# HORMONAL CONTROL OF SEED DORMANCY AND GERMINATION: DRAWING CONNECTIONS BETWEEN *ARABIDOPSIS THALIANA* L. AND *TRITICUM AESTIVUM* L.

By

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Chair

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Abstract

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This thesis studies the hormonal control of seed dormancy and germination by examining the action of two plant hormones, gibberellins (GA) and abscisic acid (ABA). This work also aims to transfer known *Arabidopsis* hormone signaling data into hexaploid bread wheat. The purpose of this transfer is to gain greater understanding of these hormone's actions on seed dormancy and germination. This thesis work extends our understanding of GA signal transduction through the cloning and characterization of *SLEEPY1* (*SLY1*) and the exploration of genetic interactions with its homologue *SNEEZY* (*SNE*). *SLY1* and *SNE* both encode F-box proteins, which are subunits of SCF E3 ubiquitin ligase complexes. SLY1 and SNE affect the disappearance of RGA protein, a negative regulator of GA signal transduction, indicating that RGA is the target of $SCF^{SLY1}$ and possibly $SCF^{SNE}$. Characterization of the genetic interaction between *SLY1* and *SNE* reveals that the recessive-interfering mutations in *SLY1* are rescued by overexpression of *SNE*. In further studies, ABA signal transduction mutants were

identified in wheat, which will allow for the dissection of commonalities in signaling between the model system *Arabidopsis* and a hexaploid crop plant. Both wheat ABA-insensitive and ABA-hypersensitive mutants were isolated in germination screens, with some secondary phenotypes examined.

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# CHAPTER ONE

# GENERAL INTRODUCTION

"Since the function of a seed is to establish a new plant, it seems curious that dormancy-an intrinsic block to germination-should exist!"

Bewley & Black, 1994

This thesis studies the hormonal control of seed dormancy and germination by two plant hormones, gibberellins (GA) and abscisic acid (ABA). Dormancy and germination are important physiological processes to study because of their key role in the survival of a plant species. While these processes are vital to plant growth and species survival, they are not well understood. ABA and GA function in the control of seed dormancy and germination, with ABA setting up dormancy during seed maturation, and GA stimulating germination. In addition to improving our fundamental understanding of these processes, this work also aims to transfer known *Arabidopsis* hormone signaling data into hexaploid bread wheat. The purpose of this is to gain greater understanding of these hormone's actions on seed dormancy and germination in a complex crop plant.

**Control of Dormancy and Germination**

As sessile organisms, plants rely on complex regulatory mechanisms to assure their survival. Seeds are the main dispersal unit for most higher plants, and thus must have the ability to germinate into a new plant efficiently. However, not all seeds will germinate immediately upon dispersal. Seed dormancy is one mechanism plants use to avoid extinction. Poor environmental conditions can cause a total loss of vegetation, due to fire, freezing, drought, flood, or disease. Dormancy is a state in which a seed fails to

germinate, even though all conditions are met to allow germination. Because dormant seeds fail to germinate; they can survive extreme conditions and germinate later, ensuring the perpetuation of the species.

Although seed dormancy is not well understood, many factors contributing to dormancy have been identified and studied (reviewed in Bewley and Black, 1994). These factors fall into two major categories: seed coat imposition and embryo dormancy. The seed coat, or testa, can confer dormancy upon a seed by inhibiting water uptake or gas exchange, and by providing a mechanical restraint to check radicle emergence. Further, the testa can hinder germination by preventing the exit of inhibitors from the embryo, or even by supplying germination inhibitors. A completely different set of factors forms the basis of embryo dormancy. Three major hypotheses on the cause of embyo dormancy are: (1) dormant seeds have some defect in metabolism preventing the mobilization of storage reserves; (2) there are gene expression differences between dormant and nondormant seeds; and (3) cell membrane state determines embryo progression in germination. Coat-imposed dormancy and embryo dormancy are not mutually exclusive. Most species exhibit dormancy caused by a combination of the above factors.

The study of dormancy inception has revealed that many different species-dependent factors affect dormancy onset. Environment is a key determining factor in depth of dormancy. Temperature during seed maturation has been implicated in many species as a component that influences the degree of dormancy. In *Rosa* spp. and in most cereal grasses, cooler temperatures promote dormancy, while in *Syringa vulgaris*, dormant seeds are produced under high temperature conditions (Stokes, 1965; van

Abrams and Hand, 1956). Both light quality and duration influence dormancy levels in a number of species. For example, *Chenopodium album* produces seed with strong dormancy when the plants are grown under long days, but produces nondormant seed when grown under short days (Karssen 1970). Further, seeds from *Arabidopsis* that mature in white fluorescent light have little dormancy, whereas seeds that mature under incandescent light exhibit strong dormancy (Hayes and Klein 1974). The well-studied dormancy inducer abscisic acid has been shown to be necessary for dormancy onset in *Arabidopsis thaliana.* Additionally, induction of dormancy in *Arabidopsis* by ABA is by embryonic, and not maternal, ABA biosynthesis (Karssen *et al.* 1983). Although there has not been a strong correlation between ABA and dormancy in all plant species, ABA sensitivity has been shown to be central to dormancy induction in such species as *Triticum aestivum* (Walker-Simmons, 1987) and *Arabidopsis thaliana* (Koornneef *et al.*, 1984). This work has focused on ABA to make connections between our model and test case, since sensitivity to this hormone is a common factor in *Arabidopsis* and wheat dormancy induction.

Dormant seed will not germinate, even if conditions are favorable. Therefore, many methods, both natural and artificial, have been employed to "break" or reduce seed dormancy. These germination activating factors act in varying ways to allow the seed to germinate. Dormancy is lost with afterripening, a natural process in which the dry seed becomes nondormant over a period of time. While the physiological processes that occur during this afterripening period are not understood, the rate of afterripening is dependent on water content and temperature during seed storage. For some species, a seed treatment with light (e.g. lettuce) or cold (e.g. *Avena fatua*) is sufficient to eliminate

dormancy. Treatment of seeds with chemicals such as cyanide, dithiothreitol, hypochlorite, nitrate, and ethanol have been shown to break dormancy (reviewed in Bewley and Black, 1982). Growth regulators, such as gibberellins, brassinosteroids, and ethylene have also been shown to stimulate germination.

As stated earlier, ABA is often necessary for dormancy onset. However, it is not the only phytohormone involved in the processes of dormancy and germination. Germination begins with seed imbibition and terminates with radicle emergence, allowing an embryo to develop into a seedling. A key growth regulator of seed germination is gibberellin. Seeds of the GA-deficient *Arabidopsis* mutant *ga1-3* are unable to germinate unless they are supplied with exogenous GA or the seed coat is cut (Koornneef and van der Veen, 1980). Further, GA insensitivity, as in the *sleepy1* mutants, also confers an increase in seed dormancy (Steber *et al.*, 1998). GA and ABA act antagonistically with each other. Not only does GA promote germination, but exogenous GA application during seed development blocks dormancy onset in such species as *Avena fatua* (Metzger, 1983). ABA, in return, antagonizes GA action both by inhibiting germination and by inhibiting GA-stimulated α-amylase production (reviewed in Bethke *et al.*, 1997). Brassinosteroids stimulate germination and can rescue the germination phenotypes of GA biosynthetic mutants (Steber and McCourt, 2001). Ethylene production by *Xanthium* and peanut embryos has been implicated in dormancy loss (Hasegawa *et al.*, 1995). This thesis focuses on the signaling of two of these hormones, abscisic acid and gibberellins.

**Abscisic Acid Signaling**

Abscisic acid (ABA) is a sesquiterpenoid phytohormone involved in such physiological processes as stimulation of embryo maturation, induction of seed dormancy, inhibition of germination, regulation of stomatal closure, and induction of stress responses to drought, cold, and salt (reviewed in Leung and Giraudat, 1998). Genetic studies have identified many genes involved in ABA biosynthesis and response. Plants synthesize ABA indirectly from carotenoids, using zeaxanthin as the source for the ABA biosynthetic pathway. ABA biosynthesis will not be discussed in detail here, but is extensively reviewed by Finkelstein and Rock (2002). ABA biosynthetic mutants fail to induce seed dormancy and exhibit a vegetative wilty phenotype (Koornneef *et al.*, 1982). These phenotypes have been used to define and prove the function of ABA in seed dormancy and water relations. ABA response mutants resemble ABA biosynthetic mutants, but their phenotypes cannot be rescued by ABA application.

ABA signaling genes have been identified in screens for mutants with increased or decreased response to ABA in germination. ABA-insensitive mutants isolated based on germination on concentrations of ABA normally inhibitory to wild-type germination include *aba insensitive1* (*abi1*), *abi2*, *abi3*, *abi4*, *abi5*, and *abi8* (reviewed in Finkelstein and Rock, 2002). The semi-dominant *abi1-1* and *abi2-1* mutations result in nondormant seed, a vegetative wilty phenotype, and leaf temperatures 1°C cooler than wild-type, due to transpirational cooling (Koornneef *et al.*, 1984; Merlot *et al.*, 2002). The *ABI1* and *ABI2* genes encode homologous ser/thr protein phosphatases in the 2C class (PP2C) (Leung *et al.*, 1994; Meyer *et al.*, 1994; Leung *et al.*, 1997; Rodriguez *et al.*, 1998).

Other mutants isolated in these screens do not exhibit a vegetative wilty phenotype, but do produce nondormant seed and exhibit various other vegetative phenotypes (reviewed in Finkelstein and Rock, 2002). *ABI3*, *ABI4*, and *ABI5* encode B3-, APETALA2-, and bZIP transcription factors, respectively (Giraudat *et al.*, 1992; Finkelstein, 1994; Finkelstein and Lynch, 2000, Lopez-Molina and Chua, 2000).

ABA hypersensitive mutants have been identified in *Arabidopsis* based on inability to germinate at concentrations of ABA normally permissive to wild-type germination. Mutations in *ENHANCED RESPONSE TO ABA1* (*ERA1*), *ERA3* / *EIN2*, and *ABA HYPERSENSITIVE1* (*ABH1*) result in seed with a hypersensitive response to ABA in germination (Hugouvieux *er al.* 2001; Cutler *et al.* 1996; Ghassemian *et al.* 2000). The *era1* and *abh1* mutants also exhibit a vegetative drought tolerant phenotype (Pei *et al.*, 1998; Hugovieux *et al.*, 2001). *ERA1* encodes the β-subunit of a farnesyl transferase (Cutler *et al.*, 1996); *ERA3* encodes a membrane-bound metal sensor and is allelic to *ETHYLENE INSENSITIVE2* (Alonso *et al.*, 1999; Ghassemian *et al.*, 2000); and *ABH1* encodes an mRNA CAP-binding protein (Hugouvieux *et al.*, 2001). The above-mentioned genes are apparently involved in mediating the ABA response in seed germination; however, coordination of their activities is unclear. The current model for ABA signal transduction in seed germination is shown in Figure 1 (based on Finkelstein and Rock, 2002).

**GA Signaling**

Gibberellins (GAs) are a large family of diterpenoid compounds, some of which are bioactive in the developmental processes of seed germination, stem elongation, leaf

expansion, trichome development, and flower and fruit development. Briefly, biosynthesis of GAs takes place in three stages; geranylgeranyl diphosphate is used in the production of *ent*-kaurene, which is then converted into $GA_{12}$, and finally altered into $C_{19}$ GAs (reviewed in detail by Olszewski *et al.*, 2002). GA biosynthetic mutants exhibit varying degrees of phenotypes. The most severe GA mutant, *ga1-3*, is a deletion of the *ent*-kaurene synthase gene, which is the first stage of GA biosynthesis. The *ga1-3* mutants exhibit the severe phenotypes of lack of germination, dwarfism, dark green leaf color, and inability to flower. These phenotypes are rescued by exogenous application of GA (Koornneef and van der Veen, 1980).

A number of negative regulators of GA response have been characterized in *Arabidopsis*. Mutations in *SPINDLY* (*SPY*), an O-linked N-acetylglucoseaminyl transferase cause erect rosette leaves with a pale green color, early flowering, and reduced seed set (Jacobsen and Olszewski, 1993; Jacobsen *et al.*, 1996). Overexpression of the RING finger transcription factor encoded by *SHORT INTERNODES* (*SHI*) produces a dwarf phenotype not reversed by GA treatment (Fridborg *et al.*, 1999). Further *Arabidopsis* mutants exhibiting a supersensitive response to GA include members of the DELLA family of putative transcription factor-encoding genes. There are five members of this DELLA domain family of transcription factors: REPRESSOR OF ga1-3 (RGA), GA-INSENSITIVE (GAI), RGA-LIKE1 (RGL1), RGL2, and RGL3 (Sanchez-Fernandez *et al.*, 1998; King *et al.*, 2001; Wen and Chang, 2002). Loss-of-function mutations in *RGA* and *GAI* result in taller plants (Dill and Sun, 2001; King *et al.*, 2001). Gain-of-function mutations in these genes cause an accumulation of their respective protein and result in decreased stem growth (Koornneef *et al.*, 1985; Dill *et al.*, 2001).

Thus, RGA and GAI are negative regulators of stem elongation. To stimulate stem elongation, GA triggers the disappearance of these DELLA proteins (Silverstone *et al.*, 2001; Dill *et al.*, 2004; Fu *et al.*, 2004). Gain-of-function semidwarf mutations in the homologous wheat *Rht* DELLA genes resulted in the "green revolution" by improving resistance to lodging (Peng *et al.*, 1999). These negative regulators gave the first insight into how the GA signal was transduced.

Positive regulators of GA response have been identified through screens for GA-insensitive mutants. The *Arabidopsis PICKLE* (*PKL*) gene encodes a CH3 chromatin-remodeling factor (Ogas *et al.*, 1999) implicated in GA signaling. Although its role in GA signal transduction is unclear, loss-of-function mutations in the *PKL* gene result in GA-insensitive dwarves that overproduce GAs (Ogas *et al.*, 1997). The *SLEEPY1* (*SLY1*) gene encodes the F-box subunit of an SCF E3 ubiquitin ligase complex (McGinnis *et al.*, 2003; Fu *et al.*, 2004). The recessive *sly1-2* and *sly1-10* mutants have GA-insensitive phenotypes including dark-green leaves, dwarfism, reduced fertility, delayed germination, and overaccumulation of RGA, GAI, and RGL2 (Steber *et al.*, 1998; Steber and McCourt, 2001; McGinnis *et al.*, 2003; Fu *et al.*, 2004; Dill *et al.*, 2004; Tyler *et al.*, 2004). It is becoming apparent that the DELLA family of transcription factors are targeted by $SCF^{SLY1}$ for degradation by the 26S proteasome (McGinnis *et al.*, 2003; Fu *et al.*, 2004; Dill *et al.*, 2004). Thus, SLY1 positively regulates the GA signal by negatively regulating negative regulators of GA signal transduction. Although still unidentified, the current hypothesis is that a protein kinase transduces the GA signal, phosphorylating the DELLA proteins and enabling DELLA recognition by $SCF^{SLY1}$ for ubiquitylation

(reviewed in Itoh *et al.*, 2003). The current model for GA signal transduction is shown in Figure 2 and described in more detail in Chapters 2 and 3.

The ubiquitin-proteasome pathway is emerging as a key regulator of many plant hormone response pathways. Auxin (Gray *et al.*, 2001), jasmonic acid (Xu *et al.*, 2002), GA (McGinnis *et al.*, 2003), and ethylene (Guo and Ecker, 2003; Potuschak *et al.*, 2003) responses have been linked to SCF complex activity. SCF (Skp1, Cullin, F-box) complexes are one type of E3 ubiquitin ligase. This complex, identified in animals, yeast, and plants (Patton *et al.*, 1998) is composed of a SKP1 homologue, a CULLIN homologue, an F-box protein, and a RBX homologue (Zheng *et al.*, 2002). The F-box component of this complex recognizes a specific substrate protein, allowing the SCF complex to catalyze its ubiquitylation. Polyubiquitylation of the substrate targets it for degradation by the 26S proteasome.

### *Arabidopsis* as a model and wheat as a test case

*Arabidopsis thaliana* has been established as the "model" system for plant studies. A number of characteristics make this species an ideal choice for study. It has a small genome (200 Mbp; Bennet and Smith, 1991) that has now been fully sequenced. Further, *Arabidopsis* is small, has a short life cycle (6-8 weeks), and produces a large number of progeny (~10,000 / plant), making it perfect for genetic studies.

Hexaploid bread wheat (*Triticum aestivum*, $2n = 6x = 42$, genomes AABBDD), on the other hand, is a genetically complex organism, with a large genome at 16 billion bp (Arumuganthan and Earle, 1991). Genetic studies are challenging in wheat because many genes are present in multiple copies. Wheat's life cycle is relatively long, ranging

from 3-6 months, with a relatively small number of progeny (~300 / plant) produced by the end of this life cycle. Despite these challenges, however, wheat molecular genetics is advancing rapidly. Many researchers have successfully identified genes in wheat, through a variety of methods (reviewed in Appendix).

We have identified homologues of many of the *Arabidopsis* ABA signaling genes in wheat (Table 1). We have chosen to draw connections between the hormonal control of seed germination and dormancy in *Arabidopsis* and wheat, though they are quite divergent species. In Chapter 2, we describe the cloning of *SLEEPY1* in *Arabidopsis*. In Chapter 3, we continue to study the nature of known *SLEEPY1* mutations and explore the genetic interactions with its homologue *SNEEZY*. In Chapter 3, we describe a screen for wheat mutants with increased and decreased response to ABA. Due to the tight link between ABA and GA in the control of seed germination, the screen for ABA hypersensitivity recovered putative GA-insensitive mutants which may be homologues of the GA response genes *SLEEPY1* or *SNEEZY*. We have continued to discover more about the GA signaling pathway using *Arabidopsis*, while identifying mutant resources in wheat to be used in further studies of ABA signal transduction and dormancy control.

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**Table 1.** ABA response mutants of *Arabidopsis* and their wheat homologues

| Gene | Phenotype | Gene Product | AGI | Wheat homologue** | Ref |
|---|---|---|---|---|---|
| ABH1 | ABA hypersensitive germination, drought tolerant | mRNA CAP-binding protein | At2g13540 | TC130967 | (30) |
| ABI1 *abi1-1 is semi-dominant* | ABA-insensitive germination, non-dormant seeds, drought sensitive, early flowering | Protein phosphatase type 2C | At4g26080 | TC129964, TC134607, TC133771, TC107921, | (41, 52) |
| ABI2 *abi2-1 is semi-dominant* | ABA-insensitive germination, non-dormant seeds, drought sensitive | Protein phosphatase type 2C | At5g57050 | TC135406, TC112274, TC126033, TC127052 | (42, 64) |
| ABI3 | ABA-resistant germination, pleiotropic defects in seed maturation, Effects on plastid differentiation | Vp1-like B3-domain transcription factor | At3g24650 | TC116316 | (26) |
| ABI4 | ABA-resistant germination, sugar- and salt-resistant germination and seedling growth | APETALA2-domain transcription factor | At2g40220 | | (19) |
| ABI5 | ABA-resistant germination, slightly sugar-resistant germination and seedling growth | bZIP domain transcription factor | At2g36270 | TC131941 | (16) |
| ABI8 | ABA-resistant germination, severely stunted growth, defective stomatal reg., male sterile | Protein of unknown function | | | |
| ERA1 | Enhanced response to ABA in germination, enhanced stomatal response / drought tolerance, meristem defect | Farnesyl transferase, β-subunit | At5g40280 | TC140520 | (11) |
| ERA3/ EIN2 | Enhanced response to ABA in germination, ethylene insensitive | Membrane-bound metal sensor? | At5g03280 | TC113499 | (23) |
| FRY1 | ABA hypersensitive | Inositol polyphosphate-1-phosphatase | At5g63980 | TC123879 | (82) |
| HYL1 | ABA hypersensitive | dsRNA-binding protein | At1g09700 | TC128172 | (45) |

**Based on Finkelstein and Rock, 2002.

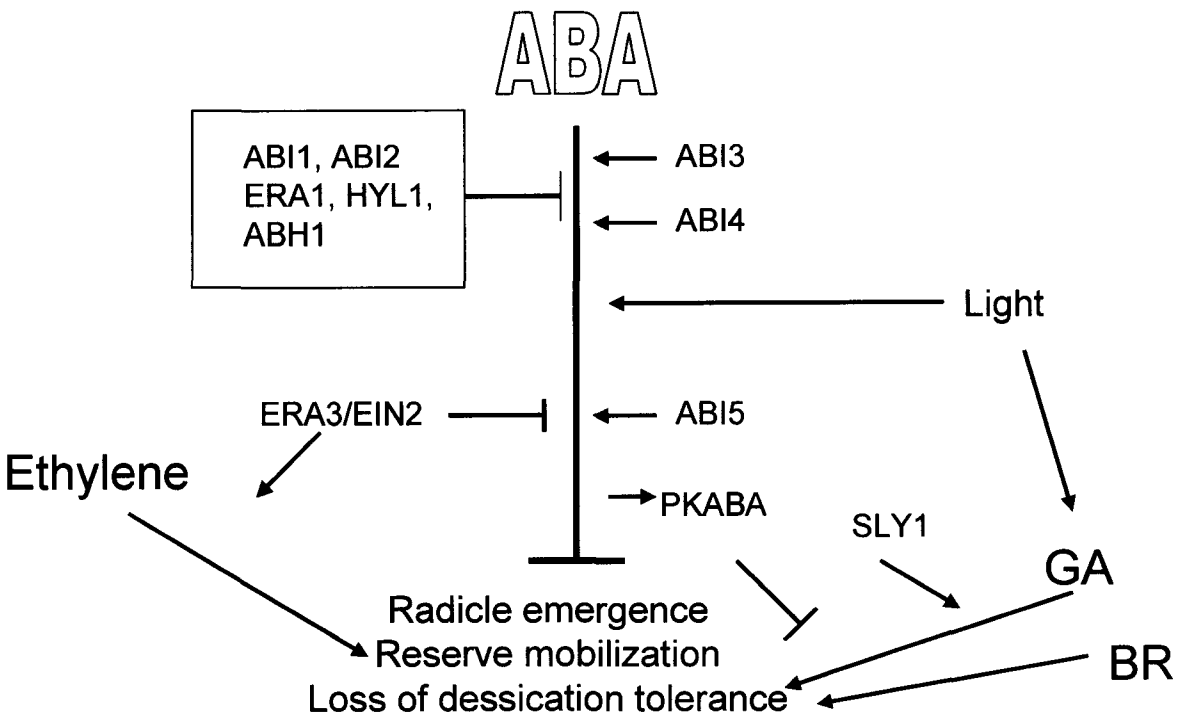


**Figure 1.** ABA signaling and interactions with other hormones affecting seed dormancy and germination. Abscisic acid antagonizes germination by blocking radicle emergence, reserve mobilization, and loss of dessication tolerance. ABI1, ABI2, ERA1, HYL1, and ABH1 are all negative regulators of the ABA signal. ABI3, ABI4, and ABI5 are positive regulators of the ABA signal. Ethylene promotes germination, possibly through ERA3 / EIN2 action, a cross-over point between ethylene and ABA signal transduction. The GA signal, positively regulated by SLY1, also stimulates germination; its activity is inhibited by protein kinases, such as PKABA. Brassinosteroids also stimulate germination, but cross-talk between this pathway and ABA has not been characterized. Based on Finkelstein and Rock, 2002.

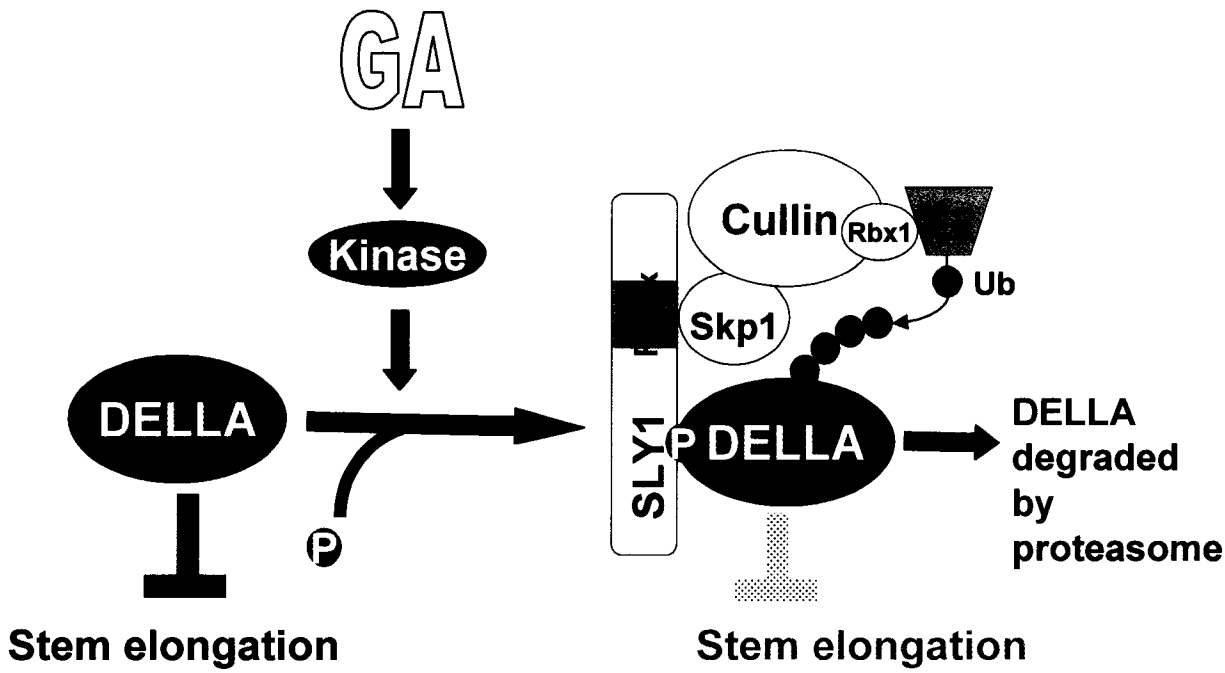


**Figure 2.** GA signal transduction model, depicting DELLA proteins targeted for degradation by $SCF^{SLY1}$. In this model, the GA signal is mediated by some unidentified kinase. This kinase phosphorylates the $SCF^{SLY1}$ targets, such as the DELLA proteins, which allows them to be recognized by $SCF^{SLY1}$ to be targeted for degradation. Thus, GA is able to elimate its inhibitors, allowing the GA response. Based on Itoh *et al.* (2003).

# CHAPTER TWO

# THE ARABIDOPSIS SLEEPY1 (SLY1) GENE ENCODES A PUTATIVE F-BOX SUBUNIT OF AN SCF E3 UBIQUITIN LIGASE

This chapter describes the map-based cloning of the *SLEEPY1* gene in *Arabidopsis thaliana*. My contributions to this paper included part of the genomic sequencing in final steps of cloning *SLY1*, and the mRNA expression analysis of the *SLY1* transcript. This paper was published in Plant Cell with authorship in the following order: Karen M. McGinnis, Stephen G. Thomas, Jonathan D. Soule, Lucia C. Strader, Janice M. Zale, Tai-ping Sun, and Camille M. Steber.

**Abstract**

The Arabidopsis *SLEEPY1* (*SLY1*) gene positively regulates gibberellin (GA) signaling. Positional cloning of *SLY1* revealed that it encodes a putative F-box protein. This result suggests that SLY1 is the F-box subunit of an SCF-E3 ubiquitin ligase regulating GA responses. The DELLA domain protein RGA (repressor of *ga1-3*) is a repressor of GA-response that appears to undergo GA-stimulated protein degradation. RGA is a potential substrate of SLY1 because *sly1* mutations cause a significant increase in RGA protein accumulation even after GA treatment. This result suggests $SCF^{SLY1}$-targeted degradation of RGA through the 26S proteasome pathway. Further support for this model is provided by the observation that an *rga* null allele partially suppresses the *sly1-10* mutant phenotype. The predicted SLY1 amino acid sequence is highly conserved among plants pointing to a key role in GA response.

# INTRODUCTION

Bioactive gibberellins (GAs) are tetracyclic diterpenoid phytohormones required for germination, stem elongation, induction of flowering, and fertility (Richards et al., 2001). Severe mutants defective in GA biosynthesis (e.g., the deletion of the *GA1* gene in *ga1-3*) display failure in germination, dwarfism, delayed flowering, and reduced fertility. All of these phenotypes are rescued by GA treatment. In contrast, mutants impaired in GA signaling display similar phenotypes, but are not GA-rescued.

Elements of the GA-response pathway in *Arabidopsis thaliana* have been defined through genetic analysis of mutants (Richards et al., 2001; Olszewski et al., 2002). Positive regulators of GA signaling include *SLY1* (SLEEPY1) and *PKL* (PICKLE). *PKL* encodes a putative CHD3 chromatin remodeling factor (Ogas et al., 1999). Recessive mutations in this gene result in adult plants where the primary root meristem retains embryonic characteristics. This phenotype is enhanced by GA biosynthetic inhibitors.

Negative regulators of GA signaling include *SHI, SPY, RGA, GAI, RGL1* and *RGL2*. Overexpression of the *SHI* (*SHORT INTERNODES*) gene results in a GA-insensitive semi-dwarf phenotype. The predicted *SHI* gene product contains homology to the RING finger domain that mediates protein-protein interaction in ubiquitylation and in transcription (Fridborg et al., 1999; Fridborg et al., 2001). Loss of *SPY* function results in a GA-overdose phenotype including increased internode length, parthenocarpy, and increased resistance to the GA biosynthetic inhibitor paclobutrazol during vegetative growth and germination (Jacobsen and Olszewski, 1993). *SPY* (*SPINDLY*) encodes an *O*-GlcNAc transferase (OGT, (Jacobsen et al., 1996; Swain et al., 2002)). OGTs may

regulate the target protein function by competing with protein kinases for modification of phosphorylation sites. *RGA* (repressor of *ga1-3*) and *GAI* (GA-insensitive) encode members of the DELLA (VHIID) domain subfamily of the GRAS family of putative transcription regulators (Richards et al., 2001; Olszewski et al., 2002; Peng and Harberd, 2002). Loss of *RGA* or *GAI* function results in decreased sensitivity to the GA biosynthetic inhibitor paclobutrazol during vegetative growth. Conversely, mutations in the DELLA domain of *GAI* and *RGA* result in a gain-of-function (semi-dominant) semi-dwarf phenotype (Peng et al., 1997; Dill et al., 2001). *RGA* and *GAI* share 83% amino acid identity, and act as GA-repressible repressors of stem elongation in Arabidopsis (Dill and Sun, 2001; King et al., 2001). Recent evidence shows that the RGA protein accumulation decreases in response to GA treatment. This suggests that RGA is subject to GA-induced proteolysis (Silverstone et al., 2001). Other members of the DELLA gene family in Arabidopsis include *RGL1*, *RGL2*, and *RGL3* (RGA-Like) (Sanchez-Fernandez et al., 1998). *RGL2* is a negative regulator of germination whose transcript levels are transiently increased during dormant seed imbibition (Lee et al., 2002). *RGL1* appears to be a negative regulator of more diverse GA responses including germination, stem elongation, leaf expansion, flowering and flower development (Wen and Chang, 2002).

The DELLA family of GA-response genes is a highly conserved gene family of considerable agronomic importance (Peng et al., 1999). Introduction of the DELLA-domain semi-dwarf mutations into crop plants resulted in the 16-31% increase in yield referred to as the Green Revolution (Allan, 1986; Peng et al., 1999). These genes are negative regulators of GA response. Loss of function leads to increased GA signaling, while gain-of-function results in reduced GA signaling and dwarfism. Whereas there are

five members of the DELLA family in Arabidopsis, there is only a single DELLA gene in rice (*SLENDER-RICE*, *SLR1*) and in barley (*SLENDER*, *SLN1*). Like RGA, both SLR1 and SLN1 are apparently subject to GA-regulated proteolysis (Chandler et al., 2002; Gubler et al., 2002; Itoh et al., 2002). Recently, proteolysis of SLN1 was shown to depend on the 26S proteasome pointing to a role for ubiquitin in GA signal transduction (Fu et al., 2002).

The *sly1* mutants were isolated as recessive GA-insensitive dwarf mutants in two independent screens based on their increased seed dormancy, a property expected in a GA-response mutant. The first screen recovered the EMS-induced *sly1-2* allele that suppressed the ability of *abi1-1* (ABA-insensitive) to germinate on 3 μM ABA. The second screen identified *sly1-10* based on brassinosteroid-dependent germination (Steber et al., 1998; Steber and McCourt, 2001). Loss of *SLY1* function results in all of the phenotypes expected of a GA-response mutant, including increased seed dormancy, growth as a dark green dwarf, delayed flowering, and reduced fertility.

This paper reports map-based cloning of the *SLY1* gene, a putative F-box subunit of an SCF E3 ubiquitin ligase. BLAST search revealed SLY1 homologues in many plant species suggesting that its role as a positive regulator of GA response is also conserved. In addition, mutations in the *SLY1* gene result in a high level RGA protein accumulation even in the presence of GA. This result indicates that the *SLY1* gene is needed for GA-stimulated proteolysis of RGA protein. The dwarf phenotype of *sly1* plants is suppressed by the *rga-24* null mutation. Thus, accumulation of high levels of RGA protein is required for the dwarf phenotype of *sly1* mutants. Together these results suggest that an $SCF^{SLY1}$ complex mediates GA-induced degradation of RGA.

# RESULTS

## Effect of *sly1-10* mutant on *GA3ox1* expression

GA biosynthesis is subject to feedback regulation. Decreased GA biosynthesis or response results in increased mRNA accumulation for most of the late GA biosynthetic genes encoding GA-20-oxidase (GA20ox) and GA-3-oxidase (GA3ox), while application of exogenous GA results in decreased expression of the same GA biosynthetic genes (reviewed by Hedden and Phillips, 2000). Since mutations in *SLY1* result in reduced GA response, we would expect increased expression of these GA biosynthetic genes in the *sly1-10* mutant. To test this hypothesis, we compared the transcript accumulation of one of the GA-3-oxidase genes in Arabidopsis, *GA3ox1,* in wild-type, *ga1-3*, and *sly1-10* (Fig. 1). As previously shown, in the absence of GA the *GA3ox1* transcript levels are 5-fold higher in the severe GA biosynthetic mutant *ga1-3* than in wild-type (Chiang et al., 1995; Cowling et al., 1998; Yamaguchi et al., 1998). Similar to *ga1-3*, the *sly1-10* mutant accumulated 5-fold higher *GA3ox1* mRNA levels compared to wild type in the absence of exogenous GA. Application of $GA_4$ resulted in a 10-fold decrease in *GA3ox1* expression in *ga1-3*, but only a 2-fold decrease in *sly1-10.* This result indicates that the *sly1-10* mutant retains some residual sensitivity to $GA_4$.

## Map-based Cloning of *SLY1*

The *SLY1* gene was cloned using a map-based approach in order to further elucidate its function in GA signal transduction. The *sly1-2/sly1-2* mutant in the Landsburg *erecta* (L*er*) ecotype was crossed to wild-type ecotype Columbia (Col) to generate an $F_2$

mapping population of *sly1-2/sly1-2* plants segregating for Ler and Col specific physical markers. The *sly1-2/sly1-2* $F_2$ seeds were germinated by cutting the seed coats. Due to the poor germination and poor fertility of *sly1-2*, all mapping was performed relative to PCR-based physical markers that differ between the two ecotypes (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). *SLY1* was 7.7 cM from g3883 and 0.2 cM from *RPS2* (Fig. 2). *RPS2* was used as a point from which to begin a chromosome walk towards g3883. A population of 805 *sly1-2/sly1-2* $F_2$s (1,610 chromosomes) was scored for the *RPS2* CAPS marker (Bent et al., 1994). Four $F_2$s heterozygous for the *RPS2* marker were recovered and used as a basis for a chromosome walk of 0.9 Mb. The recombination rate across this region was very low with a ratio of 4500 kb/cM compared to the Arabidopsis average of 200 kb/cM (Schmidt et al., 1995). *SLY1* was localized to a 70kb region between the markers T19F6.7 and T22A6.D2.

There were 20 predicted genes in the 70-kb region containing *SLY1* (Arabidopsis Genome Initiative, 2000). Transformation of *sly1-2* and *sly1-10* plants with BAC subclones revealed that an 11.7-kb subclone, T22A6.2G10, rescued the dwarf (data not shown) and germination (Fig. 3A) phenotypes of *SLY1* mutants. This subclone contains three predicted ORFs, T22A6.30 (At4g24200), T22A6.40 (At4g24210) and T22A6.50 (At4g24220). Transformation with the T22A6.40 ORF alone rescued the *sly1-10* mutant phenotypes (Fig. 3B). DNA sequence analysis of *sly1-2* and *sly1-10* revealed that these alleles contain mutations within this 453-bp ORF (Fig. 4A). *sly1-2* has a 2-bp deletion (C337-T338) causing a frameshift that eliminates the last 40 amino acids. *sly1-10* contains a 23-bp deletion (433-456) followed by an ~8-kb insertion. This causes loss of only the last 8 amino acids and addition of 46 random amino acids. Thus, T22A6.40

(At4g24210) is the *SLY1* gene. The *SLY1* gene contains no introns and encodes a small predicted protein of 151 amino acids containing a putative F-box domain.

**Expression of the *SLY1* Gene**

Sequencing of several full length SLY1 cDNAs has identified 5'-UTRs of 87-nt and 105-nt (Fig. 4A, http://signal.salk.edu/). The major ORF in this transcript is the *SLY1* gene. The long 380-nt 3'-UTR shows a high probability of secondary structure (99.6 kcal/mol) according to Vienna RNA Secondary Structure Prediction (http://www.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The Arabidopsis F-box gene *TIR1* mRNA also contains a long structured 3'-UTR (Genbank AF327430). Structured 3'-UTR have been implicated in control of mRNA stability and localization (Decker and Parker, 1995). RT-PCR analysis of wild-type L*er* plants showed that the *SLY1* transcript is present in all tissues examined (Fig. 4B) including rosette leaves, green siliques, flowers, stems, cauline leaves, and seedlings. The *SLY1* mRNA was not detected in the *sly1-10* mutant due to an 8-kb insertion within the PCR product. However, a band of 9-kb is detected in *sly1-10* but not in wild-type L*er* by northern analysis indicating that the gene is still expressed (data not shown).

**Analysis of the *SLY1* Gene Structure**

BLAST search revealed that the predicted SLY1 protein sequence contains homology to the Cyclin F-box family of proteins (Fig. 5A, Altschul et al., 1990; Zhang et al., 1998; Gagne et al., 2002). The F-box protein is one of the four main subunits of the SCF (SKP1, Cullin, F-box) complex, one type of E3 ubiquitin ligase (Conaway et al., 2002).

The F-box subunit directs interaction of the complex with a specific target for ubiquitylation. Thus, *SLY1* may transduce the GA signal by regulating the stability of GA-response proteins through ubiquitin-mediated proteolysis.

SCF-mediated proteolysis regulates many aspects of plant development including senescence, flower development, circadian rhythm, light receptor signaling, and hormone signaling (Hellmann and Estelle, 2002). The $SCF^{TIR1}$ and $SCF^{COI1}$ complexes are needed for auxin and jasmonate signal transduction respectively (Gray et al., 2001; Xu et al., 2002). $SCF^{TIR1}$ controls AXR2/IAA7 stability, and is the only SCF in Arabidopsis for which a target has been identified thus far (Gray et al., 2001). The predicted TIR1 and COI1 F-box proteins share 34% amino acid identity with each other, and contain an F-box at the N-terminus and an LRR domain at the C-terminus. The predicted SLY1 protein has no homology to these proteins outside of the F-box motif. F-box proteins often contain a C-terminal protein-protein interaction domain such as Leucine Rich Repeats (LRRs), WD40 repeats, or Kelch repeats for interaction with its target protein (del Pozo and Estelle, 2000). While SLY1 lacks such a conserved protein-protein interaction domain, its C-terminus is clearly important for function. Both *sly1-2* and *sly1-10* alleles leave the F-box intact, but alter the C-terminus (Fig. 4A). Other examples of F-box proteins lacking a conserved C-terminal protein-protein interaction domain include the mammalian gene Fbx8 and the Arabidopsis gene *SON1* (Cenciarelli et al., 1999; Ilyin et al., 2000; Kim and Delaney, 2002).

SLY1 is highly conserved in the plant kingdom. Many members of the *SLY1* gene family were identified by tBLASTn search of plant ESTs (Fig. 5B). While homologues were detected in many plant species, none were detected outside the plant kingdom.

Amino acid homology to the predicted SLY1 protein ranged from 57% identical/67% similar in the dicot soybean (*Glycine max*) to 42% identical/57% similar in the monocot barley (*Hordeum vulgare*). The rice orthologue of *SLY1* was independently identified by Sasaki et. al. (Sasaki et al., 2003) by positional cloning of *GID2* (GA-insensitive dwarf). *GID2* encodes a predicted 212 amino acid protein 43% identitcal/56% similar to SLY1. The regions of highest amino acid identity are the F-box and the 37 amino acid GGF region (Fig. 5B). Two variable regions (Fig. 5B, VR) are absent in At*SLY1*. BLAST search detected a single *SLY1* homologue (154 amino acids, 26% identical) in Arabidopsis, MIF21.6 (At5g48170) on chromosome V (Fig. 5B).

**Accumulation of RGA protein is increased in *sly1* mutants**

The fact that GA causes reduced accumulation of RGA protein (Silverstone et al., 2001) invited the question "Is RGA a target of $SCF^{SLY1}$ ubiquitin ligase?". To explore this hypothesis we examined the stability of RGA protein in *sly1-2* and *sly1-10* mutants. RGA protein accumulates at a much higher level in both *sly* alleles than in wild type, even after GA treatment (Fig. 6A). In contrast, the *RGA* mRNA level was not dramatically altered in the *sly1-10* mutant (Fig. 6B). These results show that *SLY1* is directly or indirectly required for GA-stimulated degradation of RGA. Given that SLY1 contains an F-box domain, we speculate that RGA may be a target of an $SCF^{SLY1}$ complex.

**Analysis of the *sly1-10 rga-24* double mutant**

If the dwarf phenotype of *sly1* mutants is due to over-accumulation of RGA protein, we would expect loss-of-function mutations in the *RGA* gene to rescue the *sly1* dwarf phenotype. The *sly1-10* allele was crossed to *rga-24* and used to generate a *sly1-10 rga-24* double mutant. Fig. 3C shows a comparison of *sly1-10*, the *sly1-10 rga-24* double mutant, and L*er* after 60 days growth on soil. The phenotype of the *rga-24* mutant is almost identical to L*er* (Silverstone et al., 1997). The *rga-24* mutation clearly results in a partial rescue of the *sly1-10* dwarf phenotype, but does not significantly suppress the germination or fertility defects of *sly1-10*. This result indicates that *rga-24* is epistatic to *sly1-10* for stem elongation, and that *RGA* acts downstream of *SLY1* in GA signal transduction.

## DISCUSSION

This paper reports the map-based cloning of the *SLY1* gene of Arabidopsis. *SLY1* is a positive regulator of GA-response. Recessive mutations in the *SLY1* gene affect the full range of GA phenotypes, including feedback regulation of *GA3ox1* biosynthetic gene (Fig. 1). Thus, the fact that *SLY1* encodes a putative F-box protein suggests that the GA signal is transmitted via an $SCF^{SLY1}$ E3 ubiquitin ligase.

Ubiquitylation controls target protein activity at multiple levels including proteolysis and the potentiation of transcriptional activation domains (Conaway et al., 2002). Major members of the SCF complex include homologues of SKP1, Cullin, and the RING-finger domain protein Rbx1 (Zheng et al., 2002). The F-box subunit directs interaction of the

complex with a specific target for ubiquitylation. The conserved F-box domain allows the protein to interact with the SKP1 subunit of the SCF. SKP1 tethers the F-box protein to the N-terminus of cullin. The RING-finger protein Rbx1 binds the C-terminus of cullin and the E2 ubiquitin conjugating enzyme. The E3 ubiquitin ligase catalyzes transfer of ubiquitin from E2 to the substrate. Formation of a polyubiquitin chain on the substrate targets it for proteolysis by the 26S proteasome.

In Arabidopsis, there are at least 21 Skp1 homologues (ASK for Arabidopsis Skp1), 11 cullin homologues, and 2 RBX homologues (Farras et al., 2001; Lechner et al., 2002; Shen et al., 2002). Recent work has characterized the superfamily of 694 putative F-box protein genes in Arabidopsis (Gagne et al., 2002; Kuroda et al., 2002). The *SLY1* gene falls into the C2 family of F-box proteins according to (Gagne et al., 2002). Representatives of all of the Arabidopsis F-box family including C2 were found to interact with Arabidopsis SKP1 homologues using yeast two-hybrid indicating that they are part of functional SCF complexes. A representative of the C2 F-box family was shown in interact with ASK1, ASK2, ASK4, ASK11, and ASK13 (Gagne et al., 2002). SCF complexes in Arabidopsis may be regulated by self-ubiquitylation of the F-box, or by RUB modification of the Cullin subunit (Conaway et al., 2002; del Pozo et al., 2002). It was recently shown that RUB1 modification of AtCUL1 is required for normal auxin signal transduction. But the precise role of RUB modification is unknown.

A number of previously published results support the notion that the GA signal is transmitted by ubiquitylation and proteolysis. Reynolds and Hooley (Reynolds and Hooley, 1992) reported isolation of a GA-regulated tetraubiquitin cDNA from *Avena fatua*. It is possible that an increased pool of ubiquitin is needed for GA response. Chen

et. al. (1995) identified a GA-induced cDNA encoding a ubiquitin conjugating enzyme in rice. Finally, the negative regulator of GA-response, RGA, appears to be subject to GA-stimulated proteolysis (Silverstone et al., 2001). The barley and rice homologues of RGA, SLN1 and SLR1 respectively, also appear to be subject to GA-stimulated proteolysis (Chandler et al., 2002; Gubler et al., 2002; Itoh et al., 2002). This result suggests that this aspect of GA signal transduction is conserved among dicots and monocots. Recently, inhibitor studies suggested that GA-stimulated proteolysis of barley SLN1 was dependent on the 26S proteasome, pointing to a role for the 26S proteosome in GA-induced proteolysis of the RGA/SLN1/SLR1 family (Fu et al., 2002).

This paper proposes that GA causes ubiquitylation of RGA via an $SCF^{SLY1}$ E3 ubiquitin ligase. According to this hypothesis, SLY1 acts positively in GA-response because it is the negative regulator of RGA, a negative regulator of GA response. Evidence in favor of this model include that 1) *sly1* mutants have a GA-insensitive phenotype, 2) the predicted SLY1 protein has homology to an F-box, 3) RGA accumulates at high levels in *sly1* mutants even in the presence of GA, and 4) the *rga-24* mutation suppresses the dwarf phenotype of *sly1-10*. Fig. 6 shows that mutations in *SLY1* result in high level accumulation of RGA protein. While this evidence does not directly prove an interaction between SLY1 and RGA, it is highly suggestive that an $SCF^{SLY1}$ complex may direct GA-stimulated degradation of RGA. If this hypothesis is correct, SLY1 will be the second F-box protein in plants for which a target has been identified. It is possible the DELLA motif of RGA is needed for regulation by $SCF^{SLY1}$, because this motif is essential for GA-induced degradation of RGA (Dill et al., 2001).

Like the *sly1-10* mutation, deletion of the DELLA motif stabilizes RGA even in the presence of GA (Dill et al., 2001).

It is tempting to speculate that an $SCF^{SLY1}$ complex may target the entire DELLA family including GAI, RGL1, RGL2, and RGL3 for destruction. However, *RGL2* appears to be regulated in transcription (Lee et al., 2002), and it was shown that RGL1-GFP and GAI-GFP translational fusions are not subject to GA-regulated proteolysis (Fleck and Harberd, 2002; Wen and Chang, 2002). While this work has not yet been confirmed by examination of native RGL1 and GAI proteins, it suggests that RGL1 and GAI are not subject to regulation by classic ubiquitin-directed proteolysis. Nevertheless, the *sly1* mutants are defective in the full range of GA responses, while *RGA* affects stem elongation, leaf expansion, and flowering time. Thus, the *sly1* phenotypes of increased seed dormancy and reduced fertility likely result from a mechanism other than increased levels of RGA protein. It will be important to determine if *SLY1* regulates other GA-response genes, including other members of the DELLA family.

*sly1* mutant phenotypes are not as strong as those of the GA biosynthetic mutant *ga1-3*. The *ga1-3* seeds have an absolute requirement for added GA to germinate. Although *sly1* mutants show increased seed dormancy (5% germinate, Fig. 2), they do eventually afterripen and hence germinate (C. Steber, unpublished). The *ga1-3* mutant has a stronger dwarf phenotype than *sly1* mutants (Steber et al., 1998). Whereas *ga1-3* plants are fully infertile, *sly1* mutants are partially infertile. One possible explanation for this may be that the *SLY1* homologue MIF21.6 may be functionally redundant with *SLY1*. This may also explain why GA can cause some reduction in *GA3ox1* transcript levels in the *sly1* mutant background. In contrast, GA cannot cause reduction of RGA protein

levels in a *sly1* mutant background. This suggests that *SLY1* is more directly involved in the regulation of RGA than of *GA3ox1*.

While the *sly1* dwarf phenotype is not as strong as that of *ga1-3*, the *sly1* mutants result in a considerably higher (approximately 5-fold) level of RGA protein accumulation than *ga1-3* (Fig. 6). If plant height were a direct function of RGA protein levels, we would expect *sly1* mutants to be smaller than *ga1-3*. There are two possible explanations for this. One is that the plant may compensate for RGA overabundance in *sly1* mutants by downregulating other DELLA family proteins. Another explanation is that the RGA protein that accumulates in *sly1* mutants may not be fully functional.

How does GA control the activity of $SCF^{SLY1}$? Most SCF ubiquitin ligase-regulated proteins are targeted for ubiquitylation and degradation by phosphorylation (Willems et al., 1999). However, there are examples where ubiquitin ligase regulated proteins are targeted for ubiquitylation and destruction by proline hydroxylation or by glycosylation (Huang et al., 2002; Yoshida et al., 2002). Phosphorylation was recently implicated in the regulation of 26S proteosome-mediated proteolysis of barley DELLA protein SLN1 (Fu et al., 2002). In addition Saskaki et al (2003) show evidence that the RGA homologue SLR1 is targeted for degradation by phosphorylation. Given that proteolysis is a conserved mechanism for regulating the DELLA family of proteins in plants, it is reasonable to speculate that RGA may also be regulated by phosphorylation. However, there is currently no direct evidence of this. In this model $SCF^{SLY1}$ would only recognize RGA protein when it is phosphorylated by a GA-stimulated kinase. Thus, a GA-regulated kinase and/or phosphatase may play a crucial role in GA signaling.

In summary, we propose a model for the role of SLY1 in GA signal transduction (Fig. 7). Wild-type plants (+GA) reach normal height because GA stimulates $SCF^{SLY1}$ to target RGA for degradation, thus alleviating RGA-inhibition of stem elongation. In GA biosynthetic mutants (no GA), there is insufficient GA to stimulate the $SCF^{SLY1}$ complex to target RGA for proteolysis. Overabundance of RGA inhibits stem elongation leading to a dwarf phenotype. Mutations in *SLY1* prevent degradation of RGA both in the presence and absence of GA leading to RGA-inhibition of stem elongation and a dwarf phenotype.

## MATERIALS AND METHODS

### Material and Growth Conditions

*Arabidopsis thaliana* ecotypes Landsberg *erecta* (L*er*) and Columbia (Col), and BAC clones used in this study were obtained from the Arabidopsis Biological Research Center (http://www.arabidopsis.org/ ) Plants were grown under 16 hr day/22°C 8 hr night/16°C under halide lights at 100-150 μE $m^{-2}$ $sec^{-1}$. *sly1* mutants are less fertile if grown under continuous light. Before plating on 0.5x Murashige and Skoog basal salt mixture (Sigma) plus 0.8% (g/v) agar, seeds were surface sterilized by incubation in 10% (v/v) bleach/0.01% SDS for 10 min, followed by 6 sterile water washes. Seeds were imbibed for 4 days at 4°C to ensure synchronous germination, then moved to continuous fluorescent light at 50 μE $m^{-2}$ $sec^{-1}$ and 22°C. Seeds with emerging radicals were scored as germinated after 7 days under lights. Stock solutions of $GA_4$ (Sigma or a gift of Dr. Tadao Asami, RIKEN, Japan) were made in ethanol. Plant hormones were added to

autoclaved media cooled to 55°C. Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial use.

For the analysis of RGA protein levels and expression of *RGA* and *GA3ox1* transcript levels, Arabidopsis seedlings were grown as described previously (Silverstone et al., 2001). To facilitate germination, *ga1-3* seed was pretreated with 100 μM $GA_4$ during the stratification period then washed 5 times with water. Treatments of $GA_4$ or water were performed by adding 1 mL of solution directly to the surface of the agar. Whole seedlings were harvested and ground directly in liquid nitrogen, using a pestle and mortar.

**Map-based cloning**

SSLP and CAP markers were used to localize *SLY1* to chromosome IV (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). CAPS markers used for fine mapping the *SLY1* gene are listed online (supplementary table). Those clones marked "cereon" were based on the Cereon markers at the TAIR web site (http://www.arabidopsis.org/). All other markers were derived in this study. The germination and dwarf phenotypes of the *sly1* mutant necessitated using PCR based mapping markers. 19 new CAPS markers were defined by screening 56 2kb-PCR products using 4-bp cutters (see supporting online material). In order to identify the *SLY1* gene by complementation, sublibraries were generated from BAC clones T19F6 and T22A6 by partial digestion with Sau3AI. Inserts ranging from 5 to 20 kb were inserted into the BamHI cloning site in pBIN19. Subclones representing the region were transformed into *sly1-2* or *sly1-10* (Bechtold et al., 1993; Clough and Bent, 1998). Sequence alignments were performed using tBLASTn to search the TIGR EST and Genbank databases (Altschul et al., 1990; Smith et al., 1996; www.ncbi.nlm.nih.gov/, 2002; www.tigr.org, 2002).

**The *SLY1* Transcript**

The *SLY1* cDNA 3' end was determined by sequencing the 3' ends of full length cDNAs recovered from an *Arabidopsis thaliana* Col uni-Zap XR cDNA library (Strategene, La Jolla, CA) to identify a polyadenylated sequence. The 3' end was amplifed [2μL cDNA library, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 mM primer, 1X reaction buffer, 5U Ex-Taq polymerase (Takara Bio Inc., Shiga, Japan) for 40 cycles of 96° 25sec, 60° 40sec, and 72° 80sec] using sly1-2f (5'-AGACGAGCGGCTTTGGGAGC-3') for 5 cycles before the addition of the T7 primer (5'- TAATACGACTCACTATAGGG-3'). This PCR product was used as template for the reaction using 2-63f (5'-TCTCTCTAAACCCAATCCG-3') for 5 cycles before the addition of T7 primer. PCR products were gel purified (Qiaex II, Qiagen, Inc., Valencia, CA) and cloned using the TOPO-XL PCR cloning kit (Invitrogen, Carlsbad, CA). Clones were sequenced using fluorescence-based dideoxy terminators and Ampli-Taq polymerase and run on an Applied Biosystems sequencer model 377 (Perkin Elmer Applied Biosystems, Inc., Norwalk, CT).

Due to the presence of the MIF21.6 *SLY1* homologue on chromosome V, it was difficult to specifically detect the *SLY1* transcript by northern analysis. Thus, qualitative RT-PCR was used to look for the presence or absence of *SLY1* mRNA in various aerial tissues and in *sly1* mutants. Tissue was harvested from 8-week old Arabidopsis plants or from seedlings (where indicated). Total RNA was extracted from 0.1 g of plant tissue using the RNeasy® Plant Mini Prep Kit (Qiagen) according to the manufacturer's instructions with addition of an RNAse-free DNAseI treatment (Roche). RT-PCR was conducted on 100 ng of total RNA using a Roche LightCycler™ and the LightCycler

RNA Amplification Kit – SYBR Green I (Roche) according to the manufacturer's instructions using an annealing temperature of 56°C and 5 mM $MgCl_2$. A no reverse transcription control was included for all RNA preps to confirm absence of genomic DNA contamination. The primers used for *SLY1* amplification were 5'TCTCTCTAAACCCAATCCG-3' and 5'-CCAGCATTGAACATCACATCTGAC-3'. The primers used for *ACT2* amplification were 5'CTGGATTCTGGTGATGGTGTGTC-3' and 5'-TCTTTGCTCATACGGTCAGCG-3' (An et al., 1996). The products of amplification were separated on a 2% agarose gel for 3 hr at 60V (Sambrook et al., 1989).

**Immunoblot Analysis**

Total protein was extracted from water or $GA_4$ treated seedlings as described previously (Silverstone et al., 2001). For each sample, 20 μg of total protein was fractionated in an 8% SDS polyacrylamide gel and analyzed by immunoblotting (Silverstone et al., 2001) using a 2000-fold dilution of an anti-RGA antibody from rat and a 7500-fold dilution of peroxidase-coupled goat anti-rat IgG. Immunoreactive species on the blots were detected using Supersignal Dura Reagent (Pierce). Ponceau staining was performed by incubating the blot with 0.2% (w/v) Ponceau S (Sigma) in 1% (v/v) acetic acid.

**RNA blot analysis of *GA3ox1* and *RGA* transcripts**

Total RNA was isolated and *GA3ox1* mRNA detected using an antisense *GA3ox1* RNA probe as described previously (Yamaguchi et al., 1998). The *RGA* transcripts were

analyzed using a random-primed labeled 2.3-kb *RGA* DNA probe as described (Silverstone et al., 1998). As a loading control, blots were reprobed with a labeled 18S oligonucleotide probe as described previously (Dill and Sun, 2001). The RNA blots were exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale. CA) and quantified on a PhosphorImager (Model 455Si; Molecular Dynamics), using Imagequant v5.1 software.

**Generation of the *sly1-10 rga-24* mutant**

We isolated the *sly1-10 rga-24* homozygous double mutant by crossing *rga-24* and *sly1-10*. The genotypes of homozygous $F_2$ plants were confirmed using allele-specific PCR primers. Genotyping of the *rga-24* allele was performed as described previously (Dill and Sun, 2001). Primers sly1-10f (5'-TCGTCACTGGACTAACATCGGCTG-3') and 2-63r (5'-GCTAACAGTCTGGCTTATGGATAC-3') amplify a 350-bp product in *SLY1* but not *sly1-10*. Primers sly1-10f and sly1-10r2 (5'-GAGCATGCTTGATCCTAGGA-3') amplify a 320-bp product in *sly1-10* but not in *SLY1*. PCR reactions were performed as described (Dill and Sun, 2001).

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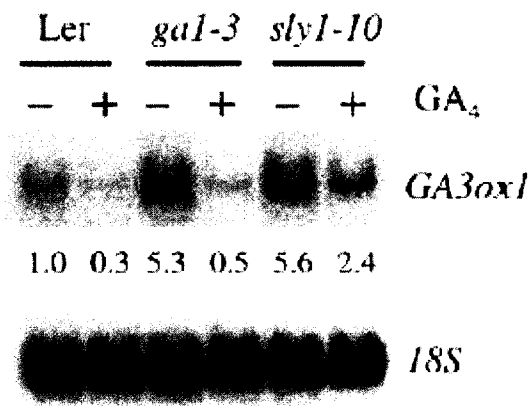


**Figure 1.** Effect of *sly1* mutations on *GA3ox1* transcript.

An RNA blot, containing 10 μg of total RNA isolated from 8-day-old Ler (wild-type), *ga1-3* and *sly1-10* seedlings treated with (+) or without (–) 1 μM $GA_4$ for 2 h, was hybridized with a labeled *AtGA3ox1* antisense RNA probe. The blot was reprobed using a labeled 18S oligonuclueotide probe. The numbers below the blot indicate the relative levels of *AtGA3ox1* mRNA after standardization, using the 18S RNA as a loading control. The level of *AtGA3ox1* mRNA in the wild type (water treated) was arbitrarily set as 1.0.

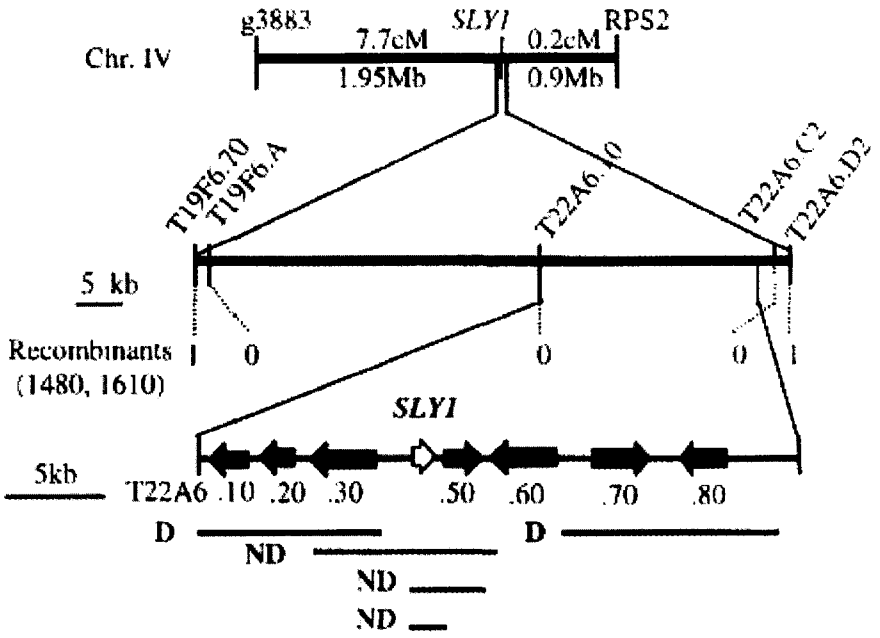


**Figure 2.** Map-based cloning of *SLY1*.

Fine mapping delineated a 70-kb region containing *SLY1* between markers T19F6.70 and T22A6.D2. Single recombinants were identified at T22A6.D2 (in 1610 chromosomes), and at T19F6.70 (in 1480 chromosomes). Overlapping BAC clones T22A6 and T19F6 cover this region (Arabidopsis Genome Initiative, 2000). Transformation with subclones of these BACs identified an 11.7-kb complementing subclone, T22A6.2G10 (D=dwarf, ND=Non-dwarf). Transformation with T22A6.40 (from -1347 to +666 relative to the ATG) rescued *sly1*.

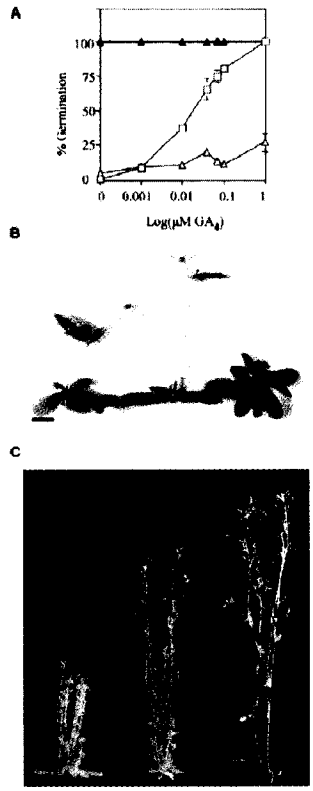


**Figure 3.** Complementation of *sly1* mutants.

**(A)** GA dose-response in germination. Transformation with the T22A6.2G10 subclone rescues the GA-insensitive germination phenotype of *sly1-2*. Percent germination of dormant *sly1-2* seeds (Δ), the GA biosynthesis mutant *ga1-3* (□), and *sly1-2* transformed with T22A6.2G10 (▲) are shown. Wild-type L*er* germination was identical to T22A6.2G10 transformed *sly1-2*. Error bars are SE for triplicate samples of 50-100 seeds. **(B)** Transformation with T22A6.40 (-1347 to +666) rescues the dwarf phenotype of *sly1-2*. Wild type Ler (left), *sly1-2*+ T22A6.40 (center), and *sly1-2* (right) plants. Bar is 1 cm. **(C)** Suppression of *sly1-10* by *rga-24*. Figure shows *sly1-10*, the *sly1-10 rga-24* double mutant, and wild-type L*er*. *rga-24* partly rescues the dwarf phenotype of *sly1-10*, but not poor fertility. L*er* and homozygous mutant plants were grown on soil for 60 days under a long day photoperiod. Bar is 15 mm.

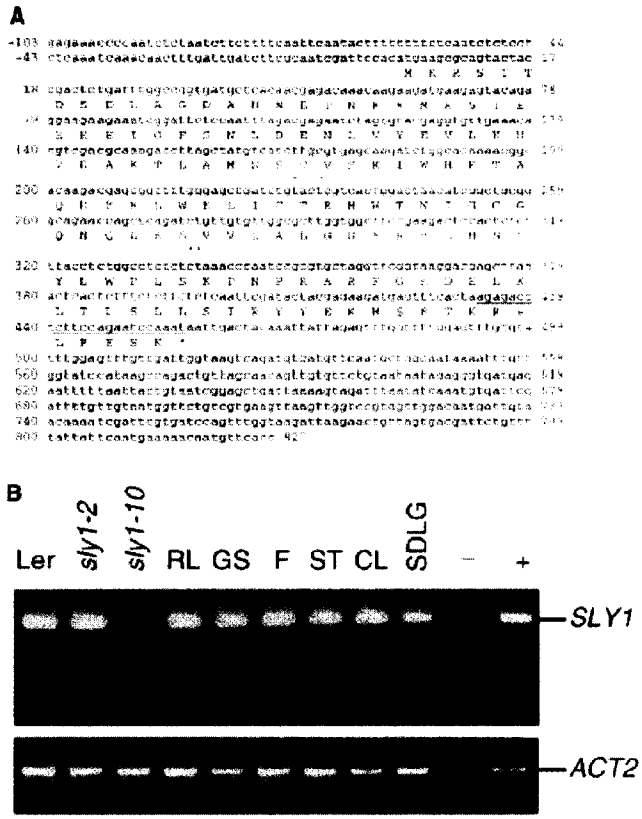


**Figure 4.** *SLY1* mRNA.

**(A).** Sequence of full length *SLY1* cDNA. The *SLY1* gene (Genbank NM_118554) contains no introns and predicts a protein of 151 amino acids. The first ATG (+1) in the transcript is the *SLY1* translational start site. *sly1-2* is a 2-bp deletion (**). *sly1-10* contains a 23-bp deletion (underlined) and an 8-kb insertion. **(B)** RT-PCR analysis of *SLY1* mRNA accumulation. Ethidium stained 2% agarose gel of RT-PCR using 100 ng of total RNA for each sample. *SLY1* (250-bp) plus an *ACT2* (471-bp) accumulation are shown for wild-type Ler, *sly1-2* (-2), *sly1-10* (-10) whole aerial plants, and wild-type Ler rosette leaves (RL), green siliques (GS), flowers (F), stems (ST), cauline leaves (CL), and seedlings (SDLG). But for seedlings, tissue was harvested from 4-week old plants. (-) is no RNA template, (+) is genomic DNA.

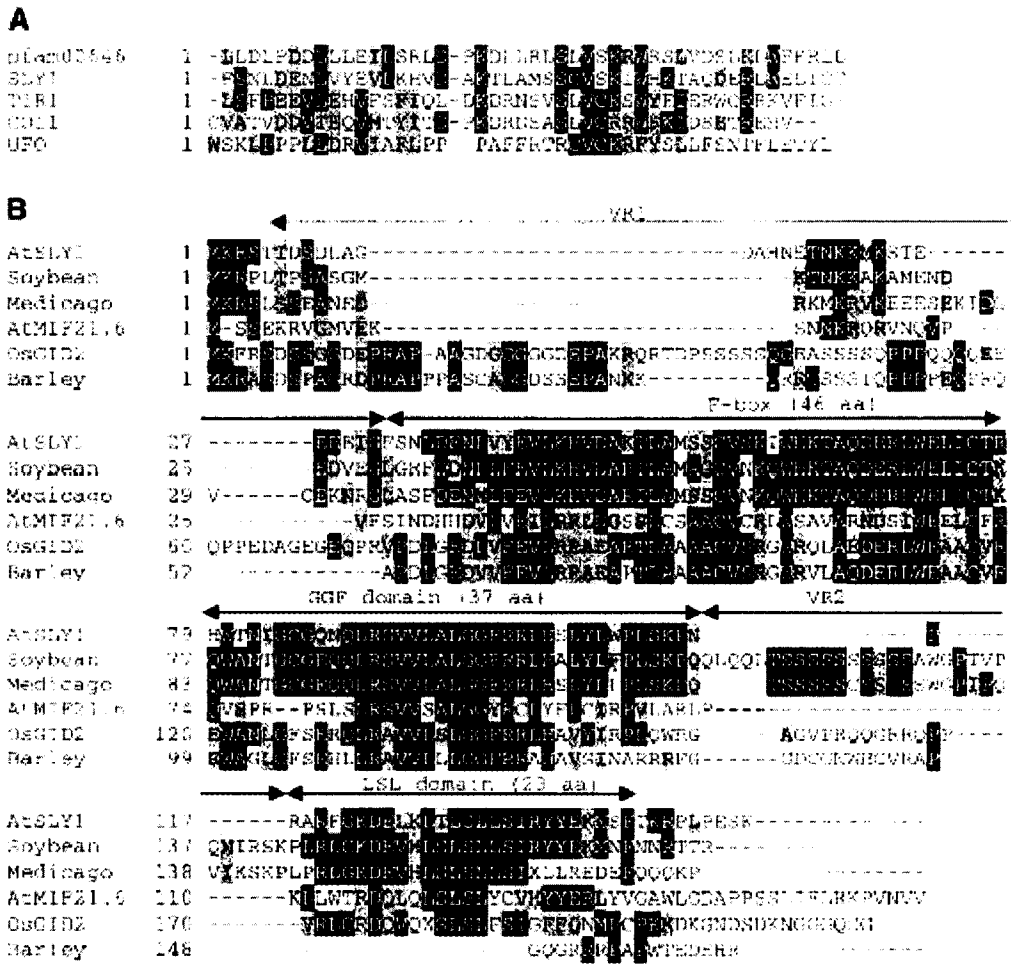


**Figure 5.** SLY1 sequence alignments.

(**A**) Alignment of the SLY1 F-box with NCBI consensus Pfam00646 (an F-box protein family) and other Arabidopsis F-box proteins. (**B**) Alignment of SLY1 with plant homologues from *Glycine max* (soybean) BI785351, *Medicago trunculata* BQ239225, *Arabidopsis thaliana* MIF21.6 AB023039, OsGID2 (rice), and *Hordeum vulgare* (barley) BF622212. Amino acid sequence predicted from the largest open reading frame in each EST is listed. VR stands for variable region, GGF and LSL refer to conserved residues.

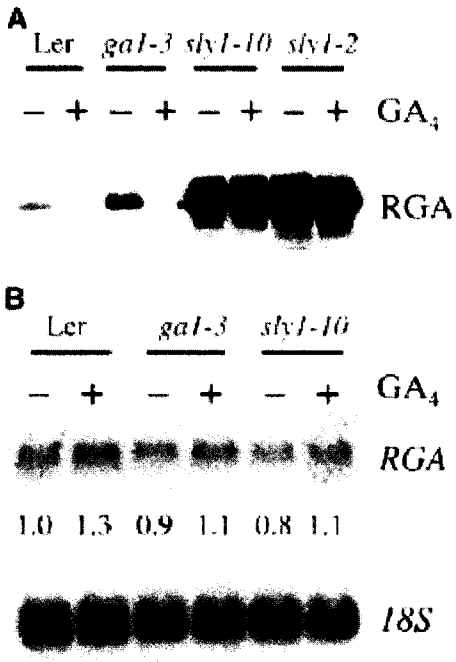


**Figure 6.** RGA protein levels, but not the *RGA* mRNA, are highly elevated in the *sly1* mutants.

**(A)** Eight-day-old seedlings were treated with (+) or without (–) 1 μM $GA_4$ for 2 h, and protein extracts fractionated in an 8% SDS-PAGE. The protein blot was probed with an anti-RGA antibody. Ponceau staining was used to confirm equal loading of the blot (data not shown). **(B)** A duplicate RNA blot described in Fig. 1A was hybridized with a labeled *RGA* DNA probe. The numbers below the blot indicate the relative levels of *RGA* mRNA after standardization, using the 18S as a loading control. The level of *RGA* mRNA in the wild type (water treated) was arbitrarily set as 1.0.

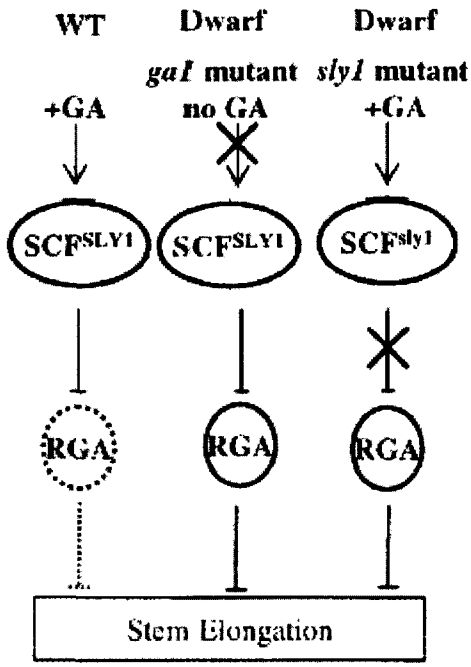


**Figure 7.** A model for the role of SLY1 in GA signaling.

The dwarf phenotype of GA biosynthetic mutants results from accumulation of active RGA protein. In wild-type plants (+GA), GA stimulates $SCF^{SLY1}$ (directly or indirectly) to target RGA for degradation resulting in normal height. The *ga1* biosynthetic mutant is a dwarf because there is insufficient GA to stimulate $SCF^{SLY1}$ to target RGA for proteolysis. The *sly1* mutant is a dwarf because lack of functional $SCF^{SLY1}$ results in increased accumulation of RGA protein

# CHAPTER THREE

## RECESSIVE INTERFERING MUTATIONS IN THE GA-RESPONSE GENE *SLEEPY1* ARE RESCUED BY OVEREXPRESSION OF ITS HOMOLOGUE *SNEEZY*

This chapter describes the determination that the *sly1-2* and *sly1-10* mutations are recessive interfering, as well as describing the initial study of the genetic interactions of *SLEEPY1* and *SNEEZY*. Karen McGinnis performed the original screen for suppressors of *sly1-10*. Sian Ritchie retested these suppressors and performed ABA dose-response curves on JS53 and JS54 lines. Jon Soule aided in the construction of the overexpression and antisense constructs. This paper has been submitted and accepted with revision to the Proceedings of the National Academy of Sciences-USA journal, with authors in the following order: Lucia C. Strader, Sian Ritchie, Jon Soule, Karen McGinnis, and Camille M. Steber.

**Abstract**

This paper reports the genetic interaction of two F-box genes, *SLEEPY1* (*SLY1*) and *SNEEZY* (*SNE*), in *Arabidopsis thaliana* GA (gibberellin) signaling. The *SLY1* gene encodes an F-box subunit of an SCF E3 ubiquitin ligase complex that positively regulates GA signaling. The *sly1-2* and *sly1-10* mutants have recessive, GA-insensitive phenotypes including delayed germination, dwarfism, reduced fertility, and overaccumulation of DELLA proteins including RGA, GAI and RGL2. The DELLA proteins are putative transcription factors that negatively regulate GA signaling. *SLY1* is required for the GA-stimulated disappearance of DELLA proteins, which suggests that GA targets them for destruction via $SCF^{SLY1}$-mediated ubiquitylation. Overexpression of *SLY1* in *sly1-2* and *sly1-10* plants rescues the recessive GA-insensitive phenotype of these mutants. Surprisingly, antisense expression of *SLY1* also suppresses these mutants. This result caused us to hypothesize that the *SLY1* homologue *SNEEZY* (*SNE*) can functionally replace *SLY1* in the absence of the recessive interfering *sly1-2* or *sly1-10* genes. This hypothesis was supported because overexpression of *SNE* suppresses *sly1-10*. In addition to rescuing the *sly1-10* dwarf phenotype, *SNE* overexpression also restored normal RGA protein levels, suggesting that the SNE F-box protein can replace SLY1 in the proteolysis of RGA. If the C-terminal truncation in the *sly1-2* and *sly1-10* alleles interferes with *SNE* rescue, we reasoned that overexpression of *sly1-2* may interfere with wild-type *SLY1* function. Indeed, overexpression of *sly1-2* in wild-type L*er* (Landsberg *erecta*) yields dwarf plants.

## Introduction

Gibberellins (GAs) are a family of tetracyclic diterpenoid phytohormones needed to induce seed germination, stem elongation, transition to flowering, and fertility (1). Many positive and negative regulators of GA signaling have been identified as mutants with increased and decreased GA response (extensively reviewed by 1, 4). In *Arabidopsis*, known negative regulators of GA signaling include the *O*-linked *N*-acetylglucosamine transferase *SPINDLY* (*SPY*) (5), and the putative transcription factors *SHORT INTERNODES* (*SHI*) (6), *RGA* (7), *GA INSENSITIVE* (*GAI*, 8), and *RGL1*, *RGL2*, and *RGL3* (9-11). Known positive regulators of GA signaling in *Arabidopsis* include the chromatin remodeling factor *PICKLE* (*PKL*) (12) and the F-box domain gene *SLEEPY1* (*SLY1*) (13).

Mutations in the *SLEEPY1* gene were originally isolated in screens designed to detect a GA-insensitive increase in seed dormancy (14). The *sly1-10* allele was isolated based on brassinosteroid-dependent germination (15). The *sly1-2* and *sly1-10* mutations result in a recessive GA-insensitive phenotype that includes increased seed dormancy, increased sensitivity to ABA during germination, dwarfism, and reduced fertility. No other recessive alleles have yet been characterized.

Map-based cloning revealed that *SLY1* encodes an F-box domain protein of 151 amino acids (13). F-box proteins are subunits of SCF (Skp1, cullin, F-box) E3 ubiquitin ligases (16). The F-box protein confers specificity on the complex by direct interaction with the substrate via its C-terminus. F-box proteins often contain consensus protein-protein interaction domains at the C-terminus such as Leucine rich repeats, Kelch repeats,

and WD repeats. While SLY1 does not contain such a consensus sequence, it is clear that the C-terminal domain is functionally important. The *sly1-2* allele is a frameshift mutation resulting in loss of the last 40 amino acids, whereas the *sly1-10* allele is a complex rearrangement resulting in loss of the last eight amino acids and addition of a random 23 amino acids (13). The N-terminal F-box domain binds to a Skp1 homologue which tethers the F-box protein to N-terminus of cullin, the backbone of the SCF complex. The C-terminus of cullin binds the RING-finger domain protein Rbx1, which binds the E2 ubiquitin conjugating enzyme. The SCF complex catalyzes transfer of a ubiquitin moiety from the E2 to the substrate (17). Formation of a polyubiquitin chain on the substrate targets it for proteolysis by the 26S proteasome. The *SLY1* gene belongs to the 17-member C2 subfamily of the 694 superfamily of Arabidopsis F-box genes (18). The *SLY1* gene is highly conserved in higher plants (13, 19). The two homologous F-box genes, *SLY1* in Arabidopsis and *GID2* (*GA-INSENSITIVE DWARF 2*) in rice, are both needed for GA response and for the GA-stimulated disappearance of the DELLA proteins (13, 20-22).

GA responses are repressed by the DELLA subfamily of the GRAS (GAI, RGA, and SCARECROW) family of putative transcription factors (23). It appears that GA causes GA responses by triggering the disappearance of DELLA proteins (24-26). Whereas only a single DELLA protein is known in barley (*SLN1*, *SLENDER1*, 24) and in rice (*SLR1*, *SLENDER RICE1*, 27), there are five DELLA genes in Arabidopsis. *GAI* (*GA-INSENSITIVE*) and *RGA* (*REPRESSOR OF ga1-3*) act redundantly to negatively regulate stem elongation, leaf expansion, and apical dominance (28, 29). *RGL1*, *RGL2*, and *RGA* appear to be the main negative regulators of floral development (9, 10). *RGL1*

also appears to be involved in stem elongation and leaf expansion (9). *RGL2* is the key DELLA repressing seed germination, but *RGL1* may also play a role (9, 11). The precise role of *RGL3* (*RGA-Like*) in GA signaling remains to be elucidated.

That GA stimulates disappearance of DELLA proteins via the ubiquitin-proteasome pathway was first suggested by the requirement for F-box proteins Arabidopsis *SLY1* and rice *GID2* for the GA-regulated disappearance of RGA and SLR1 protein, respectively (13, 22). SLY1 and GID2 are each part of an SCF E3 ubiquitin ligase (20, 21). Thus far, SLY1 has been shown to regulate the GA-stimulated disappearance of RGA and GAI (13, 20, 30), while GID2 regulates SLR1 (21, 22). The interaction between rice GID2 and SLR1 depends on phosphorylation of SLR1, suggesting that GA-stimulated phosphorylation is the signal causing $SCF^{GID2}$ to ubiquitylate SLR1, thereby targeting it for destruction (21, 22). Accumulation of ubiquitylated SLR1 is dependent on GID2 (22). The strength of the interaction between SLY1 and the gai-1 mutant protein depends on phosphorylation of gai-1, suggesting that a similar mechanism is at work in Arabidopsis (20).

There is one homologue of *SLY1* in Arabidopsis named *SNEEZY* (*SNE*, At5g48170 (13). The predicted SNEEZY protein is 25% identical/42% similar to SLY1 at the amino acid level. The research described here reveals that the *sly1-2* and *sly1-10* are recessive interfering mutations, and that *SNE* can functionally replace *SLY1* in GA signal transduction.

## Materials and Methods

**Plant Growth Conditions.** Plants were grown as in McGinnis *et al.* (13). For GA treatment studies, 1 mL water or 1 mL 1 μM $GA_4$ (Sigma) were applied to the agar surface of 10-day-old seedlings for 2 h before tissue was harvested. ABA-dose response curves were generated using seeds harvested at the same time stored dry for at least two weeks as described by (14), except that germination was defined as radicle emergence.

**Plasmid Construction.** For use in plasmid construction, *SLEEPY1* (At4g24210, NM 118554), *sly1-2*, and *SNEEZY* (At5g48170, NM124191) were cloned from either L*er* or *sly1-2* genomic DNA by PCR, using 2-62f and 2-64r primers for *SLY1* and *sly1-2*, and MIf21.6f and MIf21.6r for *SNE*. These PCR products were cloned into the pTOPOXL TA® vector (Invitrogen) in both orientations. To create pJS53 (*35S:SLY1-AS*), pJS54 (*35S:SLY1-OE*), and pJS65 (*35S:sly1-2-OE*), inserts were excised using the restriction enzymes XbaI and SstI and ligated into pBI121 cut with the same enzymes, thereby replacing the GUS gene. To create pJS67 (*35S:SNE-OE*), the *SNE* insert was excised using the restriction enzymes BamHI and XbaI and ligated into pBI121, cut with the same enzymes, thereby replacing the GUS gene. Once fragments were cloned into the appropriate vectors, the inserts were sequenced to ensure correct orientation and that no mutations were introduced during PCR and cloning steps.

**Transformation and Isolation of Transgenic Lines.** Using the floral dip method (31), L*er*, *sly1-2*, and *sly1-10* were transformed with constructs carried by *Agrobacterium tumefaciens* strain GV3101. Transformants were selected on 0.5x Murashige and Skoog (MS) medium (Sigma)/ 0.8% agar containing 100 μg/ml

kanamycin. Transformation events were confirmed by PCR, using primers sly1r and nosr for pJS53 transformants, and 2-63f and nosr for pJS54 and pJS65 transformants.

**mRNA Analysis.** *SLY1* transcript accumulation was analyzed by semi-quantitative, directional reverse transcriptase-mediated PCR (RT-PCR) in *SLY1* overexpression and antisense lines. RT-PCR on 100 ng total RNA was conducted on a Roche LightCycler using the Roche LightCycler RNA Amplification Kit SYBR Green I according to the manufacturer's instructions, with 5 mM $MgCl_2$ and a 56°C annealing temperature. Reverse transcription was performed with only the sly1r primer to detect only the *SLY1* sense transcript. After reverse transcription, primer 2-63f was added, and 25 cycles of PCR amplification performed. The 2-63f and sly1r primers are designed around the *sly1-10* rearrangement, such that there should be no product in *sly1-10*. This control shows that only *SLY1*, not *SNE*, mRNA is being detected. The *ACT2* mRNA loading control was amplified with the ACT2f and ACT2r primers (13, 32).

*SNE* expression was evaluated by RNA gel blot analysis using 15 µg total RNA per lane (33). Total RNA was prepared using the hot phenol extraction method (34), except that the phenol extraction was performed at 73°C instead of 80°C. The *SNE* mRNA was detected using a random-primed $^{32}$P-labeled 1 kb *SNE* DNA probe (Amersham RediPrimeII), encompassing the SNE coding region from 142 bp upstream to 858 bp downstream of the translational start site. Signal was detected using a PhosphorImager™ (model 445Si, Molecular Dynamics). For a loading control, northern blots were rehybridized to an 18S rRNA probe generated using the primers 18Sf and 18Sr.

**Suppressor Screen.** To screen for overexpression suppressors of *sly1-10*, *sly1-10* plants were first transformed with pVICEn4HPT by the floral dip method. The activation-tagging T-DNA vector pPCVICEn4HPT has the hygromycin resistance gene for selection and four copies of the Cauliflower Mosaic Virus (CaMV) 35S promoter at the right border 35. The *sly1-10* mutant causes increased seed dormancy. Afterripened *sly1-10* seeds fail to germinate well on 0.6 μM ABA (Figure 1D). Suppressors were isolated based on their ability to germinate on 0.6 μM ABA. The four copies of the 35S enhancer cause high level constitutive overexpression of sequences downstream of the insertion site. To select dominant suppressor mutations, T1 seeds were plated on 0.6 μM ABA, 15 μg/mL hygromycin in MS-MES plates (0.5x MS, 0.8% agar, 5 mM MES pH 5.5). Seedlings that germinated and appeared hygromycin resistant were transferred to soil and retested for suppression of the germination phenotype in the T2 generation.

**Immunoblot Analysis.** Ten-day-old seedlings were treated for 2 hours with 1 μM $GA_4$ or water. Tissue was ground in liquid nitrogen and then homogenized in ice-cold 50 mM Tris-acetate, pH 8.6, 5 mM EDTA, 5 mM EGTA, 5% glycerol, 0.5% PVP, 5 mM DTT, and a Complete Mini protease inhibitor cocktail tablet (Roche), according to manufacturer's instructions. The crude protein extract was centrifuged at 20,000 x g for 20 min, 4°C. The supernatant was removed and added to SDS-PAGE sample buffer. Proteins were separated on a 10% SDS-PAGE mini-gel loaded with approximately 20 μg total protein per lane (mini protean II; Bio-Rad) and blotted (Mini Trans-blot Electrophoretic Transfer Cell; Bio-Rad) to PVDF membranes, according to the manufacturer's instructions. Protein blots were then incubated for one hour in 5% (w/v) nonfat dry milk in TBS-Tween and for 2 hr in anti-RGA (1:15,000) (25). Cross-reacting

proteins were visualized with donkey, anti-rabbit alkaline phosphatase conjugate (Jackson ImmunoResearch) and visualized with NBT/BCIP substrate (Roche).

**Oligonucleotide Sequences.** 2-62f, 5'- AAGGCATCTGAGAAACCC-3'; 2-64r, 5'- GGCTAACCATCGCAAGAATAAC-3'; MIf21.6f, 5'- TCCTCCTCTCTCTCTGCT TCTCAC-3'; MIf21.6r, 5'- TCCCCAAGAGTCAATAACTTGCTC-3'; Sly1r, 5'- CCAG CATTGAACATCACATCTGAC-3'; nosr, 5'-CGGGATCCCCCGATCTAGTAACATA GATGACAC-3'; 2-63f, 5'- TCTCTCTAAACCCAATCCG-3'; Act2f, 5'-CTGGATTCT GGTGATGGTGTGTC-3'; Act2r, 5'-TCTTTGCTCATACGGTCAGCG-3'; 18Sf, 5'-CA GACTGTGAAACTGCGAATGGCTC-3'; 18Sr, 5'-GACCCATCCCAAGGTTCAACT ACGA-3'

## Results

**Overexpression and antisense expression of *SLY1*.** In the process of characterizing the *SLY1* gene, we examined the effect of overexpression and antisense expression of *SLY1* in wild-type L*er* and in *sly1* mutants. Because *sly1*-2 and *sly1*-10 mutations in *SLY1* are recessive and cause GA-insensitivity, we expected overexpression of *SLY1* to lead to evidence of increased GA signaling, and antisense expression of *SLY1* to result in a GA-insensitive phenotype. Overexpression of the *SLEEPY1* gene complemented the dwarf and infertility phenotypes in both *sly1-2* and *sly1-10* and had no apparent effect on growth of wild-type L*er* (Figure1A). Surprisingly, antisense expression of *SLY1* also rescued the *sly1-2* and *sly1-10* dwarf and infertility phenotypes, while antisense expression in L*er* caused no decrease in plant height (Figure 1A). PCR

analysis confirmed that the lines were transformed with the correct construct (data not shown). Directional RT-PCR showed that lines containing the *SLY1-OE* (overexpression) construct overproduced the *SLY1* transcript, while those containing *SLY1-AS* (antisense) construct produced little or no *SLY1* transcript (Figure 1B). Thus, elimination of the *SLY1* mRNA by *SLY1-AS* rescues *sly1-2* dwarfism and infertility phenotypes. Even though *sly1-2* and *sly1-10* alleles are recessive, they do produce transcript (13). Thus we postulate that *sly1-2* and *sly1-10* are recessive interfering mutations such that: 1) the *SLY1* homologue *SNE* can functionally replace *SLY1* in the complete absence of *SLY1* mRNA; and 2) expression of the *sly1-2* and *sly1-10* alleles containing C-terminal truncations does not interfere with wild-type *SLY1* function, but does interfere with the ability of *SNE* to replace *SLY1*.

The DELLA protein RGA is subject to GA regulation such that the protein disappears in wild-type L*er* upon addition of GA (25). The *sly1-2* and *sly1-10* mutations lead to overproduction of RGA both in the presence and absence of GA (13 and Figure 1C). Immunoblot analysis of RGA protein accumulation showed that both the *SLY1-OE* and *SLY1-AS* constructs reduced the accumulation of RGA protein in *sly1-10* (Figure 1C) and in *sly1-2* (data not shown) in the presence and absence of $GA_4$. Expression of the same constructs in L*er* appeared to result in low level accumulation of RGA protein (Figure 1C).

The *sly1-2* and *sly1-10* mutants were isolated in screens for mutants with increased seed dormancy. The seed germination phenotype of these GA-insensitive mutants is highly variable from seed lot to seed lot, ranging from 0 to 100% germination following harvest. Those seed lots that are highly dormant do eventually germinate

following afterripening in dry storage (C. Steber unpublished). However, afterripened *sly1-2* and *sly1-10* seeds are hypersensitive to ABA, showing very low germination at 0.6 μM ABA, a concentration that does not inhibit wild-type germination. Neither the *SLY1-OE* nor *SLY1-AS* construct had a significant effect on wild-type L*er* germination (data not shown). Both the *SLY1-OE* and *SLY1-AS* significantly increased germination of *sly1-2* and *sly1-10* on 0.3 μM and 0.6 μM ABA (Figure 1D).

**Isolation of *SNE* as an overexpression suppressor of *sly1-10*.** A screen for genes that could suppress *sly1-10*, when overexpressed, recovered *SNEEZY*. Approximately 1,000 pPCVICEn4HPT transformed *sly1-10* plants were screened for the ability to germinate on 0.6 μM ABA. Transformation with the T-DNA pPCVICEn4HPT results in overexpression of downstream sequences due to four copies of the 35S promoter at the right border. When suppressor candidates were screened for levels of *SNE* mRNA accumulation, isolate 1.3 was found to be overexpressing SNE (Figure 2A). Isolate 1.3 rescues the *sly1-10* defects in germination, plant height and fertility (Figure 1E). To confirm that these effects are due to *SNE* overexpression, *sly1-10* plants were transformed with *SNE* under control of the 35S promoter. Transformation of *sly1-10* plants with this clone confirmed that *SNE* over-expression rescues the dwarf phenotype (data not shown).

**Over-expression of *sly1-2* in L*er*.** If the *sly1-2* and *sly1-10* alleles interfere with the ability of *SNE* to rescue loss of *SLY1* function, it is possible that overexpression of the *sly1-2* allele will interfere with wild-type *SLY1* activity. To test this, wild-type L*er* plants were transformed with *sly1-2* under control of the 35S promoter (*sly1-2-OE*). Of eight transformed plants recovered, four showed varying degrees of dwarfism, while four

appeared wild-type (Figure 2C). We expected the variation in phenotype to correspond with the level of *sly1-2* overexpression. RT-PCR analysis of *sly1-2* expression in two dwarf and two tall *sly1-2OE* transformants revealed that the dwarf phenotype correlated with higher levels of *sly1-2* mRNA accumulation (Figure 2B).

**The *SNE* gene.** Like *SLY1*, *SNE* is highly conserved in the plant kingdom. Members of the *SNE* gene family were identified by a tBLASTn search of plant ESTs and the rice genomic sequence (36, www.tigr.org), and are distinct from *SLY1* homologues previously identified (Figure 3). No homologues were detected outside of the plant kingdom. SNE homologues ranged from 32.3 % amino acid identity in barley, to 45.6% in orange.

*SNEEZY* (*SNE*) is a 471 bp gene encoding a predicted 157 amino acid protein. The *SNE* transcript is approximately 900 bp in length and corresponds to Genbank cDNA NM124191 (Figure 4). Like *SLY1*, *SNE* contains no intron. ClustalW alignment revealed that the *SLY1* and *SNE* genes are 53% identical at the nucleotide level, and 24% identical and 42% similar at the amino acid level (37). Developmental northern analysis of the *SNE* transcript revealed that it accumulates at high levels in stems and flowers, and at a low level in seedlings, rosette leaves, cauline leaves, and green siliques (Figure 4). *SLY1*, on the other hand, is expressed at a low-level in all examined tissues (13).

## Discussion

This paper describes a unique genetic interaction between two homologous F-box genes, *SLY1* and *SNE*, in GA signaling. Two lines of evidence converged to indicate that

the *SNE* gene can functionally replace *SLY1* and that the *sly1-2* and *sly1-10* mutations are recessive interfering with respect to *SNE*. First, antisense expression of the *SLY1* gene rescued the *sly1-2* and *sly1-10* mutations. This suggests that in the complete absence of *SLY1* transcript, the *SNE* gene is able to functionally substitute for *SLY1* in GA signaling. Second, *SNE* was recovered in a screen for genes that can suppress *sly1-10* when overexpressed. These results point to an interesting interaction between the *SLY1* and *SNE* genes in GA-signaling.

The *sly1-2* and *sly1-10* alleles are GA-insensitive recessive mutations causing increased seed dormancy, sensitivity to ABA, dwarfism and reduced fertility (14, 15). Both mutations result in C-terminal truncation, but neither mutation resulted in a loss of transcript (13) and are not true knock-outs. Since these mutations are not dominant, it is clear that they do not normally interfere with wild-type *SLY1* activity. However, it is possible that *sly1-2* and *sly1-10* can prevent *SNE* from replacing *SLY1* in GA signaling. One possibility is that the loss of the last 40 amino acids in *sly1-2* and last eight amino acids in *sly1-10* prevents the protein from interacting with the DELLA substrate, but does not prevent association with the SCF complex through the intact F-box domain. Once sly1-2 and sly1-10 proteins are no longer present, SNE might be able to join the SCF complex in the place of SLY1 for fairly efficient degradation of $SCF^{SLY1}$ targets. In this case, the affinity of wild-type SLY1 for its SCF complex is greater than the affinity of sly1-2 and sly1-10, and the affinity of sly1-2 and sly1-10 for the SCF complex is greater than the affinity of SNE.

The function of *SLY1* and *SNE* in GA signaling is exquisitely sensitive to changes in dosage. Overexpression of *SNE* in the *sly1-10* mutant background inundates the

system with excess SNE, allowing SNE to overcome the interfering effect of *sly1-10*. Moreover, overexpression of *sly1-2* at high levels in L*er* pushes the equilibrium in the other direction, allowing *sly1-2* to interfere with SLY1 protein activity causing a dwarf phenotype. The dwarfism in *sly1-2-OE* lines appears to depend on the level of *sly1-2* transcript accumulation (Figure 2B). Since antisense of *SLY1* rescues the *sly1-2* and *sly1-10* mutants, we predict that a complete knockout of *SLY1* will have no phenotype. All of the characterized alleles of *SLY1* and rice *GID2* are either point mutations or C-terminal truncations located downstream of the F-box domain. Interestingly, the *gar2* mutant is a point mutation in the *SLY1* C-terminus resulting in a dominant gain-of-function mutation that rescues the *gai-1* dwarf (20, 30). This mutation causes increased affinity of SLY1 for its substrates RGA and GAI (20, 30).

While the data here suggest that *SNE* may be normally involved in GA signaling, they do not rule out the possibility that *SNE* functions in another pathway. Cross-reactivity between F-box proteins may provide one mechanism for the crosstalk recently observed between the GA and ethylene or auxin pathways (38, 39). It is interesting to note that F-box proteins involved in hormone signaling have a tendency to cluster in similar F-box families. While SNE and SLY1 are in the C2 family, the auxin F-box TIR1 and the jasmonate F-box COI1 are in the C3 family (18, 40, 41). If *SNE* normally functions in GA signaling, expression of a truncated SNE gene would be expected to cause a GA-insensitive phenotype.

SNE is the founding member of a new family of F-box proteins in plants (Figure 3B). Like the 151 amino acid SLY1 protein, the 157 amino acid SNE protein is small and has an unusually high predicted pI of 10.38. It has recently been shown that both

SLY1 and SNE associate with the same SCF complexes (20). SNE transcript accumulation is developmentally regulated, showing the highest level in stems and flowers. This pattern of mRNA accumulation is similar to that seen in GID2 (M. Matsuoka, personal communication). The SNE family members are similar to, but distinct from, the members of the SLY1 protein family in plants (Figure 3; 13, 19). One of the SNE homologues is a *GID2*-like gene in rice temporarily referred to as OsSNE. The SLY1 amino acid sequence is 30.2% identical/ 41% similar to GID2 and 26% identical/ 39% similar to OsSNE, whereas SNE is 21.3 % identical/ 33.3% similar to GID2 and 44.8% identical/57.4% similar to OsSNE. The existence of SNE homologues in other plant species suggests that the function of SNE may be conserved among higher plants.

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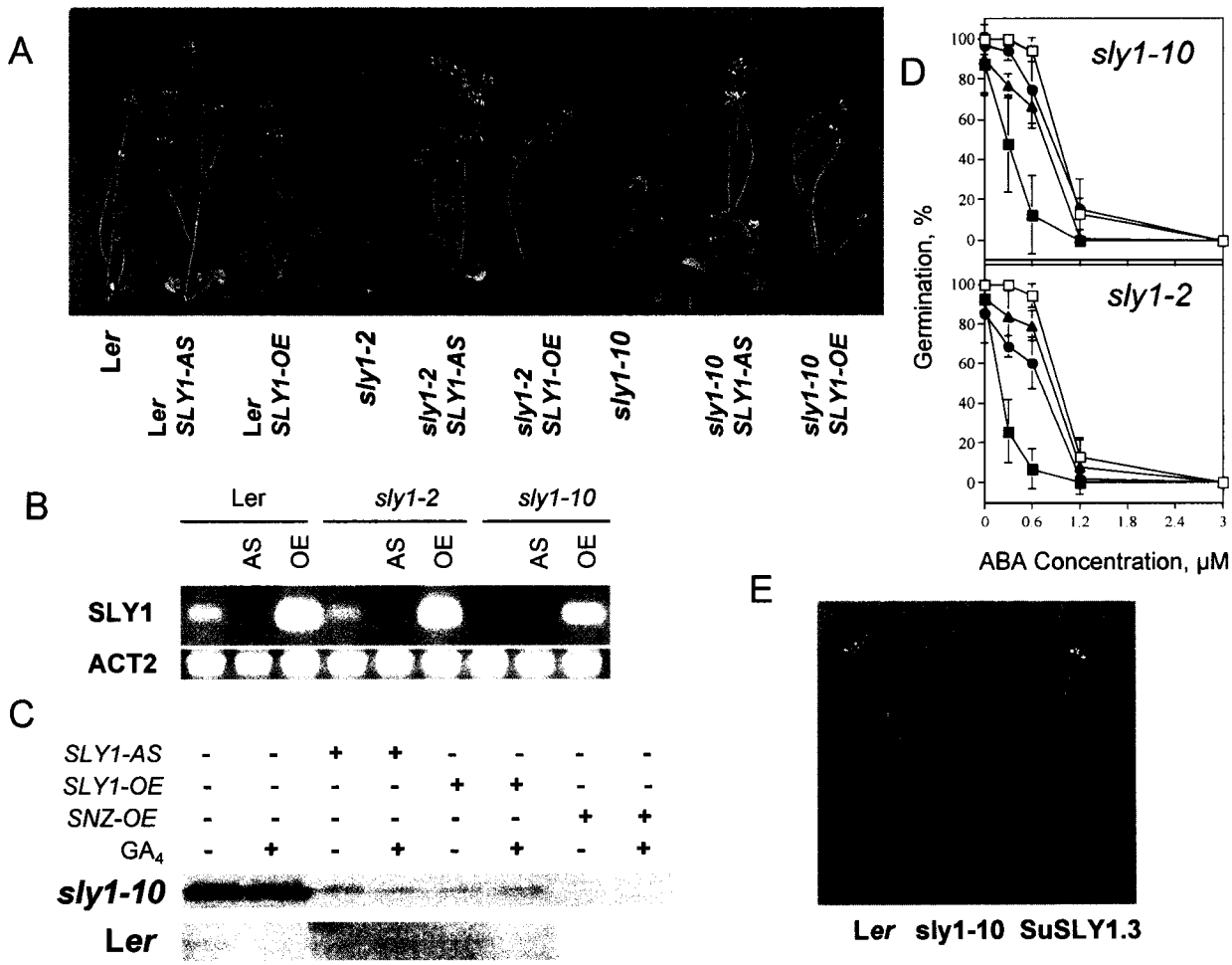

**Fig. 1.** Antisense and overexpression of *SLY1*. (*A*) Photograph of seven week old plants. (*B*) Directional RT-PCR analysis of *SLY1* and *ACT2* mRNA accumulation in antisense and overexpression lines. Primers detect no mRNA in the *sly1-10* negative control, as primers were designed around the rearrangement in this mutant. An ethidium bromide-stained 1.5% agarose gel from RT-PCR using 100 ng of total RNA for each sample is shown. (*C*) Western analysis of crude protein extracts of 10 day old seedlings with (+) or without (-) 2 h $GA_4$ treatment, fractionated on 10% SDS-PAGE, and detected with anti-RGA. Lines shown are L*er* and *sly1-10* transformed with *SLY1-AS*, *SLY1-OE*, *SNE-OE*, or untransformed. (*D*) Percent germination after 5 days on MS plates containing 0, 0.3, 0.6, 1.2, or 3.0 μM ABA for *sly1-10* (upper panel) and *sly1-2* (lower panel) seeds for the following lines: (□) L*er* wild-type control, (■) untransformed *sly1-2* or *sly1-10*, (●) *SLY1-AS* transformant, (▲) *SLY1-OE* transformant. (*E*) Photograph of four week old L*er*, *sly1-10*, and suppressor of *sly1-10* isolate1.3 (*sly1-10* OE *SNE*).

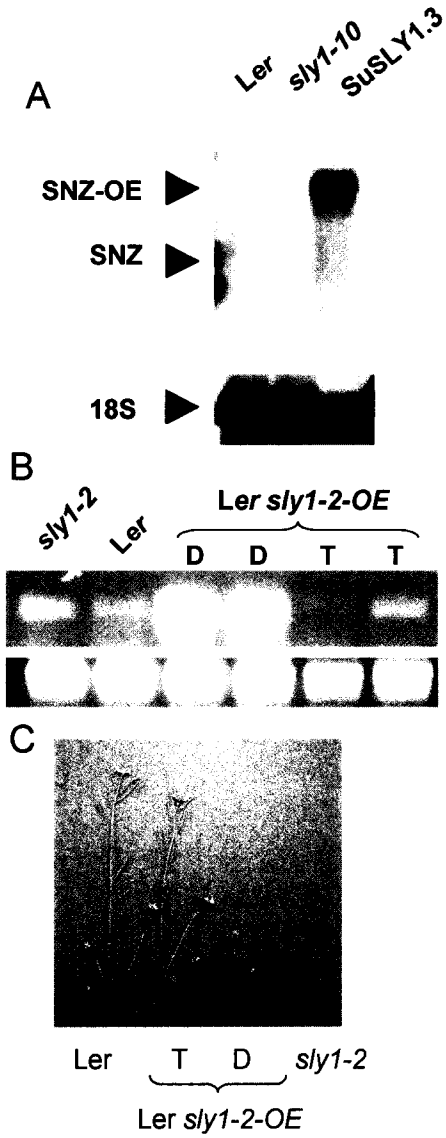


**Fig. 2.** Overexpression of *SNE* and *sly1-2*. (*A*) RNA gel blot analysis of *SNE* mRNA and *18S* rRNA accumulation in L*er*, *sly1-10*, and suppressor of *sly1-10* isolate 1.3 using 15 μg total RNA isolated from total aerial tissue of five week old plants. (*B*) RT-PCR analysis of *SLY1* mRNA in *sly1-2-OE* lines, designated as dwarf (D) or tall (T). *ACT2* mRNA levels were also monitored for a loading control. An ethidium bromide-stained 1.5% agarose gel is shown. (*C*) A photograph of two *sly1-2-OE* lines, derived from a single T1 plant, designated as dwarf (D) or tall (T).

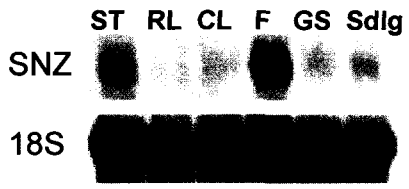


**Fig. 3.** *SNE* developmental mRNA expression. A Northern blot analysis of *SNE* mRNA and *18S* rRNA control accumulation using 15 μg total RNA isolated from wild-type L*er* stems (ST), rosette leaves (RL), cauline leaves (CL), flowers (F), green siliques (GS), and seedlings (Sdlg).

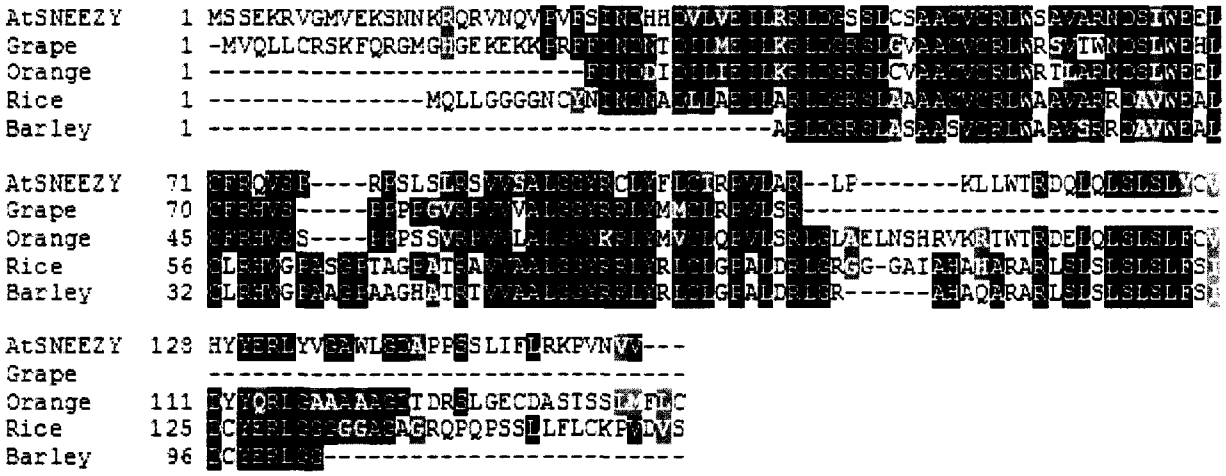

**Fig. 4.** SNE homologues. ClustalW alignment of predicted SNE protein (At5g48170) with plant homologues from grape (CF609441), orange (CK665669), rice (AC116426), and barley (CF609441) is shown. The amino acid sequence is predicted from the largest open reading frame in each sequence.

# CHAPTER FOUR

# ISOLATION OF ABA SIGNALING MUTANTS IN WHEAT

This chapter describes the isolation of wheat ABA-insensitive and wheat ABA-hypersensitive mutants. This manuscript is intended for submission to Genetics, with the following authors: Lucia C. Strader and Camille M. Steber.

ABSTRACT

This paper reports the isolation of wheat mutants insensitive and hypersensitive to the phytohormone Abscisic acid (ABA) in germination. ABA induces dormancy during grain maturation. Wheat is unique in that ABA sensitivity is dependent on the dormancy status of the grain. Wheat grains are highly sensitive to ABA when dormant, but highly ABA insensitive when afterripened. Based on this, ABA insensitive mutants were isolated using dormant grain, while ABA hypersensitive mutants were isolated in afterripened wheat. Eleven ABA-insensitive mutants were identified using EMS-mutagenized cv. Brevor. Two of these mutants were associated with a vegetative cool leaf temperature and wilty phenotypes. Further, 36 ABA hypersensitive mutants were isolated in fast-neutron mutagenized Chinese Spring. These mutants fall into five phenotypic classes based on ABA-dose response in germination. These mutants demonstrate the possibilities of isolating hormone signaling mutants in hexaploid systems.

# INTRODUCTION

Abscisic acid (ABA) is a sesquiterpenoid phytohormone regulating important traits in higher plants (Leung and Giraudat 1998). ABA is needed to induce seed dormancy embryo maturation, and to induce stress responses to drought, cold, and salt. ABA induces drought tolerance by a number of mechanisms including induction of stomatal closure, stimulation of root growth, and induction of gene expression. Mutants causing decreased ABA biosynthesis show decreased seed dormancy and a vegetative wilty phenotype. While ABA-insensitive mutants can show decreased seed dormancy and a vegetative wilty phenotype, ABA hypersensitive mutants can display increased seed dormancy and increased drought tolerance. This study explores the role of ABA in controlling responses to the environment, especially seed dormancy and germination, by identifying mutants with altered ABA response in wheat. These wheat ABA response mutants should aid in both the control of preharvest sprouting associated with insufficient grain dormancy and in the improvement of abiotic stress response by enhancing our understanding of ABA signaling in this species.

Hexaploid bread wheat (*Triticum aestivum* L., $2n = 6x = 42$, genomes AABBDD) is a genetically complex organism, due to high genetic redundancy and large genome size. Wheat arose from the convergence of three progenitor species, *Triticum urartu* (*T. monococcum*), *Aegilops speltoides*, and *Ae. tauschii* (Galili *et al.* 2000; Jiang and Gill 1994). Because bread wheat is an allohexaploid, many genes are present in multiple copies, making genetic studies challenging. Wheat has a large genome at 16 billion bp (Arumuganthan and Earle 1991) with an average ratio of 4.4 Mb / cM (Faris and Gill 2002). In spite of these challenges, mutant screens in wheat have successfully identified

mutants displaying herbicide resistance (Newhouse *et al.* 1992), altered testa color (Warner *et al.* 2000), and resistance to such diseases as powdery mildew (Kinane and Jones 2001), leaf rust and stem rust (Williams *et al.* 1992; Friebe *et al.* 1994, Kerber and Aung 1995), and yellow and brown rust (Boyd *et al.* 2002). Moreover, the disease resistance mutation identified by Kerber 1991 was recessive. These successful mutant screens encouraged us to screen for ABA response mutants in hexaploid wheat.

Mutant studies have identified ABA signal transduction genes in a number of plant species (reviewed in detail by Finkelstein and Rock 2002). Two classes of ABA response mutants have been identified based on altered response to exogenous ABA during germination. ABA-insensitive (abi) mutants have been identified in *Arabidopsis* based on the ability to germinate on concentrations of ABA normally inhibitory to wild-type germination. Some ABA mutants identified in this way, such as the semi-dominant *abi1-1* and *abi2-1*, cause both nondormant seed and vegetative wilty phenotypes (Koornneef *et al.* 1984). Further, *abi1* and *abi2* mutants have leaf temperatures 1° cooler than wild-type, due to cooling from open stomates and increased transpiration (Merlot *et al.* 2002). Other ABA-insensitive mutants result in a nondormant seed phenotype, but have no detectable effect on vegetative water relations (Finkelstein and Somerville 1990). Examples of this type include *ABI3*, an orthologue of maize *VP1* (*VIVIPAROUS1*; Bies-Etheve *et al.* 1999; Giraudat *et al.* 1992), *ABI4*, and *ABI5* (Finkelstein and Lynch 2000; Finkelstein *et al.* 1998). Additionally, a number of screens have identified mutants showing ABA hypersensitivity. The ABA hypersensitive 1 gene (*ABH1*) and enhanced response to ABA (*ERA1* and *ERA3* / *EIN2*) genes were identified as mutants that fail to germinate on ABA concentrations permissive for wild-type germination (Hugouvieux *et*

*al.* 2001; Cutler *et al.* 1996; Ghassemian *et al.* 2000). Both the *era1* and *abh1* mutants have increased drought resistance. These experiments suggest that ABA hypersensitive mutations can be used to enhance the drought tolerance of crop plants. Other examples of ABA hypersensitive mutants include the inositol polyphosphate-1-phosphatase FIERY1 (FRY1; Xiong *et al.* 2001) and the dsRNA binding protein HYL1 (HYPONASTIC LEAVES1; Lu and Fedoroff 2000). Isolating mutants such as these could be quite helpful in developing a greater understanding of ABA's influence on agronomic traits of wheat.

Drought tolerance and grain dormancy are important agronomic traits regulated by ABA. ABA mediates stomatal closure (Maser *et al.* 2003) and has a role in drought tolerance that has not been precisely defined. Though there are ABA-independent components contributing to drought tolerance, microarray studies have revealed that over half of the drought-induced genes are also ABA-induced (Seki *et al.* 2002a; Seki *et al.* 2002b). ABA induces seed dormancy and desiccation tolerance during embryo maturation. Dormant seed will not germinate, even under favorable germination conditions. Generally, dormant seed becomes nondormant with afterripening. The required period of afterripening can range from 2 weeks in *Arabidopsis* (van der Schaar *et al.* 1997) to 60 months in *Rumex crispus* (Cavers and Harper 1966).

The connection between ABA response and grain dormancy is important in wheat because of its relevance to the phenomenon of preharvest sprouting (PHS). PHS occurs when nondormant mature grain germinates while still attached to the mother plant. This generally occurs under cool, moist conditions. The degree of dormancy and length of afterripening required for wheat grain germination is variable and cultivar-dependent,

ranging from highly dormant cultivars requiring six months afterripening to slightly dormant cultivars requiring only a few days afterripening. The tendency to undergo preharvest sprouting has been correlated with white kernel color (Groos *et al.* 2002) and with decreased sensitivity to ABA (Walker-Simmons 1987). Although testa color does affect dormancy and preharvest sprouting tendencies, it is not the sole determining factor. There are red wheat varieties that still suffer preharvest sprouting under cool moist growing conditions in the United Kingdom (Flintham and Gale 1988). Warner *et al* (2000) found that red testa color enhances, but is not fully responsible for grain dormancy using sodium azide generated mutants in the *R1* gene locus of Chinese Spring. The naturally occurring Phs (Preharvest sprouting) gene is a major determinant of the dormancy difference between red and white wheat that exerts its influence through the embryo rather than through testa color (Flintham 2000). In white wheat, resistance to preharvest sprouting was found to strongly correlate with sensitivity to ABA (Walker-Simmons 1987). Thus it is important to identify ABA response genes in wheat. The wheat homologue of maize VP1 has been found to control preharvest sprouting independent from testa color (McKibbin *et al.* 2002). As VP1 is a transcription factor needed for ABA response in the grain (McCarty *et al.* 1991), this clearly implicates ABA in controlling resistance to preharvest sprouting. ABA responsiveness has been shown to positively correlate with resistance to preharvest sprouting (Morris *et al.* 1989; Walker-Simmons 1987). Thus, it should be possible to isolate ABA response and dormancy mutants that are not associated with testa color.

Here we report the recovery of ABA insensitive and ABA hypersensitive mutants in hexaploid wheat, based on the unique relationship between wheat dormancy and wheat

ABA sensitivity. Eleven ABA insensitive and 39 ABA hypersensitive mutants were isolated in this study. Isolates were characterized based on ABA dose response and vegetative phenotypes.

## MATERIALS AND METHODS

**Plant material:** The *Triticum aestivum* L. cultivar Chinese Spring (doubled haploid line Dv418 constructed by Jan Dvorak), a red spring wheat, was used as the background for all ABA hypersensitive studies. For the ABA insensitive studies, soft white wheat cultivar Brevor was used.

**Growth conditions and germination experiments:** Wheat was either field-grown in eastern Washington (Spillman Farm, Pullman, WA) or grown in a greenhouse, as indicated. For greenhouse-grown plants, growth conditions were 16h 18-22° day and 15-17° night. Supplemental high intensity sodium vapor lighting was used during winter months. Brevor plants were vernalized at the four-leaf stage for six weeks at 4° under dim lighting. For all wheat germination assays, grains were grown and harvested at the same time and were hand-threshed and either stored at 20-25° to afterripen, or stored at -20° to preserve dormancy. Grain was bisected, separating embryo from the bulk of the endosperm, for embryo germination tests. Both embryos and whole grains (caryopses) were plated in a 9 cm Petri plate lined with a single germination disc wetted with 6 mL treatment solution. Plates were lidded, sealed with Parafilm and incubated at 30° in the dark for the desired length of time. All treatment solutions were buffered to pH 5.5 with 5 mM 2-[*N*-morpholino] ethane sulfonic acid (MES; Sigma). For *Arabidopsis* germination assays, seeds from a single seedlot were harvested and stored at -20° or 23°

for 3.5 months to preserve dormancy or to afterripen, respectively. *Arabidopsis* experiments used seeds surface sterilized in 20% bleach / 0.1% SDS for 15 min, followed by four to six washes with sterile water. Seeds were plated on 0.8% agar plates containing 0.5x Murashige and Skoog basal salt mixture (Sigma) and buffered to pH 5.5 with 5 mM MES. Abscisic acid stock solutions were in DMSO and was added to autoclaved media cooled to ~55°. Seeds were imbibed on plates at 4° for 4 days to encourage synchronous germination, and then moved to lights at 22°. Percent germination was determined after 5 days under lights. Seeds were scored as germinated upon radicle emergence.

**Screen for hypersensitive ABA response:** Chinese Spring Dv418 grain were mutagenized at 4 gray with fast neutron (gift from R. Warner) (Redei and Koncz 1992). The mutagenized grain ($M_1$) were field grown and the progeny ($M_2$) resulting from self-fertilization of the $M_1$ plants were hand-harvested. Only one head from each $M_1$ plant was harvested, with heads left intact to maximize the number of alleles known to be independent.

To screen for ABA hypersensitivity, grain was afterripened at room temperature for 2 years before screening. Grains were screened by pools, with each pool representing progeny from 10 $M_1$ plants. Four $M_2$ grains from each $M_1$ head in a pool were removed by hand and plated in nine cm Petri plates containing a single germination disc wetted with 6 mL 5 μM (+)ABA (gift from S. Abrams). Plates were then sealed with Parafilm and incubated at 30° in the dark. After 96h, ungerminated grains were transferred to new Petri plates containing a single germination disc wetted with 6 mL 10 μM $GA_3$ to encourage germination. Grains that did not germinate after 72h on $GA_3$ were considered

inviable. Those grains that germinated after 72h on $GA_3$ were transferred to soil and grown to maturity in the greenhouse. The resulting $M_3$ grain was afterripened for 6 months, after which time Chinese Spring is nondormant, and retested for ABA hypersensitivity by plating on 5 μM (+)-ABA. All isolates from a single pool were considered to be potential siblings.

**Screen for ABA insensitivity:** Approximately 1500 Brevor grains were presoaked in 200 mL 50 mM phosphate buffer (pH 7.0) for six hours. Grain was then EMS mutagenized in 200 mL 0.3% (v/v) ethyl methane sulfate (EMS; Sigma) in 50 mM phosphate buffer (pH 7.0) with shaking for 16 hours at 23°. EMS was neutralized with an equal volume of 10% (w/v) sodium thiosulfate and washed 10 times over a five hour time period to remove residual EMS. The mutagenized grain ($M_1$) was grown under standard greenhouse conditions (in growth conditions section). A single head from each $M_1$ plant was hand-harvested, with heads kept intact to maximize the number of alleles known to be independent. Grain was stored at -20° to preserve dormancy.

To screen for an ABA insensitive response, dormant grains were screened by pools, with each pool representing progeny from 10 $M_1$ plants. Four $M_2$ grain from each $M_1$ head in a pool were removed by hand and plated in nine cm Petri plates containing a single germination disc wetted with 6 mL 5 μM (+)-ABA (gift from S. Abrams). Plates were then sealed with Parafilm and incubated at 30° in the dark. After 48h, germinated grain were transferred to soil and grown to maturity in the greenhouse. The resulting $M_3$ grain was retested for ABA insensitivity by plating on 5 μM (+) ABA. All isolates from a single pool were considered to be potential siblings.

**Temperature measurements:** Flag leaf temperature was measured for greenhouse grown Brevor and ABA-insensitive mutants. Temperature was determined 1 inch from the collar of the leaf using either an infrared camera (FLIR) or an infrared thermometer (VWR). For each type of measurement, emissivity was set to 1.0 (Merlot *et al.*. 2002). Infrared thermometer measurements were performed after sunset to reduce temperature fluctuations. Temperatures of all available flag leaves from each plant were measured, with Brevor temperatures measured every third plant to compensate for changing greenhouse temperatures.

## RESULTS

**ABA response depends on grain dormancy status:** Previous work in wheat suggested that dormancy status determines degree of ABA sensitivity in wheat. Morris *et al.* (1989) found that in cultivars Clark's Cream and Parker 76, ABA responsiveness of caryopses and embryos correlated with dormancy status. To determine if this is generally true for all wheat cultivars, we examined four wheat cultivars for their ABA responsiveness in the dormant and afterripened state. The four cultivars examined were Brevor (soft white winter), Chinese Spring (red spring), Zak (soft white spring), and Scarlet (hard red spring). Here the results for the cultivar Brevor are shown (Figure 1A and 1B). Once wheat grain has been afterripened, it becomes insensitive to even very high levels of ABA, germinating readily in ABA concentrations of over 100 μM (Figure 1A). Dormant whole grain will not germinate for quite a long period of time, while afterripened whole grains germinate within 24 hours, regardless of ABA concentration

(not shown). Figure 1A shows percentage germination of dormant versus afterripened whole grain at different ABA concentrations. Dormant grain failed to germinate regardless of ABA concentration, while afterripened whole grain showed 100% germination at all concentrations tested including 100 μM ABA.

Since dormant whole wheat grains normally fail to germinate even in the absence of ABA (Fig 1A), ABA sensitivity is usually measured by stimulating germination by bisection (Walker-Simmons 1987). In this process the embryo half of the grain is placed on filter paper soaked with the treatment solution. This promotes germination of dormant embryos, allowing one to measure inhibition of germination by ABA. Embryos from afterripened grain show 100% germination even in the presence of high ABA levels (Figure 1B). Embryos from dormant grain are subject to dose-dependent inhibition of germination by ABA (Figure 1B). Dormant embryos germinate to approximately 85% in the absence of ABA. Germination is slightly inhibited by imbibition in 0.1 μM ABA, reaching 60% germination after 72h incubation. Germination is diminished to 10% at 5 μM ABA and embryos fail to germinate at 50 or 100 μM ABA (Figure 1B). Similar results were obtained in cultivars Chinese Spring, Zak, and Scarlet; however, the depth of dormancy and ABA sensitivity for these cultivars was less than that of Brevor, hence slightly higher ABA concentrations were needed to completely inhibit germination (data not shown).

We also examined the effect of seed dormancy on ABA response in *Arabidopsis* germination by comparing the ABA dose-response of dormant and afterripened seeds. Although there were slight differences in the rate of germination on ABA in dormant versus afterripened *Arabidopsis* seed (not shown), overall, the final percent germination

on ABA was similar (Figure 1C). Unlike wheat, *Arabidopsis* does not lose ABA sensitivity with afterripening. After 5d of incubation in the presence of 0, 0.3, 0.6, 1.2, 3.0, or 10.0 μM (+/-) ABA, germination of dormant *Arabidopsis* ecotype Landsberg *erecta* appears to be identical to the germination of afterripened seed from the same seed lot. On 0 and 0.3 μM ABA, seeds germinated to 100%. Germination reached 85% on 0.6 μM ABA. On 1.2 μM ABA, germination was severely inhibited with little to no germination observed at 3.0 and 10.0 μM ABA (Figure 1C). Similar results were obtained with *Arabidopsis* ecotype Columbia (data not shown).

The exceptional dependence of wheat ABA response on dormancy status allowed the design of grain dormancy dependent screens for ABA response mutants. Dormant grain was used in the screen for ABA insensitivity, as wild-type grain is unable to germinate in the presence of ABA when dormant. Afterripened grain was used in the screen for ABA hypersensitivity, because wild-type grain germinates readily in the presence of ABA when afterripened. It should be noted that these screens can detect both mutants with altered ABA response and mutants with altered dormancy characteristics.

**Isolation of ABA-insensitive mutants:** ABA insensitive mutants were isolated in dormant $M_2$ EMS-mutagenized Brevor grain. The Brevor background was used for this screen because it is a white wheat with good grain dormancy, allowing us to focus on testa color-independent germination controls. As shown in Figure 1A, whole dormant grain does not germinate in the presence of ABA. We screened for mutations allowing dormant grain to germinate in the presence of 5 μM (+)-ABA. ABA-insensitive candidates were those dormant grains that germinated following 48h incubation on 5 μM (+)-ABA at 30° in the dark (see diagram, Figure 2). A total of 31 ABA-insensitive

candidates were isolated from 7,320 dormant $M_2$ grain. Of these 31 candidates, 14 died and four were infertile. Of the remaining 17 dormant $M_3$ progeny, 11 retested for ABA insensitive germination on 5 μM ABA. These mutants appeared similar to wild-type Brevor in plant height, time to flowering, and leaf color.

**ABA-insensitive mutants were associated with a wilty phenotype:** The *Arabidopsis* ABA-insensitive mutants *abi1-1* and *abi2-1* are associated with a vegetative wilty phenotype. Two independent wheat ABA-insensitive mutants, 144-6A and 144-122, show a wilty phenotype under mild drought conditions. Previous studies have shown that the wilty ABA-insensitive mutants of *Arabidopsis* exhibit leaf temperatures 1° cooler than those of wild-type using an infrared camera (Merlot *et al.* 2002). ABA insensitivity results in more open stomates. The resulting increase in transpiration rate has a cooling effect. We measured the temperature of flag leaves of wheat ABA insensitive mutants to determine if our wilty mutants behaved similarly. The flag leaf of isolates 144-6A and 144-122 were one to three degrees cooler than wild-type Brevor, as measured with an infrared thermometer and infrared camera (Table 1, Figure 3).

Further characterization of ABA-insensitive mutants revealed that many exhibited altered ABA dose-response in germination (Figure 4). Figure 4 shows the germination percentage of dormant embryos (half grains) from Brevor and four ABA-insensitive lines on increasing concentrations of ABA after 72 hours of incubation. Isolate 144-6A demonstrates higher levels of germination than wild-type at all ABA concentrations, while isolates 144-23A, 144-29A, and 144-51A only show higher levels of germination than wild-type at 10 to 25 μM ABA. These four mutants show higher levels of

germination on ABA than wild-type at all concentrations, confirming that they are indeed more ABA insensitive.

**Isolation of ABA hypersensitive mutants in wheat:** Fully afterripened fast-neutron-mutagenized $M_2$ Chinese Spring grain was employed to identify mutants with increased sensitivity to exogenous ABA in germination. We screened for mutations that hindered germination of grain incubated on 5 μM (+)-ABA for 96 hours at 30° in the dark. Grains that failed to germinate were treated with $GA_3$ to promote germination (schematic shown in Figure 5). Of the 89 ABA-hypersensitive candidates recovered from the screen of 22,250 $M_2$ grain, 44 either died or were infertile. Following afterripening, 39 of the remaining 45 $M_3$ progeny retested for failure to germinate on 5 μM ABA. Five mutants that retested for lack of germination appeared to be dark green dwarves and are most likely GA mutants. An example of a mutant with a dwarf phenotype, isolate 1314-45, is shown in Figure 6. Other dwarf isolates include 910-22, 1314-76, 910-69, and 78-69.

**Analysis of ABA dose-response in ABA-hypersensitive mutants:** ABA dose-response curves were performed on dormant embryos (half grains) of ABA-hypersensitive mutants for comparison to wild-type ABA sensitivity. Although the initial mutant isolation was performed on afterripened grain, dormant embryos were used in this experiment to establish a baseline for wild-type. Had these tests been performed on afterripened embryos, Chinese Spring would have had no response to ABA, making this comparison difficult. ABA dose-response germination curves were determined for most of the ABA-hypersensitive isolates and for wild-type Chinese Spring (Figure 7A). Mutants that were not tested had low seed set due to infertility and therefore insufficient

grain to perform the tests. Those lines not tested were 78-69, 910-69, 910-84A, and 910-84B. The ABA dose response curves of dormant embryos were divided into 5 phenotypic classes (Table 1). The first class resembled wild-type ABA dose-response. Dormant embryos from both Chinese Spring and Class 1 mutants germinate readily after 48h in the absence of ABA. Increasing ABA concentrations decreased germination, with a 25 μM ABA treatment yielding approximately 40% germination (Figure 7A and B). Interestingly, these mutants show a hypersensitive response to ABA as afterripened whole grain. Class 2 mutants exhibited wild-type germination in the absence of ABA, increased ABA sensitivity at early timepoints, and germination increasing later to appear more wild-type. An example of this pattern of germination is shown in Figure 7C. Class 1 and Class 2 mutants were less severe in their ABA dose responsiveness than the other classes.

Mutant embryos in classes 3-5 exhibited more dramatic increases in ABA sensitivity. Class 3 mutants showed wild-type germination in the absence of ABA, but increased ABA sensitivity at all timepoints. An example of this pattern of germination is shown in Figure 7D. These more classic ABA hypersensitive mutants include the dwarf isolates 1314-45, 910-22, 1314-76. Class 4 and Class 5 mutants showed reduced germination of cut grain in the absence of ABA, with germination vastly impaired by ABA application. Class 4 mutants 1314-28A and 78-68 recovered to 100% germination of both cut and whole grain in the absence of ABA after two years of afterripening (Figure 7E). The single Class 5 mutant, 1314-16 showed reduced germination of both whole and cut grain even after 3 years of afterripening (Figure 7F and 8).

## DISCUSSION

This paper describes the isolation of 11 independent ABA-insensitive germination mutants, and 36 independent ABA-hypersensitive germination mutants. These screens are unique because they may identify not only mutations resulting in altered ABA response, but also in altered grain dormancy and afterripening. For example, apparent ABA-insensitive mutants might result from more rapid afterripening, while apparent ABA-hypersensitive mutants might result from a defect in afterripening. Thus the genetic analysis, cloning, and characterization of the genes identified in this study should enhance our understanding of ABA signaling and grain dormancy in wheat.

Although this study refers to mutants with increased sensitivity to exogenous ABA as ABA hypersensitive mutants, it is important to remember that apparent ABA hypersensitivity can be caused by a number of mechanisms other than altered ABA signaling. Apparent increased ABA sensitivity could result from increased endogenous ABA levels due to either increased biosynthesis or decreased catabolism. In future studies, this possibility can be explored by measuring the endogenous levels of ABA. Mutants with decreased GA signaling like Arabidopsis *sly1-2* show increased sensitivity to ABA in germination (Steber *et al.* 1998). Indeed, five of the mutants isolated in this screen exhibited the classic GA mutant phenotypes of dwarfism and dark green leaves. Further studies will examine the GA responsiveness of these five mutants. In addition, mutants with decreased brassinosteroid signaling, *det2-1* and *bri1-1*, are hypersensitive to ABA in germination (Steber and McCourt 2001). The apparent ABA insensitive mutants could be the result of reduced dormancy or even an enhanced response to GA (Steber *et*

*al.* 1998). It will be important in the further characterization of wheat ABA response mutants to explore these other possibilities.

In addition to hormone signaling, the mutants recovered might be the result of defects in dormancy and afterripening. Dormant embryos of genuine ABA hypersensitive mutants should show complete germination in the absence of ABA and hypersensitivity on all concentrations of ABA. This description fits the ABA dose-repsonse of Class 3 embryos. Mutant embryos that fail to afterripen would have wild-type ABA sensitivity when dormant, but appear ABA hypersensitive following six months of afterripening due to failure to lose dormancy. In light of this, the Class 1 mutants are good candidates for afterripening mutants. These mutants failed to germinate in the presence of 5 μM (+) ABA when afterripened, and were presumed to be ABA hypersensitive. However, the dormant embryos from these mutants show no greater response to ABA than wild-type. Thus it is possible that Class 1 mutants have prolonged dormancy. This hypothesis can be tested by careful comparision of ABA response in embryos and whole grain before and after afterripening. Normally, wheat grain dormancy is broken by cutting the testa. Interestingly, Class 4 and Class 5 mutants appear to be insensitive to the dormancy breaking effect of cutting the grain. Embryos from these grains show reduced germination even in the absence of ABA. This insensitivity to seed cutting is relieved by two years of afterripening in Class 4, but not in Class 5.

The mutants described here will further our understanding of the ABA-dependent and ABA-independent control of preharvest sprouting. During PHS, hydrolytic enzymes are activated and endosperm constituents are broken down, resulting in lower yield and

diminished quality. PHS thus lowers the grade of wheat grain from bread-quality to feed-quality, resulting in a loss of profit for the farmer (reviewed by Wahl and O'Rourke 1993). Susceptibility to preharvest sprouting and seed dormancy have an inverse relationship. In addition, ABA insensitivity has been linked to PHS in wheat (Walker-Simmons 1987). Mutants from this screen provide promising germplasm for the control of PHS. It should be possible to identify mutants that afterripen more rapidly, but are not susceptible to PHS. For example, mutants that appear to be ABA insensitive after only two weeks of afterripening would be ideal. Future screens may use modern cultivars so that this work may be more directly relevant to wheat improvement.

The characterization of the mutants identified here has provided some clues to the nature of the genes involved. The only ABA-insensitive *Arabidopsis* mutants with showing both ABA insensitivity in germination and a vegetative wilty phenotype are the semi-dominant mutations in the PP2C genes *abi1* and *abi2* (Leung *et al.* 1994; Leung *et al.* 1997). The possibility that the wilty ABA-insensitive wheat mutants 144-122 and 144-6A are homologues of *Arabidopsis ABI1* or *ABI2* must be investigated. The fact that 144-122 and 144-6A have a vegetative wilty phenotype indicates that wheat ABA response mutants can alter the regulation of water relations. Thus it will be important to determine whether any of the ABA-hypersensitive mutants identified here result in increased drought tolerance. Such mutants could potentially correspond to the recessive Arabidopsis *era1* and *abh1* mutants (Pei *et al.* 1998, Hugovieux *et al.* 2001).

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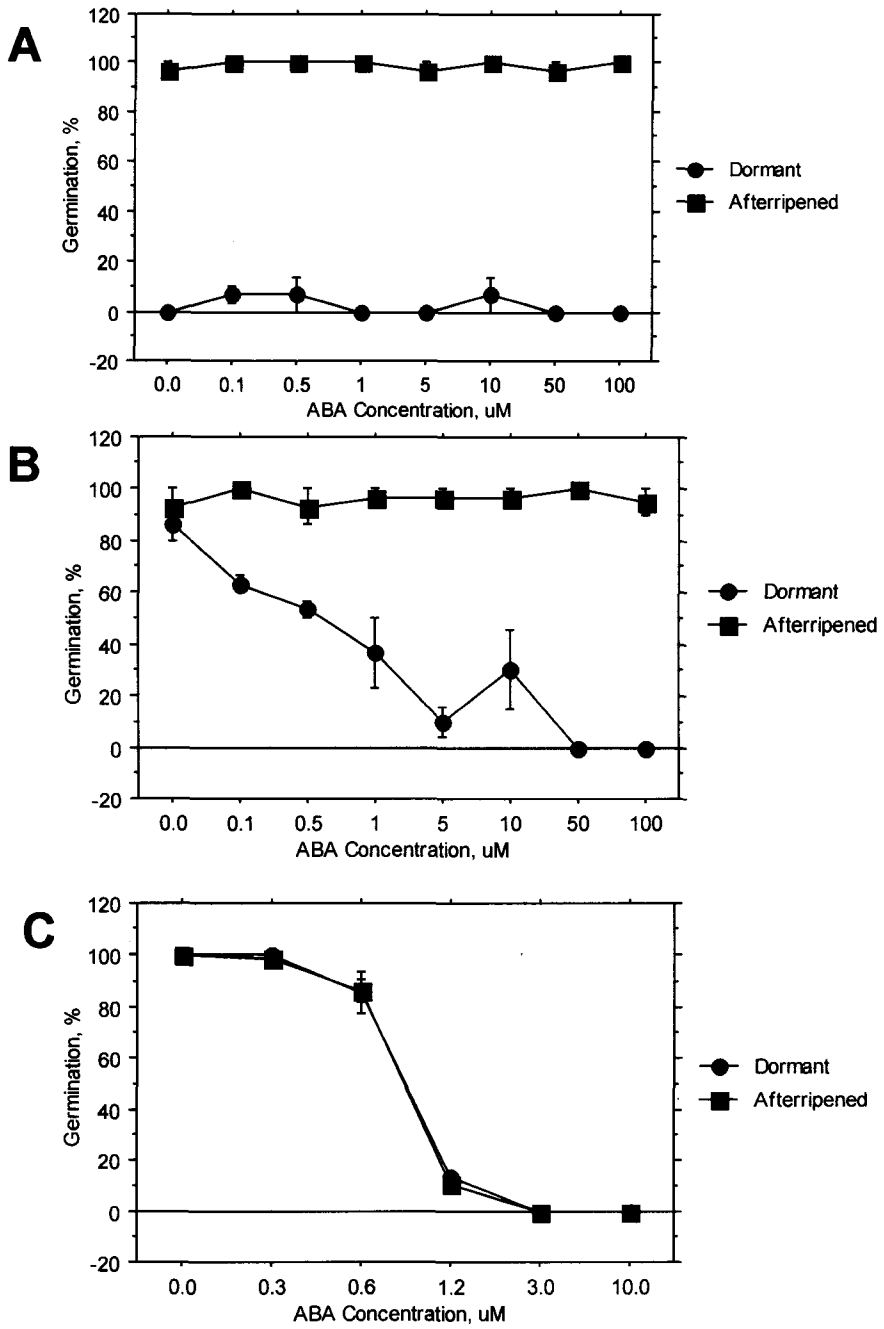


**Figure 1.** Percent germination on varying concentrations of ABA. (A) Percent germination at 72h of dormant and afterripened intact wheat caryposes on (+)-ABA. (B) Percent germination at 72h of dormant and afterripened wheat embryos on (+)-ABA. (C) Percent germination at 120h of dormant and afterripened *Arabidopsis* seed on (+/-)-ABA.

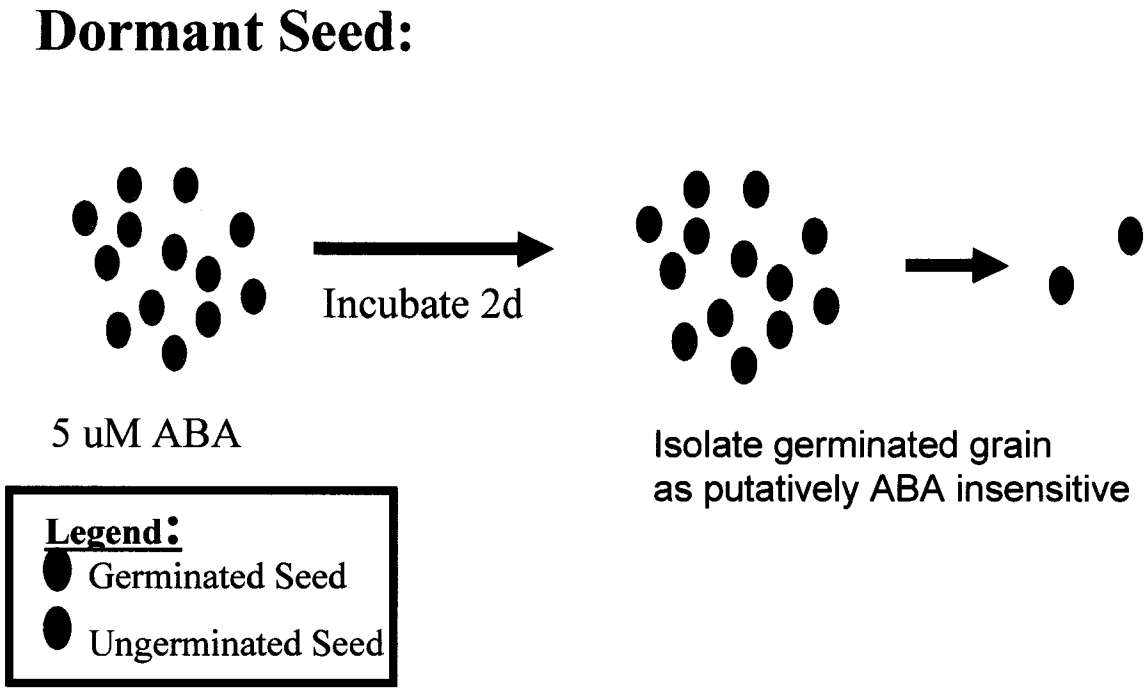


**Figure 2.** Schematic for wheat ABA insensitive screen. Intact grain was incubated 48h in 5 μM (+)-ABA. Grains that had germinated at this time were isolated as putatively ABA-insensitive.

| Isolate | Average | SE | n |
|---|---|---|---|
| Brevor | 0 | ± 0.05 | 30 |
| 144-6A | -1.49 | ± 0.48 | 18 |
| 144-23A | 0.48 | ± 0.27 | 14 |
| 144-25 | -0.13 | ± 0.24 | 30 |
| 144-29A | -.51 | ± 0.30 | 16 |
| 144-51A | 0.26 | ± 0.23 | 17 |
| 144-79 | 0.28 | ± 0.23 | 11 |
| 144-122 | -3.2 | na | 1 |

**Table 1.** Flag leaf temperature measurements of ABA insensitive mutants.

Temperatures are shown as difference from wild-type ± SE.

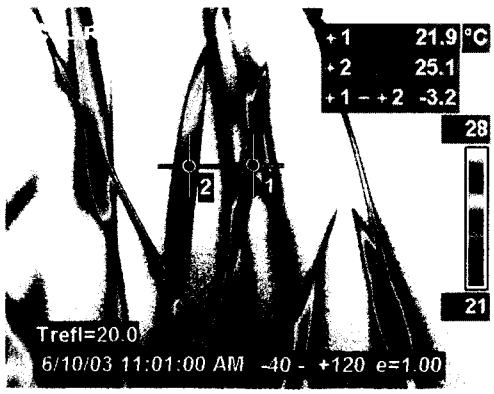


**Figure 3.** Isolate 144-122 exhibits a cool leaf temperature. Infrared measurement and photograph of 144-122 (leaf 1) and wild-type Chinese Spring (leaf 2). 144-122 had a flag leaf temperature of 21.9°, while Chinese Spring had a flag leaf temperature of 25.1°. Emissivity was set to 1.0 for these measurements.

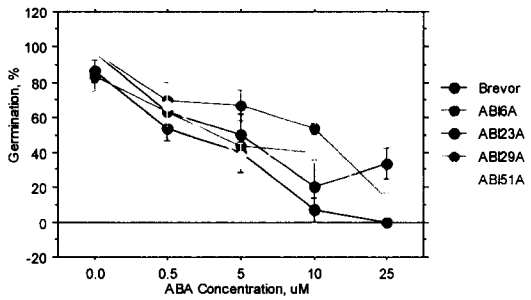


**Figure 4.** Germination of wild-type Brevor and 4 ABA insensitive lines on increasing concentrations of ABA for 72h.

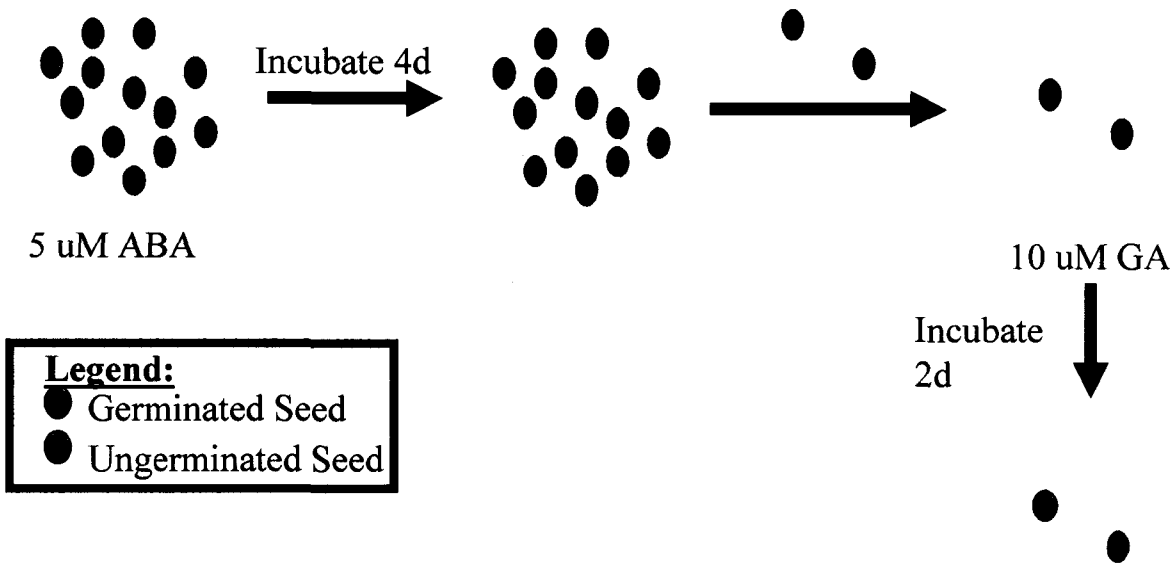


**Figure 5.** Schematic for wheat ABA hypersensitive screen. Intact grain was incubated on 5 μM (+)-ABA for 96h. Those grain that did not germinate at this time were isolated as putatively ABA hypersensitive and placed on 10 μM $GA_3$ for 48h to encourage germination.

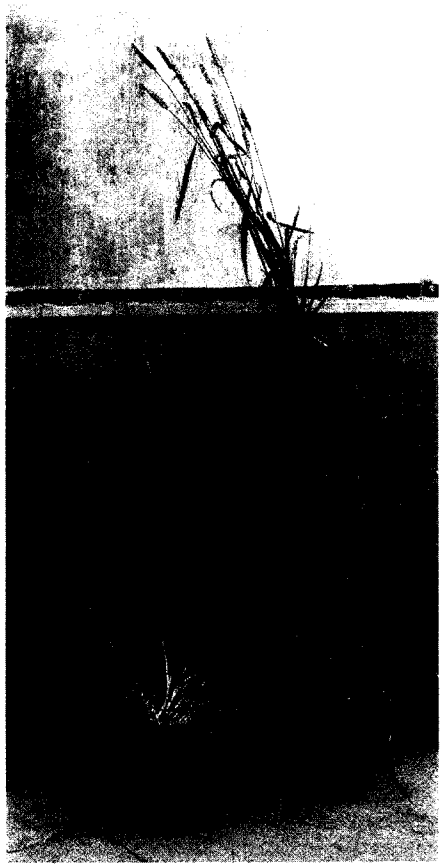

**Figure 6.** Photograph of the dwarf ABA hypersensitive line 1314-45 (left) and Chinese Spring (right).

| Mutant Group | ABA-hypersensitive Mutants | Dormant ABA Dose-response Phenotype |
|---|---|---|
| 1 | 1314-26A, 910-55A, 46-17, 1314-26B, 1314-35, 1314-1, 78-112, 1314-115C | Wild-type |
| 2 | 1314-93, 1314-34, 78-7, 78-39, 1314-42 | Increased ABA sensitivity early, germination recovers later |
| 3 | 1314-82A, 1314-76, 1314-130, 1314-45,1314-64, 910-22, 78-15, 910-13, 1314-46 | Increased ABA sensitivity at all timepoints |
| 4 | 1314-28A, 78-68 | Reduced germination in absence of ABA; Afterripened in two years |
| 5 | 1314-16 | Reduced germination in absence of ABA; Not afterripened in three years |

**Table 2.** Classification of ABA hypersensitive mutants based on ABA dose reponse curve data (examples in Figure 7).

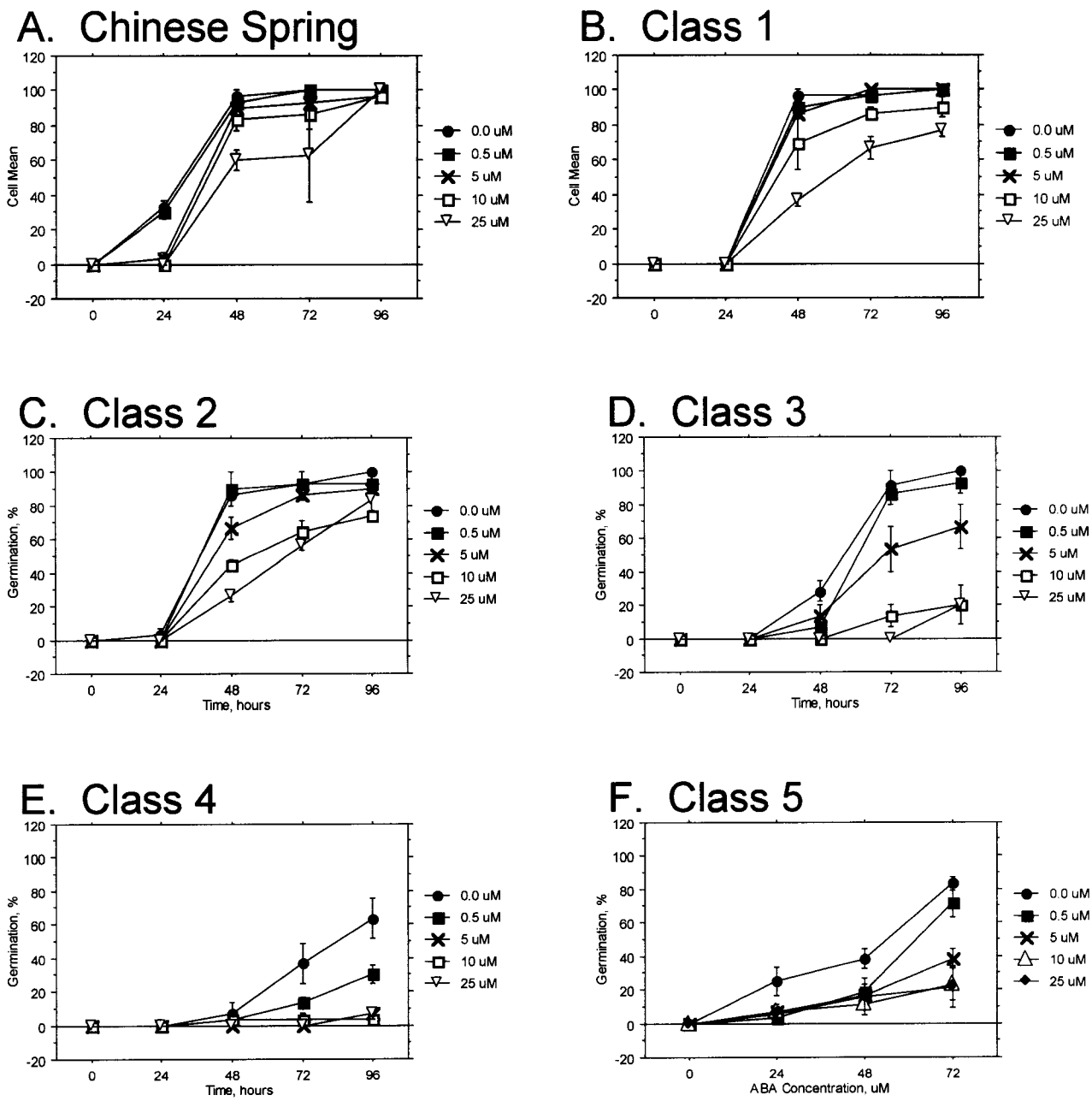

**Figure 7.** Examples of ABA dose response curves for each class of ABA hypersensitive mutant. (A) Percent germination over time in Chinese Spring with increasing concentrations of (+)-ABA. (B) Percent germination over time of line 910-55A is shown as an example of Class 1 ABA response. (C) Percent germination over time of line 1314-34 is shown as an example of Class 2 ABA response. (D) Percent germination over time of line 910-13 is shown as an example of Class 3 ABA response. (E) Percent germination over time of line 78-68 is shown as an example of Class 4 ABA response. (F) Percent germination over time of line 1314-16 is shown as an example of Class 5 ABA response.

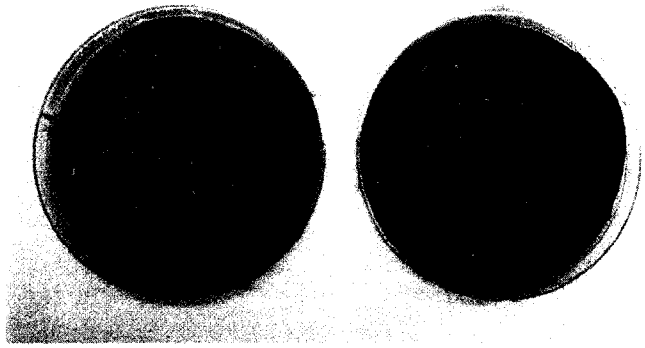

**Figure 8.** Photograph of Chinese Spring (left) and line 1314-16 (right) germination after 120 hours of incubation on 5 μM (+)-ABA.

# APPENDIX

**2003 Congress on *In Vitro* Biology Symposium on Transgenic Cereals: Mutation- and transposon-based approaches to identification of genes for pre-harvest sprouting in wheat**

Lucia C. Strader, Janice M. Zale, and Camille M. Steber

SUMMARY

This article reviews techniques for gene identification and cloning in allohexaploid bread wheat (*Triticum aestivum* L.). Gene identification and cloning in wheat are complicated by the large size and high redundancy of the genome. Both classical mutagenesis and transposon tagging are important tools for the study of grain dormancy and plant hormone signaling in wheat. While classical mutagenesis can be used to identify wheat mutants with altered hormone sensitivity, it can be difficult to clone the corresponding genes. We review the techniques available for gene identification in wheat, and propose that transposon-based activation tagging will be an important tool for wheat genetics.

WHEAT GENETICS

Bread wheat (*Triticum aestivum* L.) is an allohexaploid that arose from the convergence of three diploid progenitor species. The A genome originated from *Triticum urartu* (*T. monococcum*), the B genome from *Aegilops speltoides*, and the D genome from *Ae. tauschii* (Galili *et al.*, 2000; Jiang and Gill, 1994). The haploid number of wheat is 21, with seven chromosomes from each progenitor. Wheat has one of the largest genomes at 15,966 Mbp/1C (Arumuganathan and Earle, 1991), as compared to the human genome at 3,000 Mbp/1C (McLysaght *et al.*, 2000) or *Arabidopsis* at 145 Mbp/1C (Arumuganathan and Earle, 1991). The wheat genome's large size and high redundancy make it difficult to identify and isolate genes. Wheat genes have previously been cloned by reverse genetics (i.e. *Pina-D1*; Giroux and Morris, 1997; Giroux and Morris,1998), by homology to genes from other species (i.e. *Rht-D1*; Peng *et al.*, 1999), by enrichment for cDNAs of interest (i.e. *PKABA1*; Anderberg and Walker-Simmons, 1992), and by labor-intensive map-based cloning (Yan *et al.*, 2003). We are using classical mutagenesis and transposon mutagenesis to identify wheat genes controlling grain dormancy and pre-harvest sprouting.

## DORMANCY AND PRE-HARVEST SPROUTING

Pre-harvest sprouting (PHS) is the germination of mature grain while still in the spike. This occurs under cool, moist conditions before harvest. During pre-harvest sprouting, hydrolytic enzymes are activated and endosperm constituents are broken down, resulting in lower yield and diminished quality. Flour milled from sprouted grains loses thickening power, and breads baked from these flours have decreased volume and poor crumb structure. PHS thus lowers the grade of wheat grain from bread-quality to feed-quality, resulting in a loss of profit for the farmer (reviewed by Wahl and O'Rourke, 1993). The tendency towards preharvest sprouting and seed dormancy have an inverse relationship. Understanding seed dormancy should therefore give insight on controlling PHS.

Dormant embryos will not germinate, even under favorable germination conditions. All ripe wheat grain is dormant and must pass through a period of afterripening before it can germinate. The degree of dormancy and length of afterripening required for wheat grain germination is variable and cultivar-dependent, ranging from highly dormant with a 6 mo afterripening requirement to slightly dormant, with an afterripening requirement of only a few days.

White-kernel wheats are generally more susceptible to PHS than red-kernel wheats (Groos *et al.*, 2002). The connection between testa color and PHS susceptiblity may be due to close genetic linkage between testa color genes and genes involved in seed

dormancy and PHS, or due to a pleiotropic effect of the color-controlling genes in wheat (Lawson *et al.*, 1997; Groos *et al.*, 2002).

Many other factors may contribute to a low degree of PHS and to seed dormancy: germination inhibitors present in the grain, reduced alpha-amylase activity in the grain, reduced water absorption by the grain, and variations in grain hormone response (Roy *et al.*, 1999; Zanetti *et al.*, 2000). Two plant hormones have been implicated in controlling dormancy and germination. Abscisic acid (ABA) sets up dormancy as the grain matures and dries down. ABA insensitivity has been linked to PHS in wheat (Walker-Simmons, 1987). Gibberellic acid (GA acts antagonistically on ABA signaling and seed dormancy, while stimulating a germination response. These two hormones act oppositely in the control of seed dormancy. ABA and GA signaling mutants are needed to identify genes in wheat involved in hormonal control of PHS and seed dormancy.

## USING MUTANTS FOR GENE IDENTIFICATION

Mutations can be defined as heritable changes within a gene. Before the 1920's, researchers had to rely on naturally-occuring, rare mutations to study gene function. Mutagens discovered since then have greatly increased the speed and power of genetic analysis by increasing the availability of altered genes for study. Mutagens employed for gene identification in plant species can be categorized as either classical or insertional.

Classical mutagens include chemical mutagenic agents such as ethylmethane sulfonate (EMS), diethyl sulfate, azide, nitrosguanidine, and nitrosurea; and physical mutagenic agents such as X-rays, γ-rays, and fast neutrons (Rédei and Koncz, 1992). Chemical mutagens typically work by alkylating the phosphate groups of nucleotides to cause a single base change (reviewed in Heslot, 1965). Physical mutagens generally work by chromosomal breakage and/or rearrangement. A large number of mutants have been generated with classical mutagens in *Arabidopsis thaliana* L. (McKelvie, 1962; Rédei, 1970; Anderson and Mulligan, 1992), as well as in other species. Treatment of seed with classical mutagens causes mutations within the cells of the embryo. The resulting M1 plants are chimeras. Thus, mutagenized grain must be advanced to the following generation (M2) before screening to avoid genetic chimeras and allow recovery of homozygous recessive mutations (refer to Table 1).

Plants derived from insertional mutagenesis must also be grown to the M2 generation before screening for a recessive phenotype. Insertional mutagenesis has rapidly gained popularity since the 1980's (Fedoroff *et al.*, 1984; Schell, 1987; Feldmann, 1991) and uses T-DNA (transfer-DNA) and transposon-based technologies to disrupt genes. This has great advantages over classical mutagenesis as the insert causing the phenotype is of a known sequence. This typically allows for quick and easy cloning of the interrupted gene. T-DNA is a defined segment of plasmid DNA from *Agrobacterium tumefaciens* that is transferred into the plant genome upon infection (Koncz *et al.*, 1992). These plasmids have become tools for molecular engineering in plants. Addition of selectable markers has allowed us to use T-DNA to transform genes of interest into plants, and to

mutagenize the plant genome. T-DNA insertion is fairly random. Thus, T-DNA insertion may disrupt a gene and cause a phenotype. Using T-DNA insertional mutagenesis requires a separate transformation event for each line screened. As with classical mutagenesis, mutants must be screened in the second generation (T2) to detect recessive mutations (refer to Table 1). The other method of insertional mutagenesis uses of transposable elements. Transposons are DNA elements that may move from one location in the genome to another, possibly causing a phenotypic change by insertional inactivation of a gene. Transposon-tagging systems have been used to identify genes in many plant species, including maize, *Arabidopsis*, tobacco, tomato, flax, rice (reviewed by Osborne and Baker, 1995), and barley (Koprek *et al.*, 2000).

Transposon tagging systems in plants have been based on the maize *Mutator* (*Mu*), En/Spm, and Ac/Ds transposons (Bennetzen, 1996; Enoki *et al.*, 1999; Parinov *et al.*, 1999; Weil and Kunze, 2000). Ac transposons have the fewest homologues in *Triticeae* (Zale and Steber, 2002). Therefore, this review will focus on the Ac/Ds two-component system for use in wheat gene identification. The Ac or *Activator* element encodes the transposase required for transposition. Ac can be "wings clipped" by removing the 5' and/or 3' inverted repeats (IRs) required *in cis* for transposition; making it unable to move from its original chromosomal position. The Ds (*Dissociator*) element has all of the *cis*-sequences required for transposition, but requires the Ac transposase *in trans* to jump. In constructs for transposon tagging, the Ds element is inserted into the 5'-UTR of a selectable marker. The marker is only expressed once the Ds element transposes out, allowing selection of plants containing transposition events. To induce transposition for

generating mutants, Ac- and Ds-carrying lines are crossed. The Ac-expressed transposase induces Ds to transpose in the F1 generation. Transposition events can be identified in the F2; however, homozygous recessive mutants can only be identified in the F3 generation (Figure 1).

## PROGRESS IN DEVELOPING MUTATION SYSTEMS IN WHEAT

Mutation breeding has been used in wheat since 1951 (reviewed in Konzak, 1987). Early screens recovered useful mutants for reduced height and lodging resistance. Mutation breeding of this type in wheat is of great historical significance, as mutated *Rht-D1* and *Rht-B1* genes produced dwarf wheat varieties that started the "green revolution" and increased production of wheat worldwide (Allan, 1986). More recently, wheat mutant screens have been for resistance to powdery mildew (Kinane and Jones, 2001), leaf rust and stem rust (Williams *et al.*, 1992; Friebe *et al.*, 1994; Kerber and Aung, 1995), and yellow and brown rust (Boyd *et al.*, 2002). Success in previous mutant screens in wheat encouraged us to screen mutagenized lines for altered ABA sensitivity in an effort to understand pre-harvest sprouting.

### *Classical Mutagenesis*

Screens for altered ABA sensitivity in germination have yielded many different types of mutations in *Arabidopsis thaliana.* ABA-insensitive germination screens yielded several mutants with various phenotypes. Mutants such as *Ab*scisic acid-*i*nsensitive *1-1* (*abi1-1*) and *abi2-1* produce non-dormant seeds and have wilty vegetative phenotypes (reviewed

in Finkelstein and Rock, 2002). On the other hand, other *Arabidopsis* mutants selected for the same ABA-insensitive germination phenotype (*abi3*, *abi4*, and *abi5*) have non-dormant seeds, but little to no vegetative phenotype change. We have performed an ABA-insensitive germination screen in wheat, based on this previous work in *Arabidopsis*. From screening 7,320 chemically mutagenized (EMS) M2 wheat grains, we have obtained 11 independent ABA-insensitive lines (Strader and Steber, unpublished). Some mutant lines from this screen demonstrate only the ABA-insensitive germination phenotype, while others demonstrate both germination and wilty vegetation phenotypes.

The complementary screen for increased ABA response has also been done in *Arabidopsis*. From these screens, seeds displaying an *e*nhanced *r*esponse to *A*BA (*era*) during germination were isolated. The mutants *era1* and *era3*, as well as *abh1* (*AB*A-*h*ypersensitive), have increased seed dormancy. Both *era1* and *abh1* also have increased drought tolerance (reviewed in Finkelstein and Rock, 2002). A previous study isolated a reduced grain dormancy mutant in wheat, but the identity of this single, dominant mutation remains unknown (Kawakami *et al.*, 1997). We performed an ABA-hypersensitive germination screen in wheat, based on this previous work. From screening 22,520 fast-neutron mutagenized M2 wheat grains, we obtained 39 independent lines with this phenotype (Strader and Steber, unpublished).

Both of these screens have limitations in a polyploid system. Multiple copies of the same gene represented on different genomes make it difficult to isolate recessive, loss-of-function mutants with the desired phenotype. However, the fact that recessive mutants

can be recovered (Kerber, 1991) causes one to wonder if intricate gene regulation of the multiple copy genes is occurring, and whether the plants will activate expression of homeologous genes on the other genomes to recover from the loss of function of one copy. Following the phenotypes of isolated recessive mutants through several generations should give an indication as to whether this type of control is occurring. A solution to this limitation is to find a way to create stable, dominant mutations that can be made independent of genome copy number. An activation tagging system should allow one to overcome polyploid problems in mutant searches.

*Insertional Mutagenesis and TAT System Development*

Activation tagging was first performed in plants by Hayashi *et al.* (1992) using a T-DNA vector containing four copies of the CaMV 35S promoter in tandem near the right border of the T-DNA. This caused overexpression of sequence downstream of the T-DNA, allowing them to identify dominant tobacco mutants for auxin-independent growth. Since then, activation tagging via T-DNA insertion has been used to develop a number of stable dominant and semi-dominant tagged mutants in *Arabidopsis* (Weigel *et al.*, 2000). Activation tagging has also been performed in *Arabidopsis* using the Ac/Ds transposon system containing a single copy of the CaMV 35S promoter at the 3'-IR (Long *et al.*, 1997). This was the beginning of transposon-based activation tagging (TAT).

We feel that transposons are the best method for accomplishing activation tagging in wheat. Activation tagging enriches for dominant mutations, because genes downstream of the insertion carrying the 35S promoter are overexpressed, producing the phenotypic

change. This is useful in a species with a large genome, where gene redundancy is able to mask most deletion mutations. Transformation in wheat can be laborious and time-consuming, taking several months to a full year to regenerate plants from tissue culture after the transformation event. Activation tagging using the T-DNA systems requires a separate transformation event for each gene tagged, whereas generating TAT mutants by cross-pollinating wheat Ac- and Ds-carrying lines is a more expedient means of producing activation-tagged mutants. We are currently in the process of creating an Ac/Ds TAT system in wheat. Preliminary data suggests that the Ac/Ds system is operational in wheat (Zale and Steber, unpublished data). We believe both traditional mutant screens and activation tagging can be successful in polyploids. Though creating classical mutants is more quickly achieved than creating insertional mutants, cloning of activation tagged genes should be far easier to accomplish in a polyploid system.

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Table 1. Comparison of Plant Mutagenesis Methods

| Mutagen | Lesion | Type of Mutation | Generation Screened |
|---|---|---|---|
| X-ray, fast neutron, gamma-ray | Chromosome breakage | Deletion and chromosome rearrangement | M2 |
| EMS[a], DES[b], Azide | Single base change | Missense and nonsense | M2 |
| T-DNA | Insertion | Disruption | T2 |
| Transposon | Insertion | Disruption | T3 |
| Activation tagging via T-DNA or Transposon | Insertion | Disruption or overexpression of downstream gene | T2 or T3 |

[a]Ethylmethane sulfonate, [b]Diethyl sulfonate

FIGURE 1. Generating Mutants in Plants Using Transposons