMYBs*. MicroRNAs can regulate gene expression by directing cleavage or by inhibition of translation of target transcripts (Llave et al., 2002; Chen, 2004; Aukerman and Sakai, 2003). In *Arabidopsis*, miRNA159 was regulated by GA through DELLA protein. It was able to direct the cleavage of mRNA encoding GAMYB-related proteins (e.g. AtMYB33). Over-expression of miRNA159 caused a reduction of *LEAFY* transcript levels, delayed flowering in short day and perturbed anther development (Achard et al., 2004). Translational GUS fusion study indicated that miRNA159 target sequence in *MYB33* was important for restricting the expression of *MYB33* in anther for its proper function (Millar and Gubler, 2005). In rice, it was suggested that the regulation of *OsGAMYB*s was different in aleurone and flowers. MiRNA159 was co-expressed with *GAMYB* or -like genes in anthers rather than aleurone. Thus, it seemed that miRNA159 might only regulate *OsGAMYB* and -like genes in flowers rather than in aleurone (Tsuji et al., 2006). However, recent report showed that ABA induced the expression of miR159 to control transcript levels of two *MYBs* (*MYB33* and *MYB101*) during *Arabidopsis* seed germination (Reyes and Chua, 2007).

Microarray analysis revealed that OsGAMYB and its upstream regulator SLR1 were involved in the regulation of almost all GA-mediated gene expression in

rice aleurone cells and different sets of genes were regulated by *GAMYB* in aleurone cells and anthers, indicating that OsGAMYBs function differently in aleurone and anther (Tsuji et al., 2006).

#### 1.2.2.4 Heterotrimeric G protein- DWARF 1 (D1) in rice and G PROTEIN in *ARABIDOPSIS* (GPA1)

Heterotrimetric G proteins mediate the transmission of the external signals via receptor molecules to effector molecules. The G proteins consist of three different subunits: alpha, beta and gamma subunits. Evidences showed that alpha subunit of G proteins (Gα) were involved in GA signaling at low GA concentrations. Rice *dwarf 1* (*d1*) mutant is a GA-unresponsive dwarf mutant resulting from a defect in *DWARF 1*(*D1*) gene which encodes Gα protein (Fujisawa et al., 2001; Ashikari et al., 1999). In the presence of low GA concentrations, the GA induction of *OsGAMYBs* and alpha-amylase was dramatically reduced in *d1* aleurone. However, at higher GA concentrations, response to GA in *d1* mutant seemed to be normal, indicating that *D1* was involved in GA signaling and an alternative GA-signaling pathway that independent of Gα protein might also be present in rice (Mitsunaga et al., 1994; Ueguchi-Tanaka et al., 2000; Kaziro et al., 1991; Ashikari et al., 2003)..

Null mutation in Gα protein in *Arabidopsis* (GPA1) has resulted in100-fold less response to GA. Meanwhile, Plants with over-expression of *GPA1* had increased sensitivity to GA, yet GA was still required for its germination, indicating that GPA1 might indirectly operate on GA pathway to control germination (Ullah et al., 2002a).

*Gα* gene was a single copy gene in rice and *Arabidopsis*. Analysis of the mutants for *Gα* gene indicated that *Gα* gene might also be involved in many other signaling pathways such as auxin, ABA and Brassinolide (BR). Studied on *gpa1*

mutant indicated that BR signaling pathway seemed to be more directly coupled by heterotrimetric G protein at various points in plant development (Wang et al., 2001; Ullah et al., 2001; Ullah et al., 2003; Ullah et al., 2002b).

#### 1.2.2.5 PICKLE (PKL)

*PKL* gene was identified during screening for *Arabidopsis* mutants exhibiting abnormal root development. The *pkl* plants displayed several shoot phenotypes that were characteristics of *Arabidopsis* GA-deficient mutants including dwarfism, dark green leaves with short petioles, delayed bolting and reduced apical dominance (Ogas et al., 1997). *pkl* plants exhibited reduced GA responsiveness and increased endogenous GAs (Henderson et al., 2004). These phenotypes suggested that *PKL* may play a role in GA response. The synergistic effect of combining the *pkl* and *gai* mutations in flowering further supported that *PKL*, like *GAI*, may be involved in GA signaling pathway (Ogas et al., 1997).

Roots of *pkl* mutant exhibited embryonic traits after germination. This "pickle root" phenotype was dependent on GA. In *pkl* seedling, GA repressed the penetration of "pickle root" phenotype while inhibiting GA biosynthesis strongly enhanced this phenotype (Ogas et al., 1997). Although the penetrance of the "pickle root" phenotype was strongly response to GA, it is only marginally responsive to ABA and mutation on *SPY* (Henderson et al., 2004). These data suggested that GA response pathway that mediates the repression of embryonic identity in *pkl* seedling may be distinct from previously characterized GA response pathway that regulates seed germination (Henderson et al., 2004).

Cloning of *PKL* revealed that it encodes a CHD3 chromatin remodeling factor, suggesting that the inability of *pkl* seedling to repress embryonic identity might result

from a defect in transcription regulation (Ogas et al., 1999). Several embryonic identity genes which were repressed by *PKL* were identified through microarray studies (Rider et al., 2003). However, these genes were not affected by GA biosynthesis inhibitor. Therefore, further characterization of genes that are regulated by both GA and *pkl* might be needed to elucidate the function of *PKL* in GA signaling (Sun and Gubler, 2004).

### 1.2.3 Additional potential components

With Genome-sequencing efforts and microarray technology, a number of genes that potentially act in GA signaling have been indicated.

#### 1.2.3.1 WRKY proteins

The WRKY proteins are a superfamily of transcription factors that control diverse developmental and physiological processes. The WRKY domain is defined by the conserved amino acid sequence WRKYGQK at its N terminus (Eulgem et al., 2000). Two wild oat (*A. sativa* subsp.*fatua*) WRKY proteins (ABF1 and ABF2) were able to bind to the promoter of GA-regulated gene, *α-Amy2*. This data indicated that WRKY proteins might modulate the GA response in aleurone (Rushton et al., 1995).

Analysis of rice (Oryza sativa) genomic sequences identified at least 105 *WRKY* genes, among which *OsWRKY71* is highly expressed in aleurone cells. *OsWRKY71* expression was repressed by GA application. It was suggested that OsWRKY51 and OsWRKY71 may be transcriptional repressors of GA signaling in aleurone cells by functionally interfering with OsGAMYB (Zhang et al., 2004; Xie et al., 2006; Xie et al., 2007).

#### 1.2.3.2 Others

Whole genome expression profiling and proteomic technology provided powerful tools to facilitate the identification of new GA signaling components and tissue specific GA regulated genes. Microarray analysis in *Arabidopsis* and rice have identified a number of GA- and DELLA-regulated genes in seed germination and floral development (Ogawa et al., 2003; Bethke et al., 2006; Cao et al., 2006; Tsuji et al., 2006). Further studies on these genes and their respective mutants would be of great help to our understanding of GA response pathway.

## 1.3 GA induced proteolysis of the DELLA proteins via the ubiquination-proteasome pathway

In plants, targeted degradation of regulatory proteins by the ubiquitin/26S proteasome pathway contributes significantly to development by affecting a wide range of processes including embryogenesis, hormone signaling and senescence. Based on genetic and biochemical studies on DELLA proteins, the "relief-of-restraint" model proposed that DELLA proteins restrain plant growth, while GA promotes plant growth by relieving this DELLA-mediated growth restraint via triggering degradation of DELLA proteins (Harberd, 2003)**.**

As mentioned earlier, DELLA proteins are key repressors of GA response. Studies of the effect of GA on *Arabidopsis* RGA revealed that the levels of GFP-RGA fusion protein and endogenous RGA were reduced rapidly by GA treatment, indicating that GA promotes plant growth by degrading the repressor protein RGA (Silverstone et al., 2001). Similarly, GA regulates the appearance and disappearance of rice SLR1 in nuclei (Itoh et al., 2002). Using specific inhibitors of proteasome function, it was found that proteasome-mediated protein degradation was necessary

for GA-mediated destabilization of barley SLN1 (Fu et al., 2002). Further experiments with protein kinase and protein phosphatase inhibitors indicated that phosphorylation and dephosphorylation steps were also involved for GA-mediated degradation of SLN1 and RGL2 (Fu et al., 2002; Hussain et al., 2005). Site-directed substitution studies of conserved serine/threoine and tyrosine residues suggested that dephosphorylation of serine/threonine and phosphorylation of tyrosine were critical in GA induced degradation of RGL2 via the proteasome pathway (Hussain et al., 2007; Hussain et al., 2005).

17 amino acid in-frame deletions in DELLA domain of gai mutant protein made it a constitutive repressor of GA response (Peng et al., 1997). Similar internal deletions in other DELLA proteins from different species also resulted in a GA-unresponsive dwarf phenotype ( Peng et al., 1999; Boss and Thomas, 2002; Chandler et al., 2002). It was revealed that mutation in DELLA motif in RGA, SLR1 and SLN1 made the mutant protein resistant to GA-induced degradation, indicating that DELLA motif was essential for GA-induced degradation of DELLA proteins (Dill et al., 2001; Gubler et al., 2002; Itoh et al., 2002). Recent experiment showed that the N-terminal sequence containing DELLA domain was necessary for GID1 binding (Griffiths et al., 2006). In addition, studies on transgenic plants with over-expression of different kind of truncated SLR1 revealed that additional domains for example LHR1 and C terminus (Fig. 1.4) were also required for GA induced DELLA protein degradation (Itoh et al., 2002; Gubler et al., 2002).

F-box proteins SLY1 and GID2 are positive regulators of GA response. $SCF^{SLY1/GID2}$ positively regulates GA signaling by causing the destruction of the negative regulators, DELLA proteins. This conclusion is based on the fact that RGA and SLR1 accumulated at higher levels in *sly1* and *gid2* mutants than in wild type,

respectively. Null mutation of RGA could rescue the dwarf phenotype of *sly1* mutants. Similarly, null mutation of SLR1 also restored the normal height of *gid2* mutants. DELLA proteins have been shown to interact with F-box proteins SLY1 and GID2. It was found that GA induced an accumulation of phosphorylated SLR1 in *gid2* mutant, suggesting that GID2 may preferentially target the phosphorylated SLR1 for degradation (McGinnis et al., 2003; Sasaki et al., 2003; Gomi et al., 2004). In *Arabidopsis*, $SCF^{SLY1}$ also interacted more strongly with phosphorylated DELLA proteins (Fu et al., 2004). Based on these results, DELLA proteins are believed to be degraded through the 26S proteasome pathway once ubiquitinated by $SCF^{SLY1/GID2}$.

### 1.4 Model of GA signaling pathway

The process of GA signaling in the nucleus depends directly on presence or absence of DELLA protein activity. DELLA proteins are putative transcription regulators that inhibit the expression of GA-activated genes. GA releases the repression of DELLA proteins through proteolysis of DELLA proteins. The GA-induced proteolysis of DELLA proteins is dependent on $SCF^{SLY1/GID2}$ E3 ligase. An $U^{PHOR1}$ E3 ligase might also be involved in these proteolysis process (Fig. 1.6) (Thomas and Sun, 2004; Gomi and Matsuoka, 2003; Sun and Gubler, 2004).

SPY may act as negative regulator of GA response through activating DELLA protein by GlcNAc modification. OsGID1 and AtGID1-like were soluble GA receptors. Binding on GA, GID1 binds to DELLA domain of DELLA proteins to stimulate the ubiquitination of DELLA proteins by promoting binding of DELLA to the $SCF^{SLY1/GID2}$ E3 ligase (Willige et al., 2007). Through an unknown mechanism, GA induced the phosphorylation of DELLA proteins. Binding affinity of DELLA protein and $SCF^{SLY1/GID2}$ was increased by GA-induced-phosphorylation of DELLA

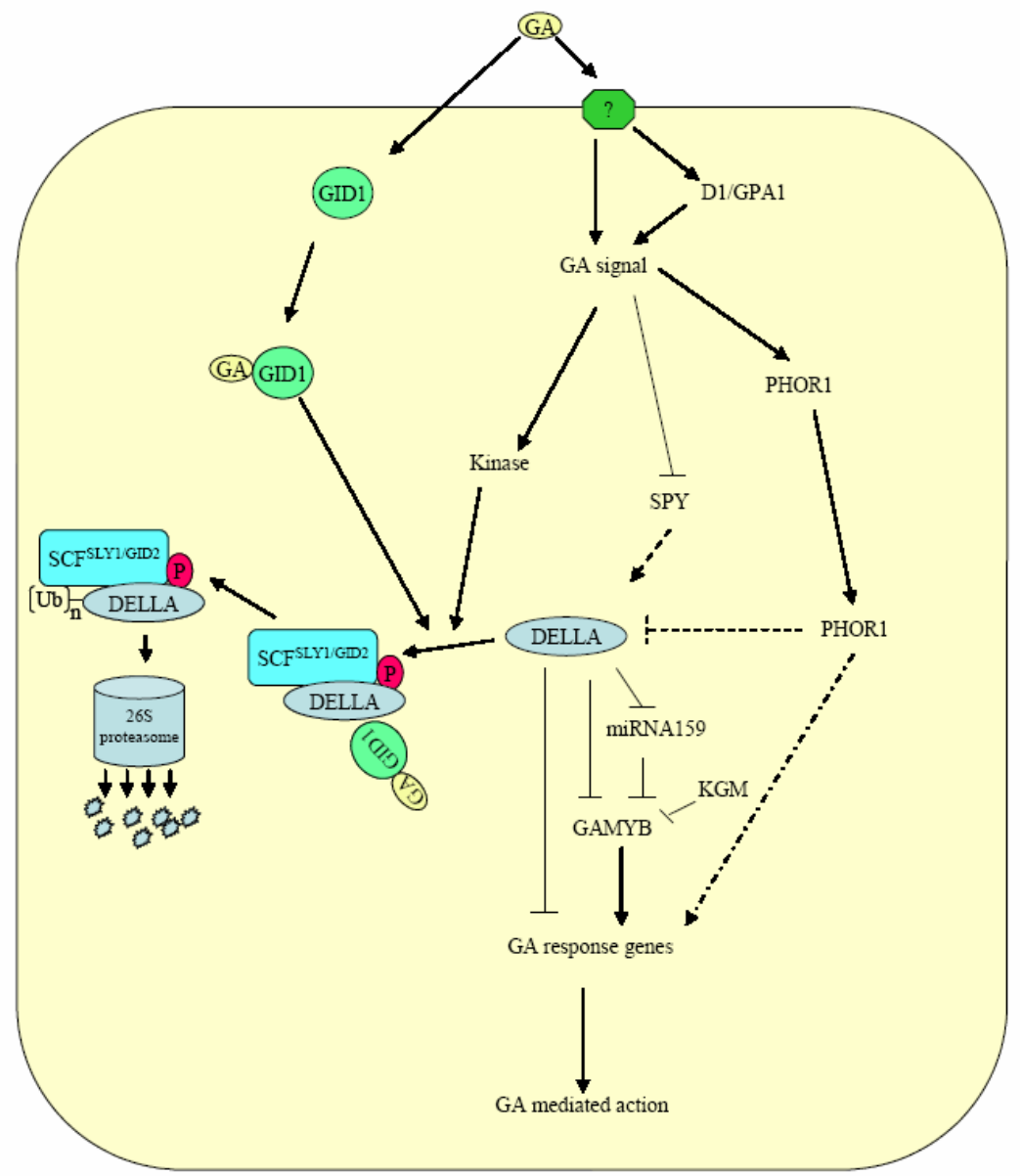


**Fig. 1.6. Model of GA signaling pathway**. Arrows and T-bars indicate positive and inhibitory effects, respectively. Weak and strong effects are represented by dotted and solid lines, respectively. [Ub]n represents polyubiquitination.

proteins (Fig. 1.6).

GAMYBs are GA activated genes that act downstream of DELLA proteins. In *Arabidopsis* GA regulates GAMYB through miRNA159, which acts downstream of DELLA protein. In barley aleurone cells, GAMYB activity is repressed by KGM (Fig. 1.6) (Woodger et al., 2003).

## 1.5 GA signaling and GA metabolism

The genes encoding GA biosynthesis enzymes have been well characterized in numerous species. GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox), enzymes catalyzing the final steps of the biosynthesis of GA, are particular important for controlling bioactive GA levels. In addition, GA2-oxidase (GA2ox) which is responsible for the deactivation of GA also plays important role in the modulation of bioactive GA levels.

GA response pathway is tightly linked to its biosynthesis and catabolism (Olszewski et al., 2002). The constitutive GA-response mutants (e.g., rice *slr1*, barley *sln1* and *Arabidopsis rga/gai-t6*) contain lower bioactive GA levels than in wild type. It was found that mRNA levels of GA20ox and/or GA3ox genes were present at lower levels in these mutants than in wild type (Croker et al., 1990; Dill and Sun, 2001; Silverstone et al., 2001). On the other hand, in reduced GA-responsive mutants, such as in gain-of-function mutant (*gai*) and loss-of-function mutant (*sly1*), higher levels of bioactive GA and *GA20ox/GA3ox* mRNA were accumulated (Ueguchi-Tanaka et al., 2000; Talon et al., 1990; McGinnis et al., 2003). These results indicated that the biosynthesis and catabolism of GA were subject to feedback regulation by GA signaling pathway.

The molecular mechanism involved in feedback regulation is not elucidated yet. REPRESSION OF SHOOT GROWTH (RSG), a tobacco trans-activator with a bZIP domain, regulates endogenous GA levels by controlling *ent*-Kaurene Oxidse gene in GA biosynthetic pathway. GA regulates the intracellular translocation of RSG. Over-expression of dominant negative mutant form of RSG confers a GA deficient phenotype and feedback regulation of *GA20ox* was affected in this transgenic tobacco. These data suggested that RSG might play a role in feedback regulation of *GA20ox* (Fukazawa et al., 2000; Ishida et al., 2004; Takahashi et al., 2003).

## 1.6 Interaction between GA and other hormone signaling pathways

The GA metabolism and response pathways integrate with other signaling pathways to regulate plant growth and development. GA and ABA antagonized in regulating developmental transition from embryogenesis to seed germination. In barley aleurone cells, GA induces while ABA inhibits the expression of α-amylase. α-amylase expression in *sln1* mutant was blocked by ABA, indicating that ABA signaling acts downstream of SLN1 in barley aleurone (Gomez-Cadenas et al., 2001; Zentella et al., 2002; Olszewski et al., 2002). On the other hand, evidences showed that miR159 played a role in the ABA repression of GA induction of GAMYB (Reyes and Chua, 2007).

SPY is a negative regulator of GA response. However, *spy* mutants also showed reduced response to cytokinin. Both GA and *spy* mutation suppressed numerous cytokinin responses. But cytokinin had no effect on GA biosynthesis or responses. These data suggested that SPY may act as a repressor of GA response and a positive regulator of cytokinin signaling. Hence SPY might play a central role in the regulation of the cross talk between GA-cytokinin response pathways during plant

development (Greenboim-Wainberg et al., 2005). *OsSPY* antisense and RNAi plants showed increased lamina joint bending, which is a brassinosteroid-related phenotype, indicating that OsSPY might play roles in both GA signaling and brassinosteroid pathway (Shimada et al., 2006).

DELLA proteins have been proposed to act as a point of intersection for several hormone-signaling pathways as well as environmental cues. It was reported that lower concentration auxin was necessary for GA-mediated *Arabidopsis* root growth by promoting GA-dependent degradation of DELLA proteins (Fu and Harberd, 2003), whereas ethylene inhibits *Arabidopsis* root growth and flowering by delaying the GA-induced DELLA destabilization (Achard et al., 2003; Achard et al., 2007; Achard et al., 2003). In addition, DELLA proteins also integrate responses to independent hormonal and environmental signals of adverse conditions (Achard et al., 2006).

## 1.7 Gibberellins and flower development

Flower development starts from the specification of the floral meristem identity followed by the floral organ identity. In response to both environmental and endogenous signals, the plant switches from vegetative growth to reproductive growth. This process is controlled by a large group of flowering time genes. Signals from various flowering time pathways are integrated and lead to the activation of a small group of meristem identity genes that specify the floral identity (Jack, 2004). The promotion of flowering by GA is one of the four major flowering time promotion pathways. The GA deficient mutant such as *ga1* in *Arabidopsis* never flowering in short days and delays flowering in long days with the retarded growth of all floral organs (especially the development of petal and stamen) (Koornneef and van der

Veen, 1980). Application of exogenous GA can restore all the flower defects of *ga1*, indicating that GA is an important stimulator of flowering in the absence of long day promotion and plays an important role in the floral organs development in the long days (Koornneef and van der Veen, 1980).

It has been demonstrated that GA promotes flowering of *Arabidopsis* by activation of flower meristem identity gene *LEAFY* (*LFY*) and controls floral organ development through induction of the expression of the floral homeotic genes: *APETALA3* (*AP3*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) (Blazquez et al., 1998; Yu et al., 2004; Eriksson et al., 2006; Yanofsky, 1995). As shown previously, lack of GAI and RGA substantially suppressed the effect of the *ga1-3* mutation on flowering time (a measure of time of floral initiation) in SD (Dill and Sun, 2001). SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1), an integrator of GA and verbalization pathways, seems to function downstream of DELLA protein in controlling flowering (Moon et al., 2003).

Although little is known about how GA controls floral organ development, previous reports have suggested that GA signaling component may modulate the GA-regulated floral development. Over-expression of *SPY* inhibited postmeiotic anther development in petunia (Izhaki et al., 2002). In addition, transgenic expression of wild type or mutant forms of *GAI* in tobacco and *Arabidopsis* resulted in male sterile phenotype owing to the delayed stamen development (Huang et al., 2003; Hynes et al., 2003). Furthermore, lacking of rice or barley DELLA proteins SLR1 and SLN1 impaired the floral development (Ikeda et al., 2001; Chandler et al., 2002). Recently, genetic studies have revealed that GA regulates flower development via suppression of DELLA proteins. It was shown that GA promotes floral organ development via

suppressing the function of RGA, RGL2 and RGL1 synergistically (this thesis, Cheng et al., 2004, Tyler et al., 2004; Yu et al., 2004).

## 1.8 Conclusions

During last decade and during the course of our studies, significant progresses have been achieved in understanding of GA signaling pathway. These achievements include the identification of signaling components including positive regulators (e.g. F-box proteins, GID1 and GID1-like), negative regulators (e.g. DELLA proteins), and mechanisms of the proteolysis of DELLA negative regulator. DELLA proteins have been indicated to act as a control molecule in modulating information from several hormone pathways and environmental cues (Fu and Harberd, 2003; Achard et al., 2003; Achard et al., 2005). Recent report showed that GID1 interact with DELLA protein to promote the degradation of DELLA proteins (Nakajima et al., 2006; Griffiths et al., 2006), indicating that GID1 may play a central role in controlling the stability and perhaps activity of DELLA proteins. However, the regulation of DELLA proteins has been indicated to be more complicate than just destabilization (Itoh et al., 2005). It was indicated that intrinsic activity of DELLA proteins may be regulated posttranslationally via phosphorylation or O-linked GlcNAc (O-GlcNAc) (Silverstone et al., 1998; Dill et al., 2001; Itoh et al., 2002; Itoh et al., 2005b; Hussain et al., 2005; Hussain et al., 2007).

Despite all these achievements, the molecular mechanism of GA function in regulating many aspects of plant growth and development such as seed germination, stem elongation, and flower development is still unclear. Based on what is known about GA signaling, it should be interesting to identify more new GA signaling pathway components that interact with GA receptor (e.g. GID1 or GID1-like) or

negative regulators (e.g. DELLA proteins ). DELLA proteins are believed to be a putative transcription regulator (Ogawa et al., 2000). However, wild-type DELLA proteins have not been shown to interact with chromatin. Therefore, it is important to identify DELLA-regulated downstream genes, and to learn if DELLA proteins interact with chromatin at specific genes and if this interaction affects the expression of these genes.

## 1.9 Objectives of this study

GA is an endogenous regulator of floral development. Severe GA-deficient mutant, *ga1-3* exhibits retarded reproductive growth of floral organs, especially petals and stamens (Koornneef and van der Veen, 1980; Fu and Harberd, 2003). Previous reports have suggested that GA signaling components, SPY, GAI, SLR1 and SLN1, may modulate these processes (Izhaki et al., 2002). However, the mechanism via which GA regulates petal and stamen development remained unclear.

Although *Arabidopsis* DELLA genes *GAI*, *RGA*, *RGL2* and *RGL1* are all expressed in inflorescences, no obvious suppression of *ga1-3* mutant floral phenotypes was observed in *ga1-3* mutants lacking GAI, RGA, RGL1, RGL2, or GAI and RGA together (Dill and Sun, 2001; King et al., 2001a; Lee et al., 2002). However, based on the fact that transgenic *RGL1* loss-of-function line was resistant to PAC-induced arrestment of floral organ development, it was suggested that RGL1 may play a role in regulating floral development (Wen and Chang, 2002). These observations highlight the importance of determining systematically the respective roles of various DELLA proteins in GA-mediated regulation of *Arabidopsis* floral development. Therefore, one of the purposes in this thesis is to determine if GA regulates floral development through different combinations of DELLA proteins in *Arabidopsis*.

DELLA family proteins belong to the GRAS family of putative transcriptional regulator (Pysh et al., 1999; Richards et al., 2000; Pharis and King, 1985). However, as a group of putative transcription regulators (Pysh et al., 1999; Richards et al., 2000), the molecular mechanism of DELLAs repressing plant growth is largely unknown. Does it control the transcription of other transcription factors or instead directly affect the transcription of the structure proteins? To address this question, one strategy would be to identify the components downstream of DELLA proteins. Microarray analysis allows the simultaneous detection of thousands of transcripts in a single experiment. Thus Affymetrix *Arabidopsis* GeneChip (ATH1, carrying 22,810 genes) will be used to identify the targets of DELLA proteins.

By comparing the expression profiles of the inflorescence of *ga1-3* and *ga1-3* mutants lacking different combination of DELLA proteins (quadruple and penta mutants), a subset of genes that possibly act downstream of DELLA protein(s) will be identified. Detailed functional studies of these genes will help us to decipher the network of GA signaling pathway in controlling flower development, especially stamen development.

# Chapter 2

# General Materials and Methods

## 2.1 Plant materials and growth conditions

*Arabidopsis thaliana* wild type controls used in this study were Ler, Col-0, and Ws-0 ecotypes. The *ga1-3* and *Ds* insertion lines (DELLA-related mutants) were in Landsberg *erecta* background (Lee et al., 2002; Cheng et al., 2004). T-DNA insertion lines for DELLA regulated genes were in Col-0 background. *opr3* mutant was in Ws-0 background (Stintzi and Browse, 2000).

For plants grown in soil, seeds were imbibed on water-moistened filter paper at 4°C for 7d (to break dormancy). The seeds were planted on Florobella potting compost in growth room (16h light/8h darkness photoperiod, 20-23°C). For the *ga1-3* mutant and *ga1-3* mutation-containing lines that does not germinate without exogenous GA, seeds were imbibed on filter paper wetted with a $10^{-4}$ M solution of $GA_3$ (Sigma, St. Louis) and planted as described in the above. For anatomical analysis of anthers of *ga1-3* mutants, seeds were chilled for 7 days without GA application, following which the seed coat was manually removed and the seeds were sown on soil.

## 2.2 Genotyping of mutant plants

Mutant plants were identified through PCR-based methods as described in section 2.3.2. Primer pairs used for genotyping were listed in Table 2.1, Table 2.2 and Table 2.3. *Ds* (or T-DNA) primer pairs (Table 2.1, Table 2.2) contain one *Ds* (or T-DNA) border primer and one gene specific primer. Full length primer pairs (Table 2.1, Table 2.2) contain two gene specific primers that spanning the *Ds* (or T- DNA) insertion. Therefore, for homozygous mutant plants, only the DNA band produced by the *Ds* (or T-DNA) primer pairs will be detected. No band will be detected with full

length primer pairs because the DNA fragment (Ds or T-DNA) inserted between these primers was too long to be amplified by the PCR condition used for genotyping. For heterozygous plants, Both Ds (or T-DNA) primer pairs and full length primer pairs will generate bands. For WT plants, only the DNA band will be detected with full length primer pairs.

**Table 2.1. Primers used for genotyping *Ds* insertion mutants and mutants in *ga1-3* background**

| **Mutant lines** | **Ds insertion primer pairs** | **Full length primer pairs** |
|---|---|---|
| *rgl1-1* | CCGGTATATCCCGTTTTCG<br>5'CCACAGAGCGCGTAGAGGATAAC | AAGCTAGCTCGAAACCCCAAAT<br>CCACAGAGCGCGTAGAGGATAAC |
| *rgl2-1* | CGGTCGGTACGGGATTTTCC<br>ACGCCGAGGTTGTGATGAGTG | GCTGGTGAAACGCGTGGGAACA<br>ACGCCGAGGTTGTGATGAGTG |
| *rga-t2* | CCGGTATATCCCGTTTTCG<br>GCCGGAGCTATGAGAAAAGTGG | AAGAATTTTAAACAAGTGAACG<br>GCCGGAGCTATGAGAAAAGTGG |
| *gai-t6* | CGGTCGGTACGGGATTTTCC<br>TAGAAGTGGTAGTGGAGTG | CCCAACATGAGACAGCCG<br>TAGAAGTGGTAGTGGAGTG |
| | **Primer pairs used to detect *ga1-3* mutation** | **Primer pairs used to detect WT** |
| *ga1-3* | TGTATGCACGTTAACGATCAAT<br>TTTCTTCATACCACCTGCGTTC | TTTGGCCCAACACACAAACCTT<br>AAGCCTCGAACTCAAGGTTCTA |

*Ds* insertion primer pairs were used to determine if there is a *Ds* inserted in the respective genes. Full length primer pairs were used to determine if the plants are heterozygous or homozygous for *Ds* insertion.

**Table 2.2. Primer pairs for confirming T-DNA insertion in selected DELLA-D and DELLA-U candidate genes**

| Gene ID | T-DNA insertion lines | T-DNA insertion primer pairs | Full length primer pairs |
|---|---|---|---|
| At2g34790 | SALK_072827(Intron) | LBa1+3111F | 3111F+3776R |
| | SALK_069340(Intron) | LBa1+5452R | 4682F+5452R |
| At4g18780 | SALK_026812(Intron) | LBa1+2906F | 2906F+3723R |
| At5g44030 | SALK_084627(Exon) | LBa1+3571R | 2485F+3571R |
| At3g62020 | SALK_065289(Exon) | LBa1+2591F | 2591F+3779R |
| | SALK_023872(300-UTR5) | LBa1+2591F | 2591F+3779R |
| At1g78440 | SALK_020228(Exon) | LBa1+3688F | 3688F+5094R |
| At2g17950 | SALK_114398(300-UTR3) | LBa1+5316R | 4489F+5316R |
| At1g09610 | SALK_050883(Exon | LBa1+3244F | 3244F+4097R |
| At3g18660 | SALK_063763(Exon) | LBa1+4645F | 4645F+5630R |
| | SALK_046841(Exon) | LBa1+3397F | 3397F+4331R |
| At3g27810 | SALK_003625(300-UTR5) | LBa1+3273R | 2492F+3273R |
| | SALK_042711(Intron) | LBa1+5355R | 4334F+5577R |
| | SALK_039465(300-UTR3) | LBa1+6471R | 5326F+6471R |
| | Gabi-Kat (N311167) (intron) | 08409+5355R | 4334F+5577R |
| At5g40350 | SALK_065218(Intron) | LBa1+4975R | 4566F+4975R |
| | SALK_017221(Intron) | LBa1+4975R | 4566F+4975R |
| At4g34990 | SALK_132874(Exon) | LBa1+3477F | 3477F+4449R |
| At1g17950 | SALK_118938(Intron) | LBa1+4318R | 3697F+4318R |
| | SALK_138624(Exon) | LBa1+3977F | 3977F+4679R |
| At3g01530 | SALK_065776(Exon) | LBa1+3411F | 3411F+4511R |
| At2g38080 | SALK_051892(300-UTR5) | LBa1+2490R | 2092F+2490R |
| At4g12730 | SALK_001056(Exon) | LBa1+4210R | 3267F+4210R |
| At1g69490 | Salk_005010(Exon) | LBa1+4596R | 3188F +4596R |
| | Salk_049717(UTR3) | LBa1+3188F | 3188F +4596R |
| At3g04070 | Salk_066615(UTR5) | LBa1+3543R | 2868F+3543R |
| At2g43620 | Salk_056680(intron) | LBa1+3894R | 3245F+3894R |
| At1g21520 | Salk_045038(UTR3) | LBa1+2550F | 2550F+3501R |
| At5g65040 | Salk_106042(UTR5) | LBa1+2793F | 2793F+3321R |

T-DNA insertion primer pairs were used to determine if there is T-DNA inserted in the respective genes. Full length primer pairs were used to determine if the plants are heterozygous or homozygous for T-DNA insertion. Primer sequences were listed in Table 2.3.

**Table 2.3. Primers for confirming T-DNA insertion in selected DELLA-D and DELLA-U candidate genes**

| | | | |
|---|---|---|---|
| LBa1 | TGGTTCACGTAGTGGGCCATCG | 5316R | TTGAGATTGATTTTGACCGTTCG |
| 08409 | ATATTGACCATCATACTCATTGC | 5326F | CCAGGAAGAACGGACAACGAGA |
| 2092F | CGACCTACCACCTAAAACGAC | 5355R | TTGATATGATGTCGGTGTAGGAGA |
| 2485F | GCCAGCTTCGACGATGAGGTA | 5452R | TGAAATAATGACGCGAATGAAGAG |
| 2490R | TCCACTACGAAGCCACAAACA | 5577R | CGCGGCCGAATAGTTACCATAGT |
| 2492F | TTAGAAACAAACAAAACGGACCAC | 5630R | TGAATCTCGGGTCCTTTACTCTTA |
| 2550F | CCTTGCACCGATGGGAGAGT | 6471R | GTTTAAGATCCGCCGCATACA |
| 2591F | AAGTGGGCCTATTTGTTTATCTAC | | |
| 2793F | AGGGGCAAAAATGGGGGTAT | | |
| 2868F | GATCTTTTCCTCCGGTCTACTTTA | | |
| 2906F | CTCCAAATTTAGCTTACCCATCAG | | |
| 3111F | TCCTCCACGCTACAACAAGA | | |
| 3188F | TGTCTAAACCATGCCCTGTCTCC | | |
| 3244F | CAAAGAGATAGCGGTAAGCAGTAG | | |
| 3245F | GGTGGTGCAGGCGGTCTAAA | | |
| 3267F | GGCCGTCGATAACTCAGCAATGT | | |
| 3273R | GTCCATGGTCCTTTTCTCACTTCT | | |
| 3321R | CACGATGATGGCAGAGACTTC | | |
| 3397F | TTATCATCATTCTCTTTCCCACTC | | |
| 3411F | CATGGTGAAGGTCTTTGGAACT | | |
| 3477F | CTTATTGCGACGAGATTACCA | | |
| 3501R | ACCATAATTAATCACAACGCATCA | | |
| 3543R | TACCGCAATCAATTTATCTGTTCC | | |
| 3571R | GATCACGATGCGGTAAGGACTG | | |
| 3688F | TCTGACCAAAACACGGACTCG | | |
| 3697F | GGCAACCACAACCGCTATC | | |
| 3723R | TTGCCATATTCATCATTCAGTTCT | | |
| 3776R | TTCCCCAAATGGCAATAGACA | | |
| 3779R | AACGACGCGATTTTTAGACTT | | |
| 3894R | AGGCAACTTGGGGGCTACGA | | |
| 3977F | AATGTGCAAGTAAATGAGTAATGG | | |
| 4097R | TATATGTTCTGCTCTTTTCTCCTG | | |
| 4210R | CGGCGCCGTTTTTATCGTCA | | |
| 4318R | ATCTTCGAATCCGTGTTTACTTGA | | |
| 4331R | CTGAGTGAAGAATTGTTGCGTAAG | | |
| 4334F | ATCGTGCCTATTTCTCCTCCAT | | |
| 4449R | ACGAAATATGCCAAAAGGTT | | |
| 4489F | GGTGGAGGATGGGCAAACA | | |
| 4511R | TAAACAATAACAACGTCCCTTCCT | | |
| 4566F | TGCCGATTCTACCACAAC | | |
| 4596R | AAATTAGCAAAAAGGGTCCACATA | | |
| 4645F | AGCTACAGGAAACAATGGAACTCT | | |
| 4679R | CCGAATGGTGATGAGTCTGA | | |
| 4682F | TACCCAAATGCAAAAACCTAATA | | |
| 4975R | CTACATCTACGTCGAGCAATAA | | |
| 5094R | CCATTGGAACCGGTTTAGGATT | | |

## 2.3 DNA handling

### 2.3.1 Plasmid DNA isolation

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAgen, Germany) according to the manufacturer's recommendations. The bacterial cells were first centrifuged and the pellet was resuspended in Cell Resuspension Solution. The Cell Lysis Solution was then added to the suspension, after which the solution was neutralised by addition of Neutralisation Solution. The lysate was centrifuged to remove cell debris. The clear supernatnant was then added to the QIAprep spin column for binding of the plasmid DNA to the resin. After the supernatant had passed through the column by centrifugation, the column was washed with Buffer PE to remove the debris. The Buffer PE was removed completely from the resin by brief centrifugation. The plasmid DNA was then eluted from the column with 100 µl of $ddH_2O$.

### 2.3.2 Polymerase chain reaction (PCR)

Reaction was set up in 50 µl total volume in a 0.2 ml PCR tube as follow: 1X reaction buffer, 0.2 mM of dNTPs, 0.4 µM of primers, 0.1 µg template DNA and 1.25 units of *Taq* (Roche, Germany) or *Pfu* DNA polymerase (Stratagene). Thermal cycling was carried out in a Programmable Thermal Controller (PTC-100, MJ Research) using the following program: initial denaturation of 94$^{o}$C for 3 minutes, followed by 35 cycles of 94$^{o}$C for 30 seconds, 45-55$^{o}$C (depending on the annealing temperature for each primer pairs) for 30 seconds, 72$^{o}$C (*Taq*) or 68$^{o}$C (*Pfu*) for 30 seconds to 10 minutes (depending on the length of amplified DNA fragments), and final extension of 72$^{o}$C (*Taq*) or 68$^{o}$C (*Pfu*) for 5 minutes. PCR products were separated by electrophoresis on agarose gels and purified as described in section 2.3.3.

### 2.3.3 Purification of DNA fragment from agarose gels

DNA from agarose gels were purified using Qiagen Gel Extraction Kit as described by the manufacturer (QIAgen, Valencia, CA, USA). The DNA fragment was excised from the agarose gel under long wavelength UV light and the gel slice was weighed, mixed with Buffer QG (3 gel volumes of Buffer QG to 1 volume of gel, 100 mg ~ 100 µl) and incubated at 50$^{o}$C until the gel slice dissolved completely. 10 µl of 3 M sodium acetate (pH5.0) was added to the mixture if the color of the mixture was not as yellow as Buffer QG. The mixture was mixed with 1 gel volume of isopropanol, applied to the QIAquick column and centrifuged. The column was washed with Buffer PE and DNA was eluted from the column with 50 µl Buffer EB.

### 2.3.4 Preparation of plasmid vectors

#### 2.3.4.1 Blunt-ending of DNA templates with T4 DNA polymerase

Blunting ends of restricted plasmid DNA for cloning was performed using T4 DNA polymerase according to the manufacturer's recommendations (New England Biolabs). Restricted plasmid DNA was dissolved in 1X restriction enzyme reaction NEBuffer supplemented with 100 µm dNTPs. T4 DNA polymerase (1 unit T4 DNA polymerase per microgram DNA) was added and the reaction mixture was incubated for 15 minutes at 12$^{o}$C. The reaction was stopped by adding EDTA to a final concentration of 10 mM and heating to 75$^{o}$C for 20 minutes.

#### 2.3.4.2 Dephosphorylation of restricted plasmid DNA by shrimp alkaline phosphatase (SAP)

The restricted plasmid DNA was dephosphorylated using SAP (Roche, Germany). Digested DNA was mixed with SAP (0.5 U/µg DNA) and incubated at 37$^{o}$C for 1 hour in the presence of 1X SAP buffer. The dephosphorylated DNA was purified using QIAgen Gel Extraction Kit as described in section 2.3.3.

### 2.3.5 Ligation of DNA inserts into plasmid vectors

50 ng of linearized vector DNA was mixed with insert DNA at a molar ratio of (1:3) (vector: insert). The ligation was carried out with the Rapid DNA Ligation Kit (Roche, Germany) at room temperature for 5-10 minutes.

### 2.3.6 DNA sequencing

Fluorescence-based cycle sequencing reactions were performed using BigDye® Terminator v 3.1 Cycle Sequencing Kit as described in manufacturer's protocol (Applied Biosystems, Perkin-Elmer, USA). The dye-labeled DNA samples were purified and run on ABI PRISM® 3700 DNA Analyzer by the IMCB Sequencing Facility.

### 2.3.7 Preparation of *E.coli* competent cells for heat-shock transformation

*E.coli* DH5α competent cells were prepared as described (Inoue et al., 1990). Single colony of DH5α from a freshly streaked plate was inoculated into 250 ml of LB medium (2% Bacto trytone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$) in a 1-liter flask, and grown with vigorous shaking (200-250 rpm) at 18˚C until $OD_{600}$ reached 0.6. The culture was placed on ice for 10 minutes to cool and centrifuged at 2500g for 10 minutes at 4˚C. The pellet was resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl) and incubated on ice for 10 minutes, and spun down as above. The cell pellet was gently resuspended in 20 ml of TB, and DMSO was added with gentle swirling to a final

concentration of 7%. After incubating on ice for 10 minutes, the cell suspension was dispensed into 1.5 ml microfuge tubes and immediately immersed in liquid nitrogen. The frozen competent cells were stored at -80˚C.

### 2.3.8 Transformation of *E.coli* cells using heat-shock method

*E. coli* DH5α competent cells (100 µl) were mixed with 10 µl of ligation reaction mixture and kept on ice for 30 minutes and subjected to heat shock at 42˚C for 90 seconds. After chilling on ice for 2 minutes, 800 µl of LB medium was added and the mixture was shaken at 37˚C for 1 hour. 200 µl of bacterial culture was streaked on LB plate containing appropriate antibiotics and incubated at 37˚C overnight.

### 2.3.9 Preparation of electro-competent *Agrobacterium*

Single colony of *Agrobacterium* AGL1 strain was inoculated into 5 ml of LB medium containing appropriate antibiotics and shaken overnight at 28$^{o}$C. The culture was diluted in 100 ml LB medium and incubated at 28$^{o}$C for approximately 4 hours to an $OD_{600}$ of 0.5. The culture was spun down at 10,000g for 10 minutes at 4$^{o}$C. The pellet was washed with 40 ml of 1 mM HEPES (pH 7.0), then resuspended with 40 ml of 1 mM HEPES (pH 7.0), 10% glycerol. The suspended cells were aliquoted (100ul/tube) and immediately immersed in liquid nitrogen. The frozen competent cells were stored at -80$^{o}$C.

### 2.3.10 Transformation of *Agrobacterium* cells by electroporation method

Miniprep plasmid DNA (0.1 µg) was added to 50 µl of frozen competent cells and incubated on ice until completely thawed. The mixture was placed in a cold 0.2

cm electroporation cuvette (BioRad) and electroporation was performed using a BioRad Gene Pulser. The parameters were set at 25 μF, 400 Ω for a 2.5 kV pulse, followed with an 8-9 ms delay. The electroporation mixture was added with 1 ml LB, transferred to a sterile culture tube, and shaken at 28$^{o}$C for 2 hours to allow cell recovery and antibiotic resistance gene expression. 20 μl of the bacterial culture was spread on a LB plate containing appropriate antibiotics and grown at 28$^{o}$C for 2 days.

## 2.4 Generation of binary vectors

For the *pMYB21::GUS* construct, a 2570bp (112bp upstream of *AtMYB21* start codon ATG) fragment was PCR amplified from Col-0 genomic DNA using primers 323F (*Pst*I) and 2890R. For the *pMYB24::GUS* construct, a 3098bp (68bp upstream of *AtMYB24* start codon ATG) fragement was PCR amplified from Col-0 genomic DNA using primers 18F (*Pst*I) and 2934R. PCR amplifications of promoter regions were performed using *PfuTurbo* DNA polymerase (Stratagene). The amplified DNAs were digested with *Pst*I and then cloned into *Pst*I/*Nco*I-cleaved pCambia 1301 vector. Their sequences were confirmed by sequencing. Primer pairs used for cloning were listed in Table 2.4. These promoter-GUS fusion constructs were then introduced into *Arabidopsis thaliana* ecotype Col-0 plants as described in section 2.5. More than three independent lines were examined for each construct at various stages of development.

**Table 2. 4. Primers used for amplification of promoters of *AtMYB21* and *AtMYB24***

| Constructs | Primers |
|---|---|
| *pMYB21::GUS* | 323F (*Pst* I): 5' TTGACATT**CTGCAG**TAGGGAAGTGC 3'<br>2890R: 5' GTAGAGAAAGATGTGGGTGAGTTGAT 3' |
| *pMYB24::GUS* | 18F (*Pst* I): 5' TTCTAGG**CTGCAG**CTAAACGACTTC 3'<br>2934R: 5' GTAATAGAAAGGGAGAGTTGTGAAAG 3' |

## 2.5 Transformation of *Arabidopsis* by *Agrobacterium* vacuum-infiltration transformation method

Transformation of *Arabidopsis* plants by *Agrobactrium* was performed by the floral dip method as described previously (Clough and Bent, 1998). Primary inflorescences of *Arabidopsis* plants were clipped at their bases and secondary inflorescences were allowed to grow until they started to show open flowers. *Agrobacterium* AGL1 cells carrying appropriate Ti plasmid were grown in LB medium with appropriate antibiotics until $OD_{600}$ reached 2. The bacteria were spun down at 4,000g for 10 minutes. The pellet was resuspended in ½ MS liquid medium (pH5.7) with 5% sucrose, 44 nM 6-benzyl aminopurine and 0.005% silwet L-77. The final concentration of bacterial was an $OD_{600}$ of 0.8. The plants were submerged in the *Agrobacterium* suspension and vacuum was drawn until solution began to bubble vigorously. The vacuum was then quickly released. Plants were covered with plastic cover overnight and cultivated in the growth room until seeds matured and dried.

## 2.6 Plant genomic DNA isolation

### 2.6.1 Plant genomic DNA for genotyping

One *Arabidopsis* leaf was cut and briefly grounded by plastic pestle in 1.5 ml centrifuge tube. 200 µl of DNA extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, and 0.5% SDS) was added and mixed well. The plant tissue debris was removed by centrifuging at 14,000 rpm for 5 minutes at room temperature. The supernatant was transferred to a new tube and mixed with equal volume of isopropanol. The DNA was precipitated by centrifuging at 14,000 rpm for 10 minutes. The pellet was washed with 70% ethanol and air-dried. 50 µl of $ddH_2O$ was used to dissolve the pellet. Typically 1-2 µl was used for genotyping by PCR.

### 2.6.2 Plant genomic DNA for promoter cloning or southern blots

Plant genomic DNA (for promoter cloning or southern blots) was extracted using Plant DNAZOL® Reagent (Invitrogen) following the manufacturer's recommendation. *Arabidopsis* leaves or flowers (0.1 g) were pulverized in liquid nitrogen using a mortar and pestle. The frozen powder was transferred to a microcentrifuge tube containing Plant DNAZOL (use 0.3 ml Plant DNAZOL for 0.1 g of plant tissue). The solution was mixed thoroughly by gentle inversion a few times and incubated at 25°C with shaking for 5 minutes. 0.3 ml chloroform was then added. The solution was mixed vigorously and further incubated at 25°C with shaking for another 5 minutes. The extracts were centrifuged at 12,000*g* for 10 minutes and the resulting supernatant was transferred to a new tube. DNA was precipitated by centrifugation after mixing of supernatant with 0.225 ml of 100% ethanol. The DNA pellet was vortexed with 0.3 ml Plant DNAZOL-ethanol wash solution (1 volume of Plant DNAZOL with 0.75 volume of 100% ethanol). The samples were incubated for 5 minutes at room temperature and centrifuged at 5,000*g* for 4 minutes to remove the DNAZOL wash solution. The DNA pellet was washed by vigorous mixing with 0.3 ml of 75% ethanol, followed by centrifugation at 5,000*g* for 4 minutes. The ethanol wash was removed by decanting. The DNA pellet was air dried and dissolved in 70 µl TE buffer (pH 8.0).

## 2.7 RNA isolation

Total RNA was isolated from plant tissues using Tri Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Briefly, 0.1 g of inflorescences was grounded into powder with liquid nitrogen. The powder was transferred to a 2 ml microcentrifuge tube containing 1 ml of Tri Reagent followed by 20 seconds of vortexing. 200 µl of chloroform was added and the mixture

was homogenized by 20 seconds of vortexing. After centrifuging at 12000g for 15 minutes at 4°C, the aqueous phase was transferred to a new tube and mixed with 500 µl of isopropanol and incubated at room temperature for 10 minutes. The RNA pellets were obtained by centrifuging at 12000g for 8 minutes at 4˚C. The RNA pellet was washed with 70% ethanol, air dried, and dissolved in RNase-free $H_2O$.

## 2.8 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA for RT-PCR was extracted as described in section 2.7. The residue genomic DNA in the total RNA was removed via treatment with RNase-free DNase I (Roche, Germany) and the total RNA further purified with the RNeasy Mini kit (QIAgen, Valencia, CA, USA).

SuperScript™II RNase $H^-$ Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA. 20 µl reactions were set up in 0.2 ml PCR tubes as follow: 4 µg of total RNA, 1 µl of 200 ng/µl random hexamer, and 1 µl of 10 mM dNTP were mixed and topped up to 12 µl with $ddH_2O$. The mixture was heated to 65˚C for 5 minutes and quickly chilled on ice. 4 µl of 5x reaction buffer, 2 µl of 0.1 M DTT and 1 µl of RNase Inhibitor (Promega) were added to the reaction. The contents were mixed gently and incubated at 42˚C for 2 minutes. 1 µl (200U) of SuperScript™II was added and the reaction was incubated for 50 minutes at 42˚C. The mixture was heated to 70˚C for 20 minutes to inactivate the enzymes. This first strand cDNA was used as template for PCR in the gene expression studies.

Using first strand cDNA as template, gene expression was examined by PCR in different genotypes and different floral organs. The primers used for expression studies of DELLA regulated genes, GA and JA responsive and biosynthesis genes were listed in Table 2.5 and Table 2.6.

## 2.9 Southern blot analysis

PCR products were separated on 1.2% agarose gel by electrophoresis in 1X TAE buffer. The DNA was transferred overnight onto Hybond-N+ nylon membrane using 0.4 N NaOH. The membrane was cross-linked, pre-hybridized at 50°C for 2 h in DIG™ Easy Hybridization buffer (Roche, Germany), and hybridized overnight at 50°C in the same buffer containing 25 ng/ml of DIG-labeled gene specific DNA probe prepared as described in Section 2.11.1. The membrane was washed in 2X SSC/0.1% SDS twice, each for 5 minutes at room temperature, followed by 0.5X SSC/0.1% SDS twice, each for 15 minutes at 68°C, and 0.1X SSC/0.1% SDS for 15 minutes at 68°C. Chemiluminescent detection of the probe was performed by incubating the membrane in 1 X Blocking Reagent (Roche, Germany) for 30 minutes at room temperature with shaking. The membrane was then incubated for another 30 minutes in the anti-DIG-alkaline phosphatase Fab fragments with a 20,000 times dilution in blocking reagent. The membrane was washed twice at room temperature in washing buffer, each for 15 minutes. The membrane was then rinsed in 1 x detection buffer for 2 minutes. Ready-to-use CDP-star solution was layered onto the membrane in a plastic bag. The membrane was incubated for 5 minutes at room temperature and the CDP-star solution drained from the bag. Hyperfilm™ MP (Amersham Biosciences) was used for autoradiography. All the buffers and reagents were from Roche, Germany.

**Table 2.5. Primers used in RT-PCR confirmation of microarray expression data**

| Gene ID | Primer Pairs |
|---|---|
| At 5g65040 | 5’ GCCGTCCGATCATCTAACCTT 3’<br>5’ AACAAATCACACGAACAAATCAAA 3’ |
| At1g02340 | 5’ GCGAAGGAGGATTTATTGGTTGTT 3’<br>5’ ATGTCGCCGGAAGAAAATAAGGAT 3’ |
| At1g02850 | 5' AAGGCGCATTTGATTTCGTT 3'<br>5' GATGGTGCAGTGTTCCCTTGAG 3' |
| At1g02930 | 5’ CTCTCAACTGGCAAGGACAT 3’<br>5’ CGGCAGCAGAAAAACAGAGTAAA 3’ |
| At1g05560 | 5' GTTTTAAGTCACCGAGCCGTAGG 3'<br>5' TAGCAAATAACACTCCGCCACTTC 3' |
| At1g09610 | 5’ TACCACGTTCCCTCGCACAAG 3’<br>5’ GATCCCAACCGGTCTCATAAAT 3’ |
| At1g09970 | 5’ GATCACTCCCGACGCTCAA 3’<br>5’ GACCGCAACTTCTTTACCATCA 3’ |
| At1g17950 | 5’ GTGGCTGCGACTGGGATGATT 3’<br>5’ TTCTCGTTAGGAATTCGGTTG 3’ |
| At1g21520 | 5’ ACACAAACACATACATTCTTACTT 3’<br>5’ TCATTCATCATCTCCCTTTCTCGT 3’ |
| At1g27030 | 5' TCTTTCTAGTACTTTGGGGTCTTT 3'<br>5' CGTGGTTGGCGTTATCG 3' |
| At1g50420 | 5’ ACTGCGCTTTACGGTTTC 3’<br>5’ CTCGCAGGATATGATGTTCTT 3’ |
| At1g52690 | 5’ AGCCCAGTCAGCCCAACAA 3’<br>5’ CGAACGCAACAAACACTAATCAAA 3’ |
| At1g53160 | 5' ACGGATGAAGAAGAGGAAGTAGG 3'<br>5' TTTGGCATAGGAAGTGTCATCTCT 3' |
| At1g56120 | 5’ AGAGTGGCAGGGACGATTGGGTAT 3’<br>5’ TTCGATTCGCCGTCTCTAAGTGG 3’ |
| At1g69490 | 5' GAAAGCAACCGGTACAGACAAAG 3'<br>5' GAACCAACTAGACTCCGAATCAGG 3' |
| At1g70690 | 5’ GCGGCGACCTAGACCCAACC 3’<br>5’ GCGGCTAACATTTCGACAAGTAA 3’ |
| At1g74670 | 5’ TGGCCAAACTCATAACTTCTT 3’<br>5’ TAAATGATACCTGCAAAAACACTA 3’ |
| At1g75820 | 5' CCGGCGGTATTCCAGATTC 3'<br>5' AGCTCCGCCTTTACCGATTATGTT 3' |

| Table 2.5. Continued | |
|---|---|
| **Gene ID** | **Primer Pairs** |
| At1g75880 | 5’ CGTTCCGGCGGTAATAGTG 3’<br>5’ GGGGGAGAGTAAAGAAGTCGTT 3’ |
| At1g75900 | 5’ CAGCGACGACATAGCCAATACATA 3’<br>5’ AGACACGTCGGGACATACAGAAGA 3’ |
| At1g76240 | 5’ AGCCGAAGACAAAACACCAACACT 3’<br>5’ AACGCAAATAGACAGGAAACAAGA 3’ |
| At1g78440 | 5’ CGGTTCGGGTCCACTATTTC 3’<br>5’ ACCTCCCATTTGTCATCACCTG 3’ |
| At2g04240 | 5’ TCTCGCCGTCTTCATCCTC 3’<br>5’ TCCCCGGTTTAAATCTGCTC 3’ |
| At2g17950 | 5’ ACCATCTTCATCACCCAACTCG 3’<br>5’ ATAAGCATCGCCACCACATTCT 3’ |
| At2g34790 | 5' TTCACCGGCCAGTAAAACCACCAA 3'<br>5’ TTTCCTCGCTCACTTTCCCATCTT 3’ |
| At2g34810 | 5’ TTCAAGCACCAATCTCAAAACAGG 3’<br>5’ AGAACCGGAATCCCTTGCTGAG 3’ |
| At2g34870 | 5’ AACCGTCGCTCGTCGTCTTACTG 3’<br>5’ ATCGATCGGTCCTTTATTTCTTAG 3’ |
| At2g38080 | 5’ CTACAGCGGAGGATCAGTCACGAA 3’<br>5’ ATCCTTAGGCGGCGGCAAAAT 3’ |
| At2g40610 | 5’ GTGGCGGGTGCTATGAGATGAAG 3’<br>5’ CCGAACTGCCAATTAGAAGGAG 3’ |
| At2g42990 | 5’ ATCGGAGAGCTGCCAAAATCATCA 3’<br>5’ TGTCCTGGCCGCAAAGAAATCC 3’ |
| At2g43620 | 5' TACACCGGGAAAAGACTACTACG 3'<br>5' GACAACCGATTCAAACAACACA 3' |
| At2g46220 | 5' CGCCGTTCGTAGTATCAGAGA 3'<br>5' AGTAGGTCGGCTTGGGAGTC 3' |
| At3g01530 | 5’ GTGCGGCGAGGGAACATAA 3’<br>5’ TCAGCAATAGAAAAACCAAATAAC 3’ |
| At3g04070 | 5' TCTTCTAGGAGCGGTGGTA 3'<br>5' CAACTGCGGTAACTTCTGAT 3' |
| At3g07450 | 5’ GGTTATGGTCCAGTCAGGTCT 3’<br>5’ TACATGGAAGAAAATTGGCAGAAC 3’ |

**Table 2.5. Continued**

| Gene ID | Primer Pairs |
|---|---|
| At3g12000 | 5’ TGGTCGGGATCTCAAAACAGG 3’ |
| | 5’ AAGCCCAAGCCGTCACATTCT 3’ |
| At3g16920 | 5’ CCGTCGGCTTTTGGGATTAC 3’ |
| | 5’ TGCTTCTTCGGTGGTGACAT 3’ |
| At3g18660 | 5’ TTTGGATTGGCGATGAAGATGACG 3’ |
| | 5’ CAAGTTATGGCCGGGAAGTGATGA 3’ |
| At3g18780 | 5' TGCTGACCGTATGAGCAAAG 3' |
| | 5' CAGCATCATCACAAGCATCC 3' |
| At3g20520 | 5’ TTCCCGGGCTGTACTGACTTG 3’ |
| | 5’ GTACCGCATCGACCACGCTAAT 3’ |
| At3g22800 | 5’ TATCGCCGGTTATCTCCCTTTAG 3’ |
| | 5’ ACCGGTTAGTTGTGAGTTCGTGAT 3’ |
| At3g27810 | 5’ AAAATCGCCAAACATCTTCC 3’ |
| | 5’ AATTATAACCCCAAACCTCTACAA 3’ |
| At3g52130 | 5’ TGAAAGCTATGAGAGTTGGGTTGG 3’ |
| | 5’ GAAGAGCCATGGAGCGGTCAA 3’ |
| At3g54720 | 5' CTGGGTCGGCGCAAGGTAAC 3' |
| | 5' CCCGAATTCCGCCACTCCA 3' |
| At3g54770 | 5’ GGTGGCCGTCTTCGTAAATC 3’ |
| | 5’ TATGGCCTTCTTCTGTATGCTCTC 3’ |
| At3g58780 | 5’ GAAAACCCATAAACCAAAATAGAT 3’ |
| | 5’ CACTGTTTGTTGGCGTACTCATAGA 3’ |
| At3g60690 | 5' CAAGCCGAGAGCCATCACAAAA 3' |
| | 5' TCATCTTCTTCCACCATCGTTATT 3' |
| At3g62020 | 5’ TCGCACTTCAGGGATAAAA 3’ |
| | 5’ CCGGAGTGGCTGTGAATA 3’ |
| At3g63010 | 5' CCTCGACGGCGTTTTCTCCT 3' |
| | 5' ATAAGCCCTCCAATACCAATCTCG 3' |
| At4g08850 | 5’ GCGCCGGAATCTTCATCTGTT 3’ |
| | 5’ TCTTCGCTTCATCATCATTCTCCA 3’ |
| At4g09960 | 5’ ACTCGTGGCCGTCTCTATGA 3’ |
| | 5’ AGTTATTGCAGCTCGGTTTTTC 3’ |
| At4g12730 | 5’ CTCCGGCGAACAAGACTGC 3’ |
| | 5’ CGGCGCCGTTTTTATCGT 3’ |
| At4g12960 | 5’ CCGATCAGAAATCACAATACTCGTT 3’ |
| | 5’ AAGCTTCCATTTCCTCTCAG 3’ |

**Table 2.5.Continued**

| Gene ID | Primer Pairs |
|---|---|
| At4g18780 | 5’ CCGCCTCAAGTTGCTCCAGA 3’<br>5’ ATCACCCAAAAGGCGAAAAATACC 3’ |
| At4g24000 | 5’ GAGCCTCCGATGATGGTGGTTA 3’<br>5’ TTTGGCATCATCTCTGTTTCGTTA 3’ |
| At4g28240 | 5' TGGCAGCATCGTTCGTAG 3'<br>5' TGCATCCAGAAAATCCGACTCAA 3' |
| At4g34990 | 5’ GGTTGTTGGCGTTCTCTTCCTA 3’<br>5’ CACGCACTGCACCTATACTTCACT 3’ |
| At5g08260 | 5' AAATCGTCGTGTCTCCTCGTCTCC 3'<br>5' AGTCCACCGGCATAAGTCTCAACC 3' |
| At5g12870 | 5’ AACCCTTCCTTGACCCACATA 3’<br>5’ CTCAAGCCCTAGTACGAAAAGATT 3’ |
| At5g17420 | 5’ TTGGGCGCCGGAGAAAGA 3’<br>5’ GAGGACTGTGCCGGCTGAAAAAT 3’ |
| At5g17540 | 5’ AATGCGGCGGTTTTATCTTC 3’<br>5’ AGCCGCATCTCCTTGTCCT 3’ |
| At5g17760 | 5' CCGTGGCGAGTCTCAGGTTAG 3'<br>5' GGCATCGCAGCATCACTCAA 3' |
| At5g23970 | 5' CCATCACGGCGCTGCTGTTA 3'<br>5' ACCGGCTTCCCCCATCCA 3' |
| At5g28030 | 5' ACTCCCGGTGGTTACATAC 3'<br>5' TTGAATCCTACGTTACTACTACTC 3' |
| At5g33370 | 5’ AGCGGGTTGGTCGGAGAAGAAGAG 3’<br>5’ TTGTGGGTCGCTAATGAAGTCC 3’ |
| At5g40350 | 5’ CCAGAAATACATCATCAAGAGCGG 3’<br>5’ GCCAAAGATCATCGACGCTCC 3’ |
| At5g43270 | 5’ TTTTGGGAAGTGGAGAATGCTAA 3’<br>5’ CTCGGTATCGGAAATCTCAGTCTT 3’ |
| At5g44030 | 5’ CGGAGGCGACGAACACGA 3’<br>5’ AGAAGACAAACGGCCGGAATAGTA 3’ |
| At5g44630 | 5’ AAGCCTGGCCGATTGTTT 3’<br>5’ TTCTCTACTGGCTTCTTCTTTTGT 3’ |
| At5g59120 | 5’ AGAATCCGCAGGGCTTTGTGAG 3’<br>5’ GAT GCGATGCCAGTTCCAGTAGCA 3’ |
| At3g15270 | 5’ ACGCCGGGGTTACTTGAAA 3’<br>5’ TTGACCGCTAAACCCTCTCC 3’ |

**Table 2.6. Primers used in checking genes in GA and JA treatment studies**

| Genes | primers |
|---|---|
| *AtMYB21* | 5’ AAAATCGCCAAACATCTTCC 3’<br>5’ AATTATAACCCCAAACCTCTACAA 3’ |
| *AtMYB24* | 5’ ATGCAAAATGGGGAAATAGGTG 3’<br>5’ AAGATCATCGACGCTCCAATAGTT 3’ |
| *AtMYB57* | 5’ GTGCGGCGAGGGAACATAA 3’<br>5’ TCAGCAATAGAAAAACCAAATAAC 3’ |
| *GA2ox1* | 5’ CGGTTCGGGTCCACTATTTC 3’<br>5’ ACCTCCCATTTGTCATCACCTG 3’ |
| *GA3ox1* | 5’ GGCCCCAACATCACCTCAACTACT 3’<br>5’ GGACCCCAAAGGAATGCTACAGA 3’ |
| *GA20ox1* | 5’ AGCCGCTTCTTTGATATGCCTCTC 3’<br>5’ TTGGGGTTGGGACGAATGGA 3’ |
| *GA20ox2* | 5’ CCGGCAGAGAAAGAACACGAA 3’<br>5’ TACGCCTAAACTTAAGCCCAGAA 3’ |
| *DAD1* | 5’ GGGCCTACTGGAGCAAATCTAAAC 3’<br>5’ GTCTCCTCCACGCGTCTCTGTAT 3’ |
| *LOX1* | 5’ GGGCTTGAGGTTTGGTATGCTATT 3’<br>5’ AACGCCTCCAACGCTTCTTTCT 3’ |
| *LOX2* | 5’ CCCGGCCGTTTATGGTG 3’<br>5’ GTCTATTTGCCGCTATTATGTATG 3’ |
| *AOS* | 5’ GGCGGGCGGGTCATCAAGT 3’<br>5’ TCGCCGGAAAATCTCAATCACAAA 3’ |
| *AOC1* | 5’ CACGCCCAAGAAGAAACTCACTC 3’<br>5’ GCTGGCTCCACGTCCTTAGA 3’ |
| *AOC2* | 5’ CTCGGAGATCTCGTACCATTCAC 3’<br>5’ ACTTATAACTCCGCTAGGCTCCAG 3’ |
| *AOC3* | 5’ CAATGGCTTCTTCTTCTGCTGCTA 3’<br>5’CTTCGAATCTGTCACCGCTCTTTT 3’ |
| *AOC4* | 5’ TCCCCTTCACAAACAAACTCTACA 3’<br>5’ GGACGGGACACATTACGCTTACG 3’ |
| *OPR3* | 5’ ACGGCGGCACAAGGGAACTCTAAC 3’<br>5’ GGGAACCATCGGGCAACAAAACTC 3’ |
| *THI2.1* | 5’ GGGTAAACGCCATTCTCG 3’<br>5’ GCTAAGTCGCATCTGTGTCA 3’ |

## 2.10 Northern blot analysis

For northern analysis, 25 µg total RNA was treated with glyoxal/DMSO mixture containing 1.3 M glyoxal, 70% dimethyl sulfoxide (DMSO) and 0.01 M $NaPO_4$. RNA was separated on a 1% agarose/$NaPO_4$ gel by electrophoresis, and transferred overnight onto a Hybond-N nylon membrane. The membrane was washed for 5 minutes with 2xSSC, cross-linked and stained with methylene blue (0.03% w/v methylene blue in 0.3 M sodium acetate pH 5.2) to monitor the equal loading. Pre-hybridization, hybridization and detection were performed as described in section 2.9.

## 2.11 Probe labeling

### 2.11.1 DNA probe labeling

The DIG-labeled DNA probes were prepared by PCR amplification using DIG-labeled dNTP mix (Roche, Germany). 20 µl reaction mixture was set up in a 0.2 ml PCR tube as follow: 1x reaction buffer, 0.2mM DIG-labeled dNTP mix, 0.2 mM of T7 primer (5'-TAATACGACTCACTATAGG-3'), 0.2 mM of SP6 primer (5'-ATTTAGGTGACACTATAG-3'), 2 ng of plasmid DNA containing target gene fragment cloned in pGEM®-T or pGEM®-T Easy vectors and 1 Units of *Taq* DNA polymerase (Roche, Germany).

### 2.11.2 RNA probe labeling

The DIG-labeled RNA probes were prepared by *in vitro* transcription as described in following sections.

#### 2.11.2.1 Template preparation

Approximately 10 μg of plasmid containing the target gene fragment cloned in pGEM®-T or pGEM®-T Easy vectors were digested with appropriate restriction enzymes. The linearized plasmids were purified using phenol: chloroform. Equal volume of phenol: chloroform (pH 7.9) was mixed with the linearized plasmids by vortexing vigorously. After centrifuging at 10000g for 1 minute at room temperature, the aqueous phase was removed and mixed well with 1/10 volume of 3 M sodium acetate (pH5.2) and 2.5 volume of 100% ethanol. The mixture was stored at -20˚C for 3 hours. DNA pellets were obtained by centrifuging at 10000g for 10 minutes at 4˚C. After washing with 70% ethanol, the pellets were air dried and dissolved in RNase-free $H_2O$.

#### 2.11.2.2 *In vitro* transcription

The purified linearized plasmid (1 μg) was mixed with 2 μl of 10 x DIG RNA labeling mix (Roche, Germany), 2 μl of 10 x transcription buffer, 2 μl of T7 RNA polymerase or SP6 RNA polymerase (NEB), 1 μl of RNase Inhibitor (Promega), adjusted to a final volume of 20 μl with RNase-free $H_2O$, and incubated for 2 hours at 37˚C. The reaction was stopped by incubation with 2 μl of RNase-free DNase I (Roche) at 37$^{o}$C for 30 minutes. The reaction mixture was mixed with 100 μl 5 M ammonium acetate, 600 μl of 100% ethanol, 1 μl glycogen (20 mg/ml, Roche), 60 μl DEPC-H2O and left at -20$^{o}$C overnight. After centrifuging at 14,000 rpm at 4$^{o}$C for 15 minutes, the pellet was washed with 200 μl of 75% ethanol, air-dried and dissolved in 50 μl DEPC-$H_2O$.

#### 2.11.2.3 Probe quantification

The yield of DIG-labeled RNA probe was estimated by dot blot method using control RNA supplied by the manufacturer (Roche, Germany). A series of dilutions of both the control RNA and the DIG-labeled RNA probe were prepared, and 1 μl of each dilution was applied onto Hybond-$N^+$ nylon membrane. The RNA on the membrane was cross-linked. Chemiluminescent detection method was used as described in section 2.9.

### 2.12 Histology and *in situ* hybridization

*Arabidopsis* inflorescences were fixed in fixation solution (4% paraformaldehyde containing 0.1% Tween 20 and 0.1% Triton X-100) under vacuum for 15 minutes and shaken overnight at 4$^{o}$C. The tissues were dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 95% and 100%), each for 3 hours. The tissues were then infiltrated and embedded according to the protocols for anther sectioning or *in situ* hybridization as below.

For anther sectioning, the inflorescences were infiltrated and embedded using Jung HistoResin$^{TM}$ Plus according to manufacturer's recommendation (Leica). Briefly, the inflorescences were first immersed in a series of ethanol/infiltration solution (75% ethanol/25% infiltration solution, 50% ethanol/50% infiltration solution, 25% ethanol/75% infiltration solution) for 1 hour each, then soaked in 100% HistoResin$^{TM}$ Plus three times for 5 hours each. The samples were embedded in LEICA HISTORESIN embedding medium and mounted in LEICA HISTORESIN mounting medium. Sections (2.5 μm) were made using a Leica RM 2055 microtome and stained with 0.25% Toluidine Blue O (Sigma).

For *in situ* hybridization, the inflorescences were immersed in 50% ethanol/50% Histo-Clear$^{TM}$ II (National diagnostics, USA) for 1 hour and three times

in 100% Histo-Clear™ II for 1 hour each. The inflorescences were transferred to 50% Histo-Clear™ II /50% paraplast and incubated at 60°C overnight. The inflorescences were then immersed in 100% paraplast for 3 days with changes of fresh paraplast once a day. Sections (10 µm) were made using a Leica RM 2055 microtome and deposited on poly L-lysine coated slides (Sigma).

*In situ* hybridization was performed as described previously (Luo et al., 1996; Coen et al., 1990). Each slide was incubated at 50°C overnight with 200 µl of hybridization buffer containing DIG labeled RNA probe (800ng/ml), 50% formamide, 300 mM NaCl, 10 mM Tris (pH7.5), 1 mM EDTA, 1X Denhardt's solution, 10% dextran sulphate, 100µg/ml Herring sperm DNA and 100 µg/ml tRNA. Slides were washed in several changes of 2xSSC, 50% formamide at 50°C, followed by two rinses with 0.5 M NaCl, 10mM Tris-HCl (pH 7.5), 1mM EDTA, and treated with 20 µg/ml RNAase A in this buffer at 37°C for 30 minutes. The slides were then washed again in 2xSSC, 50% formamide for 1 hour and several times in 1x PBS buffer (pH 7.0). The slides were stored overnight at 4°C in 1x PBS buffer. Immunological detection of the hybridized probe was carried out as described by manufacturer (Roche, Germany) with some modification. Slides were incubated with gentle agitation for 45 minutes in 1% blocking reagent diluted in 1X maleic acid (pH 7.5) (Roche, Germany), followed by 45 minutes in 1X maleic acid containing 1% bovine serum albumin (Sigma), 0.3% Triton X-100 (buffer A). This was followed by 2 hours incubation in diluted anti-DIG-alkaline phosphatase Fab fragments (1 in 2500) in buffer A followed by four washes with buffer A for 20 minutes each. Slides were briefly washed in 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2 (pH 9.5) (buffer B), and incubated for 1-2 days in 0.34 mg/ml nitroblue tetrazolium salt and 0.175 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt in buffer B. The color reaction was stopped with 10

mM Tris, 1 mM EDTA (pH 8), and sections were mounted in CRYSTAL/MOUNT™ Aqueous/Dry Mounting Medium (Biomeda, CA).

### 2.13 Callose staining and chromosome spread analysis

Callose that surrounded the pollen mother cells (PMCs) was stained with aniline blue as describe previously (Regan and Moffatt, 1990). Sections or manually dissected microspores on slide were stained with 0.05% aniline blue (Sigma) in 0.067 M $NaPO_4$ (pH8.5) at room temperature for 5 minutes. The slide was then mounted with *Vectashield* antifade mounting medium containing DAPI (1µg/ml) (Vector Labs, UK).

Chromosome spread analysis was performed as described previously (Ross et al., 1996). *Arabidopsis* inflorescences were immersed in Carnoy's fixative (ethanol: chloroform: glacial acetic acid at 6: 3: 1) at room temperature for 4 hours with occasional agitation, followed by 8 hours without agitation and then stored at -20$^{o}$C. The fixed inflorescences were transferred to black watch glass and rinsed in two changes of distilled water, followed by two 5-minute changes in citrate buffer (10 mM sodium citrate pH4.5), The inflorescences were incubated with enzyme mixture (0.3% (W/V) pectolyase (Sigma), 0.3% (W/V) cytohelicase (Sigma), and 0.3% (W/V) cellulase (Sigma) in citrate buffer) at 37$^{o}$C for 1-2 hours in humid chamber. After sufficient digestion, buds were transferred to citrate buffer and maintained at 4$^{o}$C.

A single enzyme-digested bud was transferred to a clean slide and excess buffer was removed. A small drop of 60% acetic acid was added to the bud and the whole bud was macerated with a needle under dissecting microscope in a minimum amount of liquid, taking care to avoid the material drying out. About 7 µl of 60% acetic acid was then added to the slide and the droplet stirred gently on a hotplate

(45$^{o}$C) for 1 minute. The slide was then flooded with freshly made ice-cold Carnoy's ethanol-acetic acid fixative (ethanol: acetic acid at 3: 1). Finally, the slide was titled to remove the fixative, shaken to remove any droplets and air dried using a hairdryer.

Air dried slides were stained by adding a small drop of DAPI (1µg/ml) in *Vectashield* antifade mounting medium and covered with a cover slip. The mounted slide was blotted between filter paper sheets to remove excess stain and mountant.

## 2.14 Histochemical localization of GUS activity

Histochemical detection of the GUS activity in plant tissues was performed as described previously (Jefferson et al., 1987; Topping and Lindsey, 1997). Tissues were first vacuum-infiltrated in the staining solution consisting of 2 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-Gluc), 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide. The treated tissues were incubated at 37°C overnight, followed by removal of chlorophyll by treatment with increasing concentrations of ethanol (20%, 50%, 70%, 90%, and 100% ethanol). The dehydrated tissues were examined microscopically.

## 2.15 Microarray

Total RNA from inflorescences was extracted as described in Section 2.7. cDNA synthesis, cRNA amplification, RNA probe labeling, GeneChip hybridization, washing, staining and scanning were performed by the IMCB Affymetrix Microarray Facility following the manufacturer's instructions (Affymetrix, Santa Clara, California, USA). GeneChip (*Arabidopsis* ATH1) arrays were scanned on an Affymetrix probe array scanner. Data were preliminarily analyzed using a statistics software MAS5.0 from Affymetrix (https://www.affymetrix.com/analysis/netaffx/index.affx).

## 2.16 Cross-comparing DELLA-dependent transcriptomes and ontology analysis

We obtained the signal intensities of individual genes using the statistical algorithms on Affymetrix Microarray Suite Version 5.0 (MAS5). The presence or absence of a reliable hybridization signal for each gene was determined by the detection call on MAS5. Genes were classified as GA responsive if the signal intensities deviated either positively or negatively two-fold or more between *ga1-3* and WT. Genes for which transcripts were determined to be undetectable (absent or marginal present) in *ga1-3* samples were eliminated from the list of up-regulated genes in *ga1-3*. Similarly, genes for which transcripts were determined to be undetectable (absent or marginal present) in WT samples were eliminated from the list of down-regulated genes in *ga1-3*. When the transcript was undetectable in only *ga1-3* or WT sample, we gave the background signal intensity to the undetectable transcript. If the signal intensity from the other sample was greater by two-fold or more relative to the background value, this gene was regarded as being GA-regulated. A gene is regarded as DELLA-down, if this gene is down-regulated in *ga1-3*, the signal intensity of *ga1-3* was less by two-fold or more relative to the intensity of *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*, and the signal intensity did not deviate negatively more than two-fold between *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* and WT. Similarly, a gene is regarded as DELLA-up, if this gene is up-regulated in *ga1-3*, the signal intensity of *ga1-3* was greater by two-fold or more relative to the intensity of *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*, and the signal intensity did not deviate positively more than two-fold between *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* and WT. In the flower samples, genes that were GA-responsive in any four of the six independent replicates were classified as GA-responsive.

The Gene Ontology (GO) information was retrieved through the NetAffx Gene Ontology (GO) Mining Tool, based on Molecular Function and Biological Process. Throughout the data sets, genes were identified by the AGI gene code, which was linked to Affymetrix Probe Set ID based on the gene annotation information in the NetAffx Analysis Center.

# Chapter 3

# Gibberellin Regulates *Arabidopsis* Floral Development via Suppression of DELLA Protein Functions

## 3.1 Introduction

Although little is known about how GA controls stamen filament elongation and anther development, previous reports have suggested that GA signaling components may modulate these processes. Recently, DELLA proteins has been identified to be crucial for the regulation of stem elongation growth in response to GA (Peng et al., 1997; 1999; Silverstone et al., 1998; Ikeda et al., 2001; Boss and Thomas, 2002; Chandler et al., 2002). The *Arabidopsis* genome encodes five distinct DELLA proteins (GAI, RGA, RGL1, RGL2 and RGL3). Genetic studies have shown that GAI and RGA function overlap in repressing plant stem growth (Dill and Sun, 2001; King et al., 2001), while RGL2 controls seed germination (Lee et al., 2002). RGL1 may play a role both in stem elongation and seed germination (Wen and Chang, 2002). Although *GAI*, *RGA*, *RGL2* and *RGL1* are all expressed in inflorescences, no obvious suppression *ga1-3* floral phenotype was observed in *ga1-3* mutants lacking GAI, RGA, GAI and RGA, or RGL2 (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002). However, a transgenic *RGL1* loss-of-function line was resistant to the arrest of floral organ development induced by paclobutrazol (PAC, an inhibitor of GA biosynthesis), suggesting that RGL1 might play a role in regulating floral development (Wen and Chang; 2002). These observations underscore the importance of determining systematically the respective roles of various DELLA proteins in GA-mediated regulation of *Arabidopsis* petal and stamen development.

In order to understand how GA regulates floral development, we first carried out experiments to determine at which stage of the flower and anther development in

*ga1-3* mutant was arrested. Then we use novel combinations of loss-of-function mutations of DELLA proteins to determine if DELLA proteins are repressors of stamen filament elongation and microsporogenesis. Results showed that GA is crucial both for filament cell elongation and for the developmental progression from microspore to mature pollen grain during pollen development. We also found that the DELLA proteins, RGA, RGL2 and RGL1, work together to repress stamen and anther development in GA deficient plants.

## 3.2 Materials and methods

### 3.2.1 Plant materials

*Arabidopsis thaliana* Landsberg *erecta* was used as the WT control. All mutants described in this chapter were derived from Landsberg *erecta*. Single mutant (*gai-t6* and *ga1-3*) and double mutant (*ga1-3 gai-t6*) were obtained as described previously (Peng et al., 1997; King et al., 2001). Three *Ds*-insertion lines (*rgl1-1, rgl2-1,* and *rga-t2*) were obtained from a previously described *Ds*-tagging population (Parinov et al., 1999; Sundaresan et al., 1995). Double mutants (*ga1-3 rgl2-1*, *ga1-3 rgl1-1*, and *ga1-3 rga-t2*) were obtained from crosses between the relevant single mutants and *ga1-3*. The triple mutants (*ga1-3 gai-t6 rga-t2* (T1), *ga1-3 rgl2-1 rga-t2* (T2)*, ga1-3 rgl2-1 gai-t6* (T3), *ga1-3 gai-t6 rgl1-1*, *ga1-3 rga-t2 rgl1-1*, and *ga1-3 rgl1-1 rgl2-1*), quadruple mutants (Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6*, Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*, Q3: *ga1-3 rgl1-1 gai-t6 rga-t2* and Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*) and penta mutant (*ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*) were made via cross-pollination (this thesis, Cheng et al., 2004). Primer pairs used for genotype verification were listed in Table 2.1.

### 3.2.2 Histology and in situ hybridization

For scanning electron microscopy (SEM), individual flower buds from fresh wild-type or mutant inflorescences were dissected; outer organs (sepals and petals) were removed using stainless steel needles. Buds were attached to a mounting plate, plunged into liquid nitrogen, and immediately transferred to a specimen chamber and scanned at 10 KV (JSM-5310LV, JEOL, Japan). Pollen grains were mounted on scanning electron microscopy stubs and coated with gold using previously described techniques (Bozzola and Russell, 1999).

DAPI (4', 6-diamidino-2-phenylindole) staining of pollen grain nuclei was performed as described (Chen and McCormick, 1996) and pollen numbers were counted under a microscope (Leica DM RXA2) with 40X or 20X objectives. Color photos were taken using a Spot Insight QE digital camera (Diagnostic Instruments).

Both antisense and sense probes of *ATA7* and *SDS* were synthesized from the pMC1577 and pMC2317 plasmids, respectively (Zhao et al., 2002) and *in situ* hybridization was performed as described previously in chapter 2.

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## 3.3 Results

### 3.3.1 Characterization of floral development in *ga1-3* plant

#### 3.3.1.1 GA regulates epidermal cell elongation during filament elongation

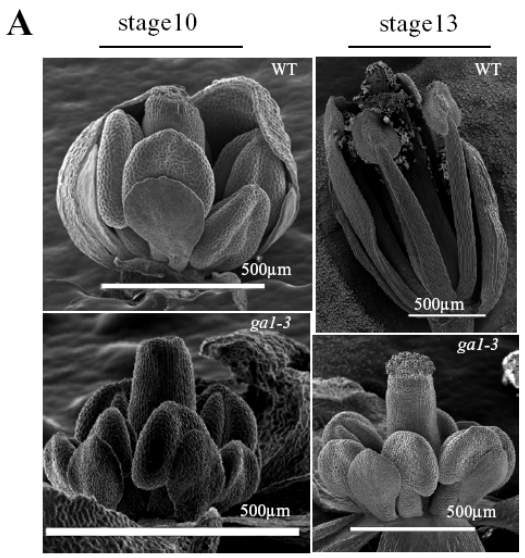
The early flower development of *Arabidopsis* from initiation until the opening of the bud has been divided into 12 stages according to a series of landmark events (Smyth et al., 1990). To determine the developmental stage at which *ga1-3* flower buds become arrested, the relative growth of stamens versus gynoecium in inflorescence flower buds (starting with the outmost and ending with the innermost dissectible bud) was compared in *ga1-3* and wild type via SEM. All floral organs were properly initiated in *ga1-3* plants, and appeared to develop normally until around

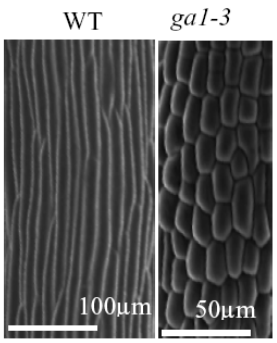
floral stage 10 (as defined by Symth et al., 1990, petals level with short stamens at stage 10). Comparing wild type and *ga1-3* flowers at stage 13, subsequent petal and stamen development beyond stage 10 was arrested in *ga1-3* (Fig. 3.1A). The ultimate stamens and pistils of *ga1-3* flower buds were much shorter than the mature stamens and pistils of the wild type control (Fig. 3.1C). In addition, GA deficiency had stronger effect on stamen filament length than on pistil length: the arrestment of stamen development resulted in significantly shorter stamens versus pistils in *ga1-3* (Fig. 3.1C). SEM of stamen filament epidermal cells showed that the arrestment of stamen filament growth in *ga1-3* was due to reduced cell length rather than to the reduction in cell number (Fig. 3.1B, D). These results indicated that GA regulates epidermal cell elongation during stamen filament elongation.

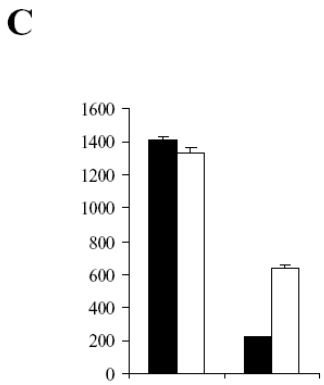
#### 3.3.1.2 *ga1-3* plants fail to produce tricellular pollen grains

*Arabidopsis* anther consists of four lobes with identical cell type architecture. Microsporogenesis initiates within the reproductive locule of each lobe. The sporogenous cells divide to generate microspore mother cells (MMC). MMC undergo meiosis to generate tetrads of free haploid microspores (MSp). The MSp are released from tetrads and undergo two rounds of cell devision to form mature tricelluar pollen grains (Sanders et al., 1999).

The surface structure of the mature pollen grains released by wild type plants was compared with that of pollen grains manually dissected from *ga1-3* anther locules. All wild type pollen grains were oval shaped with long indented lines. Very few oval shaped pollen grains were observed in *ga1-3*. Instead, the pollen grains from *ga1-3* plants were spherical in shape (Fig. 3.2A). Wild type and *ga1-3* anthers were dissected and stained with DAPI. As expected, the mature pollen grains from



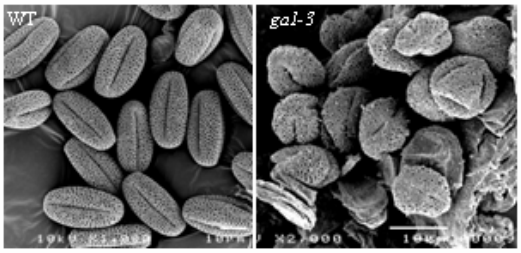




D

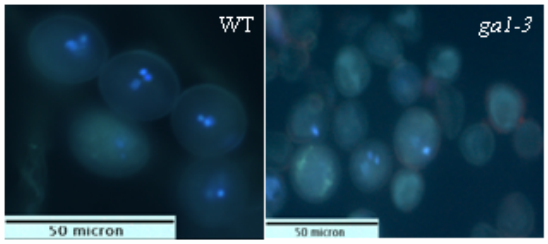
| | WT | *ga1-3* |
|---|---|---|
| Cell No. | 16±0.4 | 14±0.2 |

**Fig. 3.1. GA regulates stamen filament length via control of cell elongation.** (A) SEM of wild-type and *ga1-3* mutant flowers at floral stages 10 and 13. (B) SEM of wild-type and *ga1-3* mutant stamen filament epidermal cells. (C) Comparison of filament and pistil lengths between WT and *ga1-3* mutant. (D) Comparison of filament cell numbers between WT and *ga1-3* mutant.

A




B




C

| Genotype | 2-3 nuclei (%) | 1 nuclei (%) | No nuclei (%) | Pollen numbers examined | Number of flowers |
|---|---|---|---|---|---|
| WT | 97.5 | 0.7 | 1.8 | 2159 | 6 |
| *ga1-3* | 6.5 | 48 | 45.5 | 4408 | 27 |

**Fig. 3.2. *ga1-3* plants fail to produce tricellular pollen grains.** (A) SEM of WT and *ga1-3* mutant pollen grain surfaces. (B) DAPI staining showed that the majority of pollen grains from *ga1-3* plants had either no or only a single condensed nucleus. (C) Frequencies of tricellular pollen grains in anther locules as revealed by DAPI staining as shown in B.

plants were tricelluar, and contained three nuclei (Fig. 3.2B). However, fewer than 10% of the developing grains examined in *ga1-3* pollen sacs were found to be bicellular/tricellular (Fig. 3.2B, Fig. 3.2C). In fact, about 48% of *ga1-3* pollen grains contained only a single nucleus and 46% had no nucleus. Clearly, *ga1-3* fails to produce mature pollen, and this probably results from an arrest or impairment in pollen development prior to or during pollen mitosis in *ga1-3* (Fig. 3.2C).

#### 3.3.1.3 Microsporogenesis is arrested before pollen mitosis in *ga1-3*

In wild type, floral organs development is a highly programmed and coordinated process. *Arabidopsis* anther development can be divided into 14 stages according to major events and morphological markers. Meiosis in MMC occurred between stages 5 and 7 within each of the four locules with the generation of free tetrads of haploid microspores at stage 7. Free microspores were released from tetrads at anther stage 8 and differentiated into tri-cellular pollen grains between stages 9-12. Coordinated with pollen development was the growth and expansion in anther size, degeneration of several cell layers such as tapetum, and visible changes in specific anther cell types preceded the release of pollen grains during dehiscence (Sanders et al., 1999).

Stage 10 flowers contained anthers corresponding to stage 7-8 of anther development (Sanders et al., 1999). Petal and stamen growth arrests at around floral stage 10 in *ga1-3*, suggesting that anther development may arrest at stage 7-8 in this mutant. To determine the specific stage of anther development arrestment in *ga1-3* mutant, transverse sections of anthers from whole inflorescences of *ga1-3* plants were compared with that of wild type. No obvious differences were observed between *ga1-3* and wild type up to anther stage 7 when tetrads are formed as defined by Sanders et

al., 1999 (Fig. 3.3). However, after stage 7, it appeared that the microspores in *ga1-3* were appeared clustered and egenerated. Furthermore the anther in *ga1-3* failed to expand so significantly as that in WT and not dehisce at the later stage (Fig. 3.3, Fig. 3.12). In addition, the *ga1-3* tapetum remained at the vacuole stage (at anther stage 6) and then degenerated together with the microspores (Fig. 3.3, Fig. 3.12). As a result, the later stages of anther development (stages 9-12) could not be convincingly determined in *ga1-3*, suggesting that anther development may be arrested at stages 7-8 when microsporogenesis is arrested prior to pollen mitosis.

To confirm that microsporogenesis was arrested prior to pollen mitosis in *ga1-3*, we used the gene expression markers *SDS* and *ATA7*. *SDS* is a marker gene that specifically expressed in meiotic cells (Azumi et al., 2002). *In situ* hybridization analysis revealed near-identical expression patterns for *SDS* in anthers of *ga1-3* mutants and wild type, suggesting that the early stages of microsporogenesis were not affected in *ga1-3* (Fig. 3.4A). In fact, chromosome spread experiments confirmed that meiosis in *ga1-3* was successful, resulting in the formation of tetrads (Fig. 3.4B). To further confirm this, aniline blue staining was used. Aniline blue stains the callose wall by binding specifically to β-1, 3-glucan (Regan and Moffatt, 1990). It was known that microspore mother cells were surrounded by a callose wall. After meiosis, the callose wall degenerated and the microspores were released at stage 8. Aniline blue staining showed that the formation and degeneration of callose wall in anthers of *ga1-3* was normal. However, the tetrads in *ga1-3* anthers were not properly separated from one another, as is seen in wild type (Fig. 3.4C). *ATA7* is an early tapetum-specific molecular maker (Rubinelli et al., 1998). As expected, *ATA7* was highly expressed in wild type between stages 7 and 9 (Fig. 3.4A). The *ATA7* signal then gradually disappeared during the later stages. *ATA7* was also strongly expressed in *ga1-3*

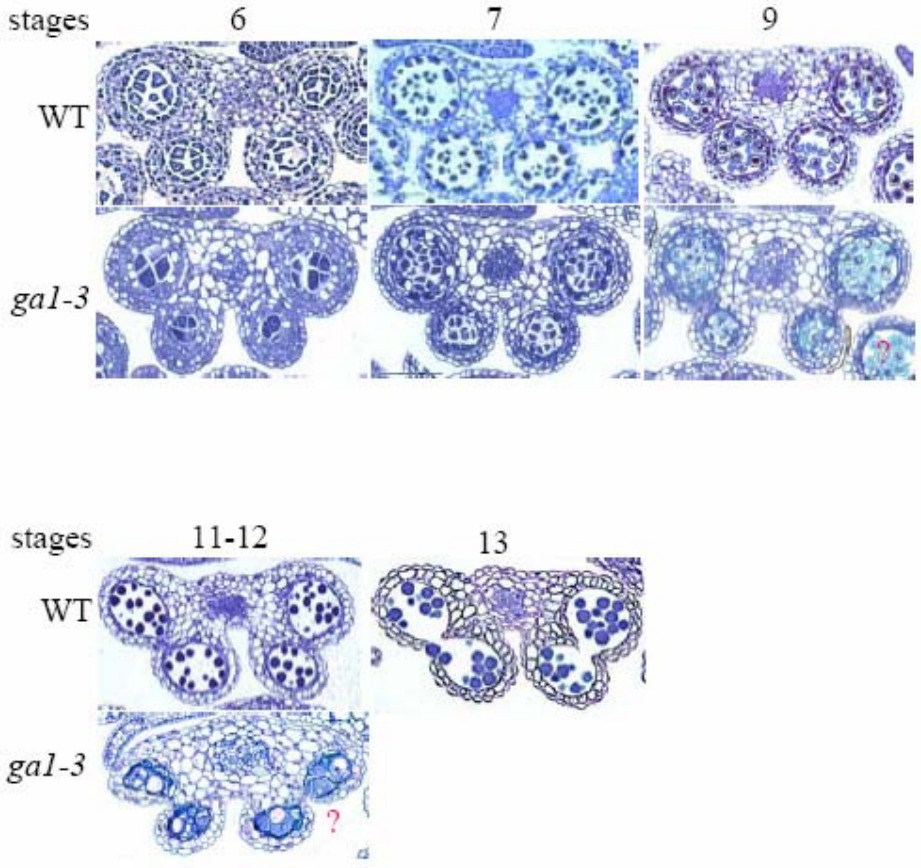



**Fig. 3.3. Histological analysis of microsporogenesis in *ga1-3*.** Transverse sections of anthers from WT and *ga1-3* mutant are displayed in developmental sequence, showing the progress in microsporogenesis in *ga1-3* and WT. *ga1-3* anthers developed normally up to the tetrad formation stage (stage7) but after this, they diverted from the normal (compared with stages 9 and 12 in wild type; *ga1-3* stages highlighted with red question mark). Eventually, all *ga1-3* pollen sac aborted.

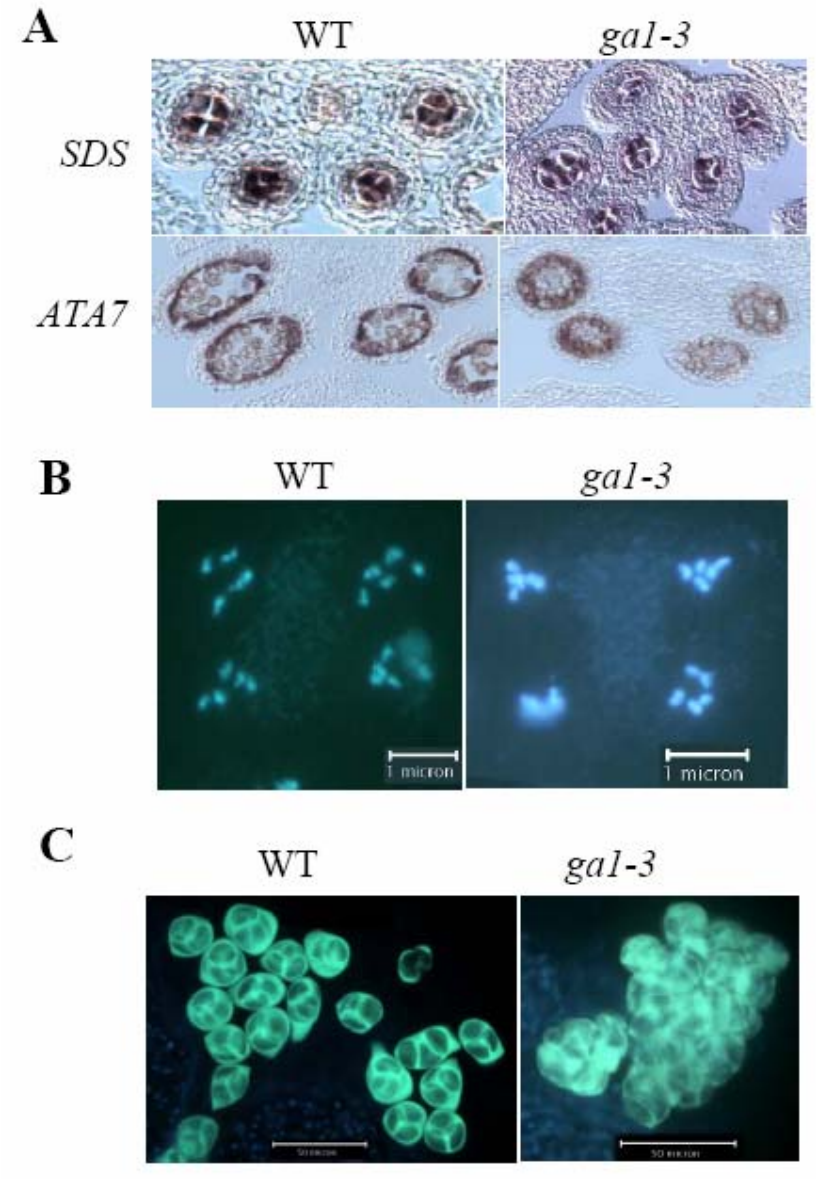



**Fig. 3.4. Pollen development is arrested in *ga1-3*.** (A) The anther-specific markers *SDS* and *ATA7* were used for in *situ* hybridization analysis to compare anther development in *ga1-3* and wild type. (B) Chromosome spread experiments confirmed that pollen meiosis is successfully completed in *ga1-3*, resulting in tetrad formation. (C) Aniline blue staining showed that tetrads in *ga1-3* tend to be clustered and are not found in the form of free microspores as is seen in the wild type. Scale bars: 1µm in B; 50µm in C.

anthers (Fig. 3.4A), implying that tapetum initiation may not be affected by GA deficiency. However, the *ga1-3* tapetum marked by expression of *ATA7* was irregular in shape. The microspores in *ATA7* marked pollen sacs had also become deformed, indicating that both tapetum and microspore were severely degenerated at a developmental stage prior to the disappearance of the *ATA7* signal (Fig. 3.4A). All of the above observations strongly suggested that microsporogenesis might be arrested prior to mitosis.

### 3.3.2 Absence of specific DELLA combinations suppresses *ga1-3* floral phenotype

#### 3.3.2.1 RGL2 and RGA are the key GA response regulators in repressing floral development

To investigate if DELLA proteins are repressors of floral development, floral phenotype of *ga1-3* plants lacking various combinations of GAI, RGA, RGL1 and RGL2 were studied. Previous studies have shown that absence of GAI or RGL1 had little effect on phenotype of *ga1-3* (Fig. 3.6). Although absence of RGA partially suppressed the stem elongation phenotype of *ga1-3* and absence of RGL2 rescued seed germination phenotype of *ga1-3* (Silverstone et al., 1998; Lee et al., 2002), absence of RGA or RGL2 did not restore normal floral development or fertility to *ga1-3* (Fig. 3.5A, Fig. 3.6).

*ga1-3* plants lacking all possible pair-wise combinations of RGL1, RGL2, GAI or RGA were analyzed. Lacking GAI and RGA completely suppressed the dwarf phenotype conferred by *ga1-3* (Fig. 3.5B) (Dill and Sun, 2001; King et al., 2001). All other combinations caused a phenotype that was indistinguishable from that of *ga1-3* (plants that lack RGL1 and RGL2, or RGL1 and GAI, or RGL2 and GAI) or from

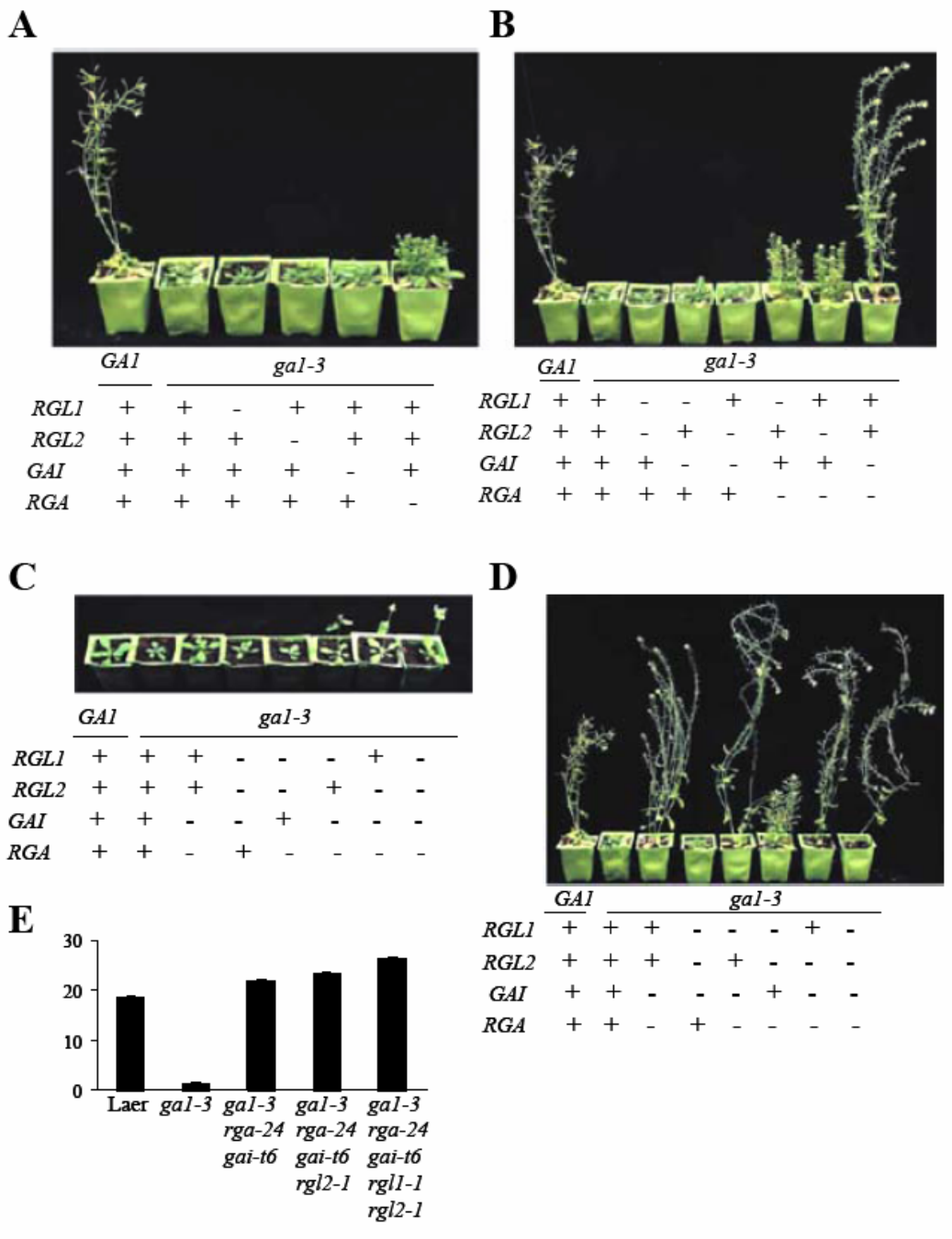

A
B
GA1
ga1-3
RGL1 + + - + + +
RGL2 + + + - + +
GAI + + + + - +
RGA + + + + + -
GA1
ga1-3
RGL1 + + - - + - + +
RGL2 + + - + - + - +
GAI + + + - - + + -
RGA + + + + + - - -
C
D
GA1
ga1-3
RGL1 + + + - - - + -
RGL2 + + + - - + - -
GAI + + - - + - - -
RGA + + - + - - - -
GA1
ga1-3
RGL1 + + + - - - + -
RGL2 + + + - + - - -
GAI + + - - - + - -
RGA + + - + - - - -
E
30
20
10
0
Laer
ga1-3
ga1-3 rga-24 gai-t6
ga1-3 rga-24 gai-t6 rgl2-1
ga1-3 rga-24 gai-t6 rgl1-1 rgl2-1

**Fig. 3.5. RGA and RGL2 are key GA-response regulators of floral development.** (A,B) Comparison of growth of *ga1-3* plants lacking single (A) or pairs (B) of DELLA proteins at 48 days. (C, D) Wild type plants were compared with *ga1-3* plants containing loss-of-function mutations causing lack of various combinations of RGL1, RGL2, GAI or RGA at 22 days (C) or 48 days (D). (E) Plant height of various genotypes at 28 days old under LD growth condition. Results are presented as mean ± standards error (n=30). Presence of wild-type gene is indicated by + and presence of loss-of function mutation is indicated by.

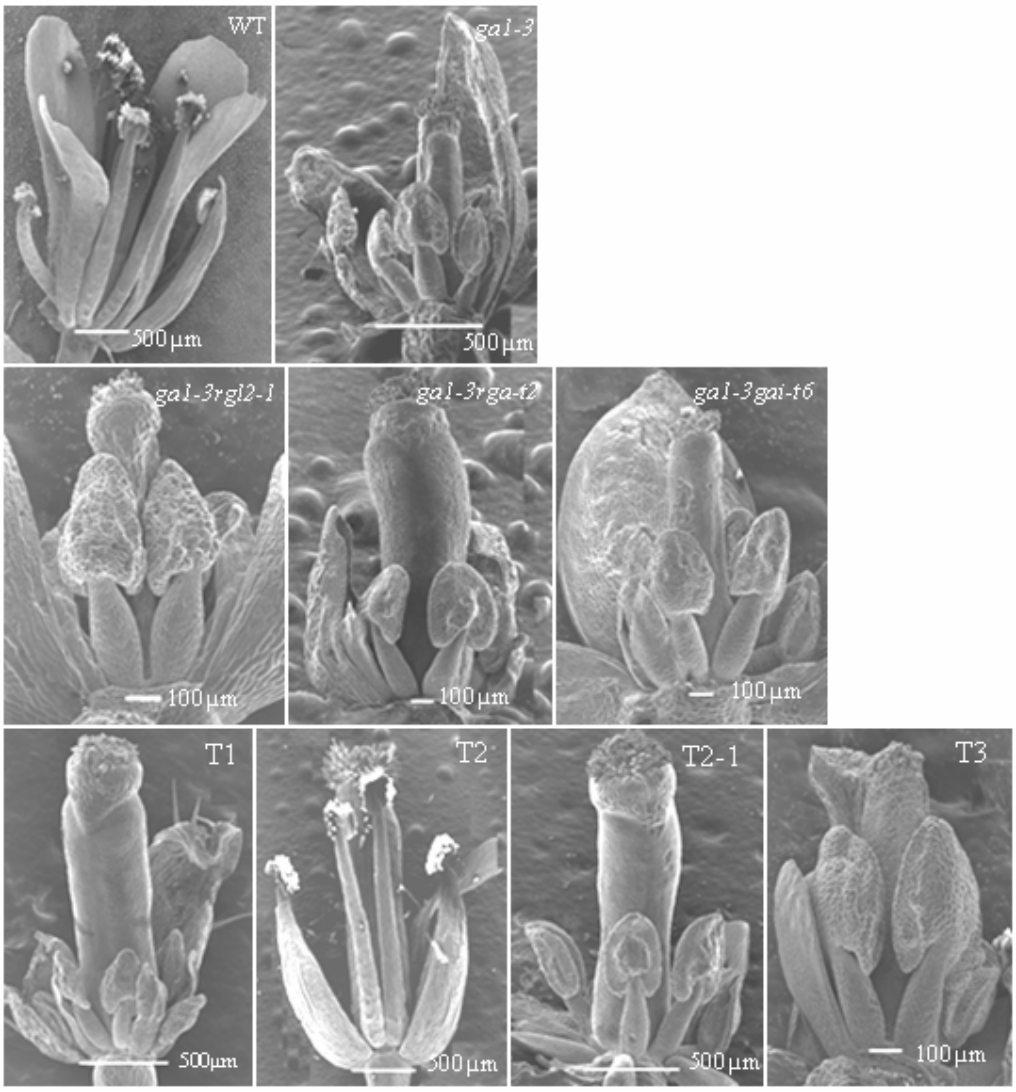



**Fig. 3.6. RGA and RGL2 are key regulators to repress the stamen and petal development.** Comparison of flower development in different genotypes (T1: *ga1-3 gai-t6 rga-t2*; T2-a: *ga1-3 rgl2-1 rga-t2* (~50d); T2-b: early developed sterile flowers of T2-a (~40d); T3: *ga1-3 rgl2-1 gai-t6*).

*ga1-3* plants lacking RGA (plants lacking RGL1 and RGA, or RGL2 and RGA) (Fig.3.5B). Thus, combination of GAI and RGA played the dominant role in controlling stem growth. Absence of RGA partially suppressed the stem phenotype of *ga1-3*. However, absence of RGL1 and RGL2 did not enhance this suppression, suggesting that RGL1 and RGL2 have relatively minor roles in the regulation of stem elongation (Fig. 3.5B).

In most cases, the pair-wise DELLA absence combinations failed to confer normal flower development on *ga1-3*. All of these lines produced flowers buds, but the buds failed to open and exhibited the arrested petal and stamen growth characteristic of *ga1-3* (Fig. 3.6, Fig. 3.7A). However, some flower opening was observed in the late developed flowers of two of the pair-wise DELLA absence combination lines. Although flowers of 40-day old *ga1-3* plants lacking RGL2 and RGA were sterile, at approximately 50 days and later, these plants produced flowers that opened and were able to set seeds (Fig. 3.6, Fig. 3.7B). In addition, the late developed flowers of *ga1-3* plants lacking GAI and RGA sometimes opened, but these flowers were almost always sterile (Fig. 3.5B; Fig. 3.6; Fig. 3.7A).

Transverse section analysis of anthers from *ga1-3* plants lacking RGA or RGL2 showed that RGA or RGL2 alone could partially rescue the anther development of *ga1-3* to anther stage 11 (anther development of *ga1-3* was arrested at anther stage 7-8) (Fig 3.8A). Although anthers from early developed flowers of *ga1-3* plants lacking both RGL2 and RGA were indistinguishable from that of *ga1-3* plants lacking either RGL2, or RGA, or RGA and GAI together, anthers from the late developed flowers of *ga1-3* plants lacking both RGA and RGL2 were normal (Fig. 3.8A). *In situ* hybridization analysis using *ATA7* marker showed that the tapetums

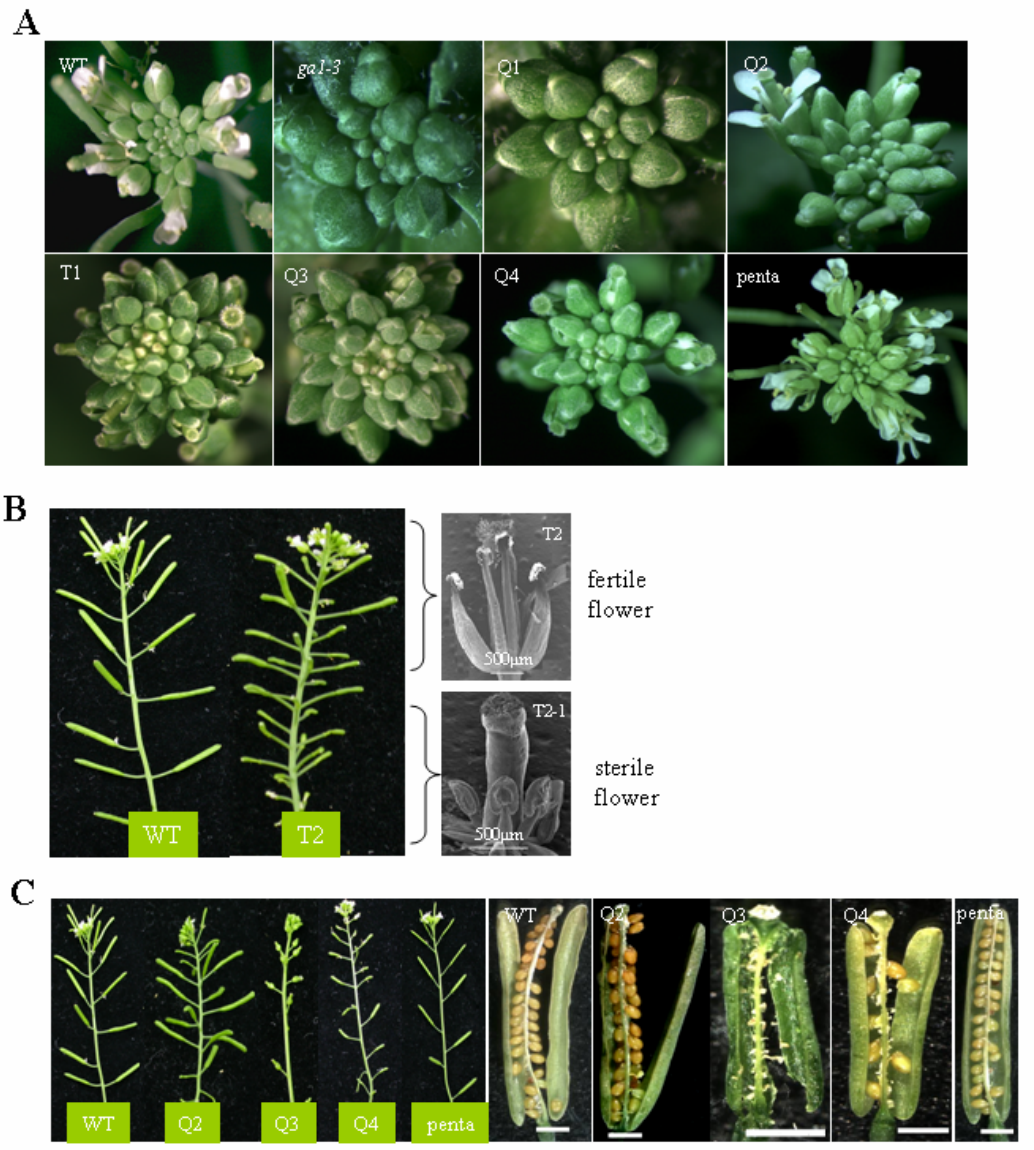



**Fig. 3.7. RGL1, RGL2 and RGA repress flower opening, petal and stamen development in *ga1-3* plants.** (A) Comparison of the flowers of 30-day-old plants of genotypes. (B) *ga1-3* plants lacking RGL2 and RGA initially produced sterile non-opening flowers, then began to produce fertile open flowers. (C) Comparison of seed set of various genotypes.(Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6*; Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*; Q3: *ga1-3 rgl1-1 gai-t6 rga-t2;* Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*; penta: *ga1-3 rgl1-1rgl2-1 gai-t6 rga-t2*; T1: *ga1-3 gai-t6 rga-t2; T2: ga1-3 rgl2-1 rga-t2* ).

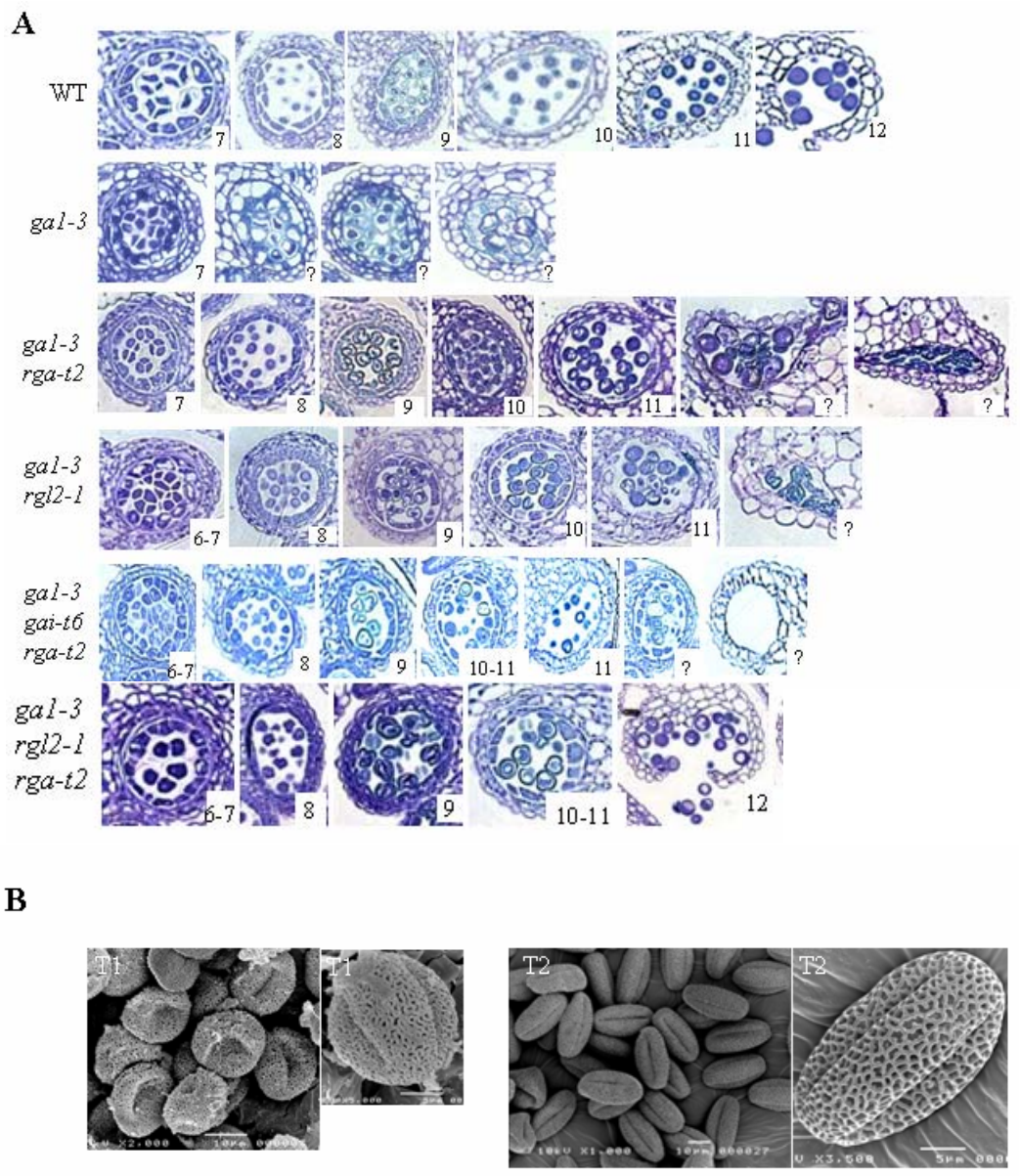



**Fig. 3.8. Microsporogenesis in double and triple mutants.** (A) Transverse sections of anthers are displayed according to developmental stages, showing the progress of microsporogenesis in *ga1-3* plants lacking RGL2 or RGA or GAI and RGA or RGL2 and RGA. (B) SEM of pollen surface of *ga1-3* plants lacking GAI and RGA or RGL2 and RGA. (T1: *ga1-3 gai-t6 rga-t2*, T2: *ga1-3 rgl2-1 rga-t2*).

of *ga1-3* plants lacking RGL1, RGL2 or RGA singularly or pair-wise were morphologically normal. This suggested that absence of any of these three DELLAs could fully restore normal tapetum development in *ga1-3* plants. Therefore, all three DELLAs RGL1, RGL2 and RGA might be required to suppress the tapetum development in *ga1-3* plants (Fig. 3.9). SEM analysis of pollen surface of *ga1-3* plants lacking RGA and RGL2 or RGA and GAI showed that *ga1-3* plants lacking RGA and RGL2 produced morphologically normal oval-shaped pollen grains. However, *ga1-3* plants lacking RGA and GAI produced *ga1-3* liked, spherical-shaped pollen grains (Fig. 3.8B).

Comparison of stamens and pistils from *ga1-3* plants lacking RGL2, GAI or RGA and *ga1-3* plants lacking RGL2 and GAI, RGL2 and RGA, or GAI and RGA indicated that absence of RGL2, GAI or RGA singularly or absence of both RGL2 and GAI did not restore the growth of stamens and pistils of *ga1-3* plants (Fig.3.10A). However, absence of GAI and RGA or RGL2 and RGA were able to fully rescue the development of pistils in *ga1-3* (Fig.3.10A), indicating that RGA is the key repressor of pistil elongation, while RGL2 and GAI enhance its function. Although early developed flowers of *ga1-3* plants lacking RGL2 and RGA were indistinguishable from that of *ga1-3* plants lacking GAI and RGA, The stamens from late developed flowers of *ga1-3* plants lacking RGL2 and RGA were morphologically normal (Fig. 3.6, Fig. 3.7, Fig. 3.10A). These observations suggested that RGL2 and RGA are likely the key repressors in GA regulated stamen development.

#### 3.3.2.2 RGL1, RGL2 and RGA act synergistically to control *Arabidopsis* stamen and petal development

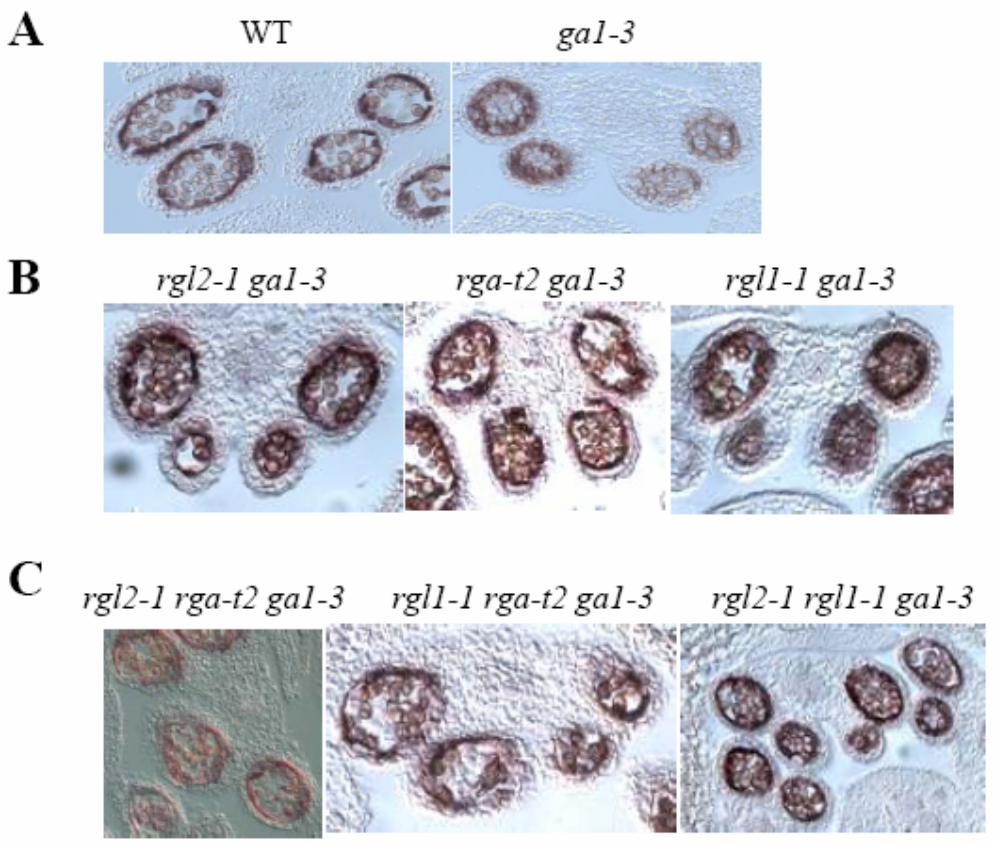



**Fig. 3.9. *ATA7* expression in different genotypes.** (A) Tapetum marked with expression of *ATA7* were abnormal in *ga1-3* mutant. (B, C) Absence of RGL1, RGL2, and RGA singularly (B) or pair-wise (C) was able to restore normal tapetum development in *ga1-3*.

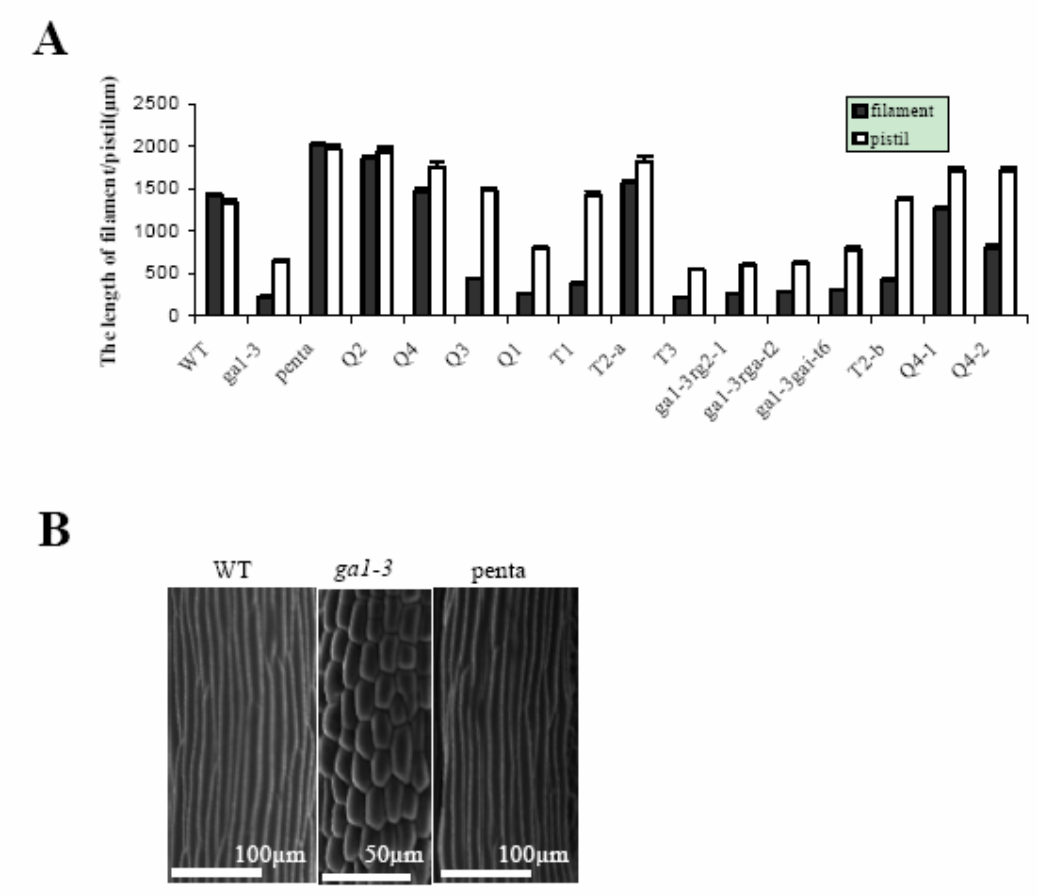



**Fig. 3.10. RGA and RGL2 are key GA response regulators in stamen filament epidermal cell elongation.** (A) Comparison of stamen and pistil lengths among different genotypes. (B) SEM of filament epidermal cells of WT, *ga1-3* and penta mutants. (Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6*; Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*; Q3: *ga1-3 rgl1-1 gai-t6 rga-t2;* Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*; penta: *ga1-3 rgl1-1 rgl2-1 gai-t6 rga-t2*; T1: *ga1-3 gai-t6 rga-t2*; T2-a: *ga1-3 rgl2-1 rga-t2;* T3: *ga1-3 rgl2-1 gai-t6;* T2-b: early developed flowers of T2-a (~40 days). Q4-1&2: early developed flowers of Q4 (~30 days).

Studies on *ga1-3* plants lacking all possible three-way combinations of RGL1, RGL2, GAI and RGA showed that absence of RGL1, RGL2 and GAI failed to suppress any detectable aspects of *ga1-3* phenotype (Fig. 3.5C & D, Fig. 3.7, Fig. 3.10A, Fig. 3.11). Absence of RGL1, RGL2 and RGA completely restored petal and stamen development of *ga1-3* and permitted normal seed set (Fig. 3.7; Fig. 3.11), despite the fact that this line was semi-dwarf and exhibited a stem elongation only slightly taller than that of *ga1-3* lacking RGA alone (Fig. 3.5A and D). Although *ga1-3* plants lacking RGL2 and RGA produced fertile flowers only in late maturity, *ga1-3* plants lacking RGL1, RGL2 and RGA produced fertile flowers at the onset of flowering, indicating that RGL1 enhances the function of RGL2 and RGA. Thus RGL1, RGL2 and RGA act in combination to control flower in response to GA (Fig. 3.5D, Fig. 3.7A and C, Fig. 3.11).

*ga1-3* plants lacking RGL1, GAI and RGA were taller than *ga1-3* plants lacking GAI and RGA (Fig. 3.5C, D), suggesting that RGL1 has a significant role in the control of stem elongation in the absence of GAI and RGA. However, absence of RGL1, GAI and RGA did not restore the normal petal and stamen development of *ga1-3* and this line were therefore sterile (Fig. 3.7A and C, Fig. 3.11). In fact, the young flower buds of this line were indistinguishable from those of *ga1-3* lacking GAI and RGA (Fig. 3.7A).

*ga1-3* plants lacking RGL2, GAI and RGA were taller at maturity than control lines lacking GAI and RGA alone (Fig. 3.5C, D and E). In contrast to what was seen with lack of RGL1, lack of RGL2 (*ga1-3* plants lacking RGL2, GAI and RGA) partially restored petal and stamen development of *ga1-3* plants lacking GAI and RGA, making this plants partially fertile (Fig. 3.7C, Fig. 3.11).

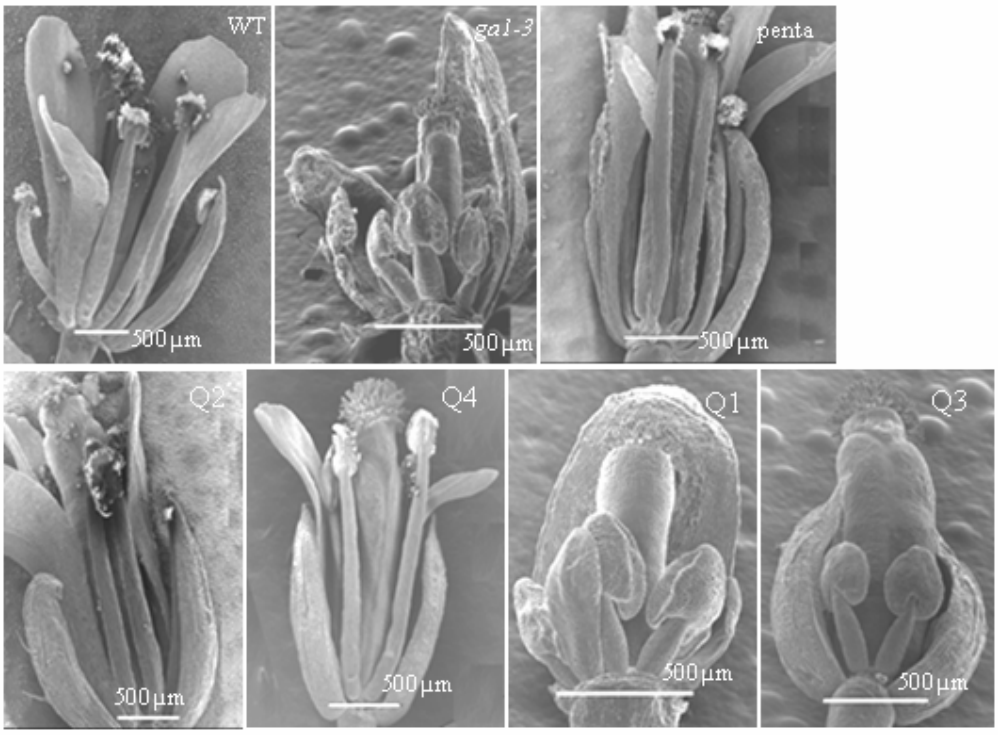



**Fig. 3.11. RGA, RGL2 and RGL1 act synergistically to repress the stamen and petal development.** Comparison of flower development among different genotypes. (Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6*; Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*; Q3: *ga1-3 rgl1-1gai-t6 rga-t2;* Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*; penta: *ga1-3 rgl1-1 rgl2-1 gai-t6 rga-t2*).

Transverse section analysis of anthers from quadruple mutants (*ga1-3* plants lacking three-way combinations of RGL1, RGL2, GAI and RGA) showed that absence of RGL1, RGL2 and RGA were able to fully restore anther development of *ga1-3* (Fig. 3.12). However, absence of RGL2, RGA and GAI partially rescued the anther development of late developed flowers with successfully completion of microsporogenesis in some of the four locules (Fig. 3.13). Lack of both RGL2 and RGA had great effect on microsporogenesis, as ~80% and ~60% of pollen grains were found to be tricellular in *ga1-3* plants lacking RGL1, RGL2 and RGA and *ga1-3* plants lacking GAI, RGL2 and RGA respectively (Table 3.1, Fig. 3.14). SEM of the pollen surface showed that *ga1-3* plants lacking RGL1, RGL2 and RGA produced WT-like oval-shaped pollen, while *ga1-3* plants lacking RGL2, GAI and RGA produced WT-like oval-shaped but wrinkled pollen grains (Fig. 3.15).

**Table 3.1 Frequencies of tricellular pollen grains in anther locules of various genotypes as revealed by DAPI staining.**

| Genotype | 2-3 nuclei (%) | 1 nuclei (%) | No nuclei (%) | Pollen numbers examined | Number of flowers |
|---|---|---|---|---|---|
| WT | 97.5 | 0.7 | 1.8 | 2159 | 6 |
| *ga1-3* | 6.5 | 48 | 45.5 | 4408 | 27 |
| Q1 | 28.3 | 7.6 | 64.1 | 2768 | 20 |
| Q2 | 78.9 | 0.3 | 20.8 | 2635 | 12 |
| Q3 | 33.9 | 14.5 | 51.6 | 4825 | 40 |
| Q4 | 58 | 5.2 | 36.8 | 2906 | 24 |
| penta | 88 | 0.1 | 11.9 | 1216 | 8 |

(Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6*, Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*, Q3: *ga1-3 rgl1-1 gai-t6 rga-t2,* Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*, penta: *ga1-3 rgl1-1 rgl2-1 gai-t6 rga-t2*)

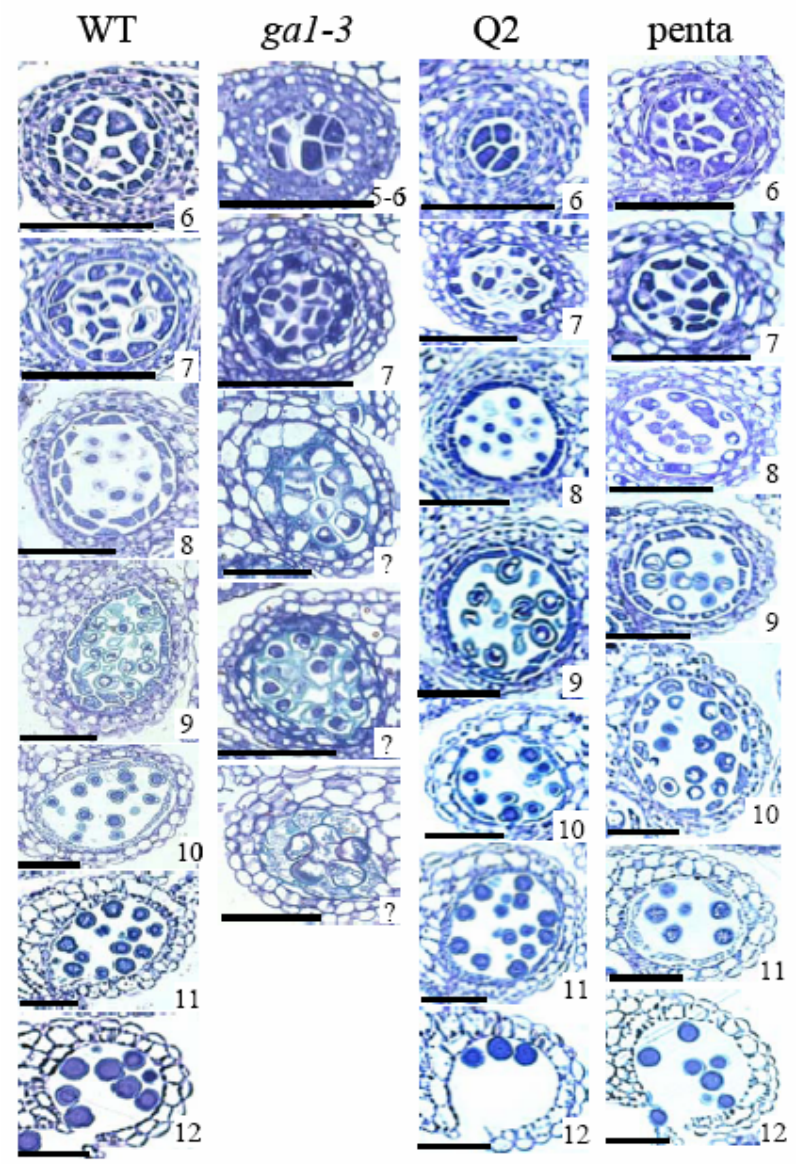



**Fig. 3.12. Absence of RGL1, RGL2 and RGA restored normal microsporogenesis of *ga1-3* mutant.** Transverse sections of anthers are displayed according to developmental stages, showing the progress of microsporogenesis in various genotypes. *ga1-3* anthers developed normally up to the tetrad formation stage (stage7) but after this, they diverted from the normal (compared with stages 9-12 in wild type; *ga1-3* stages highlighted with question mark). Eventually, all *ga1-3* pollen sac aborted. *ga1-3* plants lacking RGL1, RGL2 and RGA (Q2), or RGL1, RGL2, GAI and RGA (penta) successfully completed microsporogenesis and released mature viable pollen (scale bar: 50μm).

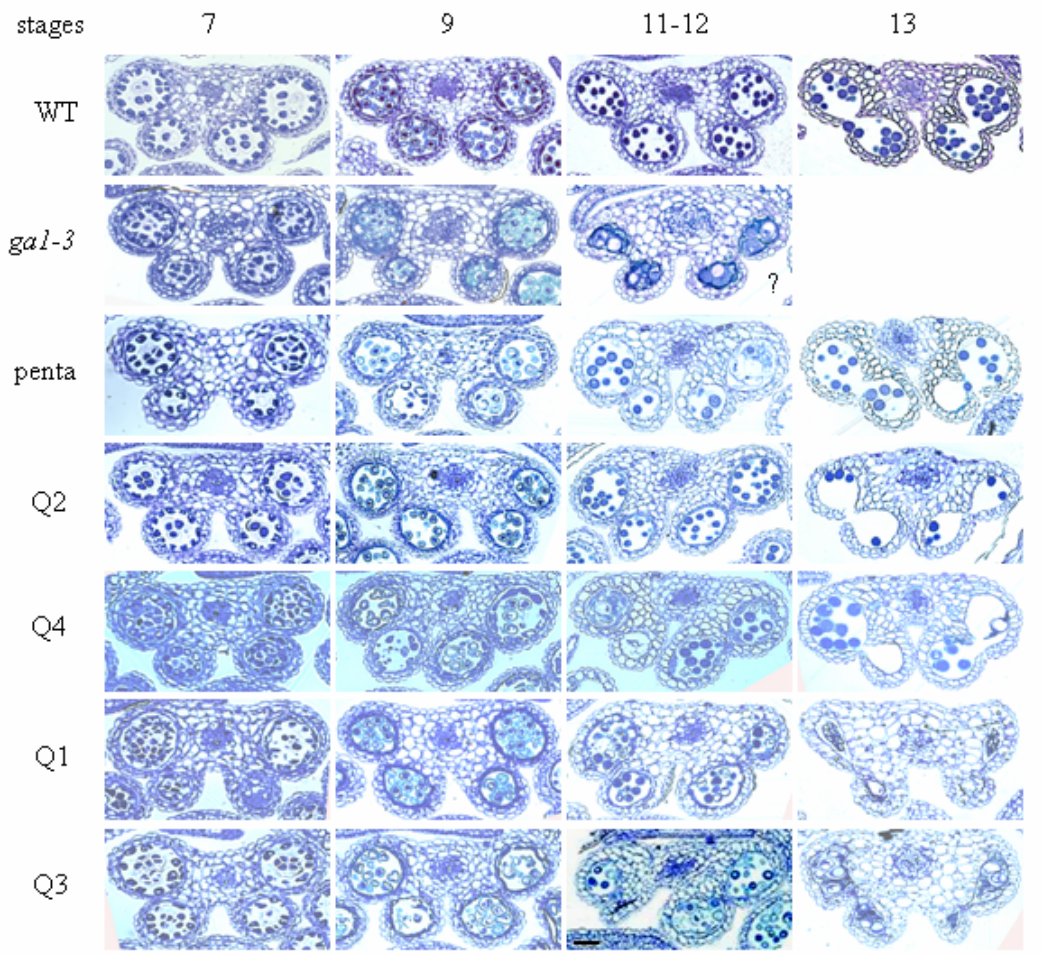



**Fig. 3.13. Histological analysis of microsporogenesis in quadruple and penta mutants.** Transverse sections of anthers of different quadruple and penta mutant are displayed according to developmental stages, showing the progress of microsporogenesis (Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6*; Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*; Q3: *ga1-3 rgl1-1 gai-t6 rga-t2*; Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*; penta: *ga1-3 rgl1-1 rgl2-1 gai-t6 rga-t2*).

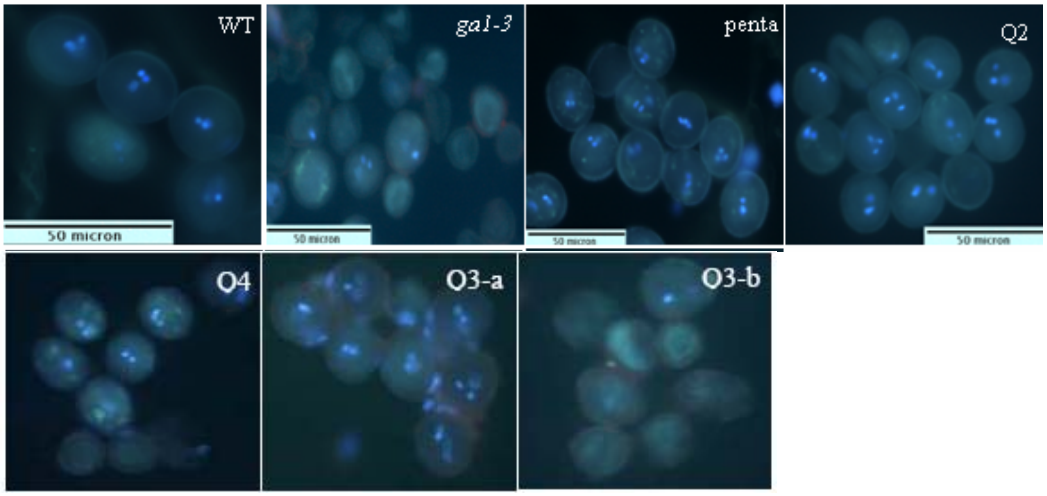



**Fig. 3.14. DAPI staining of pollen grains from various genotypes.** Pollen grains from penta and Q2 are tricellular as shown in WT. Majority of pollen grains from Q4 are also tricellular. Tricellular pollen grains can also be observed in Q3 with a relatively low frequency (Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*; Q3a&b: *ga1-3 rgl1-1 gai-t6 rga-t2;* Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*; penta: *ga1-3 rgl1-1 rgl2-1 gai-t6 rga-t2*).

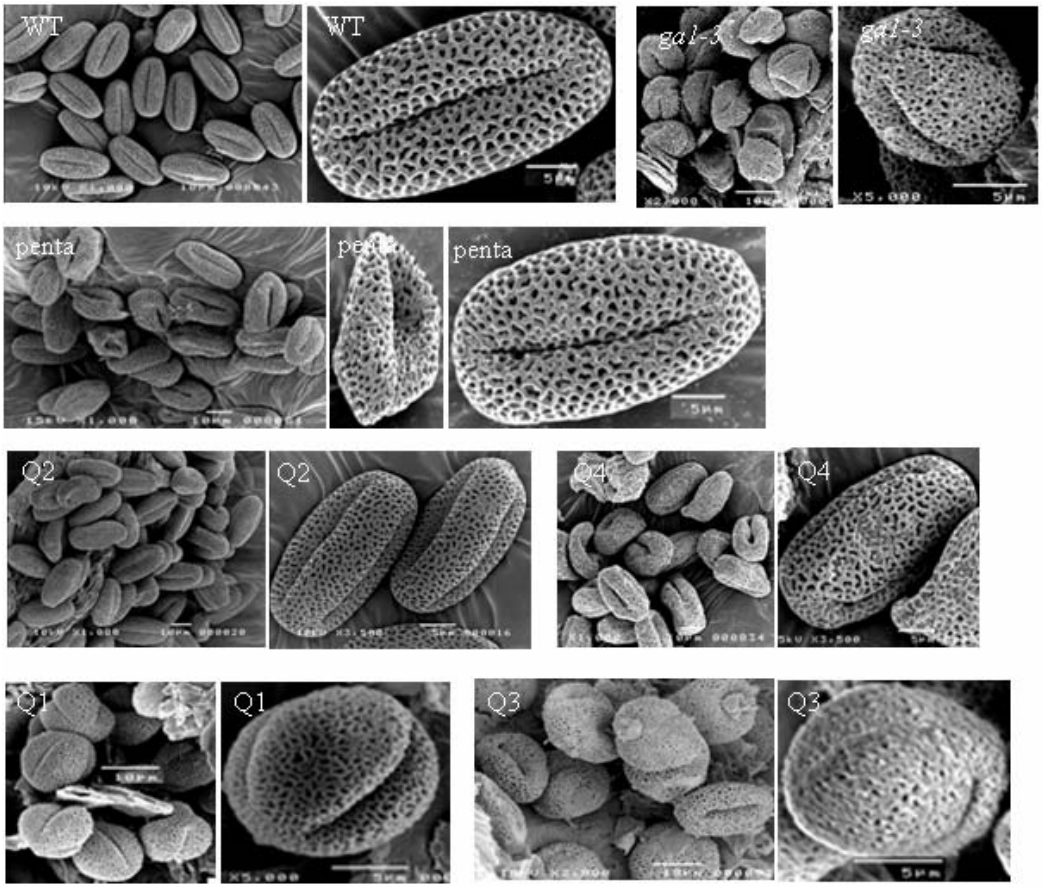



**Fig. 3.15. SEM of pollen grains from quadruple and penta mutants.** Pollen grains from Q2 mutant were almost identical to wild type pollen grains, with oval shaped and long indented lines on the surface. Pollen grains from penta and Q4 mutants were similar to WT pollen grains but were slightly more wrinkled in appearance. Pollen grains from Q1 and Q3 mutants were similar to that of *ga1-3* mutant, with round and in some cases, short indented lines on the wall surface. (Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6*; Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2*; Q3: *ga1-3 rgl1-1 gai-t6 rga-t2;* Q4: *ga1-3 rgl2-1 gai-t6 rga-t2*; penta: *ga1-3 rgl1-1 rgl2-1 gai-t6 rga-t2*).

Despite *ga1-3* plants lacking RGL1, RGL2 and GAI had hugely different stem elongation phenotypes from *ga1-3* plants lacking RGL1, GAI and RGA (Fig. 3.5 C and D), microsporogenesis of these two lines were very similar and were partially restored in these two lines. Anther development in *ga1-3* plants lacking RGL1, RGL2 and GAI or RGL1, GAI and RGA were both arrested at around anther stage 11-12 and both lines contained ~30% tricellular pollen grains (Fig. 3.13, Table 3.1). However, *ga1-3* plants lacking RGL1, RGL2 and GAI and *ga1-3* plants lacking RGL1, GAI and RGA still produced *ga1-3* liked spherical-shaped pollen (Fig. 3.14). These results further confirmed that RGA and RGL2 play dominant roles in the repression of microsporogenesis in *Arabidopsis*, and GA regulates microsporogenesis by overcoming the repressing effects of RGL1, RGA and RGL2.

Comparison of stamens and pistils of different mutants showed that *ga1-3* plants lacking RGL2, GAI and RGA had much longer stamens than that of *ga1-3* plants lacking GAI and RGA, suggesting RGL2 may play an important role in filament elongation (Fig. 3.10A). *ga1-3* plants lacking RGL1, GAI and RGA produced similar length of filament and pistil as those of *ga1-3* plants lacking GAI and RGA. However, in *ga1-3* plants lacking RGL1, RGL2 and RGA, both stamens and pistils are much longer than that of wild type, other three quadruple mutants and *ga1-3* plants lacking RGL2 and RGA. These data suggested that in the absence of RGL2 and RGA, absence of RGL1 might enhance filament and pistil elongation (Fig. 3.10A). Both the stamen and pistil were arrested in *ga1-3* plants lacking RGL1, RGL2 and GAI demonstrated that RGA play a predominant role in regulating both stamen and pistil elongation (Fig. 3.10A). SEM of stamen filament epidermal cells of different genotypes indicated that RGL1, RGL2 and RGA repressed stamen filament

cell elongation rather than cell division (Fig. 3.11A, Table 3.2). These observations suggested that RGL1, RGL2 and RGA may be the key repressors of stamen filament cell elongation, and GA regulates filament cell elongation via suppression of these DELLA proteins.

In summary, GA regulation of *Arabidopsis* petal and stamen elongation is mediated via RGL1, RGL2 and RGA, with RGL2 and RGA playing dominant roles.

**Table 3. 2. Number of epidermal cells in stamen filament**

| | WT | *ga1-3* | *ga1-3 rgl2-1* | *ga1-3 gga-t2* | *ga1-3 gai-t6* | T1 | T2 | T2-1 | T3 |
|---|---|---|---|---|---|---|---|---|---|
| Cell No. | 16±0.4 | 14±0.2 | 13±0.2 | 15±0.2 | 14±0.2 | 15±0.3 | 16±0.3 | 17±0.3 | 14±0.3 |
| | penta | Q1 | Q2 | Q3 | Q4 | Q4-1 | | | |
| Cell No | 13±0.2 | 13±0.2 | 15±0.4 | 14±0.3 | 15±0.3 | 14±0.2 | | | |

Average number of epidermal cells per stamen filament in wild type (28 days), *ga1-3* (38 days) and *ga1-3* plants lacking different DELLA combinations.(Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6* (32 days); Q2: *ga1-3 rgl1- 1rgl2-1 rga-t2* (34 days); Q3: *ga1-3 rgl1-1 gai-t6 rga-t2*(30 days)*;* Q4: *ga1-3 rgl2-1 gai-t6 rga-t2* (40 days); penta: *ga1-3 rgl1-1 rgl2-1 gai-t6 rga-t2* (22 days); T1: *ga1-3 gai-t6 rga-t2* (40 days); T2: *ga1-3 rgl2-1 rga-t2* (50 days). Cell No for T2-1 and Q4-1 represents early developed flowers from T2 (~ 40 days) and Q4 (~ 30 days) plants, respectively.

### 3.3.3 Absence of RGA, RGL2, RGL1 and GAI leads to GA-independent plant growth

*ga1-3* plants lacking RGL1, RGL2, GAI and RGA were analyzed. This mutant line bolted and flowered earlier than WT both in long day and short day (Cheng et al., 2004). In addition, these mutant plants were taller than wild type control (Fig. 3.5C, D and E). The combined absence of RGL1, RGL2, GAI and RGA were able to suppress the effects of *ga1-3* on petal and stamen development. The flowers of *ga1-3* plants lacking RGL1, RGL2, GAI and RGA exhibited fully extended stamens and petals

(Fig. 3.7A, Fig. 3.11). Anther development proceeded to completion, resulting in flowers that were fertile and able to set seeds in both LD and SD (Fig. 3.7C).

Transverse sectioning showed that, like *ga1-3* plants lacking RGL1, RGL2 and RGA, *ga1-3* lacking RGL1, RGL2, GAI and RGA achieved complete microsporogenesis (Fig. 3.12, Fig. 3.13). No obvious difference was observed between wild type and *ga1-3* plants lacking RGL1, RGL2 and RGA. However, we often observed that one or two of the four locules of the anthers of *ga1-3* plants lacking RGL1, RGL2, GAI and RGA were aborted (Fig. 3.12, Fig. 3.13). DAPI staining showed that 97.5% of the pollen grains were tri-cellular in WT, while only 88% of the pollen grains were tri-cellular in *ga1-3* plants lacking RGL1, RGL2, GAI and RGA (Fig. 3.14, Table 3.1). SEM analysis of pollen grains from *ga1-3* plants lacking RGL1, RGL2, and RGA showed that their surface structure was indistinguishable from that of wild type, with oval-shaped with long indented lines (Fig. 3.15). However, surface appearance of pollen grains from *ga1-3* plants lacking RGL1, RGL2, GAI and RGA were substantially different from those of wild type, mostly having a wrinkled appearance, and occasionally with severe deformity (Fig. 3.15).

As in *ga1-3* plants lacking RGL1, RGL2 and RGA, stamen filament growth in *ga1-3* plants lacking RGL1, RGL2, GAI and RGA were found to be slightly longer than those of the wild type (Fig. 3.10A). SEM of stamen filament epidermal cells indicated that restoration of stamen filament length in *ga1-3* plants lacking RGL1, RGL2, GAI and RGA was due to an increase in cell length elongation as opposed to an increase in cell number (Fig. 3.10B, Table 3.2), a difference similar to what was previously observed between wild type and *ga1-3* stamen filaments. Thus, the

elongation of stamen filaments became GA independent when all four DELLA proteins were removed.

### 3.4 Discussions

DELLA proteins act as repressors of plant growth whose function is opposed by GA (Richards et al., 2002). In several cases, GA opposes DELLA function by promoting DELLA disappearance (Silverstone et al., 2001; Itoh et al., 2002; Gubler et al., 2002; Fu et al., 2002). Recent studies have identified candidate F-box components of a SCF E3 ubiquitin ligase responsible for targeting DELLA proteins to the proteasome (McGinnis et al., 2003; Sasaki et al., 2003). Therefore GA stimulates GA-responses by targeting DELLA protein growth repressors for destruction in the proteasome (Harberd, 2003). Interestingly, it seems that GA opposes RGL2 function both by causing downregulation of *RGL2* transcripts and RGL2 protein levels during seed germination (Lee et al., 2002; Tyler et al., 2004).

Previous studies of *Arabidopsis* DELLA function have involved phenotypic comparisons of GA-deficient (*ga1-3*) plants with GA-deficient plants lacking GAI, RGA, RGL1, or RGL2 or a limited range of combinations of these factors (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002). In this chapter, we described the effects of a more comprehensive set of loss-of-function combinations of DELLA proteins, focusing specially on floral development.

Flower development consists of three distinct phases: floral initiation (in which the vegetative meristem is transformed into an inflorescence meristem), floral organ initiation and floral organ growth. As shown previously, absence of GAI and RGA substantially suppresses the effect of the *ga1-3* mutation on flowering time (a measure of time of floral initiation) in SD (Dill and Sun, 2001). We have shown that

an additional lack of RGL2 or RGL2 and RGL1 together further advances the flowering time (in both LD and SD) of *ga1-3* plants lacking GAI and RGA (Cheng et al., 2004). However, the magnitude of this further advance is relatively small compared with that initially caused by lack of both GAI and RGA. Thus, GAI and RGA play the predominant role in regulating flowering time in the GA signaling floral promotive pathway (Simpson and Dean, 2002), with only small contributions from RGL1 and RGL2.

By contrast, RGL1, RGL2 and RGA play key roles in floral organ development. The temporal coordination of the development of individual floral organs is essential for floral function. For example, at around the time that the pollen matures and is released from the anther, the stamen filaments of flowers of self-fertilizing species such as *Arabidopsis* elongate and bring the pollen into contact with the stigmatic papillae(Smyth et al., 1990; Bowman, 1994). We showed that the relatively short stamen filaments of *ga1-3* flowers resulted from an arrest of cell elongation rather than division and that the combined lack of RGL1, RGL2, GAI and RGA restored stamen filament cell elongation in *ga1-3* plants. We also showed that, in general, microspores do not proceed to the formation of mature pollen in *ga1-3* anthers, and that microspore development is possibly arrested prior to pollen mitosis in *ga1-3*, and that tapetum development is arrested in *ga1-3*. Whether the effect of *ga1-3* on pollen mitosis is a secondary effect of arrested tapetum development, or is independent of the effect on tapetum development is at present unclear. In addition, we occasionally observed *ga1-3* flower buds containing a significant number of tricellular pollen grains. Further investigation is needed to find out if this is a true reflection of the *ga1-3* developmental process or is caused by some other unknown environmental factors. Lack of RGL1, RGL2 and RGA or RGL1, RGL2, GAI and

RGA proteins restored microsporogenesis in *ga1-3* plants. Further genetic analysis enabled us to identify RGL2, RGA and RGL1 as the key GA-response regulators controlling stamen filament length and microsporogenesis. Interestingly, pollen grains from *ga1-3* plants lacking GAI, RGL1, RGL2 and RGA, although tricellular and viable, are deformed when compared with the wild-type-appearing pollen grains from *ga1-3* plants lacking RGL1, RGL2 and RGA. Perhaps absence of all four DELLA proteins activates the GA pathway to such high levels that pollen wall materials might be overproduced, resulting in abnormal pollen morphology.

Previous developmental genetic analyses showed that the *Arabidopsis* DELLA proteins GAI and RGA act as repressors of stem elongation and that GA exerts its promotive effects on stem growth by overcoming the effects of GAI and RGA (Dill and Sun, 2001; King et al., 2001). These observations, and additional observations on the behavior of DELLA proteins in other species, have been incorporated into a general 'release of restraint' model, which envisages DELLA proteins as general agents of restraint of plant organ growth, and GA as a means of overcoming that restraint (Peng et al., 1997; King et al., 2001; Richards et al., 2001; Harberd, 2003). However, the initial experiments (which examined the effect of lack of *Arabidopsis* GAI and RGA) showed that although stem elongation could be explained in terms of the 'release of restraint' model, other aspects of growth and development which were known to be GA regulated (in particular seed germination and floral organ growth) could not (Dill and Sun, 2001; King et al., 2001). It therefore remained possible that some other, entirely different, mechanism was responsible for the GA-mediated regulation of seed germination and floral organ growth.

It has been reported that the GA-promotion of *Arabidopsis* seed germination can be explained in terms of a GA-mediated release of the restraint upon germination

imposed by RGL2 (Lee et al., 2002) or RGL1 (Wen and Chang, 2002). The results in this thesis show for the first time that the GA-regulation of floral organ development is also DELLA mediated. However, different combinations of DELLA proteins are key players to floral organ development (RGA, RGL1 and RGL2), seed germination (RGL2 and RGL1) and stem elongation (RGA, GAI). The three key aspects of the *ga1-3* mutant phenotype (dwarfism, inhibition of seed germination, retarded floral organ development) can now be explained: the lack of GA in this mutant causes a failure to overcome the repressive effects of the DELLA protein combinations that are specific to each particular phenotypic aspect. As a consequence, the 'release of DELLA restraint' hypothesis can now be considered to be a viable model with which to understand GA responses in general. One possible explanation for how different DELLA combinations control different developmental processes (e.g. seed germination versus stem elongation versus stamen development) is that individual DELLA proteins have different temporal and spatial expression patterns. For example, *GAI* and *RGA* are ubiquitously expressed in all plant tissues, whereas *RGL1* and *RGL2* transcripts are relatively enriched in the inflorescence (Silverstone et al., 1998; Lee et al., 2002; Wen and Chang, 2002). In situ hybridization showed that *RGL1* is highly expressed in the stamen primordium (Wen and Chang, 2002). Examination of an *RGL2* promoter-GUS fusion line showed that *RGL2* transcripts are also enriched in the stamen (Lee et al., 2002). The expression patterns of *RGL1* and *RGL2* are therefore consistent with our current observation that RGL1 and RGL2 are both important for stamen development.

The nature of the arrest in flower development conferred by *ga1-3* (and restored by lack of RGL1, RGL2 and RGA) is particularly interesting. Our results identify a relatively distinct developmental stage at which arrest occurs. Before that

stage, *ga1-3* stamen and anther development proceeds in a way that is indistinguishable from that of wild type. After that stage, wild-type development continues, while *ga1-3* development is blocked. It will be interesting to determine if other GA deficiency phenotypes (e.g. the particular shape of leaves of *ga1-3* mutant plants) are also due to premature arrest of an identifiable developmental sequence.

# Chapter 4

# Identification of DELLA Regulated Genes in Flowers

## 4.1 Introduction

DELLAs act as negative regulators of GA response. However, as a group of putative transcription regulators (Pysh et al., 1999; Richards et al., 2000), the molecular mechanism of DELLAs repressing plant growth is largely unknown. Floral organ development, especially petals and stamens is impaired in severe *Arabidopsis* GA-deficient mutant *ga1-3*, resulting in male-sterile phenotype of *ga1-3*. In contrast, the floral phenotypes of the GA-deficient mutant *ga1-3* are fully restored in the *ga1-3* plant lacking four DELLAs (RGL1, RGL2, GAI and RGA), suggesting that GA signaling through these four DELLAs is the major pathway for GA-mediated floral development (Cheng et al., 2004; Tyler et al., 2004; Yu et al., 2004). Therefore, GA-regulated transcriptomes for floral development might be DELLA-dependent. However, it is currently not known if all GA-regulated genes are DELLA-dependent.

Floral development is retarded in the *ga1-3* mutant, suggesting that the GA-regulated-transcriptome for floral development in the *ga1-3* mutant might be kept at a repressive state (Ogawa et al., 2003). Meanwhile, the floral development is fully restored in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant suggested that in this mutant line the GA-regulated-transcriptomes responsible for floral development might have been constitutively activated. It is reasonable to speculate that genes that are up regulated by GA would be at a lower expression level in *ga1-3*. If stabilized high levels of DELLA repressors in *ga1-3* were responsible for a proportion of these lower expressing genes, the ones which are genuinely repressed, directly or indirectly, by DELLAs would be restored to WT levels or even higher in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant in which four DELLA proteins were knocked out (resulting in

fully fertile flowers). In contrast, genes that are down regulated by GA and supposed to be activated by DELLAs would be expressed at higher level in *ga1-3*. These group of genes would be brought back to WT levels or even lower in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant. Thus, comparing the expression profiles in *ga1-3* with that in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant would help to identify the set of DELLA-dependent transcriptomes essential for floral development.

In this chapter, we compared the global gene expression patterns in unopened flower buds of the *ga1-3* mutant with that of wild type and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant using oligonucleotide-based DNA microarray analysis (Affymetrix ATH1 GeneChip, carrying 23,000 genes). We chose to compare *ga1-3* to WT instead of *ga1-3* treated with GA to identify GA-regulated (both up- and down-regulated) transcriptomes in young flower buds because we also wished to compare WT to *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant (which showed phenotypic suppression of *ga1-3*) at the same time. GA-regulated (both up- and down-regulated) transcriptomes in young flower buds was first identified by comparing the expression patterns between the *ga1-3* mutant and WT control. Then, DELLA-dependent (both up- and down-regulated) transcriptomes were identified by finding out the subgroups of GA-regulated genes with their expression restored to the WT levels in the *ga1-3 rga-t2 gai-t6 rgl1-1 rgl2-1* mutant. Data analysis showed that, in young flower buds, approximately half of the GA-regulated genes (down- or up-regulated in *ga1-3*) were restored to the WT level in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant. These GA-regulated DELLA-dependent genes were likely responsible for mediating floral development.

## 4.2 Materials and methods

Total RNA from young unopened flower buds of WT (28 day old), *ga1-3* mutant (38 day old) and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant (22 day old) was extracted as described in section 2.7. The residue genomic DNA in the total RNA was removed as described in section 2.8. Microarray analysis was carried out as described in section 2.15 and 2.16. Primers used for genotyping and expression studies were listed in Table 2.2, Table 2.3, and Table 2.5.

## 4.3 Results

### 4.3.1 Identification of DELLA-dependent transcriptome expressed during floral development

To identify DELLA-dependent transcriptomes essential for floral development, we carried out microarray analysis of RNA samples extracted from the young and unopened flower buds of the WT control, the *ga1-3* mutant and the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant. Six microarray replicates for each of the three genotypes were performed. Only genes with the signal log ratio of WT vs *ga1-3* more than 1 (two folds higher) or less than -1 (two folds lower) in at least four replicates were referred to as GA-up or GA-down, respectively. Based on the above criteria, 826 genes were identified as GA-up in the *ga1-3* young flower buds when compared to that of WT. The transcript levels of 360 out of these 826 GA-up genes (44%) were at least 2-fold higher in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant than in *ga1-3* while the remaining 466 genes did not show significant changes in their expression. These 360 genes were expected to be DELLA-down genes in the young flower buds while the 466 genes were thought to be DELLA-independent or partially-dependent GA-regulated genes. Meanwhile, the transcripts of 422 genes were accumulated to higher levels in *ga1-3* young flower buds than in the wild-type. The transcript levels of 273

out of these 422 genes (65%) were at least 2-fold lower in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant than in *ga1-3* while the remaining 149 genes did not show significant changes in their expression. These 273 genes were determined to be DELLA-up genes in the flower buds and the 149 genes to be DELLA-independent or -partially-dependent GA-regulated genes (Table 4.1).

**Table 4. 1. Summary of GA- and DELLA-regulated transcriptomes**

| | No of genes | | No of Genes |
|---|---|---|---|
| GA-up | 826 | DELLA-down | 360 |
| GA-down | 422 | DELLA-up | 273 |

To confirm the reliability of results from expression profiles analysis, RT-PCR experiments were performed using cDNAs synthesized from RNA material identical to those used in arrays. 59 genes that consist of 38 DELLA-down genes and 21 DELLA-up genes were randomly examined. RT-PCR analysis confirmed that all 38 DELLA-down genes and 19 out of 21 DELLA-up genes exhibited the expected expression patterns (Fig. 4.1A, B), demonstrating that the microarray data obtained here was highly reproducible.

### 4.3.2 Ontology analysis of DELLA-dependent transcriptomes expressed during floral development

Out of the 360 DELLA-repressed genes, 243 genes have been assigned a putative molecular function based on amino acid homology anlaysis and 117 are recorded as functionally unassigned putative genes. Majority of DELLA-down floral genes encode enzymes (155 genes) responsible for the metabolism of protein, carbohydrate and lipid and encode proteins (89 genes) with binding activity to nucleic

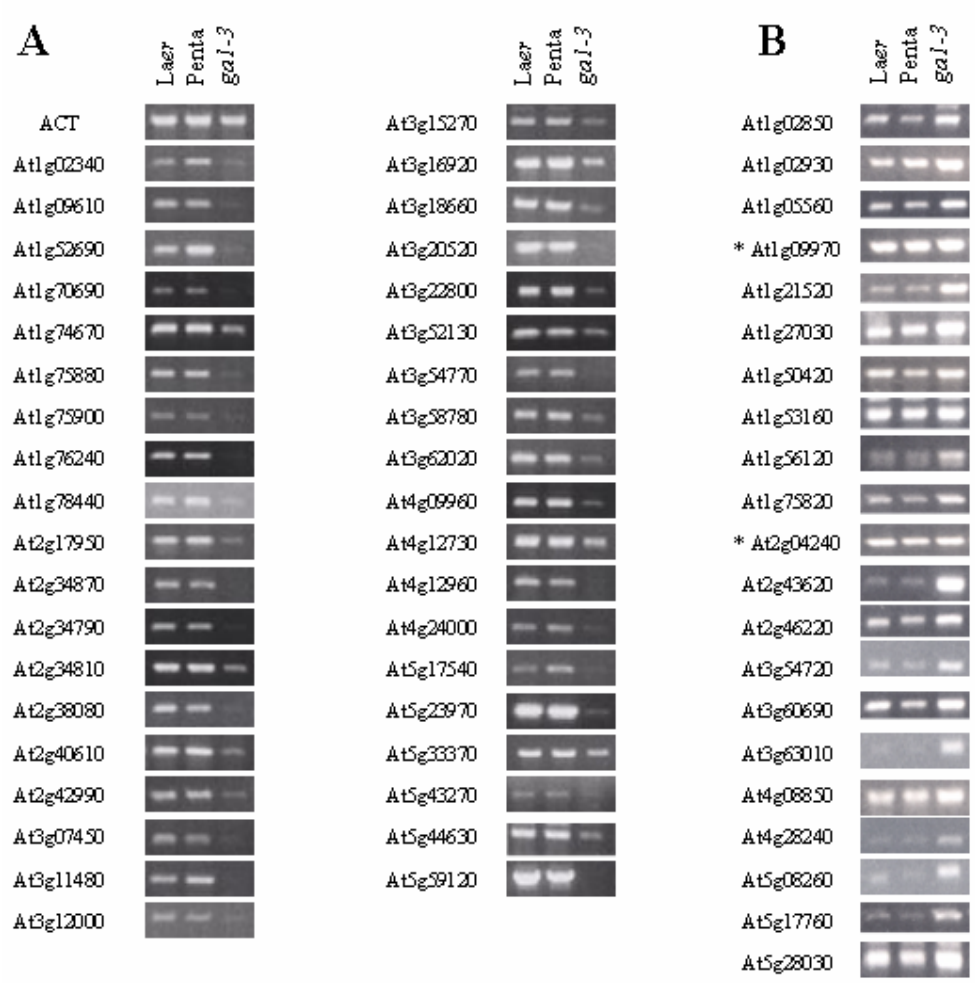



**Fig. 4.1. RT-PCR confirmation of DELLA-down and DELLA-up genes in the unopened young flower buds.** (A) DELLA-down genes. (B) DELLA-up genes. RT-PCR analysis was repeated on three independent samples and a representative ethidium bromide gel picture is shown here. Corresponding gene locus identity (Gene ID) is provided. Two genes (At1g09970 and At2g04240) in panel B didn't show obvious difference in expression and were highlighted with asterisk. penta: *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* penta mutant. *ACT2* (At3g18780) was used as the normalization control.

acid, nucleotide, ion, and protein binding, suggesting that the arrest of floral organ growth may be coupled with low metabolic activities (Table 4.2). Many types of transcription factors are known to control or regulate floral development (Krizek and Fletcher, 2005). Our microarray analysis identified seven MYB family genes, four squamosa promoter binding protein genes, three bHLH family genes, three MADS box genes and three AP2 domain-containing transcription factor genes as DELLA-down genes (Table 4.2, Table 4.3), suggesting that these factors might be the link between DELLA-mediated GA signaling and floral development.

Previous studies have shown that the impaired growth of petal and stamen filament in *ga1-3* is due mainly to the arrest of cell elongation rather than cell division (Cheng et al., 2004). In our microarray studies, four expansin genes were identified as DELLA-down genes in the young flower buds (Table 4.3), and two of them (At2g37640, At2g40610) were also identified as DALLA-down genes in seeds (Cao et al., 2006), suggesting that expansins may be crucial for the cell elongation in both seed germination and floral development. On the other hand, seven xyloglucan endotransglycosylase/hydrolase (XTH) genes and six pectinesterase genes were suggested to be the major genes responsible for cell wall loosening in the imbibed seeds (Cao et al., 2006). However, none of these two categories of genes was identified to be DELLA-down genes in the young flower buds. Instead, four cellulose synthase genes, two cellulase, and one 1, 4-beta-mannan endohydrolase were found as DELLA-down genes in the young flower buds (Table 4.3). These data suggested that DELLAs might repress the expression of genes responsible for the biogenesis and modification of cell wall components in controlling stamen development.

**Table 4. 2. Ontology analysis of DELLA-regulated genes in unopened flower buds based on molecular function assigned**

| Molecular Function | | | Unopened Flower Buds | |
|---|---|---|---|---|
| | | | DELLA-D | DELLA-U |
| **Catalytic activity** | (Total) | | (155) | (110) |
| | Hydrolase | (Subtotal) | (60) | (21) |
| | acting on: | glycosyl bonds | 17 | 7 |
| | | ester bonds | 21 | 6 |
| | | peptide bonds | 16 | 3 |
| | | acid anhydride | 2 | 5 |
| | Transferase | | 37 | 55 |
| | Oxidoreductase | | 32 | 24 |
| | Lyase | | 11 | 4 |
| | Ligase | | 5 | 4 |
| | Others | | 3 | 2 |
| **Binging activity** | (total) | | (89) | (99) |
| binding to: | Nucleic acid | | 36 | 30 |
| | Ion | | 15 | 27 |
| | Nucleotide | | 11 | 30 |
| | Protein | | 10 | 18 |
| | Oxygen | | 10 | 10 |
| | Tetrapyrrole | | 6 | 9 |
| | Lipid | | 7 | 2 |
| | Carbohydrate | | 3 | 9 |
| | Others | | 5 | 6 |
| **Transcription regulator activity** | (total) | | (32) | (27) |
| | MYB | | 8 | 4 |
| | Zinc finger | | 1 | 7 |
| | bHLH | | 3 | 3 |
| | MADS box | | 3 | 0 |
| | WRKY genes | | 0 | 3 |
| | Others | | 19 | 12 |
| **Transporter activity** | | | 24 | 15 |
| **Structure molecular** | | | 3 | 0 |
| **Antioxidant activity** | | | 4 | 0 |
| **Nutrient reservoir** | | | 1 | 1 |

In the young flower buds, 243/360 DELLA-D and 180/273 DELLA-U genes were assigned with molecular functions.

**Table 4. 3. Genes related to some important biochemical and biological processes in unopened young flower buds**

| | Unopened Flower Buds | |
|---|---|---|
| | DELLA-D | DELLA-U |
| **1. Cell growth and cell wall loosening** | | |
| XTH | | At4g37800 |
| | | At5g57560 |
| Cellulose synthase | At4g18780 | |
| | At4g24000 | |
| | At5g17420 | |
| | At5g44030 | |
| Cellulose | At1g13130 | |
| | At3g26140 | |
| 1,4-beta-mannan endohydrolase | At3g10890 | |
| Glycoside hydrolase | At3g16920 | |
| | At3g42950 | |
| Expansin | At1g20190 | At2g18660 |
| | At2g37640 | |
| | At2g40610 | |
| | At3g29030 | |
| **2. Transcription Factors** | | |
| bHLH family proteins | At1g25330 | At4g01460 |
| | At1g59640 | At5g46760 |
| | At5g39860 | At5g50915 |
| MYB family proteins | At1g17950 | At1g06180 |
| | At2g38090 | At3g11280 |
| | At3g01140 | At5g44190 |
| | At3g27810 | At5g59780 |
| | At3g27812 | |
| | At4g34990 | |
| | At5g40350 | |
| | At3g01530 | |
| Zinc-finger family proteins | At5g25830 | At1g13400 |
| | | At1g66140 |
| | | At1g68520 |
| | | At1g73870 |
| | | At2g01940 |
| | | At2g47680 |
| | | At5g25160 |

**Table 4.3-continued**

| | Unopened Flower Buds | |
|---|---|---|
| | DELLA-D | DELLA-U |
| MADS box family proteins | At2g45650 | |
| | At3g58780 | |
| | At4g09960 | |
| WRKY family proteins | | At2g23320 |
| | | At3g56400 |
| | | At4g23810 |
| AP2 domain containing protein | At1g15360 | At1g25560 |
| | At1g16060 | |
| | At5g67180 | |
| Squamosa promoter binding protein | At1g27360 | |
| | At1g53160 | |
| | At3g15270 | |
| | At5g43270 | |
| Homeodomain transcription factor | At1g62990 | |
| | At2g17950 | |
| **3. Protein Phosphorylation** | | |
| Protein kinase | At1g61590 | At1g16260 |
| | At5g57670 | At1g21250 |
| | | At1g21270 |
| | | At1g29720 |
| | | At1g65190 |
| | | At1g66880 |
| | | At1g66920 |
| | | At1g69730 |
| | | At2g26980 |
| | | At2g32680 |
| | | At3g09830 |
| | | At3g23110 |
| | | At3g45640 |
| | | At3g45780 |
| | | At4g04540 |
| | | At5g25440 |
| | | At5g38210 |
| | | At5g40540 |
| | | At5g60900 |

**Table 4. 3.-continued**

| | Unopened Flower Buds | |
|---|---|---|
| | DELLA-D | DELLA-U |
| Leucine-rich repeat proteins | At4g18640 | At1g09970 |
| | | At1g33560 |
| | | At1g35710 |
| | | At1g51805 |
| | | At1g56120 |
| | | At2g31880 |
| | | At3g11010 |
| | | At4g08850 |
| | | At5g48380 |
| Receptor protein kinase | | At1g75820 |
| | | At4g23130 |
| | | At4g23180 |
| S-locus lectin protein kinase | | At1g11350 |
| | | At2g19130 |
| | | At4g11900 |
| | | At4g27300 |
| S-receptor kinase | | At1g65790 |
| **4. Disease and stress response** | | |
| Response to disease and pathogens | At1g55020 | At1g33560 |
| | At1g72260 | At1g72930 |
| | At3g11480 | At2g32680 |
| | At3g13650 | At2g43570 |
| | At3g16920 | At2g43620 |
| | At3g21240 | At3g11010 |
| | At4g23690 | At3g20590 |
| | At5g24780 | At3g23110 |
| | | At3g50950 |
| | | At4g16990 |
| | | At4g19530 |
| | | At4g26090 |
| | | At5g45250 |

**Table 4. 3-continued**

| | Unopened Flower Buds | |
|---|---|---|
| | DELLA-D | DELLA-U |
| Water and salt stress | At1g05260 | At1g33560 |
| | At1g29395 | At2g21620 |
| | At1g52690 | |
| | At2g21490 | |
| | At5g24780 | |
| Oxidative stress | At3g59845 | At3g45640 |
| | At4g11290 | |
| | At4g30170 | |
| | At5g24780 | |
| | At5g51890 | |
| Cold | At1g05260 | At5g57560 |
| | At1g29395 | |
| Heat | At5g67180 | |
| UV | At3g21240 | |
| | At4g13770 | |
| Toxin catabolism | At1g17190 | At1g02930 |
| Multidrug transport | At3g26590 | |
| | At4g22790 | |
| | At5g49130 | |
| Wounding | At1g55020 | At2g38870 |
| | At3g11480 | |
| | At3g21240 | |
| | At5g24780 | |
| DNA damage response | At3g12710 | |
| | At5g44680 | |
| Others | At1g11000 | At1g31580 |
| | At1g52040 | |
| | At2g43550 | |
| | At5g51060 | |

**Table 4. 3-continued**

| | Unopened Flower Buds | |
|---|---|---|
| | DELLA-D | DELLA-U |
| **5. Hormone response** | | |
| ABA | At1g29395 | At1g75750 |
| | At1g52690 | At2g26980 |
| | At1g55020 | At3g22060 |
| | At5g59320 | At3g45640 |
| GA | At1g74670 | At1g15550 |
| | At1g78440 | At1g22690 |
| | | At1g75750 |
| | | At4g25420 |
| | | At5g51810 |
| Auxin | At1g29510 | At2g45210 |
| | At1g44350 | At3g60690 |
| | At2g21220 | |
| | At3g15540 | |
| | At3g23050 | |
| | At3g25290 | |
| | At4g12410 | |
| | At4g13790 | |
| | At5g47530 | |
| Ethylene | At1g15360 | At1g05010 |
| | | At1g28360 |
| | | At5g25190 |
| BR | | At1g75750 |
| Cytokinin | | At2g26980 |

Genes listed here are summary of microarray results based on information provided in Gene Title, Molecular Function and Gene Description by Affymetrix. DELLA-D: DELLA-down genes; DELLA-U: DELLA-up genes.

GA2-oxidase (At1g78440, responsible for the degradation of bioactive GAs) and a GAST1-like gene (At1g74670) have previously been shown to be up-regulated by GA (Shi and Olszewski, 1998; Ogawa et al., 2003). The restoration of expression of these two genes in *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* suggests that GA may regulate their expression by triggering the degradation of DELLA proteins and therefore they were identified as DELLA-down genes (Table 4.3). Interestingly, nine auxin-response genes including auxin responsive transcription factors *IAA19* (At3g15540) and *AUXIN RESISTANT 2* (*AXR2*) (At3g23050) (Liscum and Reed, 2002), putative IAA-amino acid hydrolase 6 (*ILL6*) (At1g44350) (LeClere et al., 2002), two auxin-responsive dopamine beta-monooxygenase (At5g47530 and At3g25290) (Neuteboom et al., 1999), and four auxin-responsive genes (At4g13790, At1g29510, At4g12410, At2g21220) (Table 4.3) were identified as DELLA-down genes in the young flower buds.

Among the 273 DELLA-up floral genes, 180 genes have been assigned a putative molecular function and 93 are recorded as expressed putative genes. Again, the two largest groups of DELLA-up genes consist of genes encoding proteins with catalytic activity (110 genes) or binding activity (99 genes) (Table 4.2). Majority of DELLA-up enzyme genes were transferase genes (55 genes) and oxidoreductase genes (24 genes) but not hydrolase genes as observed in DELLA-down floral genes (Table 4.2). GA biosynthesis is controlled by a negative feedback loop. The lower expression levels of three key gibberellin biosynthesis genes (two GA-20-oxidase genes (At4g25420, At5g51810) and one GA-3β-hydroxylase gene (At1g15550) in *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* suggest that GA down-regulates these genes by the degradation of DELLA proteins and therefore they were identified as DELLA-up genes (Table 4.3) (Ogawa et al., 2003). Further analysis showed that a great number

of transcription factors, including seven putative zinc finger family genes (*ZINC FINGER PROTEIN ZEP3*, *ZEP4*, *JAG* etc) (Tague and Goodman, 1995; Riechmann et al., 2000), four MYB family genes (*MYB59*, *MYB13*, At5g44190, At3g11280) (Riechmann et al., 2000), three putative bHLH family genes (At5g50915, At5g46760, At4g01460) (Heim et al., 2003) and three WRKY family genes (*WRKY15*, *WRKY70*, *WRKY53*) (Eulgem et al., 2000) were identified as DELLA-up genes (Table 4.3), suggesting that DELLAs might mediate a complex genetic regulatory network to repress floral development.

Recent studies have shown that DELLAs act as the integrator of environmental cues as well as endogenous phytohormonal signals to protect plants from environmental stress (Lee et al., 2002; Cao et al., 2005; Achard et al., 2003; Achard et al., 2006). In young flower buds, five genes responsive to oxidative stress were identified as DELLA-down genes (Table 4.3). Meanwhile, multidrug transport (3 genes) and wounding response genes (4 genes) were identified as DELLA-down genes in the young flower buds (Table 4.3). Interestingly, while only two protein kinase genes (At5g57670 and At1g61590) and one leucine-rich repeat kinase gene (At4g18640) were identified as DELLA-down genes, a significant number of putative protein kinase genes (19 protein kinase genes, nine leucine-rich repeat kinase genes, four S-locus protein kinase genes and three receptor protein kinase genes) are identified as DELLA-up genes in the young flower buds (Table 4.3), suggesting that protein phosphorylation modification might play a key role in controlling floral organ growth (Morris and Walker, 2003). These data implied that DELLAs are not only actively involved in protecting plant from different environmental stress but probably also in mediating disease resistance in young flower buds.

### 4.3.3 Identification of 37 stamen-enriched DELLA-down regulated genes

Studies in *ga1-3* plants showed that GA plays a more important role in petal and stamen development than in sepal and pistil development. We showed in the previous chapter that GA regulates stamen filament cell elongation via suppression of DELLA proteins. It will be interesting to determine which of these DELLA regulated genes might function in stamen development. Therefore, the spatial expression patterns of 43 DELLA-down genes were examined in four different floral organs: sepal, petal, stamen and pistil. 36 genes (out of 43) were identified to be enriched in stamen (Table 4.4).

Among these 36 stamen-enriched genes, there were six DELLA-down *MYB* genes (*AtMYB21*, *AtMYB24*, *AtMYB32*, *AtMYB46*, *AtMYB52*, and *AtMYB57*) (Table 4.4), indicating that *MYB*s might play an important role in stamen development. However, with the exception of *AtMYB32*, which has been indicated to be involved in normal pollen development (Preston et al., 2004), the functions of the rest *MYB* genes are largely unknown. Three cellulose synthase catalytic subunits (CesA04 (IRX5), CesA07 (IRX3) and CesA08 (IRX1)) were also identified to be stamen-enriched. Cellulose synthase catalytic subunits (CesAs) have been indicated in catalyzing the biosynthesis of cellulose, the major component of plant cell walls (Taylor et al., 1999; Taylor et al., 2000; Turner and Somerville, 1997; Gardiner et al., 2003; Holland et al., 2000). This result suggested that GA might regulate cell elongation via modulation of the formation of plant cell walls. Two APG (anther-specific gene)-like proteins and one putative APG isolog protein which possibly function in anther development were also identified to be stamen enriched (Roberts et al., 1993). In addition, several unknown and putative proteins were also identified to be DELLA regulated stamen-

enriched genes in this study (Table 4.4). These 36 genes would serve as candidates to understand how DELLAs regulate stamen development.

**Table 4. 4. RT-PCR examination of DELLA-down genes in different floral organs**

| Gene ID | Gene description | DELLA-Down | Expression in floral organs | | | | |
|---|---|---|---|---|---|---|---|
| | | | Se | Pe | St | Pi | Yb |
| At5g23970 | Acetyl-coA benzylacohol | confirmed | +++ | +++ | + | ++ | +++ |
| At3g58780 | Agamous like 1 | confirmed | ++ | ++ | ++ | ++ | ++ |
| *At1g75880 | APG-like | confirmed | ++ | ++ | +++ | - | + |
| *At1g75900 | APG-like | confirmed | + | - | + | + | + |
| *At2g34790 | Berberrine bridge enzyme | confirmed | + | - | +++ | + | + |
| *At2g34810 | Berberrine bridge enzyme | confirmed | - | - | + | + | - |
| *At4g18780 | Cellulose synthase (IRX1) | confirmed | + | ++ | +++ | + | + |
| *At5g44030 | Cellulose synthase (IRX5) | confirmed | + | + | +++ | ++ | + |
| *At5g17420 | Cellulose synthase (IRX3) | confirmed | ++ | ++ | +++ | + | + |
| *At3g16920 | Chitinase(GHF19) | confirmed | + | - | ++ | + | + |
| *At3g22800 | Extensin-like | confirmed | ++ | ++ | +++ | +++ | ++ |
| *At3g62020 | Germin-like protein | confirmed | ++ | ++ | +++ | ++ | ++ |
| *At1g78440 | Gibberellin 2-oxidase | confirmed | ++ | ++ | +++ | +++ | +++ |
| *At2g17950 | Homeodomain transcription factor | confirmed | + | - | +++ | + | + |
| *At3g11480 | Hypothetical | confirmed | + | + | +++ | +++ | ++ |
| *At1g09610 | Hypothetical protein | confirmed | ++ | + | +++ | + | + |
| *At1g76240 | Hypothetical protein | confirmed | ++ | + | +++ | + | + |
| *At3g18660 | Hypothetical protein | confirmed | ++ | ++ | +++ | + | + |
| *At3g20520 | Hypothetical protein | confirmed | ++ | ++ | ++ | ++ | ++ |
| *At1g52690 | Late embryogenesis abundant protein | confirmed | ++ | +++ | +++ | ++ | +++ |
| *At4g09960 | MADX-box protein (AGL11) | confirmed | + | + | ++ | +++ | ++ |
| *At3g27810 | MYB21 | confirmed | ++ | ++ | +++ | + | - |
| *At5g40350 | MYB24 | confirmed | + | +++ | +++ | + | - |
| *At4g34990 | MYB32 | confirmed | ++ | ++ | +++ | +++ | +++ |
| *At5g12870 | MYB46 | confirmed | ++ | ++ | +++ | ++ | ++ |
| *At1g17950 | MYB52 | confirmed | - | - | ++ | - | + |
| *At3g01530 | MYB57 | confirmed | +++ | + | ++ | +++ | - |
| At5g17540 | Protein hypersensitity related gene | confirmed | + | + | + | ++ | + |
| At3g07450 | Putative 5B anther specific | confirmed | + | + | - | - | +++ |
| *At2g42990 | Putative APG isolog protein | confirmed | ++ | +++ | ++ | ++ | +++ |
| *At2g38080 | Putative diphenol oxidase | confirmed | ++ | + | +++ | ++ | + |
| *At2g40610 | Putative expansin-8 | confirmed | ++ | +++ | ++ | ++ | + |
| *At4g12730 | Putative pollen surface protien | confirmed | +++ | ++ | +++ | +++ | +++ |
| *At3g54770 | RNA binding protein | confirmed | - | - | + | + | - |
| *At3g12000 | S-locus related | confirmed | - | - | ++ | ++ | + |
| At5g43270 | Squamose promoter binding 2 | confirmed | ++ | + | + | ++ | ++ |
| At3g15270 | Squamose promoter binding 5 | confirmed | ++ | + | + | + | ++ |
| *At5g59120 | Subtilisin-like serine protease | confirmed | ++ | + | ++ | ++ | ++ |
| *At5g44630 | Terpene synthase | confirmed | ++ | ++ | +++ | +++ | ++ |
| *At1g02340 | Unknown | confirmed | ++ | +++ | ++ | ++ | ++ |
| *At1g70690 | Unknown | confirmed | ++ | + | ++ | + | + |
| *At2g34870 | Unknown | confirmed | - | + | + | + | + |
| At4g12960 | Unknown | confirmed | - | - | + | ++ | + |

Se: sepal, Pe: petal, St: stamen, Pi: pistil, Yb: young flower buds that not dissectible. Expression levels in different floral organs were based on the semi-quantitative RT-PCR results. "-" not detected, "+" faintly detected, "++" detected, "+++" strongly detected. "*": genes enriched in stamen compared to other floral organs. DELLA-D: down-regulated by DELLA proteins.

### 4.3.4 Identification of RGL2-down and -up genes in flower buds

In our previous experiment, we have shown that knock-out of four DELLAs (RGL1, RGL2, RGA and GAI) was able to fully rescue plant growth of GA-deficient mutant *ga1-3* (Cao et al., 2006). In Q3 (*ga1-3 rgl1-1 rga-t2 gai-t6*) plants where three DELLAs (RGL1, RGA and GAI) were knock-out, stem elongation was restored. However, the flowers of Q3 (*ga1-3 rgl1-1 rga-t 2gai-t6*) were indistinguishable from that of *ga1-3* plants (Fig. 4.2) (this thesis, Cheng et al., 2004, Tyler et al., 2004), indicating that RGL2 plays a key role in repressing floral development. In our microarray studies, when cross-somparing the expression profile among Wt, *ga1-3* and *ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6* (penta mutant), 360 DELLA-down and 273 DELLA-up genes were identified in flowers. It will be interesting to find out if these genes were also RGL2-regulated.

It is reasonable to speculate that those DELLA-down or -up regulated genes which are essential for floral development should be also RGL2-down or -up regulated genes. To identify this subgroup of genes, expressions of 33 DELLA-down genes and 21 DELLA-up genes were examined in Q3 mutant flowers. Genes that were down regulated or up regulated in Q3 mutant flowers were referred as RGL2-down or RGL2-up genes, respectively. We identified 7 (out of 33) RGL2-down genes and 5 (out of 21) RGL2-up genes (Fig. 4.3, Fig. 4.4). RGL2-down genes encodes proteins such as transcription factors (e.g. MYB21, MYB24), enzymes related to cell wall formation and modification (e.g. Berberine bridge enzyme, putative expansin 8), as well as several hypothetical proteins (e.g. At1g76240) (Fig. 4.3). These genes would serve as good candidates for further study of DELLA-regulated floral organ development.

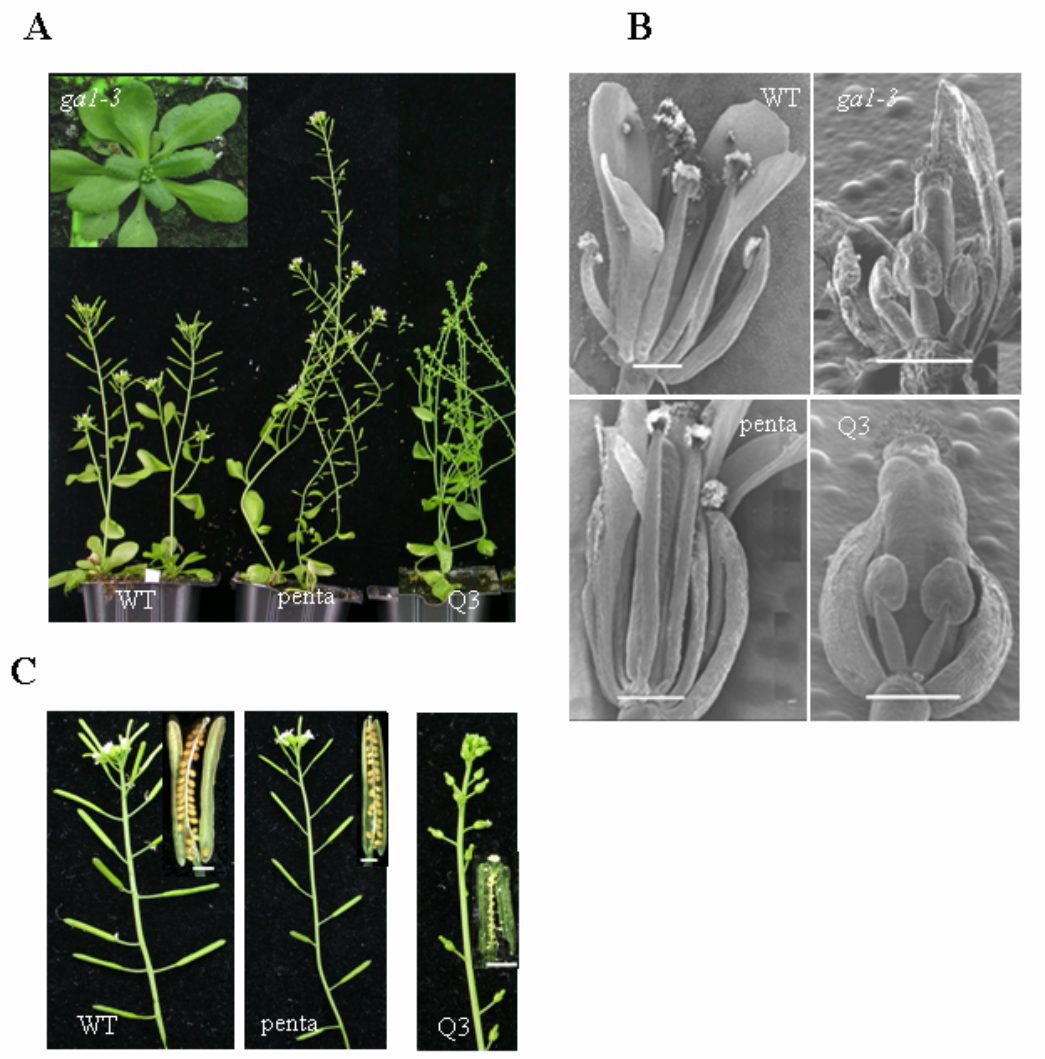



**Fig. 4.2. Characteristics of penta (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6*), Q3 (*ga1-3 rgl1-1 rga-t2 gai-t6*) and *ga1-3* mutants.** (A) Plant pictures of WT, penta, Q3 and *ga1-3* mutants. (B) Flowers of WT, penta, Q3 and *ga1-3* mutants. (C) Bolts of WT, penta and Q3 mutants.

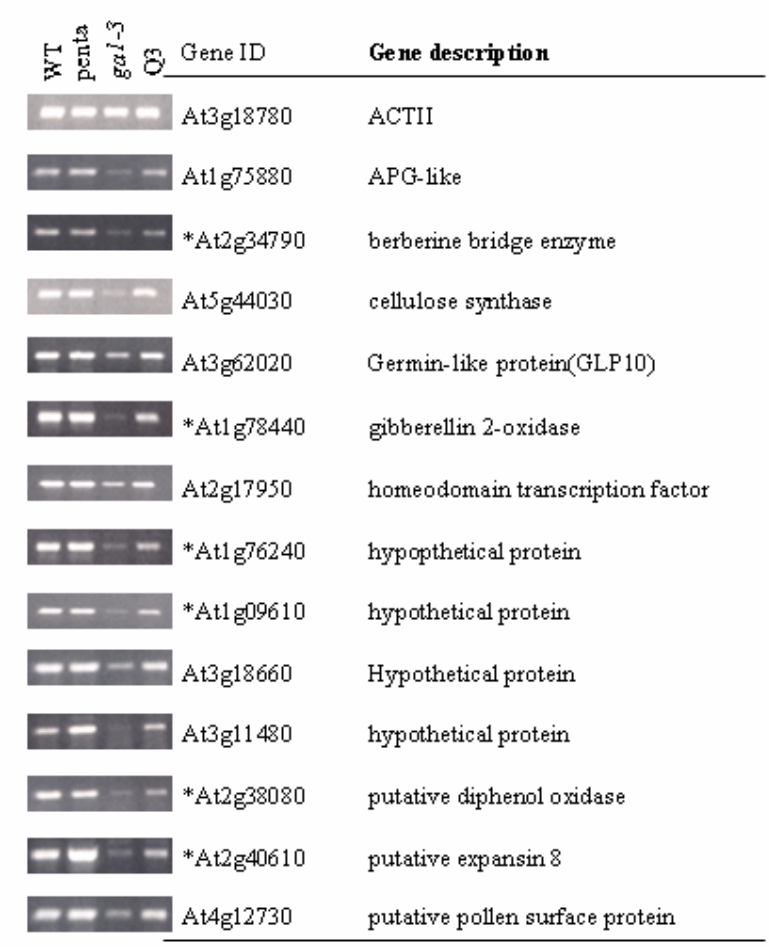
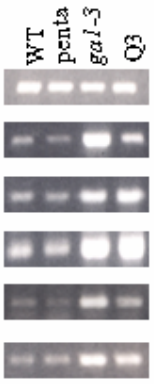



**Fig. 4.3. Identification of genes that were down-regulated in both *ga1-3* and Q3 mutants.** Representive genes that identified to be down regulated in both *ga1-3* and Q3 mutant was shown and were highlighted by "*". Expression of these genes may be repressed by RGL2.




| Gene ID | Gene description |
|---|---|
| At3g18780 | Actin 2 |
| At2g43620 | Glycosyl hydrolase family 19 (chitinase) |
| At3g04070 | NAM protein family |
| At1g69490 | NAP(NAC-like protein) |
| At5g65040 | Senescence-associated protein-related |
| At1g21520 | Unknown protein |

**Fig. 4.4. Identification of genes that were up-regulated in both *ga1-3* and Q3 mutants.** Five genes were identified to be up-regulated in both *ga1-3* and Q3 mutants. These genes might be activated by RGL2.

Among the RGL2-up regulated genes, NAP (NAC-like protein) has been shown to repress stamen development when overexpressed (Sablowski and Meyerowitz, 1998). However, it is unclear if it is GA regulated. Besides NAP, another Nam family gene was also found to be dramatically upregulated in both *ga1-3* and Q3 mutant. In addition, one glycosyl hydrolase family 19 gene (At2g43620), one senescence-associated protein (At5g65040) and one unknown gene were also identified to be RGL2-up genes (Fig. 4.4).

### 4.3.5 Isolation and characterization of T-DNA insertion lines of DELLA-regulated floral genes

Previous experiments have shown that DELLA proteins repress filament elongation by suppressing cell elongation rather than cell division (this theis, Cheng et al., 2004), suggesting that factors that promote cell elongation growth were likely down regulated in *ga1-3*. In contrast, those factors which inhibit cell elongation could be upregulated in *ga1-3* plants. In our previous studies, we identified a number of DELLA-regulated genes that might be related to the cell wall formation or modification. Expression study showed that some of them were highly enriched in the stamen. However, it is still unknown if they really function in DELLA controlling stamen filament elongation. Therefore, phenotypic analyses of loss-of-function or gain-of-function mutants of these genes are required to investigate the role of these genes. Abnormal phenotypes in the flower development in mutants should be observed if they function in this process.

To isolate loss-of-function mutants, our primary approach is to screen mutant lines with T-DNA inserted in candidate genes. T-DNA insertion lines have been

identified for 23 genes (4 unknown genes, 5 *MYBs*, 2 cellulose synthase subunit genes, 2 Nam family genes and 10 others) (Table 4.5). We obtained these T-DNA lines from ABRC (*Arabidopsis* Biological Resource Center at The Ohio State University) (Alonso et al., 2003). After two rounds of backcrossing of these lines to clean their background, these T- DNA lines were subjected to phenotypic analysis.

Mutant alleles of two cellulose synthase subunits (IRX1 (At4g18780) and IRX5 (At5g44030)) exhibited almost identical anther non-dehiscence phenotype (Table 4.5, Fig. 4.5). This observation is consistent with the fact that both of them are involved in the same complex for the secondary cell wall synthesis (Taylor et al., 1999; 2000; Holland et al., 2000). It would be interesting to study if GA regulated anther dehiscence via these cellulose synthase subunits in the future. In addition, two mutant alleles of *AtMYB21* showed male sterile phenotype (Table 4.5, Fig. 4.5). The stamen filament elongation of early developed flowers in these two alleles was arrested, resulting in male sterile phenotype. These data indicate that *MYB21* might play an important role in GA regulated stamen filament cell elongation.

In contrast, most of the T-DNA insertion lines which were supposed to be null alleles of their respective genes showed no obvious floral phenotype (Table 4.5). This might result from: 1) they are not genuine null alleles, 2) gene redundancy exists, or 3) they are not essential for normal flower development. In future, more detailed analysis, for example, construction of double or triple mutants would be a good and appropriate approach to study gene redundancy.


**Fig. 4.5. Flower phenotype in *irx5-1* mutant, *myb21-b* and *myb21-d* mutants.** (A) Flower picture of Col-0. (B) Flower picture of mutant allele of IRX5 (*irx5-1*). Anthers in *irx5-1* mutant were not able to dehisce. (C, D) Different alleles of *MYB21* (*myb21-b* and *myb21-d*) showed short stamen phenotype.

**Table 4.5. Summary of T-DNA insertion lines for genes selected from DELLA-D and DELLA-U genes**

| Gene ID | Gene description | T-DNA insertion lines | Phenotype |
|---|---|---|---|
| At2g34790 | berberine bridge enzyme | SALK_072827(Intron) | No |
| | | SALK_069340(Intron) | No |
| At4g18780 | cellulose synthase (IRX1) | SALK_026812(Intron) | yes |
| At5g44030 | cellulose synthase (IRX5) | SALK_084627(Exon) | yes |
| At3g62020 | Germin-like protein(GLP10) | SALK_065289(Exon) | No |
| | | SALK_023872(300-UTR5) | No |
| At1g78440 | gibberellin 2-oxidase | SALK_020228(Exon) | No |
| At2g17950 | homeodomain transcription factor | SALK_114398(300-UTR3) | No |
| At1g09610 | hypothetical protein | SALK_050883(Exon | No |
| At3g18660 | hypothetical protein | SALK_063763(Exon) | No |
| | | SALK_046841(Exon) | No |
| At3g27810 | MYB21 | SALK_003625(300-UTR5) | No |
| | | SALK_042711(Intron) | Yes |
| | | SALK_039465(300-UTR3) | No |
| | | Gabi-Kat (N311167) (intron) | Yes |
| At5g40350 | MYB24 | SALK_065218(Intron) | No |
| | | SALK_017221(Intron) | No |
| At4g34990 | MYB32 | SALK_132874(Exon) | No |
| At1g17950 | MYB52 | SALK_118938(Intron) | No |
| | | SALK_138624(Exon) | No |
| At3g01530 | MYB57 | SALK_065776(Exon) | No |
| At2g38080 | putative diphenol oxidase | SALK_051892(300-UTR5) | No |
| At4g12730 | putative pollen surface protein | SALK_001056(Exon) | No |
| At1g69490 | NAP(NAC-like protein) | SALK_005010(Exon) | No |
| | | SALK _049717(UTR3) | No |
| At3g04070 | NAM protein family | SALK _066615(UTR5) | No |
| At2g43620 | chitinase (Glycosyl hydrolase family 19) | SALK _056680(Intron) | No |
| At1g21520 | unknown protein | SALK _045038(UTR3) | No |
| At5g65040 | senescence-associated protein-related | SALK _106042(UTR5) | No |

All of the lines were confirmed by sequencing the PCR products derived from T-DNAinsertion border s in the genomic DNA. Genetic background was cleared by two rounds of backcross to WT and homozygous plants were used for phenotype observation. DELLA-D: DELLA down regulated genes, DELLA-U: DELLA up-regulated genes

## 4.4 Discussions

DELLAs are putative transcription regulators. Presumably, they may directly regulate the expression of some GA-response genes. However, there is currently no evidence to support this hypothesis. Alternatively, DELLAs may regulate the expression of some downstream transcription factors and these DELLA-regulated transcription factors then control the expression of GA-response genes. *GAMYB* genes are the best studied GA-regulated transcription factors. Previous studies have shown that GA regulates *GAMYB* through DELLA protein SLN1 and SLR1 in barley and rice respectively (Gubler et al., 2002; Kaneko et al., 2003). In *Arabidopsis*, *MYB33* and *MYB65* are identified as *GAMYB* genes based on homology analysis. However, *MYB33* and *MYB65* and their subfamily members are regulated at the post-transcription level by miRNA159 (Achard et al., 2004; Millar and Gubler, 2005; Reyes and Chua, 2007). In fact, *MYB33* and *MYB65* are not identified among the DELLA-down or DELLA-up genes in our dataset. In contrast, our data showed that *MYB24*, *MYB32*, *MYB52*, *MYB106*, *MYB21*, *MYB57* and *At2g38090* were the eight DELLA-down *MYB* genes involved in floral development (Table 4.2, Table 4.3). These data suggested that DELLAs might regulate a subset of *MYB* genes to repress floral development. Interestingly, four *MYBs* (*MYB59*, *MYB At5g44190*, *MYB At1g06180*, and *At3g11280*) were identified as DELLA-up genes in the young flower buds (Table 4.3). Therefore, these *MYB* genes may represent new types of *GAMYBs* and future work will focus on studying the relationship between GA and these *MYB* genes.

In addition to *MYB* genes, DELLA-down or –up *bHLH* and zinc-finger family genes are also identified in the young flower buds (Table 4.3). As expected, three types of transcription factors, namely three *MADS* box family genes (*AGL1*, *AGL6*

and *AGL11*), three *WRKY* family genes (*WRKY15*, *WRKY70*, *WRKY53*) (Eulgem et al., 2000) and four squamosa promoter binding protein-box family genes (*SPL2*, *SPL5*, *SPL11* and *SPL12*) were found among the DELLA-regulated genes for floral development (Table 4.3) (Krizek and Fletcher, 2005). Apparently, these transcription factors will regulate their own specific targets to fine-tune the regulation initiated by DELLAs. One of the future tasks will be to identify the targets controlled by these transcription factors.

As expected, the GA-response gene *GAST1* (At1g74670) and the key GA biosynthesis gene GA-3-beta-hydroxylase (At1g15550) are identified as DELLA-down and –up genes respectively in young flower buds (Shi and Olszewski, 1998; Ogawa et al., 2003). Recently, it was reported that *GID1* in rice encodes a soluble GA-receptor with homology to the consensus sequence of the hormone-sensitive lipase (HSL) homologous family (Ueguchi-Tanaka et al., 2005). Database search identified three *GID1* homologues in *Arabidopsis* and all of them have recently been shown to bind GA and DELLA proteins (Nakajima et al., 2006; Griffiths et al., 2006; Willige et al., 2007). Interestingly, two of these *GID1* homologues (At3g05120 and At3g63010) are identified as DELLA-up genes in the young flower buds, suggesting that these *GID1* homologues are probably negatively regulated by GA. However, the fact that DELLA proteins are stabilized in the *ga1-3* mutant suggests that GA is necessary to activate the GID1-like receptors to trigger the degradation of DELLA proteins.

Previous studies have shown that ABA signaling through ABI1, and ethylene signaling through CTR1, enhance the stability of DELLAs (Achard et al., 2003; Achard et al., 2006; Achard et al., 2007), suggesting that a fraction of ABA- and ethylene-signaling response genes may be identified as DELLA-regulated genes in

our dataset. Indeed, a number of ABA- and ethylene-response genes were identified as DELLA-up and -down genes in young flower buds (Table 4.3). Ethylene-related genes (3 genes) were also found as DELLA-up genes in the young flower buds (Table 4.3). In addition, low concentrations of auxin are known to promote the destabilization of DELLAs (Fu and Harberd, 2003). Accordingly, nine auxin-response genes were identified as DELLA-down genes in the young flower buds.

Both *AtMYB21* and *AtMYB24* were identified as RGL2-down genes and were enriched in stamen, indicating that RGL2 might repress stamen development via suppression of these two *MYB*s. However, no obvious phenotype was observed in *AtMYB24* mutant plants. *AtMYB21* single mutant showed very weak phenotype in stamen. Based on amino acid sequence analysis, AtMYB21 and AtMYB24 belong to the same MYB subfamily 19, suggesting they might function redundantly in controlling stamen development.

All of the three cellulose synthase subunits, which were supposed to form a complex to control the biosynthesis of secondary cell wall, were identified to be DELLA-down and stamen-enriched genes. Mutant alleles of their respective genes also showed anther no-dehiscence phenotype. However, the expression of these three genes is recovered in Q3 mutant while the stamen development in Q3 mutant was still arrested (Fig. 4.2, Fig. 4.3, and data not shown). These data implied that they might be necessary but insufficient for GA-regulated anther development in Q3 mutant.

# Chapter 5

# DELLAs Repress Flower-specific Genes *AtMYB21, AtMYB24* and *AtMYB57* through Modulation of JA Pathway in *Arabidopsis*

## 5.1 Introduction

Jasmonic acid (JA) is a lipid-derived signaling molecule that contributes to the control of metabolic, developmental and defensive processes in plants (Devoto and Turner, 2003; Weber et al., 1997). Severe JA-deficient mutant *opr3* displayed retarded filament elongation, delayed anther dehiscence, and reduced pollen viability. As a consequence, the *opr3* mutant is male sterile (Sanders et al., 2000; Stintzi and Browse, 2000; Feys et al., 1994; Xie et al., 1998; Scott et al., 2004). Application of exogenous JA can fully restore the stamen development of *opr3*, suggesting that JA plays an important role during stamen development (Stintzi and Browse, 2000). Resembling the *opr3* mutant, the *coi1* mutant is also specifically impaired in stamen development. However, in contrast to *opr3*, application of exogenous JA could not restore the *coi1* mutant phenotype to normal, suggesting that COI1 functions as a master check-point for JA-signaling (Xie et al., 1998). COI1 is an F-box E3-ligase that forms the SCF complex with SKP and CUL to mediate the degradation of its downstream targets (Xu et al., 2002). Based on the mutant phenotype displayed, it is reasonable to speculate that some of COIl's targets may be involved in controlling stamen development.

It is intriguing to know if GA-mediated and JA-mediated stamen development are via two parallel pathways or if they converge at a certain gene to regulate the same set of downstream genes to control stamen development. The known GA-response genes encoding transcription factors involved in stamen development are *GAMYBs* (*MYB33* and *MYB65*), a subset of *MYB* genes. Genetic studies showed that *MYB33* and *MYB65*

are essential to anther development but not for elongation growth of stamen filament in *Arabidopsis* (Millar and Gubler, 2005). Previous studies have shown that GA regulates *GAMYB* through DELLA protein SLN1 and SLR1 in barley and rice, respectively (Gubler et al., 1995; Gubler et al., 1999; Gubler et al., 2002; Kaneko et al., 2003; Scott et al., 2004). However, several reports failed to identify *MYB33* and *MYB65* as GA-inducible genes in *Arabidopsis* and these two *MYB* genes are in fact regulated at the post-transcriptional level by miRNA159 (Achard et al., 2004; Cao et al., 2006; Tsuji et al., 2006; Millar and Gubler, 2005). It has been suggested that JA regulates water transport in the stamens and petals to synchronize flower opening, anther dehiscence and pollen maturation (Ishiguro et al., 2001). An alternative hypothesis is that JA regulates programmed cell death in the anther as part of the dehiscence process (Zhao and Ma, 2000). A recent report showed that two *MYB* genes, *MYB21* and *MYB24*, which are responsive to JA treatment in *opr3* mutant, play crucial roles for stamen filament elongation. Interestingly, in an expression profiling study, we identified several *MYBs* including *MYB24*, *MYB21*, and *MYB57* as DELLA-down regulated genes in flower buds (Cao et al., 2006). This observation prompted us to investigate if there might be a cross-talk between GA signaling and JA signaling during stamen development.

*MYB 24* and *MYB21* are flower-specific genes (Mandaokar et al., 2006; Shin et al., 2002; Noji et al., 1998; Yang et al., 2006). In this chapter, we present data to demonstrate that expression of *AtMYB21*, *AtMYB24*, and *AtMYB57* was down-regulated in the *ga1-3* mutant but was restored to normal level in *ga1-3* plants lacking RGA, RGL1 and RGL2. This data suggested that these *MYB* genes may be DELLA-regulated downstream genes. This conclusion is further supported by the observation that the absence of four DELLAs (GAI, RGA, RGL1 and RGL2) cannot suppress the

short stamen phenotype conferred by the loss-of-function of *MYB21* and *MYB24*. In addition, we observed that application of exogenous JA onto *ga1-3* single mutant or *ga1-3 gai-t6 rgl1-1 rgl2-1* quadruple mutant flower buds could restore the expression of *MYB21* and *MYB24*. On the contrary, application of exogenous GA onto *opr3* mutant flower buds failed to induce the expression of these two *MYBs*, suggesting that JA might act downstream of GA in promoting stamen filament elongation. Analysis of the JA biosynthesis genes in different genotypes revealed that *DAD1*, one of the key JA biosynthesis genes, was down-regulated in both *ga1-3* and Q3 (*ga1-3 rgl1-1 rga-t2 gai-t6*) mutants. Furthermore, the expression of *DAD1* could be induced upon GA treatment in Q3 mutant. These data indicated that GA may regulate these three *MYB* genes through modulation of JA biosynthesis. Although application of JA could significantly restore the expression of *MYB21* and *MYB24* in *ga1-3 gai-t6 rgl1-1 rgl2-1* mutant, the mutant plant still had short stamen identical to that in the untreated control. This observation strongly suggests that *MYB21* and *MYB24* are necessary but insufficient for the normal elongation growth of stamen filament in *Arabidopsis*.

## 5.2 Materials and methods

### 5.2.1 Plant materials

*ga1-3*, Q3 (*ga1-3 rgl1-1 rga-t2 gai-t6*) and penta (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6*) mutants are in Ler background as described previously. T-DNA insertion lines for *MYB21* (*myb21-b* & *myb21-d*), *MYB24* (*myb24-a* & *myb24-b*) and *MYB57* (*myb57-1*) are in Col-0 background. Hexa1 (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb24-b*), hexa2 (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb21-b*) and hepta (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb21-b myb24-b*) mutants are in Ler background via cross-pollination of *myb21-b myb24-b* to penta mutant four times. *opr3* mutant (obtained from Dr Xie

Daoxin) is in Ws background. Primer pairs used for genotyping were as described in Table 2.2 and Table 2.3.

### 5.2.2 GUS staining, northern blot and *in situ* hybridization

Protocols for GUS staining, northern blot and *in situ* hybridization were described in chapter 2. Gene specific probes were used for northern blot and *in situ* hybridization. *AtMYB21* (+294 to +801 nt, nt stands for nucleotides, the A of the start codon ATG =1), *GA20ox2* (+28 to +627 nt), *LOX1* (+1903 to +2408 nt), *LOX2* (+1278 to +1714 nt), and *OPR3* (+4 to +439 nt) probes for Northern blots were labeled using PCR DIG probe synthesis kit (Roche, Germany) as described in Section 2.11.1. Antisense and sense probes of *AtMYB21* (+294 to +801 nt) for in situ hybridization were labeled by *in vitro* transcription as described in Section 2.11.2.

### 5.2.3 Hormone treatment

Both Q3 and *opr3* mutant plants (~27 days old) were sprayed with mock (0.1% ethanol v/v), GA3 ($10^{-4}$M) (Sigma) or MeJA (0.015% v/v) (Sigma). After treatment, whole infloresences (including two opened flowers in *opr3* mutant and two old flower buds in Q3 mutant) were collected at different time course (18h, 48h, 3d and 4d) for RNA extraction. RT- PCR was carried out according to the protocol described in Section 2.8. Primers used in expression study of GA and JA response and biosynthesis genes were listed in Table 2.6.

## 5.3 Results

### 5.3.1 DELLAs repress the expression of *AtMYB21, AtMYB24* and *AtMYB57* in the *Arabidopsis* inflorescences

Our previous microarray analysis showed that *AtMYB21*, *AtMYB24* and *AtMYB57* were DELLA down-regulated genes. The repression of these *MYBs* by DELLAs was confirmed by RT-PCR (Fig. 5.1). We found that the expressions of *AtMYB21*, *AtMYB24* and *AtMYB57* were very low or barely detectable in *ga1-3* while their expressions were restored to WT level in penta mutant (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6*). This data implied that in normal condition, GA was required for the expression of these *MYBs*. In *ga1-3* mutant where endogenous GA level was very low, DELLAs could repress the *MYBs* expressions. Meanwhile, in penta mutant, no GA was required for the expression of these three *MYBs* due to the lack of DELLA functions. Therefore, GA promotes the expression of these three *MYBs* via suppression of DELLA functions.

In order to single out which DELLA (RGL1, RGL2, RGA and GAI) is dominant in repression of these *MYBs*, *MYB* expressions were studied in four quadruple mutants in which only one of the four DELLAs remained. Results showed that their expressions were lower in Q1 (*ga1-3 rgl1-1 rgl2-1 gai-t6*) and Q3 (*ga1-3 rgl1-1 rga-t2 gai-t6*) mutants compared to Q2 (*ga1-3 rgl1-1 rgl2-1 rga-t2*) and Q4 (*ga1-3 rgl2-1 rga-t2 gai-t6*) mutants (Fig. 5.1), suggesting that RGL2 and RGA, not GAI or RGL1, were the main DELLAs in repressing the expression of these three *MYB*s. Furthermore, the high expression of *AtMYB21*, *AtMYB24* and *AtMYB57* in Q2 and Q4 mutants correlated with the normal flower phenotype in these mutants. While in Q1 and Q3 mutants, where *MYB*s expressions were relatively low, flower development were arrested (Cheng et al., 2004). This suggested that the expression of these MYBs was probably required for normal floral development.

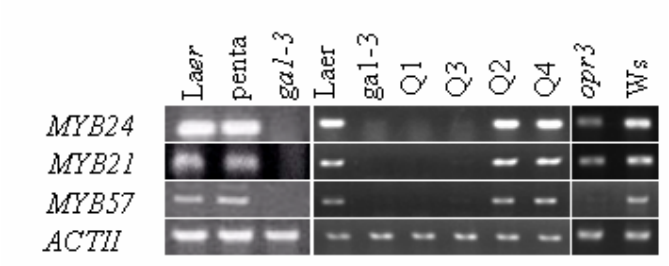



**Fig. 5.1. Expression of *AtMYB21*, *AtMYB24* and *AtMYB57* were both GA and JA dependent.** All of these three genes were down regulated in *ga1-3* and *opr3* mutants. Detailed analysis showed that these three genes may be repressed by RGL2 and RGA because their expressions were repressed in Q1 and Q3 mutants. Unopened flower buds were used for La*er*, *ga1-3*, Q1, Q2, Q3, Q4 and penta; whole inflorescences (opened and unopened flowers) were used for *opr3* mutant and Ws. (Q1: *ga1-3 rgl1-1 rgl2-1 gai-t6,* Q2: *ga1-3 rgl1-1 rgl2-1 rga-t2,* Q3: *ga1-3 rgl1-1 rga-t2 gai-t6,* Q4: *ga1-3 rgl2-1 rga-t2 gai-t6,* penta: *ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6*).

### 5.3.2 Isolation and characterization of the insertion mutants of *MYB21*, *MYB24* and *MYB57*

Both *AtMYB21* and *AtMYB24* are flower-specific genes and function in stamen development (Noji et al., 1998; Shin et al., 2002; Mandaokar et al., 2006). Expression analysis showed that *AtMYB57* is also a flower specific gene (Fig. 5.6). To further investigate their roles in GA signaling, we pursued a reverse genetic approach and identified T-DNA mutant alleles for these three *MYB*s from the Salk Institute Genomic *Arabidopsis* Laboratory (SIGnAL) database (Alonso et al., 2003). Mutant alleles were isolated and designated as *myb21-b* (SALK_042711) and *myb21-d* (Gabi-Kat (N311167)) for *MYB21*, *myb24-a* (SALK_065218) and *myb24-b* (SALK_017221) for *MYB24*, and *myb57-1* (SALK_065776) for *MYB57* (Fig. 5.2A, Table 4.5). After clearing the genetic background, we found that none of these null alleles showed any detectable differences from wild type with the exception of *myb21-b* and *myb21-d* (Fig. 5.3A-E). *MYB21* transcripts were dramatically reduced in *myb21-b* mutant allele and barely detectable in *myb21-d* mutant allele (Fig. 5.2B). The early developed flowers on homozygous *myb21* plants were sterile but the late developed flowers were fertile just as reported by Mandaokar et al., 2006. The stamen filament elongation of early developed flowers of *myb21* mutant alleles is arrested (Fig. 5.3B-C). These data demonstrated that *AtMYB21* may be the key player for the early developed flowers and its function might be dispensable for the late developed flowers due to functional redundancy.

### 5.3.3 *AtMYB24* and *AtMYB57* function additively with *AtMYB21* in controlling filament elongation, anther development and seed production

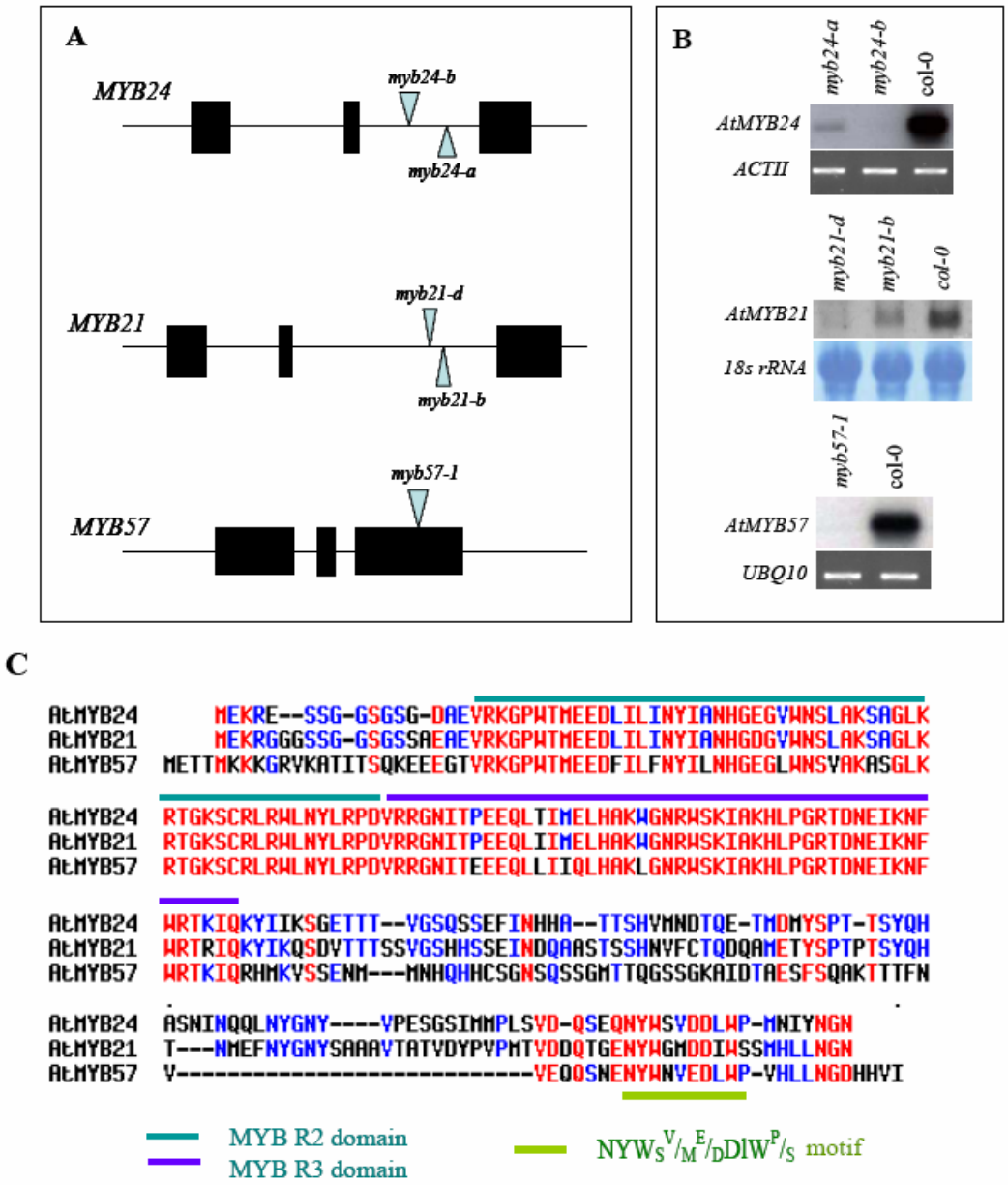



**Fig. 5.2. T-DNA insertion mutant alleles and sequence alignment of AtMYB21, AtMYB24 and AtMYB57.** (A) Schematic diagram of the genomic structure and T-DNA insertion sites for mutant alleles of three *MYB* genes (black boxes represent exons and lines represent introns). (B) Expression study in mutant alleles and WT. *MYB24* and *MYB57* expression was examined via RT-PCR and *MYB21* expression was examined via RNA gel blot hybridization. (C) Amino acid sequence alignment of AtMYB21, AtMYB24 and AtMYB57. In addition to the MYB domain, they shared another aa motif, called $NYW_S^V/_M^E/_D/DIW^P/_S$ (Kranz et al., 1998). There are 61.6% identity between AtMYB21 and AtMYB24 and 51.0% identity between AtMYB21 and AtMYB57.


**Fig. 5.3. Flower phenotype in different mutants.** (A-E) Flowers of Col-0 and single mutant alleles of *AtMYB21 (myb21-b and myb21-d), AtMYB24* (*myb24-b*) and *AtMYB57* (*myb57-1*). (F-I) Flowers of double mutants (*myb21-b myb24-b*, *myb21-d myb24-b*, *myb21-b myb57-1* and *myb24-b myb57-1*). (J-K) Flowers of triple mutants (*myb21-b myb24-b myb57-1* and *myb21-d myb24-b myb57-1*).

Based on the phylogenetic tree, *AtMYB24*, *AtMYB21*and *AtMYB57* belong to subgroup 19 of *R2R3-MYB* gene family (Kranz et al., 1998). Overall, MYB24 and MYB21 shared 61.6% identity at amino acid level and 51.0% identity between MYB21 and MYB57 (Fig. 5.2C). To elucidate if their functions are overlapping, crosses were made among homozygous *myb21-b&d, myb24-b*, and *myb57-1* plants. Four double mutants (*myb21-b myb24-b*, *myb21-d myb24-b, myb21-b myb57-1*, and *myb24-b myb57-1*) and two triple mutants (*myb21-b myb24-b myb57-1 and myb21-d myb24-b myb57-1*) were generated and used for further phenotypic analysis.

Flower development for all the double and triple mutants are normal before flower stage 12 (Smyth et al., 1990). The flower development of *myb24-b myb57-1* mutant was indistinguishable from wild type (Fig. 5.3I). The early developed flowers in *myb21-b myb57-1* mutant showed a similar short stamen phenotype to that of *myb21-b* single mutant (Fig. 5.3H). However, *myb21-b myb57-1* mutant was unable to set seeds as well as the *myb21-b* single mutant (Fig. 5.4, Table 5.1). This implied that *MYB57* had additive effects on *MYB21* for late developed flowers in either filament elongation or processes after pollination. Stamens of *myb21-b myb24-b* and *myb21-d myb24-b* mutants were typically shorter than their *myb21* and *myb24* counterparts and failed to fully extend above stigma at stage 13 (Fig. 5.3F-G). These data suggested that *MYB24* may enhance the function of *MYB21* in filament elongation. With the exception of filament elongation, pollen development appeared to be morphological normal in *myb21-b myb24-b* plants. However, in *myb21-d myb24-b* mutant, anther development was also affected (Fig. 5.3G), suggesting that *MYB24* might function redundantly with *MYB21* in controlling anther development.


**Fig. 5.4. Characteristics of bolts of different mutants.** Bolts of *myb57-1* and *myb24-b* were indistinguishable from the Col-0 wild type control. Early developed siliques of *myb21-b* and *myb24-b myb57-1* failed to set any seeds, late developed siliques were normal in these two mutants. Bolts of *myb21-b myb57-1* and *myb21-b myb24-b* exhibited sporadic setting of siliques, where majority of siliques failed to set any seeds, with occasionally fully or partially filled siliques were set. Bolts were from 45 days old plants. The triple mutant *myb21-b myb24-b myb57-1* was almost sterile except occasionally some siliques were set.

Reciprocal crosses between *myb21-b myb24-b* plants and wild type plants demonstrated that their mature pollens were viable and their female parts were fully fertile (data not shown). Although the triple mutant (*myb21-b myb24-b myb57-1*) had stamens similar to that of *myb21-b myb24-b* double mutant, its phenotype was more severe in seed production (Fig. 5.3F, G, J, K, and Fig. 5.4). Both *myb21-d myb24-b* and *myb21-d myb24-b myb57-1* plants do not set seed at all (data not shown). However, for *myb21-b myb24-b* plants and *myb21-b myb24-b myb57-1* plants, we occasionally observed that in the same inflorescence, some flowers did not set seeds; however, for other flowers they were able to develop very good siliques with seeds inside (Fig. 5.4). It is highly possible that environmental factors may influence the male fertility in the *myb21-b myb24-b, myb21-b myb57-1* as well as *myb21-b myb24-b myb57-1* mutants.

**Table 5. 1. Fertility examinations for mutants grown at LD condition**

| Col-0/ mutant | Total Number of Siliques/Primary Inflorescence | Total Number of Filled Siliques/ Primary Inflorescence | Percentage of Filled Siliques |
|---|---|---|---|
| Col-0 | 21.1±5.1 | 20.7±5.0 | 98.1±2.7 |
| *myb57* | 22.3±5.7 | 20.3±6.6 | 89.4±1.27 |
| *myb24* | 28.1±3.8 | 27.3±3.8 | 97.3±2.9 |
| *myb21* | 26.3±4.8 | 17.1±5.4 | 64.2±10.7 |
| *myb24myb57* | 26.3±6.9 | 22.6±6.8 | 85.1±6.3 |
| *myb21myb57* | 30.6±6.4 | 9.4±6.5 | 29.5±17.3 |
| *myb21myb24* | 30.8±9.5 | 5.6±4.3 | 16.9±11.8 |
| *myb21myb24myb57* | 33.4±7.5 | 1.6±1.5 | 4.1±3.6 |

[a]Siliques formed in primary inflorescence of plants were scored at 45 days

### 5.3.4 AtMYB21 and AtMYB24 act downstream of DELLA proteins in controlling filament cell elongation and anther development

Application of exogenous GA cannot rescue the stamen development in *myb21 myb24* mutant (data not shown), implying that *AtMYB21* and *AtMYB24* may be

GA-response genes. Their expressions were restored to WT level in penta (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6*) mutant, suggesting that GA regulates *MYB21* and *MYB24* through DELLA proteins. To confirm this hypothesis, we crossed *myb21-b myb24-b* mutant to penta mutant (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6*) to generate hexa1 mutant (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb24-b*), hexa2 mutant (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb21-b*) and hepta mutant (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb21-b myb24-b*). Analysis of these mutants revealed that flowers in hexa1 and hexa2 mutants are morphologically normal. However, seed production in these two mutants was dramatically reduced when compared to penta mutant ((Fig. 5.5A, data not shown). These obsevation demonstrated that *myb21-b* and *myb24-b* mutations were epistatic to mutations on DELLA.

Hepta mutant plant displayed no differences from penta mutant plant in vegetative growth. However, hepta mutant showed a short filament phenotype identical to the *myb21-b myb24-b* double mutant (Fig. 5.5A). Unlike in the *myb21-b myb24-b* double mutant, anther development in hepta mutant was affected. In most cases, anthers in hepta mutant do not dehisce. However, occasionally some anthers can dehisce (Fig. 5.4A). These results suggested that *AtMYB21* and *AtMYB24* may act downstream of DELLA genes in controlling stamen filament elongation and anther development. SEM analysis of epidermal cells of stamen filament of hepta, hexa1, hexa2, and penta mutants showed that the arrestment of filament elongation in hepta mutant is due to cell elongation rather than cell division (Fig. 5.5B &C, Table 5.2), suggesting that *MYB21* and *MYB24* act redundantly in controling the filament cell elongation instead of cell division.

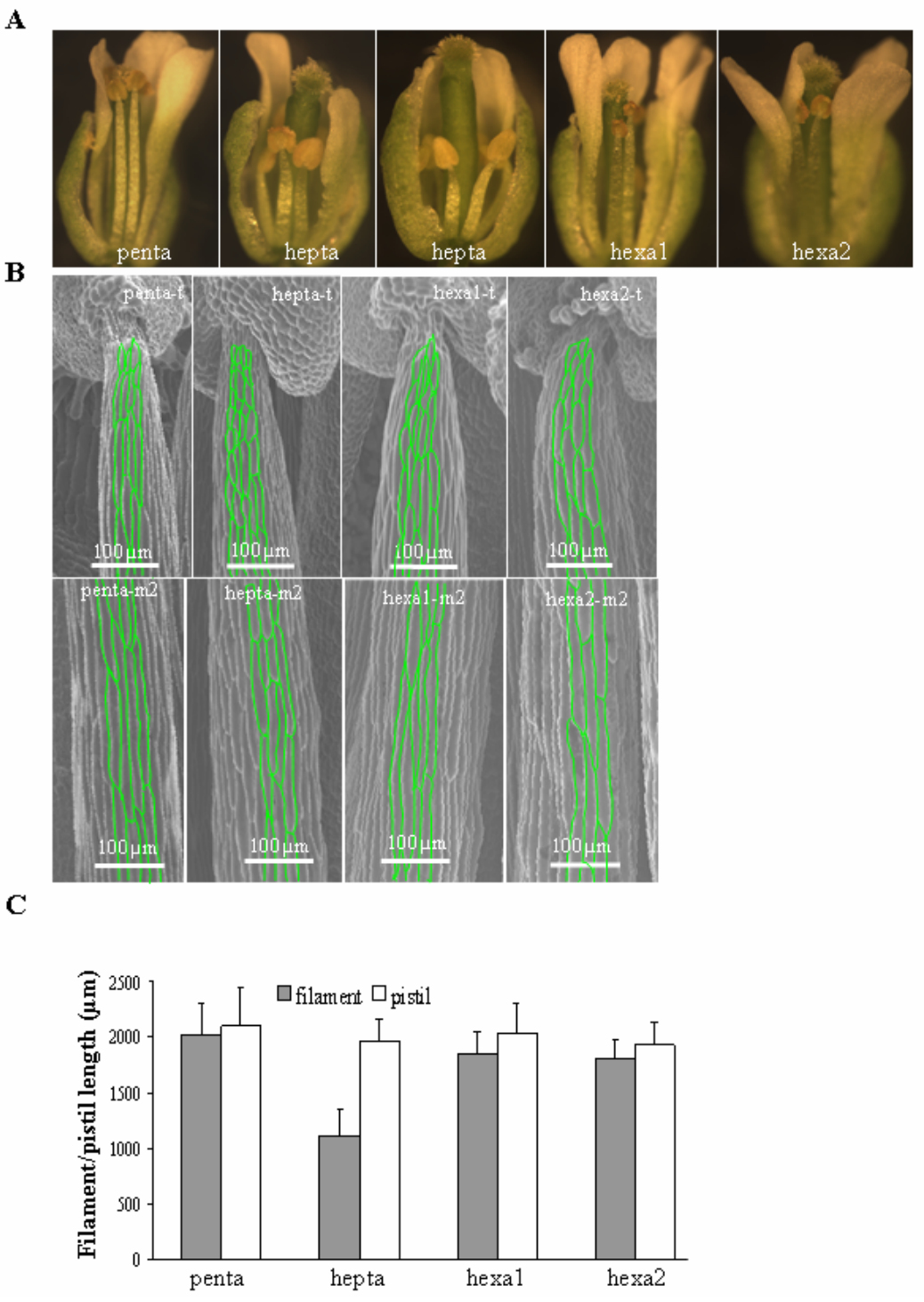

A
penta
hepta
hepta
hexa1
hexa2
B
penta-t
hepta-t
hexa1-t
hexa2-t
100 µm
penta-m2
hepta-m2
hexa1-m2
hexa2-m2
C
Filament/pistil length (µm)
2500
2000
1500
1000
500
0
filament
pistil
penta
hepta
hexa1
hexa2

**Fig. 5.5. Absence of four DELLAs (GAI, RGA, RGL1 and RGL2) was unable to suppress the short stamen phenotype conferred by the loss-of-function of *MYB21* and *MYB24*.** (A) Flower phenotype of hepta and hexa mutants. Stamen of hepta (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb21 myb24)* mutant showed a more severe phenotype than *myb21-bmyb24-b* with majority of anthers undehisced. Stamen of hexa1 (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb24)* and hexa2 (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6 myb21)* mutants were similar to penta mutant. (B) SEM of epidermal cells of top and middle part of stamen filament of different mutants. (C) Comparison of stamen and pistil lengths among different genotypes.

| Table 5. 2. Number of epidermal cells in filament | | | | |
|---|---|---|---|---|
| | penta (n=20) | hepta (n=30) | hexa1 (n=30) | hexa2 (n=30) |
| Cell number | 14±1.2 | 13±1.5 | 13±1.1 | 14±1.1 |

### 5.3.5 Expression pattern of *AtMYB21, AtMYB24* and *AtMYB57*

The absence of *AtMYB21*and *AtMYB24* together resulted in short stamen. We speculated that the expressions of these genes may be correlated with the phenotype. Firstly, we investigated the expression of *AtMYB21*, *AtMYB24*, and *AtMYB57* in tissues such as inflorescences, cauline leaves, rosette leaves, bolting stem, and roots by RT-PCR. It was found that all of these three genes were detectable only in the inflorescences (Fig. 5.6A). Meanwhile, expression of *AtMYB21* and *AtMYB24* was detected in all four floral organs (sepal, petal, stamen and pistil). Expression of *AtMYB57* was also detected in sepal, stamen and pistil (Fig. 5.6A).

Expression pattern of *AtMYB21* in flowers was also examined by *in situ* hybridization. Strong signals were detected in cells between the junction of anther and filament, where rapid filament elongation was hypothesized to occur at stage 13 for successful pollination (Smyth et al., 1990). Besides the filament, strong expression was detected in the nectaries, ovules, as well as in the vascular regions of anthers (Fig. 5.6B), indicating that *AtMYB21* may function outside of stamens.

To gain more information about *MYB21* and *MYB24*, promoters of *AtMYB21* and *AtMYB24* were fused to GUS to generate the promoter-GUS fusion constructs *pMYB21::GUS* and *pMYB24::GUS*. Similar GUS expression pattern was observed in both *pMYB21::GUS* and *pMYB24::GUS* plants. In contrast to the flower-specific expression pattern of *AtMYB21* and *AtMYB24* detected by RT-PCR and insitu hybridization, GUS staining in *pMYB21::GUS* and *pMYB24::GUS* plants was detected in the inflorescences as well as in other parts of the plants (Fig. 5.7 A-D).

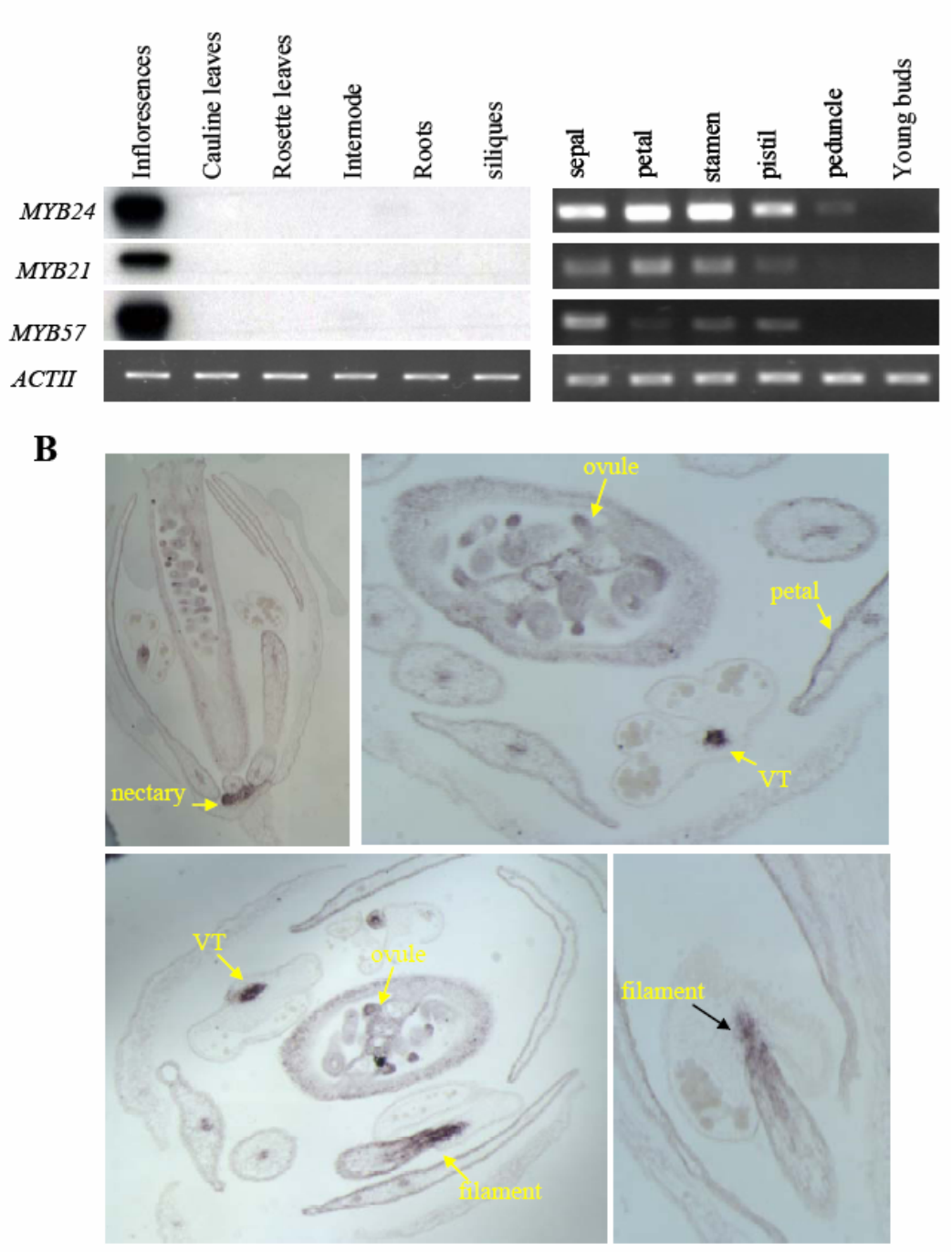



**Fig. 5.6. Expression patterns of *AtMYB21, AtMYB24* and *MYB57*.** (A) Tissue specific expression of *AtMYB21*, *AtMYB24* and *AtMYB57* was analyzed through RT-PCR in different tissues and different floral organs. (B) *AtMYB21 in situ* hybridization in flowers. (VT: vascular tissue).

Strong GUS expression was observed in the vascular bundle of sepals, upper parts of pistils, and nectaries of flowers (Fig. 5.7B). GUS expression was only detected in the vascular bundles of the long stamens at stage 12 and later (Fig. 5.7B-C). There was no GUS expression in petals at all stages of flower development. Besides in flowers, strong expression was also observed in both end of the siliques (Fig. 5.7D), the vascular bundles of stems, roots and leaves, and especially in the newly emerged rosette leaves (Fig. 5.7A). These observations suggested that there may be additional regulatory elements outside of these cloned promoter regions of *AtMYB21* and *AtMYB24* to restricted their expression in floral organs.

### 5.3.6 Expression of *AtMYB21, AtMYB24* and *AtMYB57* is dependent on JA pathway

In our previous experiments, we have shown that the expression of *AtMYB21*, *AtMYB24* and *AtMYB57* was repressed in Q3 (*ga1-3 rgl1-1 rga-t2 gai-t6*) mutant (Fig.5.1). It was also shown recently that the expression of *AtMYB21*and *AtMYB24* was downregulated in *opr3* mutant (Mandaokar et al., 2006) (Fig. 5.1). These results raised questions about the possible interaction between the JA and GA pathways in regulating stamen development. Conceptually, there are three different models for the interaction. The first model implies that GA regulates the expression of these *MYBs* through JA pathway. The second model is identical to the first but in reverse. The third model is that GA and JA do not act in a sequential manner but rather via parallel pathways, both of which are needed for the expression of these *MYBs*. To test these divergent predictions, the flowers of Q3 and *opr3* mutants were sprayed with GA and MeJA. After three independent experiments, we consistently observed that JA-treatment induced the expression of the three *MYBs* both in the *ga1-3 gai-t6 rga-t2*

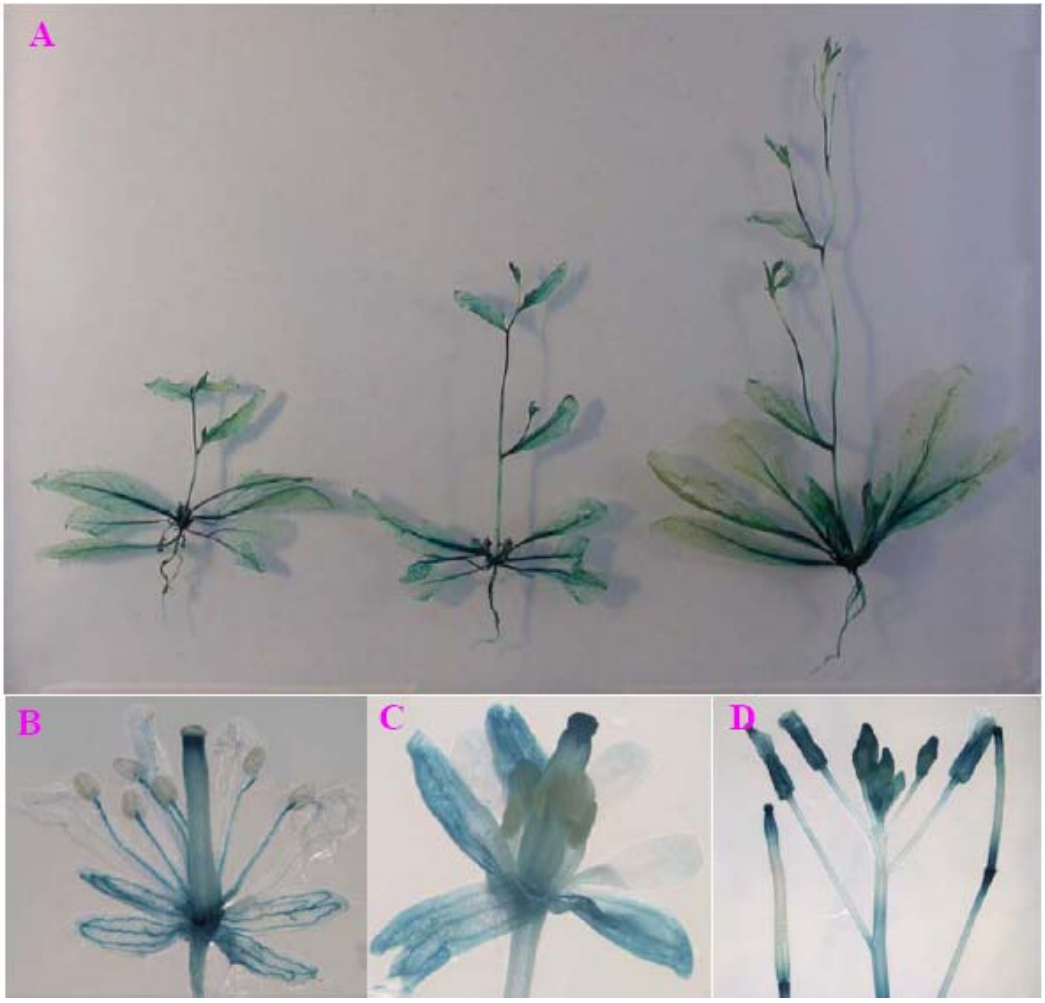



**Fig. 5.7. Expression of GUS reporter in *pMYB21::GUS* transgenic plants.** (A) GUS staining in *pMYB21::GUS* plants. (B) Flower of *pMYB21::GUS* plants at stage 12. (C) Flower of *pMYB21::GUS* plants at stage 10. (D) Inflorescence and silliques of *pMYB21::GUS* plants.

*rgl1-1* quadruple (Q3) and *opr3* mutants at 18 hrs (Fig. 5.8). On the other hand, compared to MeJA treatment, induction of *MYBs* expression by GA was much slower in Q3 mutant. The expression of these three *MYBs* was detected 3 days after GA treatment when normal flower developed in GA treated mutants (Fig. 5.8). However, GA could not induce the expression of *MYBs* in *opr3* mutant even 4 days after GA treatment (Fig. 5.9). These data demonstrated that in the absence of JA, GA was unable to induce the expression of *MYBs*, suggesting that JA pathway might act downsteam of GA pathway in regulating the expression of *MYBs*.

To exclude the possibility that GA pathway may be affected in JA deficient *opr3* mutant, we checked the expression of GA biosynthesis and responsive genes, *GA2ox1*, *GA3ox1* and *GA20ox2,* in different GA-related mutants and *opr3* mutant. *GA2ox1*, *GA3ox1* and *GA20ox2* are key GA biosynthesis genes that contribute to the biosynthesis of bioactive GA. These three genes are also under negative feedback regulation of GA signal pathway. RT-PCR results showed that *GA2ox1* expression was down-regulated, while *GA3ox1* and *GA20ox2* expression was up-regulated in GA deficient *ga1-3* and Q3 mutants, while expression of these genes was restored to normal level in penta mutant (Fig. 5.10A, B). Interestingly, similar expression level of these genes was observed in *opr3* mutant and Ws control (Fig. 5.10A, B). These results supported that GA biosynthesis and signaling pathways were normal in JA deficient *opr3* mutant.

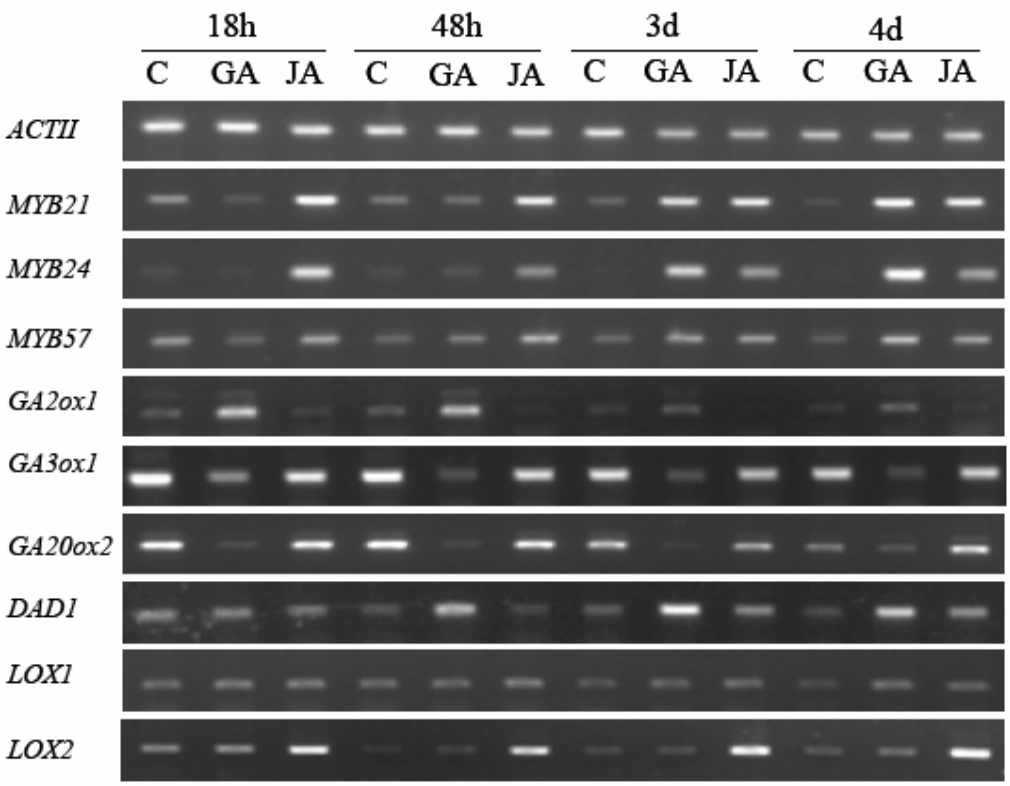



**Fig. 5.8. Induction of expression of *AtMYB21, AtMYB24* and *AtMYB57* by GA and JA in Q3 mutant.** JA induced the expression of *AtMYB21*, *AtMYB24* at 18h after treatment. Induction of expression of *AtMYB57* by JA was detected at 48h after treatment. The induction of expression of three *MYBs* by GA was detected 3 days after treatment. *DAD1* expression was induced at 48h after GA treatment (Q3: *ga1-3 rgl1-1 rga-t2 gai-t6*).

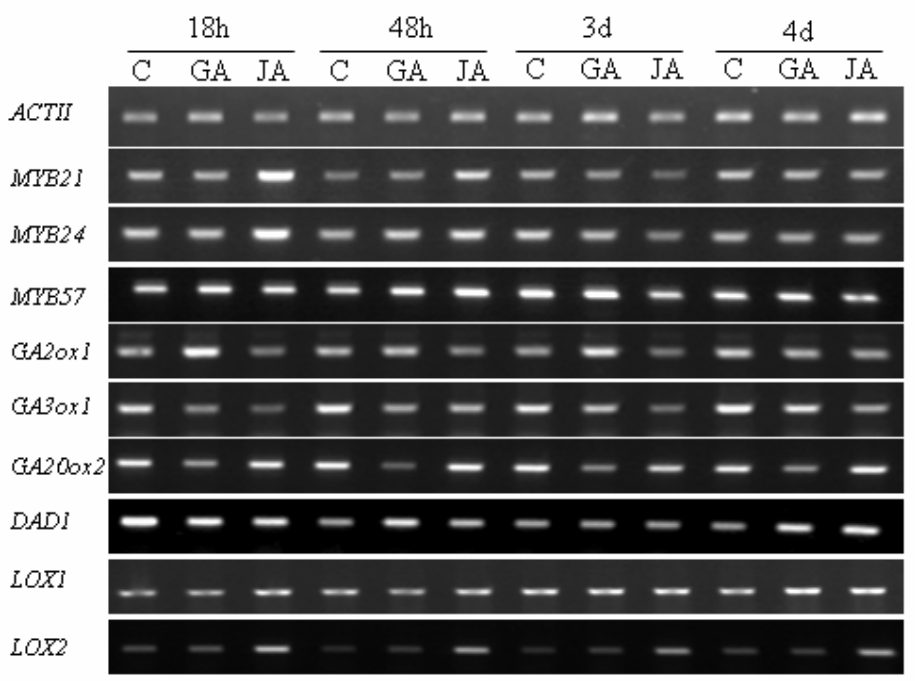



**Fig. 5.9. Induction of expression of *AtMYB21, AtMYB24* and *AtMYB57* by GA and JA in *opr3* mutant.** GA responsive genes were induced (*GA2ox1*) or repressed (*GA3ox1* and *GA20ox2*) at 18 h after GA treatment, indicating that the GA signaling pathway was not affected in *opr3* mutant. However, GA was unable to induce the expression of *AtMYB21*, *AtMYB24* and *AtMYB57* even 4 days after treatment in *opr3* mutant, suggesting that GA-induced expression of these three MYBs was JA-dependent.

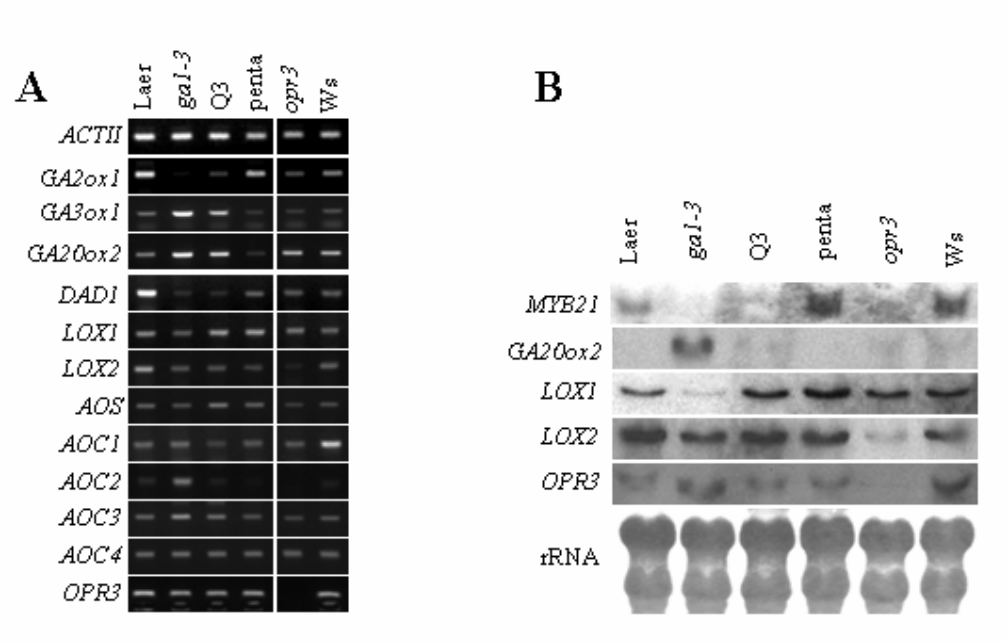



**Fig. 5.10. Expression of GA and JA responsive and biosynthesis genes in different mutants.** (A) RT-PCR results of GA and JA response and biosynthesis genes in different GA and JA related mutants. (B) Northern blot of GA and JA biosynthesis genes in different GA and JA related mutants.

### 5.3.7 *DAD1* expression was GA and DELLA dependent

Based on the above experiments, we hypothesized that GA may act upstream of JA pathway in regulating the expression of *MYBs*. The fact that JA was able to induce the expression of *MYBs* and JA response gene (LOX2) in Q3 mutant indicated that Q3 mutant could response to JA correctly.

In order to find out if JA biosynthesis pathway was affected in Q3 mutant, expression of JA biosynthesis genes including *DAD1* (*Defective in anther dehiscence 1*), *LOX2* (*Lipoxygenase 2*), *AOS* (*Allene oxide synhase*), *AOC1* (*Allene oxide cyclase 1*, At3g25760)*, AOC 2* (At3g25770)*, AOC 3* (At3g25780), *AOC 4* (At1g13280) and *OPR3* (*OPDA reductase 3*) was examined in different GA and JA mutants (Fig. 5.11, Stenzel et al., 2003b). In the JA deficient *opr3* mutant, we found that with the exception of *LOX2, AOC1,* and *OPR3,* which showed reduced expression in *opr3* mutant, all the other genes were expressed at similar levels in *opr3* mutant and Ws control. However, their expression in GA-related mutant was more complicated. We found that the expression level of *AOS*, *AOC1*, *AOC3*, *AOC4* and *OPR3* did not show significant differences in all GA-related mutants and Ler control, while the expression of *AOC2* was significantly induced in *ga1-3* mutant and then restored to WT level in Q3 and penta mutants. In contrast, the expression of *LOX1* and *LOX2* was reduced in *ga1-3* mutant and kept at a similar level in Q3 and penta mutants (Fig. 5.10A, B). These data suggested that GA and DELLA proteins might be required for the regulation of these genes.

*DAD1* expression was found to be down regulated in both *ga1-3* and Q3 mutants and partially restored in penta mutant, indicating that GA may regulate the expression *DAD1* via suppression of DELLA protein (Fig. 5.10A). *DAD1* is a stamen

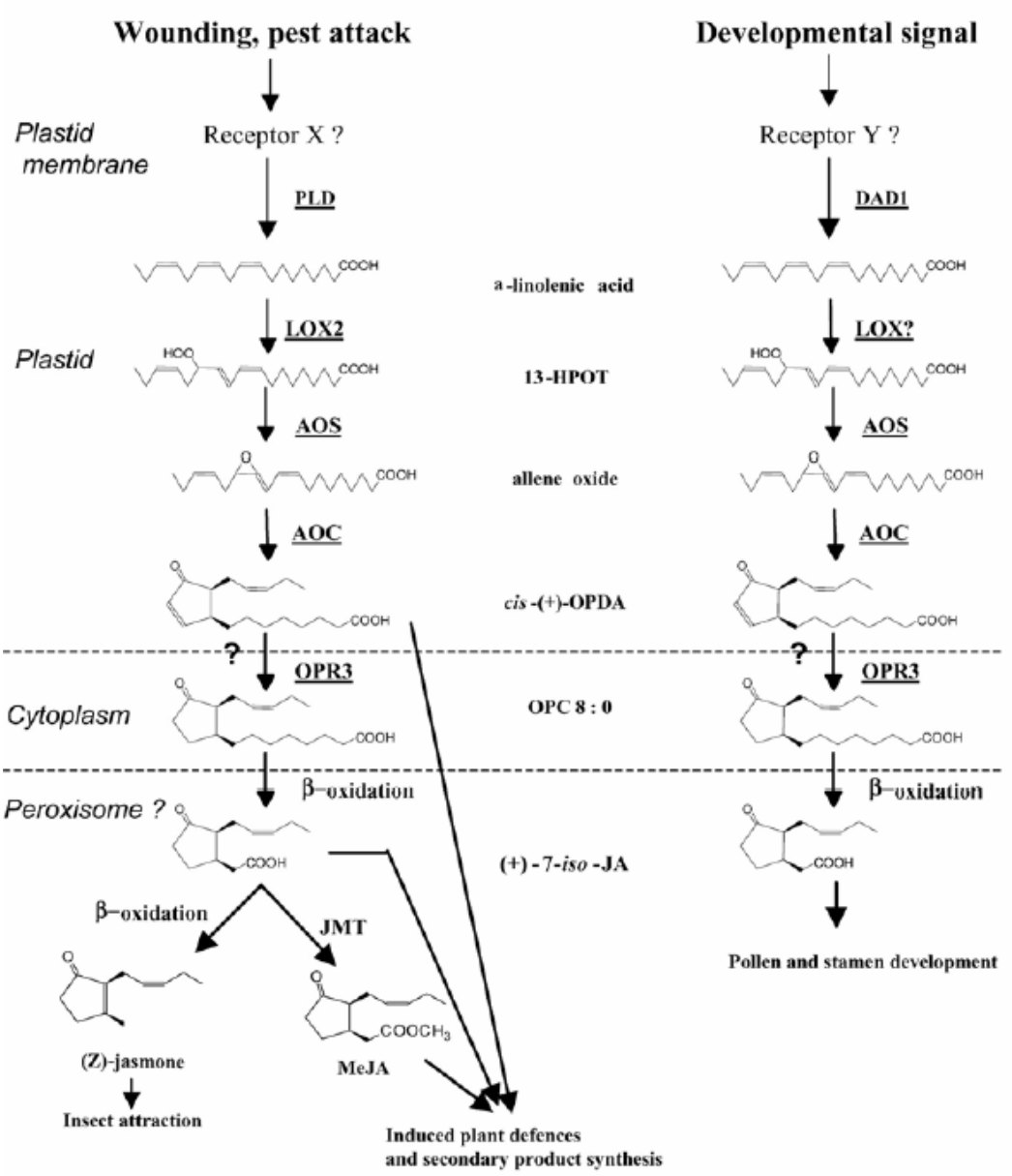



**Fig. 5.11. JA biosynthetic pathway following wounding or pest attack and in pollen development (Devoto and Turner, 2003).** The activation of a phospholipase (PLD or DAD1) may result from the elicitation of a membrane receptor. Abbreviations for enzyme names are in bold and underlined: AOC, allene oxide cyclase; AOS, allene oxide synthase; DAD1, defective anther dehiscence1; JMT, *S* -adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase; LOX, lipoxygenase; OPR3, OPDA reductase3; PLD, phospholipase. Abbreviations for names of intermediates are in bold: 13-HPOT, 13-hydroperoxylinolenic acid; OPC 8 : 0, 3-oxo-2(2'pentenyl)-cyclopentane-1-octanoic acid; OPDA, 12-oxo-phytodienoic acid.

specific gene encoding chloroplastic phospholipase A1 protein that catalyzes the initial step of JA biosynthesis. Mutation in *DAD1* resulted in a typical JA-deficient phenotype in anther dehiscence, pollen maturation and flower opening (Ishiguro et al., 2001). This phenotype is quite similar to that of *myb21-d myb24-b* double mutant. It is reasonable to speculate that GA modulates the production of JA via *DAD1* genes to regulate the expression of *MYB21* and *MYB24*. Furthermore, GA was able to induce the expression of *DAD1* prior to the induction of expression of *MYB21* and *MYB24* in Q3 mutant (Fig. 5.8), further suggesting that *DAD1* gene expression is neccessary for GA induced expression of *MYBs* in Q3 mutant.

### 5.3.8 Expression of *AtMYB21, AtMYB24* and *AtMYB57* is neccessary but insufficient for normal floral development in Q3 mutant

*AtMYB21*, *AtMYB24* and *AtMYB57* act downstream of DELLAs in controlling filament elongation. Expression of *AtMYB21*, *AtMYB24* and *AtMYB57* was repressed and floral development was arrested in Q3 (*ga1-3 rgl1-1 rga-t2 gai-t6*) mutant (Cheng et al., 2004). It is speculated that expression of *MYBs* could rescue the male sterile phenotype of Q3 mutant. Based on the observation that MeJA could restore the *MYBs* expression in Q3 mutant, we analyzed the flowers of MeJA treated Q3 mutant. Interestingly, although MeJA was able to induce *MYBs* expression in Q3 mutant similar to *opr3* mutant, we observed that the stamen development of MeJA treated Q3 plants was still arrested (Fig. 5.12). However, in GA treated Q3 plants, expression of *MYB21*, *MYB24* and *MYB57* correlated very well with the recovery of normal floral development in Q3 plants. These results suggested that only expression of *MYB21*, *MYB24* and *MYB57* were insufficient for normal floral development in Q3 mutant, suggesting that besides these JA inducible *MYBs*, other more important factors

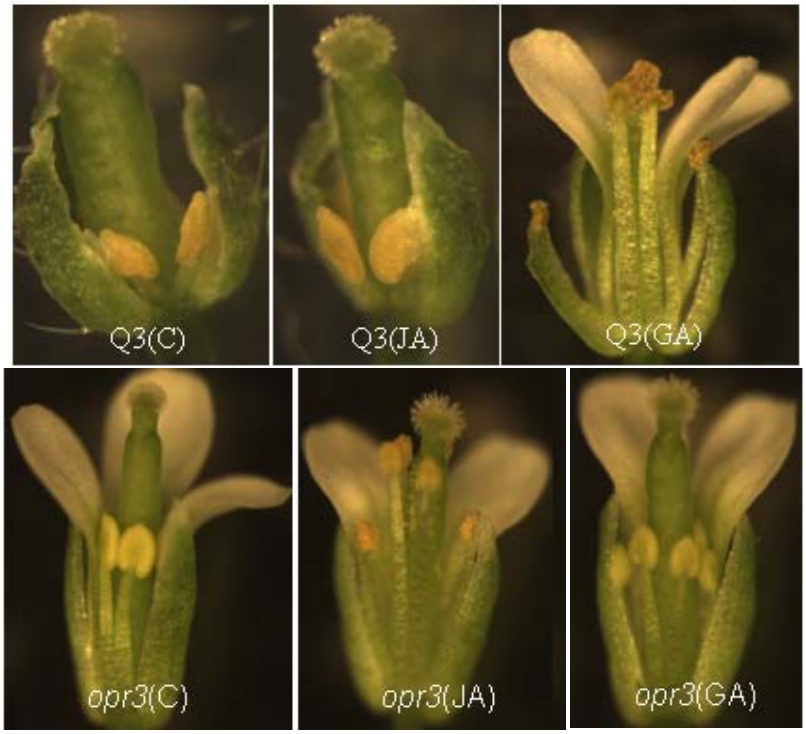



**Fig. 5.12. Flower phenotypes of Q3 and *opr3* mutants treated with mock, JA and GA.** GA was able to rescue the flower phenotype of Q3 but not able to rescue the flower of *opr3*. On the contrary, JA was able to rescue the flower phenotype of *opr3* but not able to rescue the flower of Q3. Flower pictures were taken 4 days after JA and GA treatment (Q3: *ga-13rgl1-1rga-t2gai-t6*).

which are regulated by GA may be needed for normal floral development in Q3 mutant.

## 5.4 Discussions

The presence of active cross-talk between hormone signaling pathways have been indicated in many developmental processes. GA and ABA antagonized in regulating developmental transition from embryogenesis to seed germination. Evidences showed that ABA repressed the GA induction of *GAMYB*. It was suggested that ABA block the GA response between SLN1 and GAMYB (Gómez-Cadenas et al., 2001; Zentella et al., 2002; Olszewski et al., 2002). Recently, it was reported that in germinating *Arabidopsis* seeds, ABA induces the accumulation of microRNA 159 (miR159) to mediate the cleavage of *GAMYB*: *MYB101* and *MYB33* (Reyes and Chua, 2007).

It was reported that auxin was necessary for GA-mediated *Arabidopsis* root growth by promoting GA-dependent degradation of DELLA proteins (Fu and Harberd, 2003). In contrast, ethylene inhibits *Arabidopsis* root growth by delaying the GA-induced destabilization of DELLA (Achard et al., 2003). Recently, multiple interactions between ethylene and GA signal transduction pathways were revealed in a detailed analysis of responses induced by ethylene and GA in different ethylene and GA related mutants (De Grauwe et al., 2007).

Flower development is a highly coordinated process. Many signaling pathways and genes have been implied to be involved in these processes. Hormone-hormone interaction in controlling flowering has been indicated recently. It was found that stress induced hormone ethylene control floral transition via DELLA-dependent regulation of floral meristem-identity genes *LEAFY* and *SUPPRESSOR OF*

*OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Achard et al., 2007). Although it has been known that both GA and JA are involved in stamen development, no report showed that they act independently or interactively. From our initial attempt to define the relationship between DELLA proteins and floral development, we found that there might be interactive relationship between GA and JA in controlling stamen development.

As repressors of floral development, the molecular mechanism of DELLA repressing floral organ development is largely unknown. *GAMYBs* are the well studied candidates which act downstream of DELLAs in controlling anther development (Millar and Gubler, 2005). However, this group of transcription factors is regulated post-transcriptionally by miR159. By microarray analysis we showed that GA regulates expression of *AtMYB21*, *AtMYB24* and *AtMYB57* via suppression of DELLA proteins. Flower development in Q3 mutant was arrested at flower stage 10, while flower development in Ler and penta mutant progresses well. Although we have minimized this stages effect by using unopened young flower buds in Ler and penta mutant. We could not strictly compare the flower stages in all these genotypes. Therefore, it is still possible that downregulation of *MYB21*, *MYB24* and *MYB57* in Q3 mutant may result from this stages differences among these different genotypes. Therefore these MYBs could be indirectly regulated by DELLA proteins or GA signaling.

It was reported that JA is also required for the expression of these *MYBs* (Fig. 5.1, Mandaokar et al., 2006). Genetic studies have shown that these three MYBs are required for normal stamen development. Surprisingly, JA induces the expression of these *MYBs* in the absence of GA. However, GA could not induce the *MYBs*

expression in the absence of JA. Thus GA may act upstream of JA pathway to control floral development.

It is possible that JA induces the expression of *MYBs* via modulation of stability or activity of DELLA proteins. However, if JA induces the expression of *MYBs* via destabilization or inactivation of RGL2 protein, both *MYBs* expression and flower development of JA-treated Q3 mutant (*ga1-3 rgl1-1 rga-t2 gai-t6*) should be restored to normal, as observed in penta mutant (*ga1-3 rgl1-1 rgl2-1 rga-t2 gai-t6*). The fact that the flower of JA-treated Q3 plants is identical to that of control-treated Q3 plants suggested that JA could not modify the function of RGL2 in the absence of GA. Results showed that *MYBs* are highly expressed in JA-treated Q3 mutant at 18h after JA treatment. However, Preliminary results showed that RGL2 protein level remains high at this time course (data not shown). These data suggested that destabilization of RGL2 protein may not be necessary for JA induced expression of *MYBs* in Q3 mutant. In contrast, JA may function downstream of DELLA proteins in controlling the expression of these *MYBs*. However, we could not exclude that JA could play a role for GA regulated degradation of DELLA. Future study of the GA induced degradation of DELLA proteins in JA deficient mutants would be a great help in addressing this question.

JA biosynthesis was regulated by OPDA compartmentalization and a JA-mediated positive feedback loop (Sasaki et al., 2001). Biotic and abiotic stresses also induce JA formation (Howe et al., 2000; Maucher et al., 2000; Ziegler et al., 2000). Although transcriptional up-regulation of JA biosynthesis genes (*DAD1*, *LOX*s, *AOS*, *AOCs* and *OPR3*) was observed upon treatment with JA or biotic and abiotic stresses, the accumulation of mRNA of JA biosynthesis genes was not always accompanied by endogenous formation of JA (Kramell et al., 2000; Miersch and Wasternack, 2000).

Furthermore, wounding induced JA formation prior to the accumulation of *AOS* or *AOC*s mRNA (Ziegler et al., 2001; Stenzel et al., 2003a). In our experiment, we found that JA biosynthesis gene *DAD1* was down-regulated in both *ga1-3* and Q3 mutants, implying that GA may be required for the expression of *DAD1* to control the production of JA via repression of DELLA proteins. On the other hand, the fact that the induction of *DAD1* expression prior to the expression of *MYBs* by GA in Q3 mutant strongly support our hypothesis that GA may regulate the *MYBs* expression via modulation of the biosynthesis of JA. In addition to *DAD1*, we also observed that expression of JA responsive genes *LOX1* and *LOX2* was down-regulated in *ga1-3* mutant (Fig. 6.10A, B). In contrast, some JA biosynthesis genes such as *AOC2* were up-regulated in *ga1-3* mutant. These observations suggested that GA may be one of the endogenous signal involved in the regulation of JA biosynthesis and responsive genes. In flowers of *dad1* null mutant, the JA levels were only 22% of that of WT (Ishiguro et al., 2001), indicating that limited initial substrate generation by DAD1 reaction may act as a control point for JA biosynthesis in flowers. Therefore, it is highly possible that reduced expression of *DAD1* in Q3 or *ga1-3* mutant may result in relative low JA production. Future quantification of JA concentration in these GA deficient mutants will be critical to our understanding of the relationship between JA and GA.

Genetic studies have shown that *AtMYB21*, *AtMYB24* and *AtMYB57* are indispensable for flower development. The flower phenotype of *myb21-d myb24-b myb57-1* triple mutant is similar to that of JA deficient mutants: for example *opr3* and *dad1* mutants. It is interesting to study if expression of these *MYBs* in *opr3* or *dad1* mutants could rescue the flower phenotype of these mutants. Both *ga1-3* and Q3 mutants showed a more severe flower phenotype than *myb21-d myb24-b myb57-1*

triple mutant. The fact that expression of these *MYBs* in Q3 mutant was not enough to rescue the flower phenotype of Q3 mutant indicated that these *MYBs* were not the master check-point genes for GA signaling in controlling floral development. These data indicated that modulation of JA pathway may be only one of branches of GA function in regulating floral development.

Expression pattern of *MYBs* (*AtMYB21*, *AtMYB24* and *AtMYB57*) basically correlates with their respective functions. All of these three genes are highly enriched in stamen compared to the other floral organs. The stamen enriched expression pattern is consistent with their functions in controlling stamen filament elongation and anther development. Besides in stamen, expression was also detected in other floral organs, indicating that they might play a role in these organs. However, genetic study showed that only stamen showed phenotype in *myb21-d myb24-b myb57-1* triple mutant. It is possible that these genes might be regulated at post-transcription level or other genes might function redundantly outside stamen. Promoter-GUS fusion study showed a different expression pattern with the endogenous genes, indicating that there might be other regulatory element outside the promoter regions. Therefore, further study in protein levels or translation-GUS fusion studies will greatly broad our understanding of the function of these genes.

# Chapter 6

## General Conclusions and Future Perspectives

GA is a general regulator of floral development. However, the mechanism of GA regulating floral development is largely unknown. Through this study, we found that GA promotes stamen filament cell elongation rather than cell division. In addition, GA regulates the cellular developmental pathways of anthers leading from microspore to mature pollen. Using novel combinations of loss-of-function mutations, we determined that DELLA proteins are the repressors of stamen filament elongation and microsporogenesis in *Arabidopsis*. GA promotes stamen development through repression of DELLA proteins RGA, RGL1 and RGL2.

Through microarray analysis, we identified 273 DELLA-up genes and 360 DELLLA-down genes in *Arabidopsis* flowers. Among the DELLA-down genes, there are several *MYBs* that are different from those already known *GAMYBs*. Genetic study showed that three DELLA-down *MYBs* (*AtMYB21*, *AtMYB24* and *AtMYB57*) are necessary for normal stamen development and may function downstream of DELLA proteins in controlling stamen development. It has been shown that JA is also required for the expression of these *MYBs*. This information prompts us to speculate that there might be interaction between GA and JA in controlling expression of these *MYBs*. Our results indicated that JA may act downstream of GA in controlling these *MYBs*. Further study suggested that GA possibly regulates JA biosynthesis gene, *DAD1*, to control the JA production via suppression of DELLA proteins.

Based on these results, a model was proposed on GA-regulated the petal and stamen development (Fig. 6.1). As shown in the model, GA promotes the development of petal and stamen via suppression of DELLA proteins. DELLA proteins repress the development of petal and stamen through activating or inhibiting

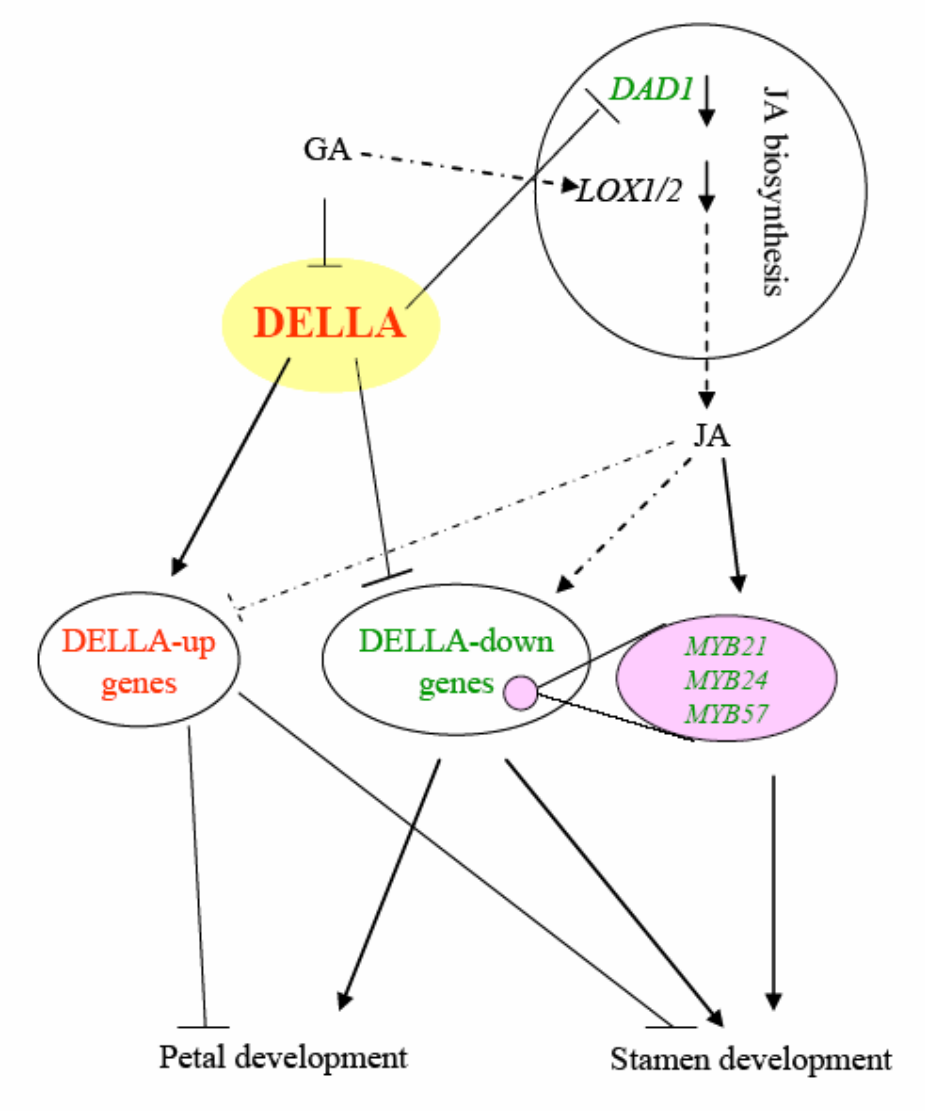



**Fig. 6.1. Model of GA regulating petal and stamen development.** Arrows and T-bars indicate induction and repression, respectively. Effects that were supported by strong evidences are represented solid lines. Dotted lines represent proposed effects. Dotted arrow for the JA biosynthesis part represents more than one step is involved.

a subset of DELLA-up genes or DELLA-down genes, respectively. There is cross-talk between GA and JA in regulating stamen development. GA is required for the expression of *DAD1*, *LOX1* and *LOX2*. GA promotes *DAD1* expression through suppression of DELLA proteins. Therefore, GA may regulate expression of *AtMYB21*, *AtMYB24* and *AtMYB57* through modulation of JA biosynthesis pathway.

Although a lot of progress has been achieved through this study, there are still many mysteries regarding how GA regulated floral development. We have identified DELLA-up and DELLA-down genes for floral development. However, the way these DELLA-regulated genes are organized is largely unknown. Further studies defining the hierarchic relationship among these DELLA regulated genes will be necessary for our understanding of the DELLAs' functions in flower development.

We suggested that JA might act downstream of GA in regulating expression of *AtMYB21*, *AtMYB24* and *AtMYB57*. However, besides *AtMYB21*, *AtMYB24* and *AtMYB57*, JA might also regulate other DELLA regulated genes in controlling stamen development. Identification of both GA and JA regulated genes would be of great help to the understanding of GA regulated flower development. Expression of *AtMYB21*, *AtMYB24* and *AtMYB57* could not rescue the flower phenotype of Q3 mutant, suggesting that these *MYBs* may not be the master check-point genes for GA regulated flower development. Therefore, further study will also be needed to find out what these genes or pathways are.

The interaction of GA and JA might be more complicated than we have reported. Like ethylene and auxin, JA could also be required for the GA induced degradation of DELLA proteins. Our results suggested that DELLA proteins might repress the expression of JA biosynthesis gene *DAD1*. However, the molecular mechanism of how DELLA proteins regulate *DAD1* expression is still unknown: is

*DAD1* the direct or indirect target of DELLA proteins? Therefore, future work would be needed to address these interesting questions.

## Chapter 7

## Reference List


Achard, P., Baghour, M., Chapple, A., Hedden, P., Van Der, S.D., Genschik, P., Moritz, T., and Harberd, N.P. (2007). The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. Proc Natl Acad Sci U S A *104*, 6484-6489.

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der, S.D., Peng, J., and Harberd, N.P. (2006). Integration of plant responses to environmentally activated phytohormonal signals. Science *311*, 91-94.

Achard, P., Herr, A., Baulcombe, D.C., and Harberd, N.P. (2004). Modulation of floral development by a gibberellin-regulated microRNA. Development *131*, 3357-3365.

Achard, P., Vriezen, W.H., Van Der, S.D., and Harberd, N.P. (2003). Ethylene regulates arabidopsis development via the modulation of DELLA protein growth repressor function. Plant Cell *15*, 2816-2825.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science *301*, 653-657.

Amador, V., Monte, E., Garcia-Martinez, J.L., and Prat, S. (2001). Gibberellins signal nuclear import of PHOR1, a photoperiod-responsive protein with homology to Drosophila armadillo. Cell *106*, 343-354.

Ashikari, M., Hironori, I., Miyako, U., Sasaki, A., Gomi, K., Kitano, H., and Matsuoka, M. (2003). Gibberellin signal transduction in rice. J Plant Growth Regul *22*, 141-151.

Ashikari, M., Wu, J., Yano, M., Sasaki, T., and Yoshimura, A. (1999). Rice gibberellin-insensitive dwarf mutant gene Dwarf 1 encodes the alpha-subunit of GTP-binding protein. Proc Natl Acad Sci U S A *96*, 10284-10289.

Aukerman, M.J. and Sakai, H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell *15*, 2730-2741.

Azumi, Y., Liu, D., Zhao, D., Li, W., Wang, G., Hu, Y., and Ma, H. (2002). Homolog interaction during meiotic prophase I in Arabidopsis requires the SOLO DANCERS gene encoding a novel cyclin-like protein. EMBO J *21*, 3081-3095.

Bethke, P.C., Hwang, Y.S., Zhu, T., and Jones, R.L. (2006). Global patterns of gene expression in the aleurone of wild-type and dwarf1 mutant rice. Plant Physiol *140*, 484-498.

Blazquez, M.A., Green, R., Nilsson, O., Sussman, M.R., and Weigel, D. (1998). Gibberellins promote flowering of arabidopsis by activating the LEAFY promoter. Plant Cell *10*, 791-800.

Blazquez, M.A. and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. Nature *404*, 889-892.

Boss, P.K., Buckeridge, E.J., Poole, D., and Thomas, A.M. (2003). New insights into grapevine flowering. Functional Plant Biology *30*, 593-606.

Boss, P.K. and Thomas, M.R. (2002). Association of dwarfism and floral induction with a grape 'green revolution' mutation. Nature *416*, 847-850.

Bowman,J. (1994). Arabidopsis: *An Atlas of Morphology and Development*. New York: Springer-Verlag).

Bozzola,J.J. and Russell,L.D. (1999). In *Electron Microscopy: Principles and Techniques for Biologist*.

Cao, D., Cheng, H., Wu, W., Soo, H.M., and Peng, J. (2006). Gibberellin mobilizes distinct DELLA-dependent transcriptomes to regulate seed germination and floral development in Arabidopsis. Plant Physiol *142*, 509-525.

Cao, D., Hussain, A., Cheng, H., and Peng, J. (2005). Loss of function of four DELLA genes leads to light- and gibberellin-independent seed germination in Arabidopsis. Planta *223*, 105-113.

Chandler, P.M., Marion-Poll, A., Ellis, M., and Gubler, F. (2002). Mutants at the Slender1 locus of barley cv Himalaya. Molecular and physiological characterization. Plant Physiol *129*, 181-190.

Chen, D., Juárez, S., Hartweck, L., Alamillo, J., Simón-Mateo, C., Pérez, J., Fernández-Fernández, M., Olszewski, N., and García, J. (2005). Identification of SECRET AGENT as the *O*-GlcNAc TRANSFERASE that participates in plum pox virus infection. Journal of Virology *79*, 9381-9387.

Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science *303*, 2022-2025.

Chen, Y.C. and McCormick, S. (1996). sidecar pollen, an Arabidopsis thaliana male gametophytic mutant with aberrant cell divisions during pollen development. Development *122*, 3243-3253.

Cheng, H., Qin, L., Lee, S., Fu, X., Richards, D.E., Cao, D., Luo, D., Harberd, N.P., and Peng, J. (2004). Gibberellin regulates Arabidopsis floral development via suppression of DELLA protein function. Development *131*, 1055-1064.

Clough, S.J. and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J *16*, 735-743.

Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G., and Carpenter, R. (1990). floricaula: a homeotic gene required for flower development in antirrhinum majus. Cell *63*, 1311-1322.

Comer, F.I. and Hart, G.W. (2000). O-Glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate. J Biol Chem *275*, 29179-29182.

Croker, S.J., Hedden, P., Lenton, J.R., and Stoddart, J.L. (1990). Comparison of Gibberellins in Normal and Slender Barley Seedlings. Plant Physiol *94*, 194-200.

De Grauwe, L., Vriezen, W.H., Bertrand, S., Phillips, A., Vidal, A.M., Hedden, P., and Van Der, S.D. (2007). Reciprocal influence of ethylene and gibberellins on response-gene expression in Arabidopsis thaliana. Planta 226, 485-498.

Devoto, A. and Turner, J.G. (2003). Regulation of jasmonate-mediated plant responses in arabidopsis. Ann. Bot. (Lond) *92*, 329-337.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A., and Benfey, P.N. (1996). The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell *86*, 423-433.

Diaz, I., Vicente-Carbajosa, J., Abraham, Z., Martinez, M., Isabel-La Moneda, I., and Carbonero, P. (2002). The GAMYB protein from barley interacts with the DOF transcription factor BPBF and activates endosperm-specific genes during seed development. Plant J *29*, 453-464.

Dill, A., Jung, H.S., and Sun, T.P. (2001). The DELLA motif is essential for gibberellin-induced degradation of RGA. Proc Natl Acad Sci U S A *98*, 14162-14167.

Dill, A. and Sun, T. (2001). Synergistic derepression of gibberellin signaling by removing RGA and GAI function in Arabidopsis thaliana. Genetics *159*, 777-785.

Dill, A., Thomas, S.G., Hu, J., Steber, C.M., and Sun, T.P. (2004). The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell *16*, 1392-1405.

Eriksson, S., Bohlenius, H., Moritz, T., and Nilsson, O. (2006). GA4 is the active gibberellin in the regulation of LEAFY transcription and Arabidopsis floral initiation. Plant Cell *18*, 2172-2181.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. (2000). The WRKY superfamily of plant transcription factors. Trends Plant Sci *5*, 199-206.

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell *6*, 751-759.

Filardo, F.F. and Swain, S.M. (2003). SPYing on GA signaling and plant development. *Journal of Plant Growth Regulation*. JOURNAL OF PLANT GROWTH REGULATION. *22*, 163-175.

Fleet, C.M. and Sun, T.P. (2005). A DELLAcate balance: the role of gibberellin in plant morphogenesis. Curr Opin Plant Biol *8*, 77-85.

Fridborg, I., Kuusk, S., Moritz, T., and Sundberg, E. (1999). The Arabidopsis dwarf mutant shi exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. Plant Cell *11*, 1019-1032.

Fridborg, I., Kuusk, S., Robertson, M., and Sundberg, E. (2001). The Arabidopsis protein SHI represses gibberellin responses in Arabidopsis and barley. Plant Physiol *127*, 937-948.

Fu, X. and Harberd, N.P. (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. Nature *421*, 740-743.

Fu, X., Richards, D.E., Ait-Ali, T., Hynes, L.W., Ougham, H., Peng, J., and Harberd, N.P. (2002). Gibberellin-mediated proteasome-dependent degradation of the barley DELLA protein SLN1 repressor. Plant Cell *14*, 3191-3200.

Fu, X., Richards, D.E., Fleck, B., Xie, D., Burton, N., and Harberd, N.P. (2004). The Arabidopsis mutant sleepy1gar2-1 protein promotes plant growth by increasing the affinity of the SCFSLY1 E3 ubiquitin ligase for DELLA protein substrates. Plant Cell *16*, 1406-1418.

Fujisawa, Y., Kato, H., and Iwasaki, Y. (2001). Structure and function of heterotrimeric G proteins in plants. Plant Cell Physiol *42*, 789-794.

Fukazawa, J., Sakai, T., Ishida, S., Yamaguchi, I., Kamiya, Y., and Takahashi, Y. (2000). Repression of shoot growth, a bZIP transcriptional activator, regulates cell elongation by controlling the level of gibberellins. Plant Cell *12*, 901-915.

Garcia-Martinez, J.L., Lopez-Diaz, I., Sanchez-Beltran, M.J., Phillips, A.L., Ward, D.A., Gaskin, P., and Hedden, P. (1997). Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. Plant Mol Biol *33*, 1073-1084.

Gardiner, J.C., Taylor, N.G., and Turner, S.R. (2003). Control of cellulose synthase complex localization in developing xylem. Plant Cell *15*, 1740-1748.

Gilroy, S. and Jones, R.L. (1994). Perception of Gibberellin and Abscisic Acid at the External Face of the Plasma Membrane of Barley (Hordeum vulgare L.) Aleurone Protoplasts. Plant Physiol *104*, 1185-1192.

Gocal, G.F., Poole, A.T., Gubler, F., Watts, R.J., Blundell, C., and King, R.W. (1999). Long-day up-regulation of a GAMYB gene during Lolium temulentum inflorescence formation. Plant Physiol *119*, 1271-1278.

Gocal, G.F., Sheldon, C.C., Gubler, F., Moritz, T., Bagnall, D.J., Macmillan, C.P., Li, S.F., Parish, R.W., Dennis, E.S., Weigel, D., and King, R.W. (2001). GAMYB-like

genes, flowering, and gibberellin signaling in Arabidopsis. Plant Physiol *127*, 1682-1693.

Gomez-Cadenas, A., Zentella, R., Walker-Simmons, M.K., and Ho, T.H. (2001). Gibberellin/abscisic acid antagonism in barley aleurone cells: site of action of the protein kinase PKABA1 in relation to gibberellin signaling molecules. Plant Cell *13*, 667-679.

Gomi, K. and Matsuoka, M. (2003). Gibberellin signalling pathway. Curr Opin Plant Biol *6*, 489-493.

Gomi, K., Sasaki, A., Itoh, H., Ueguchi-Tanaka, M., Ashikari, M., Kitano, H., and Matsuoka, M. (2004). GID2, an F-box subunit of the SCF E3 complex, specifically interacts with phosphorylated SLR1 protein and regulates the gibberellin-dependent degradation of SLR1 in rice. Plant J *37*, 626-634.

Greenboim-Wainberg, Y., Maymon, I., Borochov, R., Alvarez, J., Olszewski, N., Ori, N., Eshed, Y., and Weiss, D. (2005). Cross talk between gibberellin and cytokinin: the Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. Plant Cell *17*, 92-102.

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z.L., Powers, S.J., Gong, F., Phillips, A.L., Hedden, P., Sun, T.P., and Thomas, S.G. (2006). Genetic Characterization and Functional Analysis of the GID1 Gibberellin Receptors in Arabidopsis. Plant Cell *18*, 3399–3414.

.

Gubler, F., Chandler, P.M., White, R.G., Llewellyn, D.J., and Jacobsen, J.V. (2002). Gibberellin signaling in barley aleurone cells. Control of SLN1 and GAMYB expression. Plant Physiol *129*, 191-200.

Gubler, F. and Jacobsen, J.V. (1992). Gibberellin-responsive elements in the promoter of a barley high-pI alpha-amylase gene. Plant Cell *4*, 1435-1441.

Gubler, F., Kalla, R., Roberts, J.K., and Jacobsen, J.V. (1995). Gibberellin-regulated expression of a myb gene in barley aleurone cells: evidence for Myb transactivation of a high-pI alpha-amylase gene promoter. Plant Cell *7*, 1879-1891.

Gubler, F., Raventos, D., Keys, M., Watts, R., Mundy, J., and Jacobsen, J.V. (1999). Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. Plant J *17*, 1-9.

Harberd, N.P. (2003). Botany. Relieving DELLA restraint. Science *299*, 1853-1854.

Harberd, N.P., King, K.E., Carol, P., Cowling, R.J., Peng, J., and Richards, D.E. (1998). Gibberellin: inhibitor of an inhibitor of...? Bioessays *20*, 1001-1008.

Hartweck, L.M., Genger, R.K., Grey, W.M., and Olszewski, N.E. (2006). SECRET AGENT and SPINDLY have overlapping roles in the development of Arabidopsis thaliana L. Heyn. J Exp Bot. *57*, 865-875.

Hartweck, L.M. and Olszewski, N.E. (2006). Rice GIBBERELLIN INSENSITIVE DWARF1 is a gibberellin receptor that illuminates and raises questions about GA signaling. Plant Cell *18*, 278-282.

Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K.I. (2001). U box proteins as a new family of ubiquitin-protein ligases. J Biol Chem *276*, 33111-33120.

Hedden, P. (2003). The genes of the Green Revolution. Trends Genet. *19*, 5-9.

Hedden, P. and Phillips, A.L. (2000). Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci *5*, 523-530.

Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B., and Bailey, P.C. (2003). The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol *20*, 735-747.

Henderson, J.T., Li, H.C., Rider, S.D., Mordhorst, A.P., Romero-Severson, J., Cheng, J.C., Robey, J., Sung, Z.R., de Vries, S.C., and Ogas, J. (2004). PICKLE acts throughout the plant to repress expression of embryonic traits and may play a role in gibberellin-dependent responses. Plant Physiol *134*, 995-1005.

Hoad, G. (1995). Transport of hormones in the phloem of higher plants. J Plant Growth Regul *16*, 173-182.

Holland, N., Holland, D., Helentjaris, T., Dhugga, K.S., Xoconostle-Cazares, B., and Delmer, D.P. (2000). A comparative analysis of the plant cellulose synthase (CesA) gene family. Plant Physiol *123*, 1313-1324.

Hooley, R., Beale, M.H., and Smith, S.J. (1990). Gibberellin perception in the Avena fatua aleurone. Symp. Soc Exp Biol *44*, 79-86.

Hooley, R., Beale, M.H., Smith, S.J., Walker, R.P., Rushton, P.J., Whitford, P.N., and Lazarus, C.M. (1992). Gibberellin perception and the Avena fatua aleurone: do our molecular keys fit the correct locks? Biochem Soc Trans *20*, 85-89.

Howe, G.A., Lee, G.I., Itoh, A., Li, L., and DeRocher, A.E. (2000). Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol *123*, 711-724.

Huang, S., Cerny, R.E., Qi, Y., Bhat, D., Aydt, C.M., Hanson, D.D., Malloy, K.P., and Ness, L.A. (2003). Transgenic studies on the involvement of cytokinin and gibberellin in male development. Plant Physiol *131*, 1270-1282.

Hussain, A., Cao, D., Cheng, H., Wen, Z., and Peng, J. (2005). Identification of the conserved serine/threonine residues important for gibberellin-sensitivity of Arabidopsis RGL2 protein. Plant J *44*, 88-99.

Hussain, A., Cao, D., and Peng, J. (2007). Identification of conserved tyrosine residues important for gibberellin sensitivity of Arabidopsis RGL2 protein. Planta *226*, 475-483.

Hynes, L.W., Peng, J., Richards, D.E., and Harberd, N.P. (2003). Transgenic expression of the Arabidopsis DELLA proteins GAI and gai confers altered gibberellin response in tobacco. Transgenic Res *12*, 707-714.

Ikeda, A., Ueguchi-Tanaka, M., Sonoda, Y., Kitano, H., Koshioka, M., Futsuhara, Y., Matsuoka, M., and Yamaguchi, J. (2001). slender rice, a constitutive gibberellin response mutant, is caused by a null mutation of the SLR1 gene, an ortholog of the height-regulating gene GAI/RGA/RHT/D8. Plant Cell *13*, 999-1010.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of Escherichia coli with plasmids. Gene *96*, 23-28.

Isabel-LaMoneda, I., Diaz, I., Martinez, M., Mena, M., and Carbonero, P. (2003). SAD: a new DOF protein from barley that activates transcription of a cathepsin B-like thiol protease gene in the aleurone of germinating seeds. Plant J *33*, 329-340.

Ishida, S., Fukazawa, J., Yuasa, T., and Takahashi, Y. (2004). Involvement of 14-3-3 signaling protein binding in the functional regulation of the transcriptional activator REPRESSION OF SHOOT GROWTH by gibberellins. Plant Cell *16*, 2641-2651.

Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K. (2001). The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell *13*, 2191-2209.

Itoh, H., Sasaki, A., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Hasegawa, Y., Minami, E., Ashikari, M., and Matsuoka, M. (2005). Dissection of the phosphorylation of rice DELLA protein, SLENDER RICE1. Plant Cell Physiol *46*, 1392-1399.

Itoh, H., Ueguchi-Tanaka, M., Sato, Y., Ashikari, M., and Matsuoka, M. (2002). The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. Plant Cell *14*, 57-70.

Izhaki, A., Borochov, A., Zamski, E., and Weiss, D. (2002). Gibberellin regulates post-microsporogenesis processes in petunia anthers. Physiol Plant *115*, 442-447.

Izhaki, A., Swain, S.M., Tseng, T.S., Borochov, A., Olszewski, N.E., and Weiss, D. (2001). The role of SPY and its TPR domain in the regulation of gibberellin action throughout the life cycle of Petunia hybrida plants. Plant J *28*, 181-190.

Jack, T. (2004). Molecular and genetic mechanisms of floral control. Plant Cell *16 Suppl*, S1-17.

Jacobsen, S.E., Binkowski, K.A., and Olszewski, N.E. (1996). SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction in Arabidopsis. Proc Natl Acad Sci U S A *93*, 9292-9296.

Jacobsen, S.E. and Olszewski, N.E. (1993). Mutations at the SPINDLY locus of Arabidopsis alter gibberellin signal transduction. Plant Cell *5*, 887-896.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J *6*, 3901-3907.

Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J., and Patterson, C. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J Biol Chem *276*, 42938-42944.

Kamiya, Y. and Garcia-Martinez, J.L. (1999). Regulation of gibberellin biosynthesis by light. Curr Opin Plant Biol *2*, 398-403.

Kaneko, M., Inukai, Y., Ueguchi-Tanaka, M., Itoh, H., Izawa, T., Kobayashi, Y., Hattori, T., Miyao, A., Hirochika, H., Ashikari, M., and Matsuoka, M. (2004). Loss-of-function mutations of the rice GAMYB gene impair alpha-amylase expression in aleurone and flower development. Plant Cell *16*, 33-44.

Kaneko, M., Itoh, H., Inukai, Y., Sakamoto, T., Ueguchi-Tanaka, M., Ashikari, M., and Matsuoka, M. (2003). Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? Plant J *35*, 104-115.

Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991). Structure and function of signal-transducing GTP-binding proteins. Annu Rev Biochem *60*, 349-400.

Keith, B., Brown, S., and Srivastava, L.M. (1982). In vitro binding of gibberellin A(4) to extracts of cucumber measured by using DEAE-cellulose filters. Proc Natl Acad Sci U S A *79*, 1515-1519.

King, K.E., Moritz, T., and Harberd, N.P. (2001a). Gibberellins are not required for normal stem growth in Arabidopsis thaliana in the absence of GAI and RGA. Genetics *159*, 767-776.

King, R.W., Evans, L.T., Mander, L.N., Moritz, T., Pharis, R.P., and Twitchin, B. (2003). Synthesis of gibberellin GA6 and its role in flowering of Lolium temulentum. Phytochemistry *62*, 77-82.

King, R.W., Moritz, T., Evans, L.T., Junttila, O., and Herlt, A.J. (2001b). Long-day induction of flowering in Lolium temulentum involves sequential increases in specific gibberellins at the shoot apex. Plant Physiol *127*, 624-632.

Koornneef, M., Elgersma, A., Hanhart, C., van Loenen, M., van Riji, L., and Zeevaari, J.A. (1985). A gibberellin insensitive mutant of Arabidopsis thaliana. Physiol Plant *65*, 33-39.

Koornneef, M. and van der Veen, J.H. (1980). Induction and Analysis of Gibberellin Sensitive Mutants in Arabidopsis thaliana (L.) Heynh. Theor. Appl. Genet. *58*, 257-263.

Kramell, R., Miersch, O., Atzorn, R., Parthier, B., and Wasternack, C. (2000). Octadecanoid-derived alteration of gene expression and the "oxylipin signature" in stressed barley leaves. Implications for different signaling pathways. Plant Physiol *123*, 177-188.

Kranz, H.D., Denekamp, M., Greco, R., Jin, H., Leyva, A., Meissner, R.C., Petroni, K., Urzainqui, A., Bevan, M., Martin, C., Smeekens, S., Tonelli, C., Paz-Ares, J., and Weisshaar, B. (1998). Towards functional characterisation of the members of the R2R3-MYB gene family from Arabidopsis thaliana. Plant J *16*, 263-276.

Krizek, B.A. and Fletcher, J.C. (2005). Molecular mechanisms of flower development: an armchair guide. Nat Rev Genet. *6*, 688-698.

Lanahan, M.B., Ho, T.H., Rogers, S.W., and Rogers, J.C. (1992). A gibberellin response complex in cereal alpha-amylase gene promoters. Plant Cell *4*, 203-211.

LeClere, S., Tellez, R., Rampey, R.A., Matsuda, S.P., and Bartel, B. (2002). Characterization of a family of IAA-amino acid conjugate hydrolases from Arabidopsis. J Biol Chem *277*, 20446-20452.

Liscum, E. and Reed, J.W. (2002). Genetics of Aux/IAA and ARF action in plant growth and development. Plant Mol Biol *49*, 387-400.

Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science *297*, 2053-2056.

Lovegrove, A., Barratt, D.H., Beale, M.H., and Hooley, R. (1998). Gibberellin-photoaffinity labelling of two polypeptides in plant plasma membranes. Plant J *15*, 311-320.

Luo, D., Carpenter, R., Vincent, C., Copsey, L., and Coen, E. (1996). Origin of floral asymmetry in Antirrhinum. Nature *383*, 794-799.

Mandaokar, A., Thines, B., Shin, B., Markus, L.B., Choi, G., Koo, Y.J., Yoo, Y.J., Choi, Y.D., Choi, G., and Browse, J. (2006). Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. Plant J *46*, 984-1008.

Maucher, H., Hause, B., Feussner, I., Ziegler, J., and Wasternack, C. (2000). Allene oxide synthases of barley (Hordeum vulgare cv. Salome): tissue specific regulation in seedling development. Plant J *21*, 199-213.

McGinnis, K.M., Thomas, S.G., Soule, J.D., Strader, L.C., Zale, J.M., Sun, T.P., and Steber, C.M. (2003). The Arabidopsis SLEEPY1 gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. Plant Cell *15*, 1120-1130.

Mena, M., Cejudo, F.J., Isabel-LaMoneda, I., and Carbonero, P. (2002). A role for the DOF transcription factor BPBF in the regulation of gibberellin-responsive genes in barley aleurone. Plant Physiol *130*, 111-119.

Miersch, O. and Wasternack, C. (2000). Octadecanoid and jasmonate signaling in tomato (Lycopersicon esculentum Mill.) leaves: endogenous jasmonates do not induce jasmonate biosynthesis. Biol Chem *381*, 715-722.

Millar, A.A. and Gubler, F. (2005). The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. Plant Cell *17*, 705-721.

Mitchum, M.G., Yamaguchi, S., Hanada, A., Kuwahara, A., Yoshioka, Y., Kato, T., Tabata, S., Kamiya, Y., and Sun, T.P. (2006). Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. Plant J *45*, 804-818.

Mitsunaga, S., Rodriguez, R.L., and Yamaguchi, J. (1994). Sequence-specific interactions of a nuclear protein factor with the promoter region of a rice gene for alpha-amylase, RAmy3D. Nucleic Acids Res *22*, 1948-1953.

Monte, E., Amador, V., Russo, E., Martínez-García, J., and Prat, S. (2003). PHOR1: A U-box GA signaling component with a role in proteasome degradation? J. Plant Growth Regul. *22*, 152-162.

Moon, J., Parry, G., and Estelle, M. (2004). The ubiquitin-proteasome pathway and plant development. Plant Cell *16*, 3181-3195.

Moon, J., Suh, S.S., Lee, H., Choi, K.R., Hong, C.B., Paek, N.C., Kim, S.G., and Lee, I. (2003). The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant J *35*, 613-623.

Morris, E.R. and Walker, J.C. (2003). Receptor-like protein kinases: the keys to response. Curr Opin Plant Biol *6*, 339-342.

Murray, F., Kalla, R., Jacobsen, J., and Gubler, F. (2003). A role for HvGAMYB in anther development. Plant J *33*, 481-491.

Nakajima, M., Shimada, A., Takashi, Y., Kim, Y.C., Park, S.H., Ueguchi-Tanaka, M., Suzuki, H., Katoh, E., Iuchi, S., Kobayashi, M., Maeda, T., Matsuoka, M., and Yamaguchi, I. (2006). Identification and characterization of Arabidopsis gibberellin receptors. Plant J *46*, 880-889.

Nakajima, M., Takita, K., Wada, H., Mihara, K., Hasegawa, M., Yamaguchi, I., and Murofushi, N. (1997). Partial purification and characterization of a gibberellin-binding protein from seedlings of Azukia angularis. Biochem Biophys. Res Commun. *241*, 782-786.

Neuteboom, L.W., Ng, J.M., Kuyper, M., Clijdesdale, O.R., Hooykaas, P.J., and van der Zaal, B.J. (1999). Isolation and characterization of cDNA clones corresponding with mRNAs that accumulate during auxin-induced lateral root formation. Plant Mol Biol *39*, 273-287.

Noji, M., Urao, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). Molecular cloning of two cDNAs encoding novel MYB homologues from Arabidopsis thaliana. Plant Physiol *117*, 720.

Ogas, J., Cheng, J.C., Sung, Z.R., and Somerville, C. (1997). Cellular differentiation regulated by gibberellin in the Arabidopsis thaliana pickle mutant. Science *277*, 91-94.

Ogas, J., Kaufmann, S., Henderson, J., and Somerville, C. (1999). PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in Arabidopsis. Proc Natl Acad Sci U S A *96*, 13839-13844.

Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y., and Yamaguchi, S. (2003). Gibberellin biosynthesis and response during Arabidopsis seed germination. Plant Cell *15*, 1591-1604.

Olszewski, N., Sun, T.P., and Gubler, F. (2002). Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell *14 Suppl*, S61-S80.

Parinov, S., Sevugan, M., Ye, D., Yang, W.C., Kumaran, M., and Sundaresan, V. (1999). Analysis of flanking sequences from dissociation insertion lines: a database for reverse genetics in Arabidopsis. Plant Cell *11*, 2263-2270.

Penfield, S., Gilday, A.D., Halliday, K.J., and Graham, I.A. (2006). DELLA-mediated cotyledon expansion breaks coat-imposed seed dormancy. Curr Biol *16*, 2366-2370.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The Arabidopsis GAI gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev *11*, 3194-3205.

Peng, J. and Harberd, N.P. (1993). Derivative Alleles of the Arabidopsis Gibberellin-Insensitive (gai) Mutation Confer a Wild-Type Phenotype. Plant Cell *5*, 351-360.

Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, A.J., Pelica, F., Sudhakar, D., Christou, P., Snape, J.W., Gale, M.D., and Harberd, N.P. (1999). 'Green revolution' genes encode mutant gibberellin response modulators. Nature *400*, 256-261.

Penson, S.P., Schuurink, R.C., Fath, A., Gubler, F., Jacobsen, J.V., and Jones, R.L. (1996). cGMP Is Required for Gibberellic Acid-Induced Gene Expression in Barley Aleurone. Plant Cell *8*, 2325-2333.

Pharis, R.P. and King, R.W. (1985). Gibberellins and reproductive developement in seed plants. Annu Rev Plant Physiol Plant Mol Biol *36*, 517-568.

Pimenta Lange, M.J. and Lange, T. (2006). Gibberellin biosynthesis and the regulation of plant development. Plant Biol (Stuttg) *8*, 281-290.

Preston, J., Wheeler, J., Heazlewood, J., Li, S.F., and Parish, R.W. (2004). AtMYB32 is required for normal pollen development in Arabidopsis thaliana. Plant J *40*, 979-995.

Pysh, L.D., Wysocka-Diller, J.W., Camilleri, C., Bouchez, D., and Benfey, P.N. (1999). The GRAS gene family in Arabidopsis: sequence characterization and basic expression analysis of the SCARECROW-LIKE genes. Plant J *18*, 111-119.

Regan, S.M. and Moffatt, B.A. (1990). Cytochemical Analysis of Pollen Development in Wild-Type Arabidopsis and a Male-Sterile Mutant. Plant Cell *2*, 877-889.

Reyes, J.L. and Chua, N.H. (2007). ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. Plant J *49*, 592-606.

Richards, D., Peng, J., and Harberd, N. (2000). plant GRAS and metazoan STATs: one family? Bioessays *22*, 573-577.

Richards, D.E., King, K.E., Ait-Ali, T., and Harberd, N.P. (2001). HOW GIBBERELLIN REGULATES PLANT GROWTH AND DEVELOPMENT: A Molecular Genetic Analysis of Gibberellin Signaling. Annu Rev Plant Physiol Plant Mol Biol *52*, 67-88.

Rider, S., Henderson, J., Jerome, R., Edenberg, H., Romero-Severson, J., and Ogas, J. (2003). Coordinate repression of regulators of embryonic identity by PICKLE during germination in Arabidopsis. Plant J *35*, 33-43.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G. (2000). Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science *290*, 2105-2110.

Roberts, M.R., Foster, G.D., Blundell, R.P., Robinson, S.W., Kumar, A., Draper, J., and Scott, R. (1993). Gametophytic and sporophytic expression of an anther-specific Arabidopsis thaliana gene. Plant J *3*, 111-120.

Robertson, M., Swain, S.M., Chandler, P.M., and Olszewski, N.E. (1998). Identification of a negative regulator of gibberellin action, HvSPY, in barley. Plant Cell *10*, 995-1007.

Roos, M.D. and Hanover, J.A. (2000). Structure of O-linked GlcNAc transferase: mediator of glycan-dependent signaling. Biochem Biophys. Res Commun. *271*, 275-280.

Ross, K.J., Fransz, P., and Jones, G.H. (1996). A light microscopic atlas of meiosis in Arabidopsis thaliana. Chromosome. Res *4*, 507-516.

Rubinelli, P., Hu, Y., and Ma, H. (1998). Identification, sequence analysis and expression studies of novel anther-specific genes of Arabidopsis thaliana. Plant Mol Biol *37*, 607-619.

Rushton, P.J., Macdonald, H., Huttly, A.K., Lazarus, C.M., and Hooley, R. (1995). Members of a new family of DNA-binding proteins bind to a conserved cis-element in the promoters of alpha-Amy2 genes. Plant Mol Biol *29*, 691-702.

Sabatini, S., Heidstra, R., Wildwater, M., and Scheres, B. (2003). SCARECROW is involved in positioning the stem cell niche in the Arabidopsis root meristem. Genes Dev *17*, 354-358.

Sablowski, R.W. and Meyerowitz, E.M. (1998). A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. Cell *92*, 93-103.

Sanders, P.M., Bui, A.Q., Weterings, K., McIntire, K.N., Hsu, Y.-C., Lee, P.Y., Truong, M.T., Beals, T.P., and Goldberg, R.B. (1999). Anther developmental defects in *Arabidopsis thaliana* male-sterile mutants. Sex. Plant Reprod. *11*, 297-322.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., and Goldberg, R.B. (2000). The arabidopsis DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell *12*, 1041-1061.

Sasaki, A., Itoh, H., Gomi, K., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Jeong, D.H., An, G., Kitano, H., Ashikari, M., and Matsuoka, M. (2003). Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. Science *299*, 1896-1898.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, K., Ohta, H., and Tabata, S. (2001). Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res *8*, 153-161.

Scott, R.J., Spielman, M., and Dickinson, H.G. (2004). Stamen structure and function. Plant Cell *16 Suppl*, S46-S60.

Shi, L. and Olszewski, N.E. (1998). Gibberellin and abscisic acid regulate GAST1 expression at the level of transcription. Plant Mol Biol *38*, 1053-1060.

Shimada, A., Ueguchi-Tanaka, M., Sakamoto, T., Fujioka, S., Takatsuto, S., Yoshida, S., Sazuka, T., Ashikari, M., and Matsuoka, M. (2006). The rice SPINDLY gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. Plant J *48*, 390-402.

Shin, B., Choi, G., Yi, H., Yang, S., Cho, I., Kim, J., Lee, J., Paek, N.C., Kim, J.H., Song, P., and Choi, G. (2002). AtMYB21, a gene encoding a flower-specific transcription factor, is regulated by COP1. Plant J *30*, 23-32.

Silverstone, A.L., Ciampaglio, C.N., and Sun, T. (1998). The Arabidopsis RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell *10*, 155-169.

Silverstone, A.L., Jung, H.S., Dill, A., Kawaide, H., Kamiya, Y., and Sun, T.P. (2001). Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. Plant Cell *13*, 1555-1566.

Silverstone, A.L. and Sun, T. (2000). Gibberellins and the Green Revolution. Trends Plant Sci *5*, 1-2.

Silverstone, A.L., Tseng, T.S., Swain, S.M., Dill, A., Jeong, S.Y., Olszewski, N.E., and Sun, T.P. (2006). Functional Analysis of SPINDLY in Gibberellin Signaling in Arabidopsis. Plant Physiol.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in Arabidopsis. Plant Cell *2*, 755-767.

Steber, C.M., Cooney, S.E., and McCourt, P. (1998). Isolation of the GA-response mutant sly1 as a suppressor of ABI1-1 in Arabidopsis thaliana. Genetics *149*, 509-521.

Stenzel, I., Hause, B., Maucher, H., Pitzschke, A., Miersch, O., Ziegler, J., Ryan, C.A., and Wasternack, C. (2003a). Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato - amplification in wound signalling. Plant J *33*, 577-589.

Stenzel, I., Hause, B., Miersch, O., Kurz, T., Maucher, H., Weichert, H., Ziegler, J., Feussner, I., and Wasternack, C. (2003b). Jasmonate biosynthesis and the allene oxide cyclase family of Arabidopsis thaliana. Plant Mol Biol *51*, 895-911.

Stintzi, A. and Browse, J. (2000). The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A *97*, 10625-10630.

Stracke, R., Werber, M., and Weisshaar, B. (2001). The R2R3-MYB gene family in Arabidopsis thaliana. Curr Opin Plant Biol *4*, 447-456.

Strader, L.C., Ritchie, S., Soule, J.D., McGinnis, K.M., and Steber, C.M. (2004). Recessive-interfering mutations in the gibberellin signaling gene SLEEPY1 are rescued by overexpression of its homologue, SNEEZY. Proc Natl Acad Sci U S A *101*, 12771-12776.

Sun, T.P. and Gubler, F. (2004). Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol *55*, 197-223.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D., Dean, C., Ma, H., and Martienssen, R. (1995). Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. Genes Dev *9*, 1797-1810.

Swain, S.M. and Olszewski, N.E. (1996). Genetic Analysis of Gibberellin Signal Transduction. Plant Physiol *112*, 11-17.

Swain, S.M. and Singh, D.P. (2005). Tall tales from sly dwarves: novel functions of gibberellins in plant development. Trends Plant Sci *10*, 123-129.

Swain, S.M., Tseng, T.S., and Olszewski, N.E. (2001). Altered expression of SPINDLY affects gibberellin response and plant development. Plant Physiol *126*, 1174-1185.

Swain, S.M., Tseng, T.S., Thornton, T.M., Gopalraj, M., and Olszewski, N.E. (2002). SPINDLY is a nuclear-localized repressor of gibberellin signal transduction expressed throughout the plant. Plant Physiol *129*, 605-615.

Tague, B.W. and Goodman, H.M. (1995). Characterization of a family of Arabidopsis zinc finger protein cDNAs. Plant Mol Biol *28*, 267-279.

Takahashi, Y., Fukazawa, J., Matushita, A., and Ishida, S. (2003). Involvement of RSG and 14-3-3 proteins in the transcriptional regulation of a GA biosynthetic gene. J. Plant Growth Regul. *22*, 195-204.

Talon, M., Koornneef, M., and Zeevaart, J.A. (1990). Endogenous gibberellins in Arabidopsis thaliana and possible steps blocked in the biosynthetic pathways of the semidwarf ga4 and ga5 mutants. Proc Natl Acad Sci U S A *87*, 7983-7987.

Taylor, N.G., Laurie, S., and Turner, S.R. (2000). Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. Plant Cell *12*, 2529-2540.

Taylor, N.G., Scheible, W.R., Cutler, S., Somerville, C.R., and Turner, S.R. (1999). The irregular xylem3 locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell *11*, 769-780.

Thomas, S.G. and Sun, T.P. (2004). Update on gibberellin signaling. A tale of the tall and the short. Plant Physiol *135*, 668-676.

Thornton, T.M., Swain, S.M., and Olszewski, N.E. (1999). Gibberellin signal transduction presents ellipsisthe SPY who O-GlcNAc'd me. Trends Plant Sci *4*, 424-428.

Tomita, A., Towatari, M., Tsuzuki, S., Hayakawa, F., Kosugi, H., Tamai, K., Miyazaki, T., Kinoshita, T., and Saito, H. (2000). c-Myb acetylation at the carboxyl-terminal conserved domain by transcriptional co-activator p300. Oncogene *19*, 444-451.

Topping, J.F. and Lindsey, K. (1997). Promoter trap markers differentiate structural and positional components of polar development in Arabidopsis. Plant Cell *9*, 1713-1725.

Tregear, J.W., Primavesi, L.F., and Huttly, A.K. (1995). Functional analysis of linker insertions and point mutations in the alpha-Amy2/54 GA-regulated promoter. Plant Mol Biol *29*, 749-758.

Tseng, T.S., Salome, P.A., McClung, C.R., and Olszewski, N.E. (2004). SPINDLY and GIGANTEA interact and act in Arabidopsis thaliana pathways involved in light responses, flowering, and rhythms in cotyledon movements. Plant Cell *16*, 1550-1563.

Tseng, T.S., Swain, S.M., and Olszewski, N.E. (2001). Ectopic expression of the tetratricopeptide repeat domain of SPINDLY causes defects in gibberellin response. Plant Physiol *126*, 1250-1258.

Tsuji, H., Aya, K., Ueguchi-Tanaka, M., Shimada, Y., Nakazono, M., Watanabe, R., Nishizawa, N.K., Gomi, K., Shimada, A., Kitano, H., Ashikari, M., and Matsuoka, M. (2006). GAMYB controls different sets of genes and is differentially regulated by microRNA in aleurone cells and anthers. Plant J *47*, 427-444.

Turner, S.R. and Somerville, C.R. (1997). Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell *9*, 689-701.

Tyler, L., Thomas, S.G., Hu, J., Dill, A., Alonso, J.M., Ecker, J.R., and Sun, T.P. (2004). Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. Plant Physiol *135*, 1008-1019.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T.Y., Hsing, Y.I., Kitano, H., Yamaguchi, I., and Matsuoka, M. (2005). GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. Nature *437*, 693-698.

Ueguchi-Tanaka, M., Fujisawa, Y., Kobayashi, M., Ashikari, M., Iwasaki, Y., Kitano, H., and Matsuoka, M. (2000). Rice dwarf mutant d1, which is defective in the alpha subunit of the heterotrimeric G protein, affects gibberellin signal transduction. Proc Natl Acad Sci U S A *97*, 11638-11643.

Ullah, H., Chen, J.G., Temple, B., Boyes, D.C., Alonso, J.M., Davis, K.R., Ecker, J.R., and Jones, A.M. (2003). The beta-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell *15*, 393-409.

Ullah, H., Chen, J.G., Wang, S., and Jones, A.M. (2002a). Role of a heterotrimeric G protein in regulation of Arabidopsis seed germination. Plant Physiol *129*, 897-907.

Ullah, H., Chen, J.G., Wang, S., and Jones, A.M. (2002b). Role of a heterotrimeric G protein in regulation of Arabidopsis seed germination. Plant Physiol *129*, 897-907.

Ullah, H., Chen, J.G., Young, J.C., Im, K.H., Sussman, M.R., and Jones, A.M. (2001). Modulation of cell proliferation by heterotrimeric G protein in Arabidopsis. Science *292*, 2066-2069.

Van Huizen, R., Ozga, J.A., and Reinecke, D.M. (1997). Seed and Hormonal Regulation of Gibberellin 20-Oxidase Expression in Pea Pericarp. Plant Physiol *115*, 123-128.

Vorbrueggen, G., Lovric, J., and Moelling, K. (1996). Functional analysis of phosphorylation at serine 532 of human c-Myb by MAP kinase. Biol Chem *377*, 721-730.

Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M. (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science *292*, 2070-2072.

Washio, K. (2003). Functional dissections between GAMYB and Dof transcription factors suggest a role for protein-protein associations in the gibberellin-mediated expression of the RAmy1A gene in the rice aleurone. Plant Physiol *133*, 850-863.

Weber, H., Vick, B.A., and Farmer, E.E. (1997). Dinor-oxo-phytodienoic acid: a new hexadecanoid signal in the jasmonate family. Proc Natl Acad Sci U S A *94*, 10473-10478.

Wells, L., Vosseller, K., and Hart, G.W. (2001). Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. Science *291*, 2376-2378.

Willige, B.C., Ghosh, S., Nill, C., Zourelidou, M., Dohmann, E.M., Maier, A., and Schwechheimer, C. (2007). The DELLA Domain of GA INSENSITIVE Mediates the Interaction with the GA INSENSITIVE DWARF1A Gibberellin Receptor of Arabidopsis. Plant Cell *19*, 1209-1220.

Wilson, R.N., Heckman, J.W., and Somerville, C.R. (1992). Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. Plant Physiol *100*, 403-408.

Wilson, R.N. and Somerville, C.R. (1995). Phenotypic suppression of the gibberellin-insensitive mutant (gai) of Arabidopsis. Plant Physiol *108*, 495-502.

Woodger, F.J., Gubler, F., Pogson, B.J., and Jacobsen, J.V. (2003). A Mak-like kinase is a repressor of GAMYB in barley aleurone. Plant J *33*, 707-717.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science *280*, 1091-1094.

Xie, Z., Zhang, Z.L., Hanzlik, S., Cook, E., and Shen, Q.J. (2007). Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene. Plant Mol Biol *64*, 293-303.

Xie, Z., Zhang, Z.L., Zou, X., Yang, G., Komatsu, S., and Shen, Q.J. (2006). Interactions of two abscisic-acid induced WRKY genes in repressing gibberellin signaling in aleurone cells. Plant J *46*, 231-242.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell *14*, 1919-1935.

Xu, Y.L., Gage, D.A., and Zeevaart, J.A. (1997). Gibberellins and stem growth in Arabidopsis thaliana. Effects of photoperiod on expression of the GA4 and GA5 loci. Plant Physiol *114*, 1471-1476.

Yang, X.Y., Li, J.G., Pei, M., Gu, H., Chen, Z.L., and Qu, L.J. (2006). Over-expression of a flower-specific transcription factor gene AtMYB24 causes aberrant anther development. Plant Cell Rep *26*, 219-228.

Yanofsky, M.F. (1995). Floral meristems to floral organs: Genes controlling early events in Arabidopsis flower development. Annu Rev Plant Physiol Plant Mol Biol *46*, 167-188.

Yu, H., Ito, T., Zhao, Y., Peng, J., Kumar, P., and Meyerowitz, E.M. (2004). Floral homeotic genes are targets of gibberellin signaling in flower development. Proc Natl Acad Sci U S A *101*, 7827-7832.

Zentella, R., Yamauchi, D., and Ho, T.H. (2002). Molecular dissection of the gibberellin/abscisic acid signaling pathways by transiently expressed RNA interference in barley aleurone cells. Plant Cell *14*, 2289-2301.

Zhang, Z.L., Xie, Z., Zou, X., Casaretto, J., Ho, T.H., and Shen, Q.J. (2004). A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells. Plant Physiol *134*, 1500-1513.

Zhao, D. and Ma, H. (2000). Male fertility: a case of enzyme identity. Curr Biol *10*, R904-R907.

Zhao, D.Z., Wang, G.F., Speal, B., and Ma, H. (2002). The excess microsporocytes1 gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the Arabidopsis anther. Genes Dev *16*, 2021-2031.

Ziegler, J., Keinanen, M., and Baldwin, I.T. (2001). Herbivore-induced allene oxide synthase transcripts and jasmonic acid in Nicotiana attenuata. Phytochemistry *58*, 729-738.

Ziegler, J., Stenzel, I., Hause, B., Maucher, H., Hamberg, M., Grimm, R., Ganal, M., and Wasternack, C. (2000). Molecular cloning of allene oxide cyclase. The enzyme establishing the stereochemistry of octadecanoids and jasmonates. J Biol Chem *275*, 19132-19138.