10 DE-repression of seed germination by GA signaling

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10.1 Introduction

This chapter explores evidence that the ubiquitin–proteasome pathway plays a role in gibberellin (GA) stimulation of germination via proteolysis of DELLA proteins (named after conserved amino acids). GA stimulates germination, stem elongation, transition to flowering, and fertility. DELLA proteins are repressors of GA responses defined by the presence of conserved DELLA and GRAS (named after GAI [GA-INSENSITIVE], RGA [REPRESSOR OF GAI], and SCARECROW proteins) domain amino acid sequences. It is clear that the ubiquitin–proteasome pathway relieves DELLA repression of stem elongation in response to GA signaling. The paradigm is that GA stimulates an SCF (named after the Skp1, Cullin, and F-box subunits) E3 ubiquitin ligase complex, which in turn stimulates degradation of the DELLA proteins, negative regulators of GA response, by the proteasome pathway. This induces the downstream events necessary for seed germination, which were under repression in the absence of GA. However, interpretation of the evidence for DELLA regulation of seed germination has been somewhat contentious. Work in *Arabidopsis thaliana* suggests that the DELLA protein RGL2 (RGA-LIKE2) is the main negative regulator of GA response in germination, and that the SCF^{SLY1} E3 ubiquitin ligase complex is required for GA-stimulated disappearance of RGL2.

10.2 Control of germination by GA signaling

The plant hormone GA was first identified in 1926 based on its role in stem elongation (Tamura, 1991; Sun and Gubler, 2004; Thomas *et al.*, 2005). Eiichi Kurosawa identified gibberellin as the agent causing the excessive stem elongation in rice (*Oryza sativa*) seedlings infected with *bakanae* disease. Subsequent work demonstrated that gibberellins are growth regulators in a wide range of plant, algal, and fungal species. GA is not a single plant hormone, but actually a large family of tetracyclic diterpenes. Thus far, 136 naturally occurring GA molecules have been identified in plants and fungi (Thomas *et al.*, 2005). GA acts on many physiological events during plant growth and development (reviewed by Sun and Gubler, 2004; Thomas *et al.*, 2005). GA promotes stem elongation by stimulating both cell elongation and cell division. GA stimulates the transition to flowering in most plant

species and appears to be required for normal fertility and embryo development. A wide range of evidence has shown that GA plays a role in seed germination.

By far the strongest evidence for the role of GA in germination comes from studies of mutants defective in GA biosynthesis in dicots. In *Arabidopsis*, mutations in early GA biosynthesis genes result in a requirement for GA application to germinate (Koornneef and van der Veen, 1980). Mutations in the biosynthesis genes *GA1* (copalyl synthase), *GA2* (*ent*-kaurene synthase), and *GA3* (*ent*-kaurene oxidase) result in failure to germinate. Mutations in genes acting late in GA biosynthesis such as *ga4-1* (GA 3 oxidase or GA3ox) and *ga5-1* (GA 20-oxidase or GA20ox) do not require GA application to germinate. The explanation may lay in the fact that both of these genes are part of multigene families in higher plants. These genes show tissue-specific expression. For example, the *Arabidopsis GA3ox2* gene was found to be expressed primarily in germinating seeds and seedlings, while *GA3ox1* was expressed in all growing tissues (Yamaguchi *et al.*, 1998). Future work may use studies of multiple mutants to determine whether these GA biosynthesis genes are also required for germination in *Arabidopsis*. A mutation in *ent*-kaurene synthase called *gib-1* has been identified in tomato (*Lycopersicon esculentum*) and *gib-1* seeds require GA application for germination (Benson and Zeevaart, 1990; Karssen *et al.*, 1989). Thus, the requirement for GA in germination is not unique to *Arabidopsis*.

The plant hormones ABA (abscisic acid) and GA have tightly interwoven roles in the decision to germinate. While GA is often required for germination and for postgerminative reserve mobilization, ABA inhibits germination, promotes nutrient storage, and is required for dormancy and desiccation tolerance during embryo maturation (reviewed by Bentsink and Koornneef, 2002). Seeds are said to be dormant when they are unable to germinate even under favorable conditions. Mutants unable to synthesize ABA have nondormant seeds and a vegetative wilty phenotype. The wilty phenotype results from an inability to close stomates and conserve water in response to drought stress. Thus, in adult tissues ABA is the stress hormone needed for resistance to drought, and is also involved in cold and salt stress. The hormone balance theory postulates that the decision to germinate is a balancing act between ABA and GA signals (Karssen and Lacka, 1986). If ABA biosynthesis or signaling is reduced, a seed is more likely to germinate. If GA biosynthesis or signaling is reduced, a seed is less likely to germinate. A mutation in one pathway can compensate for a mutation in the other. For example, germination of a strong GA biosynthesis mutant can be rescued by mutants causing either reduced ABA biosynthesis or sensitivity (reviewed by Bentsink and Koornneef, 2002). Conversely, the ability of ABA-insensitive mutants to germinate in the presence of high concentrations of ABA is suppressed by reduced GA biosynthesis or signaling (Steber *et al.*, 1998).

By definition, seed germination is complete when the embryo radicle emerges from the seed coat. GA is believed to stimulate germination by inducing hydrolytic enzymes that weaken the seed coat or endosperm cap, by inducing mobilization of seed nutrient storage compounds, and by stimulating expansion of the embryo (Bewley and Black, 1994; see Chapter 11). In tomato, it has been shown that GA stimulates expression of endo-β-mannanase, an enzyme that breaks down cell wall reserves in the endosperm cap (Still and Bradford, 1997; Nonogaki *et al.*, 2000).

During *Arabidopsis* germination, genes expected to be involved in cell wall degradation and induction of cell division are induced by GA (Ogawa *et al.*, 2003). In the cereal grains of rice, barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*), GA induces the expression of genes encoding α-amylase, an enzyme needed for the breakdown of the starchy endosperm (Sun and Gubler, 2004). The mobilization of this nutrient source doubtlessly contributes to the embryo growth after germination.

The role of GA biosynthesis and signaling genes in rice germination is currently unclear. An apparent knockout mutation in an *ent*-kaurene synthase gene of rice (*OsKS1*) results in the expected dwarfism and infertility phenotypes, but in contrast to *Arabidopsis* and tomato it does not result in failure to germinate (Sakamoto *et al.*, 2004; Margis-Pinheiro *et al.*, 2005). The fact that *OsKS1* is not expressed in imbibing seeds, however, raises the possibility that one of the five to seven homologues of KS may function in rice seed germination (Margis-Pinheiro *et al.*, 2005). GA-insensitive mutations in the rice F-box gene *GID2* (*GIBBERELLIN-INSENSITIVE DWARF2*) and in the GA receptor *GID1* also result in dwarfism and infertility without compromising germination (Itoh *et al.*, 2003). While the *gid1* mutants fail to induce α-amylase during seedling establishment, the mutants germinate well (Ueguchi-Tanaka *et al.*, 2005; M. Matsuoka, personal communication). There are a number of explanations for these observations. Cereal seeds ('caryopses') including rice have highly specialized seed morphology where the embryo is not entirely enclosed by the endosperm. Tissues covering the embryo may offer relatively little resistance to penetration by the coleorhiza and coleoptile. It is possible that in rice, GA is not required for germination per se but is required only for reserve mobilization. It is also possible that the rice variety in which the *gid1* and *gid2* mutants were identified does not require GA signaling in germination either due to lack of seed dormancy or due to a defect in ABA signaling in that background. The ability of GA mutant rice seeds (caryopses), which have pale testa color to germinate, may be due to reduced dormancy resulting from lack of red pigments in the testa. Red rice has strong seed dormancy compared to pigmentless cultivars (Footitt and Cohn, 1995). Loss of red testa color causes reduced seed dormancy. For example, the reduced dormancy caused by the *Arabidopsis* transparent testa mutant *tt4* suppresses the requirement for GA in germination, allowing the *ga1-1* mutant to germinate (Debeaujon and Koornneef, 2000; Chapter 2). Another possibility is that rice varieties used in these studies have a defect in ABA signaling. For example, it has been shown that all tested wheat cultivars are unable to properly splice the *ZmVP1/AtABI3* (*VIVIPAROUS1/ABA-INSENSITIVE3*) homologue of wheat *TaVP1* (McKibbin *et al.*, 2002). Thus, bread wheat has reduced seed dormancy due to reduced ABA signaling. In *Arabidopsis*, mutations causing ABA insensitivity allow both GA biosynthesis and GA-insensitive mutants to germinate. A similar defect in rice ABA signaling might obviate the requirement for GA in germination. The fact that GA biosynthesis is required for germination of the ABA-insensitive *vp5* mutants on the mother plant in maize (*Zea mays*) (White *et al.*, 2000; White and Rivin, 2000) suggests that GA is important for cereal germination during embryo development. Future research will need to address the role of GA in cereal grain germination.

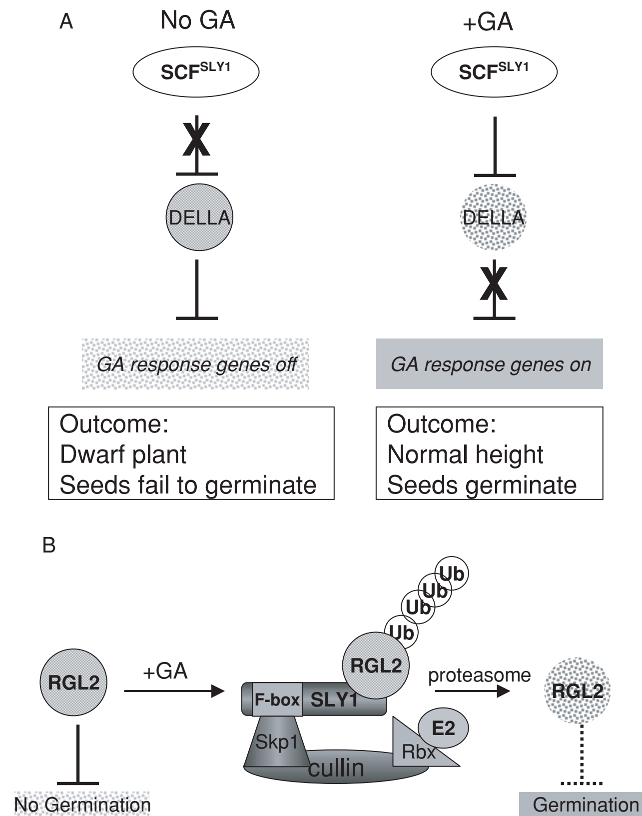

Figure 10.1 Model for control of GA responses by the ubiquitin–proteasome pathway. (A) In the absence of GA, for example in a GA biosynthesis mutant, DELLA proteins repress GA responses such as seed germination and stem elongation. When GA is added, the SCF E3 ubiquitin ligase complex causes disappearance of the DELLA protein, thereby de-repressing GA responses. (B) Destruction of the DELLA protein RGL2 is mediated by SCF^{SLY1} E3 ubiquitin ligase in *Arabidopsis*. In the absence of GA, RGL2 represses seed germination. In the presence of GA, the SCF^{SLY1} complex catalyzes the transfer of ubiquitin from the E2 ubiquitin conjugating enzyme to the target protein RGL2. Addition of at least four ubiquitin moieties allows the target to be recognized and degraded by the 26S proteasome. The configuration of the SCF^{SLY1} complex is based on Zheng *et al.* (2002).

DELLA proteins are negative regulators of GA signaling that form a subfamily of the GRAS family of putative transcription factors (Figure 10.1A). The DELLA proteins are named for a conserved amino acid sequence (D = Asp, E = Glu, L = Leu, L = Leu, A = Ala). The first DELLA genes cloned were *Arabidopsis GAI* and *RGA*. *GAI* was originally identified by Koornneef *et al.* (1985) as a gain-of-function mutation (*gai-1*) causing a GA-insensitive dwarf phenotype similar to the 'green revolution' genes of wheat (Peng *et al.*, 1999a). Loss-of-function mutations in *RGA* were identified based on suppression of the dwarf phenotype of the GA biosynthesis mutant *ga1-3* (Silverstone *et al.*, 1997). Cloning and characterization of *GAI*

and *RGA* revealed that they are homologous genes (Peng *et al.*, 1997; Silverstorne *et al.*, 1998). While loss of neither gene alone gives increased stature, the *gai-t6 rga-24* double mutant is taller than wild-type plants, indicating that together they repress stem elongation of *Arabidopsis* (Dill and Sun, 2001; King *et al.*, 2001). This increased height phenotype is reminiscent of the slender phenotype of loss-of-function mutants in *DELLA* genes of barley (*SLN1*, *SLENDER1*) and rice (*SLR1*, *SLENDER RICE1*) (Ikeda *et al.*, 2001; Chandler *et al.*, 2002). A DELLA protein has been shown to inhibit stem elongation in species as diverse as barley, rice, wheat, maize, grape (*Vitis vinifera*), tobacco (*Nicotiana tabacum*), *Brassica rapa*, and *Arabidopsis* (Peng *et al.*, 1999a; Silverstone *et al.*, 2001; Boss and Thomas, 2002; Gubler *et al.*, 2002; Itoh *et al.*, 2002; Hynes *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004; Muangprom *et al.*, 2005).

The current model for GA signaling in stem elongation is widely conserved in the plant kingdom. If the plant never produced GA, as in a GA biosynthesis mutant, repression of stem elongation by DELLA would result in a dwarf phenotype (Figure 10.1A). Addition of GA stimulates cell division and expansion within the meristem by causing disappearance of the DELLA protein. GA seems to cause DELLA protein disappearance by triggering DELLA protein polyubiquitination by an SCF E3 ubiquitin ligase (Itoh *et al.*, 2003). Addition of four or more ubiquitin moieties to a DELLA protein targets it for destruction by the 26S proteasome (Sasaki *et al.*, 2003). The interaction between the DELLA protein and SCF complex may be regulated by DELLA phosphorylation or by interaction between the DELLA protein and the GA receptor GID1 (Fu *et al.*, 2004; Gomi *et al.*, 2004; Ueguchi-Tanaka *et al.*, 2005).

Evidence from work in *Arabidopsis* suggests that GA signaling also stimulates germination via DELLA protein disappearance. There are five *DELLA* genes in *Arabidopsis*, *RGA*, *GAI*, *RGL1*, *RGL2*, and *RGL3*. *RGA* and *GAI* repress stem elongation, while *RGL1*, *RGA*, and *RGL2* repress the transition to flowering (Itoh *et al.*, 2003). *RGL2* is the main repressor of germination, although *RGL1*, *RGA*, and *GAI* also appear to contribute (Lee *et al.*, 2002; Tyler *et al.*, 2004; Cao *et al.*, in press). Two questions must be answered in order to understand the role of GA and DELLA proteins in seed germination: (1) Is RGL2 a master negative regulator of seed germination? and (2) How widely conserved is this mechanism for controlling seed germination? The evidence that DELLA and the SCF E3 ubiquitin ligase complex control germination in *Arabidopsis* will be discussed first, and then evidence in additional plant species will be considered.

10.3 The role of the ubiquitin–proteasome pathway in GA signaling

Several lines of evidence indicate that DELLA proteins are regulated by the ubiquitin–proteasome pathway (Figure 10.1). First, GA treatment causes the disappearance of DELLA proteins in *Arabidopsis*, rice, and barley (Silverstorne *et al.*, 2001; Itoh *et al.*, 2002; Gubler *et al.*, 2002; Dill *et al.*, 2004; Fu *et al.*, 2004). This disappearance requires the F-box subunit of an SCF E3 ubiquitin ligase and

the 26S proteasome. The disappearance of DELLA protein in response to GA is blocked by inhibitors of the 26S proteasome (Fu *et al.*, 2002; Hussain *et al.*, 2005) and by mutations in the F-box genes rice *GID2* and *Arabidopsis SLY1* (*SLEEPY1*) (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003). This suggests that GA causes the $SCF^{SLY1/GID2}$ E3 ubiquitin ligase to target the DELLA protein for destruction via the 26S proteasome. Mutations in *GID2* and *SLY1* cause a range of GA-insensitive phenotypes including severe dwarfism, dark green color, and reduced fertility (Itoh *et al.*, 2003). Mutations in *SLY1* also cause increased seed dormancy. SLY1 and GID2 proteins have been shown to interact with DELLA proteins, and ubiquitinated DELLA protein has been detected in rice (Sasaki *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004; Gomi *et al.*, 2004).

DELLA proteins are regulated by *Arabidopsis* SCF^{SLY1} and rice SCF^{GID2} E3 ubiquitin ligase complexes (Figure 10.1B; McGinnis *et al.*, 2003; Sasaki *et al.*, 2003). SCF complexes are one of several types of E3 ubiquitin ligases found in plants and in animals (Smalle and Vierstra, 2004). An SCF complex is identified based on the F-box subunit because it is the F-box subunit that binds to a specific target or substrate protein. Based on the crystal structure of mammalian SCF^{Skp2}, the backbone of the SCF complex is cullin (Zheng *et al.*, 2002). Cullin binds a homologue of the ring-finger protein Rbx1 at its C-terminus, and Rbx1 binds the E2 ubiquitin conjugating enzyme. At the N-terminus, cullin binds a homologue of Skp1. Skp1 binds the F-box protein via the conserved F-box domain and tethers the F-box protein to the rest of the complex. The F-box protein is generally composed of an F-box domain at the N-terminus and a protein–protein interaction domain at the C-terminus that binds to a substrate protein such as DELLA. The SCF complex catalyzes transfer of ubiquitin from E2 to the substrate, in this case a DELLA protein.

Ubiquitin is a 76-amino acid protein used in plants and animals as a tag to signal for proteolytic processing, cleavage, or destruction (Smalle and Vierstra, 2004). DELLA proteins appear to be targeted by ubiquitination for destruction by the 26S proteasome. Generally, ubiquitin is first covalently bound to a cysteine of an E1 ubiquitin-activating enzyme via a thioester bond and then transferred to a cysteine of an E2 ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin ligase catalyzes the transfer of ubiquitin from E2 to a lysine residue of the substrate protein. Formation of a chain of four or more ubiquitin moieties on the substrate allows its recognition and proteolysis by the 26S proteasome. The 26S proteasome is a multi-subunit protein complex that can be found both in the nucleus and in the cytosol. It is surmised that DELLA proteins meet their end in the 26S proteasome because the DELLA proteins barley SLN1 and *Arabidopsis* RGL2 were shown to be stabilized by compounds known to inhibit the 26S proteasome (Fu *et al.*, 2002; Hussain *et al.*, 2005). The rice DELLA SLR1 was shown to be subjected to ubiquitination by western analysis using an antibody to ubiquitin (Sasaki *et al.*, 2003). Finally, DELLA proteins in a wide range of plant species have been shown to be regulated by a highly conserved SCF E3 ubiquitin ligase (Itoh *et al.*, 2003).

Protein destruction is a recurrent theme in plant signal transduction (Smalle and Vierstra, 2004). There are almost 700 F-box proteins in the *Arabidopsis* genome. It is already clear that F-box proteins and the ubiquitin–proteasome pathway play

a crucial role in plant hormone signaling. The ubiquitin–proteasome pathway was implicated in ABA and cytokinin signaling when mutations in subunits of the 26S proteasome were shown to result in changes in hormone sensitivity (Smalle *et al.*, 2002, 2003). Recently, the ABA signaling protein ABI3 was found to be regulated by the AIP2 E3 ubiquitin ligase (Zhang *et al.*, 2005). In ethylene signaling, the transcription factors EIN3 and EIL1 are regulated by the homologous F-box proteins EBF1 and EBF2 (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Gagne *et al.*, 2004). EBF1 and EBF2 F-box proteins contain the leucine-rich repeat (LRR) type of protein–protein interaction domain at the C-terminus. The SLY1 F-box protein contains no consensus protein–protein interaction domain at the C-terminus. However, based on mutation studies, the C-terminus is required both for function and for interaction with DELLA proteins (McGinnis *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004). The COI1 F-box protein required for jasmonic acid signaling contains an LRR protein–protein interaction domain at the C-terminus (Xu *et al.*, 2002). This is similar to the LRRs found in the C-terminus of the TIR1 F-box protein (Gray *et al.*, 1999). TIR1 was recently shown to be an auxin receptor (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005).

Auxin signaling has a number of similarities to GA signaling. Auxin triggers auxin responses by targeting the IAA/AUX family of transcriptional repressors for destruction via the 26S proteasome (Gray *et al.*, 2001). The IAA/AUX proteins repress auxin responses by binding to the ARF transcription factors. DELLA proteins repress GA responses by an unknown mechanism. Auxin binds the F-box protein TIR1, thus enabling TIR1 to bind to the IAA/AUX proteins and polyubiquitinate them, thereby targeting them for destruction by the 26S proteasome (Dharmasiri *et al.*, 2005, Kepinski and Leyser, 2005). The DELLA proteins have been shown to be regulated by the F-box proteins *Arabidopsis* SLY1 or rice GID2. However, it is still unclear precisely how GA controls the interaction between the F-box protein and the DELLA protein. While interactions have been detected between F-box proteins SLY1/GID2 and DELLA proteins, no interaction between GA and SLY1/GID2 has yet been reported. However, it has been reported that GA binds to the rice GID1 protein, a GA receptor (Ueguchi-Tanaka *et al.*, 2005). GID1 shows GA-dependent interaction with the rice DELLA protein SLR1. Mutations in *GID1* lead to overaccumulation of DELLA protein, suggesting that *GID1* is required for destruction of the DELLA protein by SCF^{GID2}. Whether GID1 controls the proteolysis of DELLA proteins via direct protein–protein interaction between GID1 and GID2 F-box protein needs to be investigated.

While DELLA proteins are known to be negative regulators of GA responses, their precise function remains unknown. They are considered likely transcription factors based on amino acid homology and on yeast two-hybrid data showing that they can activate transcription (Peng *et al.*, 1999a; Itoh *et al.*, 2002). The DELLA protein family is defined by conserved amino acid sequences (Sun and Gubler, 2004). The C-terminus of DELLA proteins contains homology to the GRAS family of proteins, including a nuclear localization sequence, the VHIID domain, leucine heptad repeats (LHR), and SH2 domains. The SH2 domain of metazoan STAT (signal transducers and activators of transcription) transcription factors is involved

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in phosphotyrosine signaling (Peng *et al.*, 1999a). The DELLA subfamily of the GRAS family contains conserved domains at the N-terminus including DELLA and VHYNP. Based on mutation studies, it appears that the C-terminus is the functional domain while the N-terminus is a regulatory domain needed for response to GA (Sun and Gubler, 2004). However, this work is based on stem elongation as a GA response. Future work will need to define the protein domains needed for negative regulation of seed germination by DELLA proteins.

10.4 Is RGL2 a 'master regulator' of seed germination?

The evidence that DELLA proteins are negative regulators of germination came from studies of RGL2 in *Arabidopsis* (summarized in Figure 10.2; Lee *et al.*, 2002; Tyler *et al.*, 2004; Cao *et al.*, in press). The first study by Lee *et al.* (2002) showed that loss of *RGL2* function restores germination of the strong GA biosynthesis mutant *ga1-3* and restores germination in the presence of GA biosynthesis inhibitor paclobutrazol. This result was confirmed by Tyler *et al.* (2004). These genetic studies are the most compelling evidence that the DELLA protein RGL2 is normally needed to repress seed germination and that GA acts by alleviating RGL2 repression of seed germination.

The initial study by Lee *et al.* (2002) suggested that *RGL2* activity is controlled at the level of transcript accumulation. Northern blot analysis showed that *RGL2* transcript is induced within the first 24 h of seed imbibition, the process during which dry seeds take up water. *RGL2* mRNA levels remained high as long as the seeds were incubated at 4°C, a temperature at which seeds did not germinate. *RGL2* mRNA levels greatly decreased and germination took place within 48 h of transfer to 23°C. This correlative evidence suggested that germination is associated with a decrease

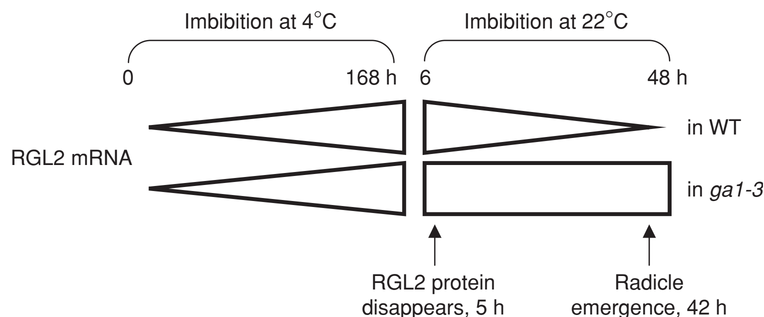

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Figure 10.2 Schematic summary of the pattern of *RGL2* expression during imbibition and germination in wild type (WT) and *ga1-3* mutant seeds. Diagram shows increasing *RGL2* mRNA accumulation during imbibition in the cold (4°C). *RGL2* mRNA levels decrease over 48 h of transfer to 23°C in wild-type, but remain high in the GA biosynthesis mutant *ga1-3* (Lee *et al.*, 2002). However, some *RGL2* mRNA remains at the time of radicle emergence in wild-type seeds (Bassel *et al.*, 2004). Radicle emergence begins within 30 h of transfer to higher temperature and is complete within 42 h (Bassel *et al.*, 2004). In the *ga1-3* mutant, RGL2 protein disappears within 5 h of adding GA to imbibing seeds (Tyler *et al.*, 2004).

in *RGL2* mRNA. This correlation between *RGL2* mRNA accumulation and failure to germinate was further supported by data showing that the strong GA biosynthesis mutant *ga1-3* accumulates high levels of *RGL2* mRNA during imbibition both at 4°C and at 23°C. *RGL2* mRNA abundance decreases within 48 h of addition of GA to imbibing *ga1-3* seeds. This suggests that one way that GA stimulates germination of *ga1-3* seeds is by decreasing the level of *RGL2* transcript. It is important to note, however, that the *RGL2* transcript did not completely disappear within 48 h of adding GA. Thus the Lee *et al.* (2002) concluded that downregulation of *RGL2* mRNA level is consistent with the notion that it is a negative regulator of seed germination, but could not conclude that complete absence of *RGL2* mRNA is required for germination to occur.

A subsequent study by Bassel *et al.* (2004) sought to determine whether downregulation of DELLA transcript is required for seed germination in *Arabidopsis*. This study very carefully examined the level of *RGL2* mRNA relative to percent germination. They found that even after *Arabidopsis* seed germination is complete, *RGL2* transcript can still be detected (Bassel *et al.*, 2004). This study clearly indicates that disappearance of *RGL2* mRNA cannot be the sole mechanism for stimulating germination. However, it does leave open the possibility that *RGL2* is a negative regulator of germination subjected to posttranscriptional regulation, such as translation, nuclear localization, protein stability, or posttranslational modification of RGL2 protein.

A study by Tyler *et al.* (2004) suggests that RGL2 function is regulated by proteolysis and that GA-dependent disappearance of RGL2 protein is regulated by the SLY1 F-box protein. Western analysis of RGL2 protein accumulation in the *ga1-3* mutant background showed that RGL2 protein disappears within 5 h after GA application to the *ga1-3* seeds, but does not disappear in the absence of GA. This correlation between GA-triggered disappearance of RGL2 protein and GA stimulation of germination suggests that disappearance of RGL2 protein is required for germination in *Arabidopsis*. The rapid disappearance of RGL2 protein would be consistent with a role during germination proper (prior to radicle emergence), rather than a role in postgermination events such as seedling establishment. Taken together with the fact that loss of *RGL2* function rescues of *ga1-3* seed germination, this study strongly suggests that RGL2 is a negative regulator of germination in *Arabidopsis*. Proof that RGL2 is a negative regulator of seed germination requires demonstration that germination fails to occur if RGL2 protein persists during germination.

RGL2 protein does persist after GA treatment of *sly1* mutant seeds (Tyler *et al.*, 2004). This suggests that the SCF^{SLY1} E3 ubiquitin ligase normally targets RGL2 for destruction in response to GA. Mutations in the *SLY1* gene result in an increase in seed dormancy and in an increase in sensitivity to ABA in germination (Steber *et al.*, 1998; Steber and McCourt, 2001; Strader *et al.*, 2004). However, *sly1* mutants do eventually after-ripen, and some seed lots do germinate (C.M. Steber, unpublished results). This suggests that the disappearance of RGL2 protein is important for germination, but is not the sole mechanism regulating germination in *Arabidopsis*. It is possible that there are additional regulators of seed germination in *Arabidopsis*, or that there are additional mechanisms for posttranslational regulation of RGL2.

Recent work by Hussain *et al.* (2005) in the tobacco BY2 cell line indicates that RGL2 protein is phosphorylated *in planta* and suggests that RGL2 protein is stabilized by phosphorylation at multiple sites. Future work will need to investigate whether posttranslational modification alters RGL2 activity in seed germination.

10.5 *Sleepy1* is a positive regulator of seed germination in arabidopsis

Mutations in the *SLY1* gene were recovered in two screens that looked for reduced ability to germinate (Figure 10.3). The first was a screen for suppressors of the ability of the ABA-insensitive mutant *ABI1-1* to germinate in the presence of 3 μM ABA (Steber *et al.*, 1998). *ABI1-1* is a semi-dominant mutation that permits seeds to germinate at up to 100 μM ABA, while germination of wild-type *Arabidopsis* seeds is inhibited by 1.2 μM ABA. Suppressor mutations were isolated in *SLY1* and in the GA biosynthesis gene *GA1*. All mutant isolates of *SLY1* from this screen were a single frameshift mutation causing loss of the last 40 amino acids, and are now referred to as *sly1-2* for historical reasons (McGinnis *et al.*, 2003). An additional allele, *sly1-10*, was recovered in a screen for brassinosteroid-dependent germination (Figure 10.3) and is a complex rearrangement resulting in loss of the last 8 amino acids (Steber and McCourt, 2001). The *sly1-2* mutation appears to result in stronger phenotypes than does the *sly1-10* mutation.

The *SLY1* gene is defined as a positive regulator of germination, because loss of function alleles lead to reduced germination and gain of function leads to increased ability to germinate. Loss of *SLY* function leads to GA-insensitive phenotypes including reduced ability to germinate, as well as dwarfism and reduced fertility. The F2 seeds resulting from crosses of *sly1-2* to wild-type plants segregated for failure

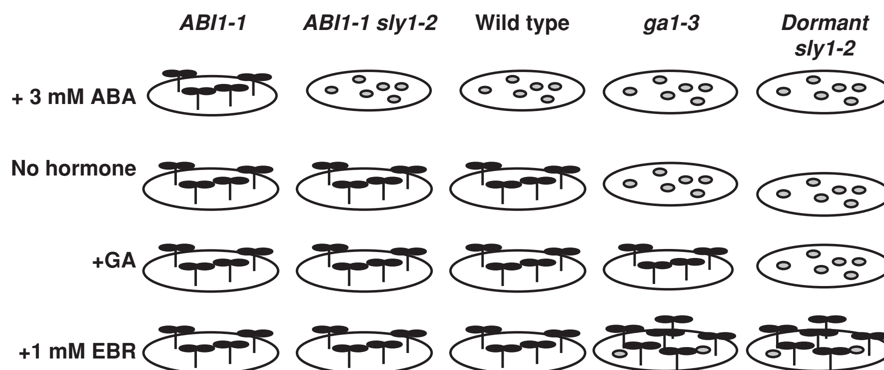

Figure 10.3 Schematic of rationale for screens that isolated mutations in the *SLY1* gene. The *sly1-2* allele was isolated as a suppressor of *ABI1-1*. *ABI1-1* is able to germinate in the presence of 3 μM ABA (Steber *et al.*, 1998). Either *sly1* or *ga1* mutations in the *ABI1-1* background cause failure to germinate on 3 μM ABA. The *sly1-10* allele was isolated based on brassinosteroid-dependent germination. Both *sly1* and *ga1* mutants are unable to germinate in the absence of hormone. Addition of GA fully rescues *ga1-3* germination, but not *sly1*. Epibrassinolide (EBR) is able to partly rescue both *ga1* and *sly1* germination.

to germinate. This suggested that even in the absence of the *ABI1-1* mutation, the recessive *sly1-2* mutation has a strong germination phenotype (Steber *et al.*, 1998). Most fresh (unaged) *sly1-2* seed lots germinate between 0 and 20% (Steber and McCourt, 2001; C.M. Steber unpublished results). Such seed stocks can require up to 3 years to after-ripen. Some rare seed lots germinate 80–100% soon after harvest. However, such seed lots show increased sensitivity to ABA in germination, suggesting that they also have a reduced capacity to germinate (Strader *et al.*, 2004). Further work is needed to determine how environmental conditions during seed development and during seed storage influence the degree of seed dormancy in *sly1* mutants. Germination of *sly1* mutants can be rescued by cutting the seed coat, suggesting that the germination phenotype is the result of seed coat-imposed dormancy (McGinnis *et al.*, 2003; see Chapters 2 and 11). The fact that *sly1-10* mutants also show reduced capacity to germinate indicates that multiple alleles of *sly1* decrease the germination efficiency of *Arabidopsis* seeds. The notion that *SLY1* is a positive regulator of seed germination is further supported by the fact that *SLY1* overexpression and a gain-of-function mutation in *SLY1*, *sly1-gar2* (*gai revertant2*), both cause increased resistance to the GA biosynthesis inhibitor paclobutrazol in germination (Peng *et al.*, 1999b; Fu *et al.*, 2004).

Evidence that the reduced germination in the *sly1* mutant is caused by failure of RGL2 protein to disappear in response to GA is strictly correlative at this point. It is not yet known whether a mutation in *RGL2* can suppress the germination phenotype of the *sly1* mutant in the same way that it suppresses the *ga1-3* germination phenotype. As described above, it is known that whereas RGL2 protein disappears within 5 h of adding GA to *ga1-3* mutant seeds, it does not disappear within 5 h of adding GA to imbibing seeds of the GA-insensitive mutant *sly1-10* (Tyler *et al.*, 2004). Thus *SLY1* is required for the GA-induced disappearance of RGL2 protein in seed germination. Since the *sly1-10* mutant has reduced ability to germinate, it is possible that this germination phenotype is due to over-accumulation of RGL2 protein. This suggests that the SCF^{SLY1} complex is responsible for relieving RGL2 repression of germination, just as it relieves DELLA repression of stem elongation (Figure 10.1). It is not yet known, however, whether *sly1* mutant seed lots that germinate well accumulate less RGL2 protein than *sly1* seed lots that do not germinate well. If RGL2 protein levels correlate with the severity of the *sly1* germination phenotype, it is possible that other ubiquitin ligases can target RGL2 protein for destruction in the absence of *SLY1*. Candidates for ubiquitin ligases that might have overlapping function with SLY1 protein in germination include the U-box protein PHOR1 (PHOTOPERIOD-RESPONSIVE1) that acts in GA signaling in potato and the F-box protein SNEEZY (SNE), which is a homologue of SLY1 in *Arabidopsis* (Amador *et al.*, 2001; Fu *et al.*, 2004; Strader *et al.*, 2004).

10.6 Do DELLA proteins have a conserved role in seed germination?

Both the *SLY1* gene and DELLA gene family are highly conserved among plant species. Homologues of *SLY1* are known in soybean (*Glycine max*), *Medicago*

truncatula, sunflower (*Helianthus annuus*), cotton (*Gossypium arboretum*), tomato, rice, barley, and wheat (Itoh *et al.*, 2003, McGinnis *et al.*, 2003). The *SLY1* homologue of rice, *GID2*, clearly functions in GA signaling in stem elongation, flowering, and fertility. However, *GID2* is not required for rice seed germination. This suggests that GA may not play an essential role in the seed germination of all plant species. Members of the DELLA gene family have also been identified in numerous plant species. A single DELLA has been identified in barley (*SLN1*; Gubler *et al.*, 2002), maize (*d*8 or *Dwarf8*; Peng *et al.*, 1999a), wine grape (Boss and Thomas, 2002), and tomato (Bassel *et al.*, 2004). Two DELLA genes have been identified in Hawaiian silversword (*Dubautia arborea*) (Remington and Purugganan, 2002), soybean (Bassel *et al.*, 2004), and hexaploid bread wheat (Peng *et al.*, 1999a).

While it is known that DELLA proteins exist in other plant species, it is not yet known whether they function similarly to *Arabidopsis* RGL2 in seed germination. Bassel *et al.* (2004) took the first steps toward exploring the role of DELLA homologues in seed germination of other plant species by identifying and characterizing the mRNA expression of DELLA homologues in tomato and in soybean. Soybean was chosen because, unlike tomato and *Arabidopsis*, it does not have seed coat-imposed dormancy. The two soybean DELLA homologues, *GmGAI1* and *GmGAI2*, identified by RT-PCR were not induced until after germination was complete. Thus, it is possible that GA and DELLA proteins are involved only in seedling establishment rather than in seed germination in this case (Bassel *et al.*, 2004). However, future work will have to determine whether these are the only DELLA or GRAS family genes expressed during soybean seed germination.

Like *Arabidopsis*, tomato seeds have seed coat-imposed dormancy and require GA to germinate. A single DELLA gene, *LeGAI*, was recovered from tomato (Bassel *et al.*, 2004). Similarly to *AtRGL2*, *LeGAI* transcript accumulated at very low levels in dry seeds and was strongly induced within 24 h of imbibition. If *LeGAI* behaved like *RGL2*, *LeGAI* mRNA should be present at high levels during imbibition of the strong GA biosynthesis mutant *gib-1*, but disappear upon imbibition in the presence of GA. In contrast, *LeGAI* mRNA failed to disappear upon imbibition in GA. This suggests either that *LeGAI* activity is not regulated at the level of transcript accumulation, or that *LeGAI* is not a negative regulator of seed germination in tomato. The main limitation in this study is that they were unable to examine LeGAI protein levels. Moreover, *LeGAI* was identified as a potential regulator of seed germination based mainly on sequence homology to *RGL2* and on transcriptional induction during imbibition. It is possible that another GRAS protein plays the role of *AtRGL2* in tomato germination. What is needed is evidence that LeGAI is functionally equivalent to *Arabidopsis RGL2*. For example, does loss of *LeGAI* function rescue *gib-1* mutant seed germination?

The example of *Arabidopsis* emphasizes the importance of genetics in determining whether a DELLA gene functions in seed germination. *RGL2* transcript is not the only DELLA transcript that accumulates during seed imbibition. *RGL3*, *RGA*, and *GAI* transcripts also accumulate to high levels during the first 24 h of imbibition (Tyler *et al.*, 2004). Loss of RGL3 does not enhance the ability of *rgl2* mutants to rescue *ga1-3* germination. However, mutations in *RGL1*, *RGA*, and *GAI* appear to

enhance *rgl2* mutant suppression of the *ga1-3* germination phenotype (Cao *et al.*, in press). Thus, induction during imbibition alone is not a sufficient criterion for determining whether homologues of *RGL2* share *RGL2* function. Future research on DELLA or GRAS gene function will need to determine whether homologues are expressed during seed imbibition and whether loss of function causes reduced requirement for GA in germination.

10.7 Future directions

It is clear that GA stimulated de-repression of germination via proteolysis of the DELLA protein RGL2 is an important component of the decision to germinate in *Arabidopsis* seeds. Further work is needed to determine whether RGL2 is a 'master regulator' of seed germination in *Arabidopsis*. Future work will also need to clarify whether GA is required for germination in other plant species, and if so, whether DELLA repression of germination is conserved within the plant kingdom. Some plant species may use other plant hormones known to stimulate germination, such as ethylene or brassinosteroids, to control seed germination (Karssen *et al.*, 1989; Leubner-Metzger, 2001; Steber and McCourt, 2001). It will be important to determine whether plant hormones that stimulate germination destabilize DELLA proteins, while hormones that inhibit germination stabilize DELLA proteins. It is known that ethylene, a hormone that stimulates germination, can delay GA-induced disappearance of DELLA proteins in seedlings (Achard *et al.*, 2003). While this is the reverse of what would be expected, the experiment needs to be performed with germinating seeds. It is possible that the role of DELLA proteins in seed germination is not as highly conserved as their role in stem elongation. It is also possible that species with seed coat-imposed dormancy like tomato and *Arabidopsis* use DELLA proteins, while species that have different mechanisms for seed dormancy such as embryo dormancy use a completely different mechanism for controlling seed germination (Bassel *et al.*, 2004).

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