

Gibberellin-Mediated Proteasome-Dependent Degradation of the Barley DELLA Protein SLN1 Repressor

Xiangdong Fu,^{a,1} Donald E. Richards,^{a,1} Tahar Ait-ali,^a Llewelyn W. Hynes,^a Helen Ougham,^b Jinrong Peng,^{a,2} and Nicholas P. Harberd^{a,3}

^aJohn Innes Centre, Colney Lane, Norwich NR4 7UJ, United Kingdom

^bInstitute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, Wales, United Kingdom

DELLA proteins are nuclear repressors of plant gibberellin (GA) responses. Here, we investigate the properties of SLN1, a DELLA protein from barley that is destabilized by GA treatment. Using specific inhibitors of proteasome function, we show that proteasome-mediated protein degradation is necessary for GA-mediated destabilization of SLN1. We also show that GA responses, such as the aleurone α -amylase response and seedling leaf extension growth, require proteasome-dependent GA-mediated SLN1 destabilization. In further experiments with protein kinase and protein phosphatase inhibitors, we identify two additional signaling steps that are necessary for GA response and for GA-mediated destabilization of SLN1. Thus, GA signaling involves protein phosphorylation and dephosphorylation steps and promotes the derepression of GA responses via proteasome-dependent destabilization of DELLA repressors.

INTRODUCTION

Bioactive gibberellins (GAs) are essential regulators of plant growth and development (Hooley, 1994). For example, during the germination of cereal grains, GA is synthesized by the embryo and secreted into the aleurone. In this situation, GA regulates the synthesis and secretion of hydrolyzing enzymes (such as α -amylase) into the endosperm. The hydrolyzing enzymes then catalyze the breakdown of endosperm storage macromolecules, releasing nutrients that are used by the establishing seedling (Bethke et al., 1997; Ritchie and Gilroy, 1998; Lovegrove and Hooley, 2000).

GA is thought to elicit GA responses in the following manner. First, GA appears to be perceived on the surface of plant cells by an unidentified outward-facing plasma membrane-associated GA receptor (Hooley et al., 1991; Gilroy and Jones, 1994). The perception of GA results in rapid increases in the levels of cytosolic calcium and calmodulin (Gilroy, 1996; Schuurink et al., 1996). G-proteins, protein phosphatases, and cGMP also may play important roles during the cytoplasmic steps of the GA signal transduction chain (Kuo et al., 1996; Penson et al., 1996; Jones et al.,

1998). Inside the nucleus, the DELLA proteins, a family of putative transcriptional regulators, mediate the GA signal (Dill et al., 2001; Richards et al., 2001; Itoh et al., 2002; Wen and Chang, 2002). Downstream of the DELLA proteins, GA regulates α -amylase synthesis in aleurone via a myb-like transcription factor (GAmYb) that binds to a specific region of the promoters of genes that encode α -amylase (Gubler et al., 1995). Recent work has shown that, in addition to genes that encode α -amylase, GAmYb can transactivate other GA-regulated genes (Gubler et al., 1995, 1999; Cercós et al., 1999).

Mutants of wheat, barley, and rice that are affected in GA signaling display an altered aleurone α -amylase response. For example, dominant mutations at the homoeoallelic wheat *Rht-B1a* and *Rht-D1a* loci confer dwarfism and a reduced growth response to GA (Börner et al., 1996; Peng et al., 1999). Severely dwarfing alleles, such as *Rht-B1c*, abolish the GA response of mutant aleurone cells (Gale and Marshall, 1975; Ho et al., 1981; Börner et al., 1996). By contrast, recessive mutations at the barley *SLENDER (SLN1)* and rice *SLENDER RICE1 (SLR1)* loci (e.g., *sln1-1* and *slr1-1*) confer a taller-than-wild-type, slender phenotype as a result of exaggerated elongation growth (Foster, 1977; Ikeda et al., 2001; Chandler et al., 2002). The aleurone cells of these slender mutants constitutively express α -amylase in the absence of GA induction (Chandler, 1988; Lanahan and Ho, 1988; Ikeda et al., 2001; Chandler et al., 2002; Gubler et al., 2002). The accelerated growth and constitutive α -amylase expression of the slender mutants is unaffected by GA

¹These authors contributed equally to this work.

²Current address: Functional Genomics Laboratory, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609.

³To whom correspondence should be addressed. E-mail nicholas.harberd@bbsrc.ac.uk; fax 44-1603-450025.

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biosynthesis inhibitors (Chandler, 1988; Lanahan and Ho, 1988; Ikeda et al., 2001). Although the phenotype of barley and rice slender mutants resembles that of wild-type plants treated with GA, the endogenous bioactive GA levels in these mutants are lower than those of the wild type (Crocker et al., 1990; Ikeda et al., 2001).

Wheat *Rht-B1a* and *Rht-D1a*, rice *SLR1*, and barley *SLN1* encode proteins orthologous with Arabidopsis *GAI*, a member of the GRAS family of putative transcriptional regulators (Peng et al., 1997, 1999; Harberd et al., 1998; Pysh et al., 1999; Richards et al., 2000, 2001; Ikeda et al., 2001; Chandler et al., 2002; Gubler et al., 2002). The Arabidopsis genome contains four other genes that encode proteins that are closely related to *GAI*: *RGA*, *RGL1*, *RGL2*, and *RGL3* (Silverstone et al., 1998; Dill and Sun, 2001; Lee et al., 2002). *GAI* and *RGA* encode proteins that act together as negative regulators of GA responses (Peng et al., 1997; Silverstone et al., 1997, 1998; Dill and Sun, 2001; King et al., 2001), and *RGL1* and *RGL2* also encode proteins that function in GA signaling (Lee et al., 2002; Wen and Chang, 2002). The proteins encoded by *GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3*, and by orthologous genes in other species, are the above-mentioned DELLA proteins, named after a motif that is highly conserved among them and that is important to their function in GA signaling (Peng et al., 1997; Wen and Chang, 2002). The DELLA proteins generally are thought to operate as repressors of GA responses, and GA is thought to induce GA responses by opposing DELLA protein action (Peng et al., 1997; Harberd et al., 1998; King et al., 2001; Richards et al., 2001).

Recent studies using DELLA proteins fused to the green fluorescent protein have shown that RGA, SLR1, and SLN1 accumulate in the nucleus of plant cells and that treatment with exogenous GA causes the disappearance of these proteins from the nucleus (Silverstone et al., 2001; Dill et al., 2001; Itoh et al., 2002; Gubler et al., 2002). These observations are compatible with previous proposals that DELLA proteins work as repressors of growth, whereas GA opposes their growth-repressing function (Peng et al., 1997; Harberd et al., 1998; Richards et al., 2001).

Here, we describe the molecular analysis of the barley *SLN1* gene and the mechanism by which its product (SLN1) mediates barley GA responses. We investigated the mechanism of GA-induced SLN1 destabilization by studying the effects of a number of different inhibitory compounds on this process. In particular, we show that specific inhibitors of 26S proteasome function block both the GA-mediated destabilization of SLN1 and GA responses (the aleurone α -amylase response and seedling leaf elongation). We also demonstrate that selected protein kinase and protein phosphatase inhibitors can block the GA induction of both SLN1 destabilization and GA responses, thus implicating protein phosphorylation and dephosphorylation steps in GA signaling. In summary, our results indicate that GA stimulates GA responses by eliciting proteasome-dependent degradation of the nuclear SLN1 GA response repressor.

RESULTS

Molecular Characterization of the Barley *sln1-1* Mutant Allele

As shown in Figure 1A, recessive mutations at *SLN1* (e.g., *sln1-1*) confer exaggerated elongation growth of barley seedlings. This phenotype persists throughout the development of the plant, resulting in adult plants that are taller than wild-type plants, with thin, pale green leaves and sterile flowers (the "slender" phenotype) (Foster, 1977; Chandler, 1988; Lanahan and Ho, 1988). In addition, the growth of *sln1-1* mutants is resistant to the growth-inhibitory effects of the GA biosynthesis inhibitor paclobutrazol, suggesting that *SLN1* encodes a repressor of GA responses and that loss-of-function mutations at *SLN1* confer a constitutive GA response (Chandler, 1988; Lanahan and Ho, 1988).

Because mutations at *SLN1* confer altered GA responses, we reasoned that *SLN1* might be a barley ortholog of the *GAI/RGA/d8/Rht-B1a/Rht-D1a/SLR1* genes (genes that encode DELLA proteins from a variety of species) (Chandler et al., 2002; Gubler et al., 2002). Therefore, we amplified *SLN1* using PCR primers derived from the wheat *Rht-D1a* sequence (see Methods). The predicted amino acid sequence of the protein encoded by the amplified *SLN1* was identical to that described previously (Chandler et al., 2002; Gubler et al., 2002; X. Fu and D.E. Richards, unpublished data). We also amplified *SLN1* from the *sln1-1* mutant (see Methods) (Figure 1A). DNA sequencing showed that the *sln1-1* mutation is a single nucleotide substitution (GAG to TAG) that converts the codon encoding Glu-250 to a stop codon (Figure 1B).

Immunoblot analysis showed that *sln1-1* plants lack detectable SLN1 protein. As shown in Figure 1C, the shoots of *SLN1* seedlings germinated and grown in the presence of exogenous GA₃ were longer than those germinated in water, whereas there was no effect of GA on *sln1-1* seedlings. Total proteins extracted from these seedlings were electrophoretically fractionated and analyzed using anti-GAI antibodies. These experiments identified an ~65-kD immunoreactive protein that was detectable in water-treated *SLN1* seedlings but not in water-treated *sln1-1* seedlings, indicating that the protein identified is SLN1 (the predicted molecular mass of SLN1 is 65.2 kD) (Figure 1C). As shown previously, SLN1 was not detectable in GA-treated *SLN1* seedlings, showing that SLN1 disappears in response to exogenous GA (Chandler et al., 2002; Gubler et al., 2002) (Figure 1C).

GA-Induced Disappearance of SLN1 Is Unaffected by the Protease Inhibitors Pefabloc SC, Aprotinin, and Phenylmethylsulfonyl Fluoride

We sought to determine a mechanism for the GA-induced disappearance of SLN1, testing initially the effects of the

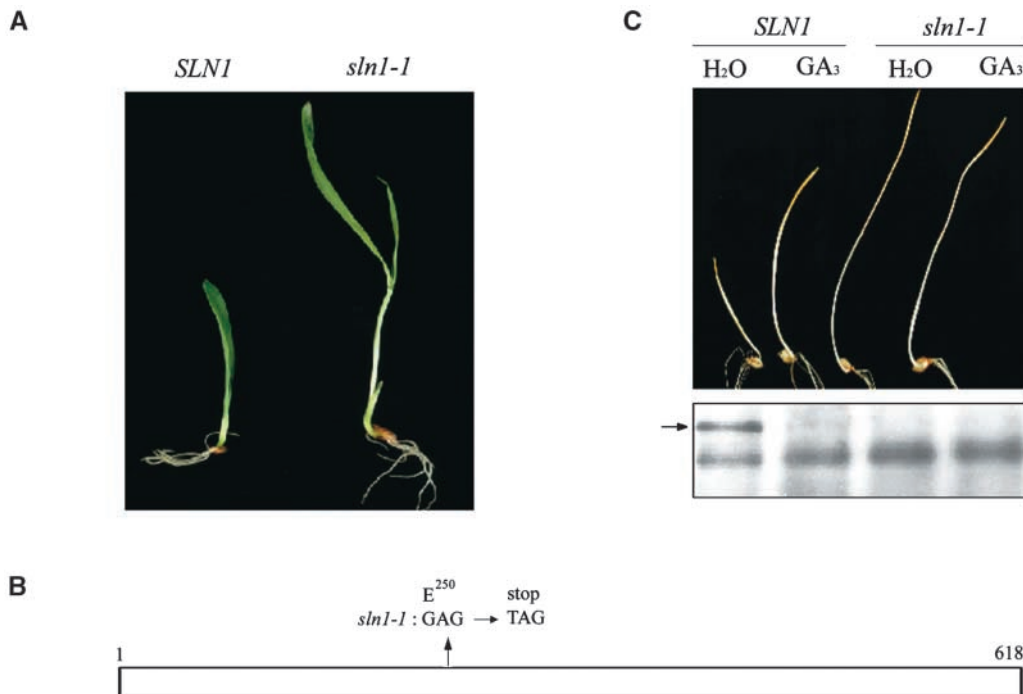


Figure 1. The *sln1-1* Mutant Allele.

(A) Five-day-old seedlings homozygous for *SLN1* or *sln1-1*.

(B) Scheme of *SLN1/SLN1* showing the site of the mutation in *sln1-1*. Numbers represent amino acid positions in *SLN1* (1 indicates the start Met, and 618 indicates the final Pro).

(C) Seedling phenotypes and protein gel blot analysis of *SLN1* and *sln1-1* plants. The seedlings were grown at 20°C for 5 days with or without 100 μ M GA₃. Proteins then were extracted from seedlings, and 15 μ g of total protein per lane was loaded and probed with anti-GAI antibodies (see Methods). The arrow indicates *SLN1*; an additional, nonspecific band served as a loading control.

cell-permeable protease inhibitors Pefabloc SC, aprotinin, and phenylmethylsulfonyl fluoride (for details of inhibitors, see Methods). As described previously, *SLN1* was detected in extracts from *SLN1* seedlings treated with water but not in extracts treated with GA (Figure 2). *SLN1* also was not detected in *SLN1* seedlings treated for 2 h with GA and Pefabloc SC, a general inhibitor of Ser proteases (Figure 2). Similar results were obtained with GA and Pefabloc SC treatments of 30 min and 24 h (data not shown). Thus, Pefabloc SC had no detectable effect on GA-induced *SLN1* disappearance (Figure 2). Similarly, treatments with other protease inhibitors, such as aprotinin and phenylmethylsulfonyl fluoride, failed to block the GA-induced disappearance of *SLN1* (data not shown).

GA-Induced Disappearance of *SLN1* Is Affected by Proteasome Inhibitors

In contrast with the results reported above, the GA-induced disappearance of *SLN1* was affected by the addition of five different cell-permeable proteasome-specific inhibitors:

MG115, MG132, proteasome inhibitor I, proteasome inhibitor II, and lactacystin (for details of inhibitors, see Methods). As shown in Figure 2, extracts from *SLN1* seedlings treated with both GA and MG115, MG132, or proteasome inhibitor I contained detectable levels of *SLN1*. Similar results were obtained with proteasome inhibitor II and lactacystin and from samples treated with GA and proteasome inhibitors for 30 min or 24 h (data not shown). These results show that proteasome inhibitors can prevent the GA-induced disappearance of *SLN1*, suggesting that this disappearance might be attributable to proteasome-mediated degradation.

GA-Regulated Seedling Leaf Extension Growth Is Dependent on Proteasome-Mediated *SLN1* Degradation

Because proteasome inhibitors block the GA-dependent destabilization of *SLN1* in barley seedlings, we tried to determine whether GA-promoted seedling leaf extension growth was affected by MG132. Three-day-old *SLN1* and *sln1-1* seedlings were grown in the presence of GA and/or

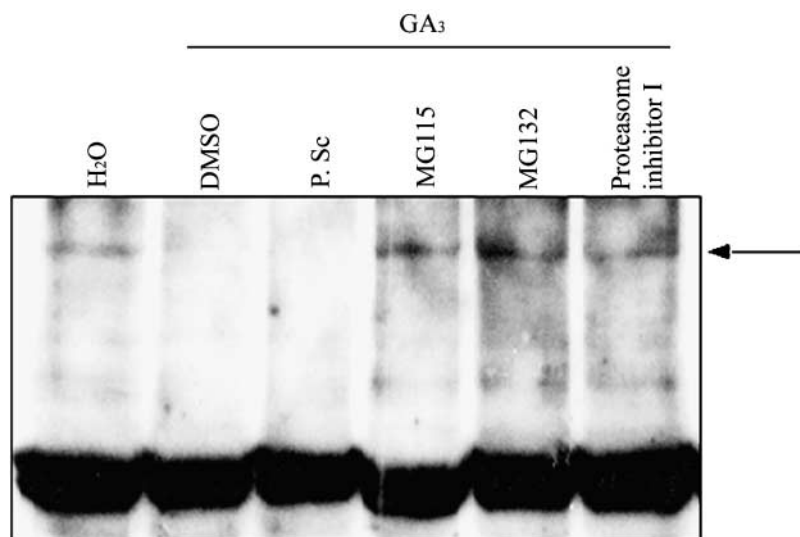


Figure 2. The Proteasome Pathway Mediates GA-Induced SLN1 Protein Degradation.

Extracts were prepared from 5-day-old *SLN1* seedlings, which had been treated for 2 h with GA₃ and 1% DMSO with or without protease or proteasome inhibitors (Pefabloc SC [P. Sc], MG115, MG132, or proteasome inhibitor I). Extracts from water-treated *SLN1* seedlings were used as a positive control. Total protein (15 μg/lane) was loaded and probed with anti-GAI antibodies. The arrow indicates SLN1. The strong bottom band in all lanes represents the nonspecific background protein described for Figure 1, and the weak lower bands may indicate SLN1 degradation products.

MG132, and first leaf lengths were measured before and after treatment (Figure 3). The leaves of *SLN1* seedlings grown in the presence of GA grew longer than those of water-treated controls, whereas MG132 blocked the response of *SLN1* seedlings to GA. The growth of *sln1-1* seedlings was little affected by either GA or MG132, indicating that the inhibitory effect of MG132 on the growth of GA-treated *SLN1* seedlings was not caused by general metabolic poisoning (Figure 3). Together, these results indicate that GA promotes the extension growth of barley seedling leaves via the proteasome-dependent destabilization of SLN1.

α-Amylase Induction in Aleurone Cells Is Dependent on Proteasome-Mediated SLN1 Degradation

GA-mediated destruction of SLN1 in aleurone cells is associated with the GA induction of α-amylase (Gubler et al., 2002). Therefore, we examined the effects of a range of inhibitors on the α-amylase responses of de-embryonated *SLN1* and *sln1-1* half-grains (see Methods). As described previously, *sln1-1* half-grains produce comparable amounts of α-amylase activity in the presence or absence of GA (Chandler, 1988; Lanahan and Ho, 1988) (Table 1). None of the inhibitors tested (Pefabloc SC, MG115, MG132, and proteasome inhibitor I) affected the production of α-amylase by *sln1-1* half-grains in the presence or absence of GA (Table 1). By contrast, the proteasome inhibitors MG115 and MG132 largely blocked the GA induction of α-amylase ac-

tivity from *SLN1* half-grains (Table 1). The fact that MG115 and MG132 blocked the α-amylase response in *SLN1* half-grains but did not inhibit the α-amylase production of *sln1-1* half-grains shows that the observed effects of these inhibitors on GA responses is not attributable to nonspecific effects or the poisoning of cellular metabolism. Rather, MG115 and MG132 block the α-amylase response of *SLN1* half-grains by inhibiting proteasome activity, and proteasome-dependent degradation of SLN1 is necessary for the induction of α-amylase activity.

Protein Kinase and Phosphatase Inhibitors Block GA-Induced SLN1 Protein Degradation and α-Amylase Production

To identify additional steps in the GA signal transduction pathway, we tested the effects of various protein phosphorylation and dephosphorylation inhibitors on GA-induced SLN1 degradation. Previous experiments have shown that the Ser/Thr protein phosphatase inhibitor okadaic acid (OA) is effective at blocking the GA-induced production of α-amylase by wheat aleurone cells (Kuo et al., 1996). We examined the effects of OA and sodium vanadate (SV), a widely used general inhibitor of protein phosphatases (for details of these inhibitors, see Methods), on the GA-induced degradation of SLN1. SLN1 was detected in extracts from *SLN1* seedlings treated with water or with GA and either OA or SV

for 2 h (Figure 4A) or 24 h (data not shown) and was not detected in GA-only controls. Thus, treatment with OA or SV blocked the GA-induced degradation of SLN1. We also showed that both OA and SV blocked the GA-mediated induction of α -amylase activity in *SLN1* half-grains but did not block the constitutive production of α -amylase by *sln1-1* half-grains (Table 1). These results show that OA and SV affect GA responses by perturbing the signaling chain associated with SLN1 (or they affect SLN1 itself), making SLN1 resistant to GA-mediated destabilization.

Staurosporine is a broad-range inhibitor of Ser/Thr protein kinases, whereas protein phosphatase 2 (PP2) is a selective protein kinase inhibitor (see Methods). Treatment with either staurosporine or PP2 failed to block the GA-induced degradation of SLN1 or the production of α -amylase activity (Figure 4B, Table 1). By contrast, treatment with two protein Tyr kinase inhibitors, genistein (a broad-range protein kinase inhibitor) and Tyroprostin B46 (AG555; for details, see Methods), blocked the GA-induced degradation of SLN1 (Figure 4B). Figure 4 shows extracts from seedlings treated for 2 h; similar results were obtained for each inhibitor after treatments for 30 min and 24 h (data not shown). Genistein and Tyroprostin B46 also blocked GA-induced α -amylase production in *SLN1* half-grains but did not block constitutive α -amylase production in *sln1-1* half-grains (Table 1). Together, the results described here suggest that protein kinases and protein phosphatases mediate GA-induced degradation of SLN1, thus eliciting GA responses.

DISCUSSION

Here, we show that several well-defined, cell-permeable inhibitors of proteasome function can block the GA-induced disappearance of SLN1. Thus, proteasome function is necessary for the destabilization of SLN1 in response to the GA signal. Furthermore, we show that two GA responses, leaf extension growth and the aleurone α -amylase response, are blocked by proteasome inhibitors. Our results suggest that GA induces GA responses in barley via proteasome-dependent degradation of SLN1.

To identify additional steps in the GA signaling pathway, we tested the effects of a range of protease, kinase, and phosphatase inhibitors on GA responses and on the GA-mediated destabilization of SLN1. None of these inhibitors (and none of the proteasome inhibitors described above) inhibited the production of α -amylase activity by *sln1-1* aleurones. In addition, the growth of *sln1-1* seedling leaves was unaffected by the proteasome inhibitor MG132. These are important observations because they enable us to discount a criticism that often is leveled at studies using inhibitors—that is, that the effects of inhibitors are nonspecific and may be the result of general poisoning of cellular metabolism. This cannot be the case in our experiments, because if it had been, the inhibitors would have blocked the constitutive GA responses exhibited by the *sln1-1* mutant. Any affect of the inhibitors used in our experiments on the GA responses

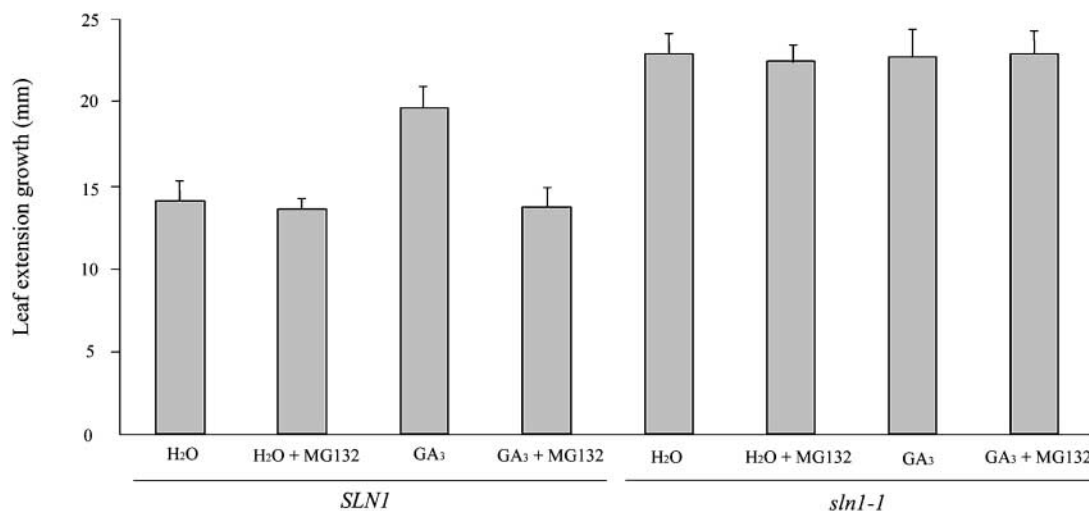


Figure 3. GA-Promoted Leaf Extension Growth Requires Proteasome-Dependent GA-Mediated SLN1 Destabilization.

Seedlings were germinated and grown for 3 days on water. The length of the first leaf (from leaf tip to seed) of each seedling was measured, and treatment (combinations of water, GA, and MG132 as shown) was begun. Twelve hours after the initiation of treatment, the length of the first leaf was measured again. The results shown are mean differences ($n = 30$; error bars represent standard errors) between the first and second measurements.

Table 1. Effects of the Different Inhibitors on α -Amylase Production in Barley Aleurone Layers

Inhibitors	α -Amylase Produced (milliunits/g)			
	<i>SLN1</i>		<i>sln1-1</i>	
	Water	GA ₃	Water	GA ₃
Control	0.08 ± 0.01	9.60 ± 0.03	6.24 ± 0.07	7.21 ± 0.05
MG115	0.07 ± 0.02	0.29 ± 0.04	5.87 ± 0.06	6.37 ± 0.07
MG132	0.07 ± 0.03	0.34 ± 0.09	5.99 ± 0.08	6.29 ± 0.10
Pefabloc SC	0.09 ± 0.02	6.48 ± 0.11	5.47 ± 0.12	6.91 ± 0.08
SV	0.08 ± 0.03	0.45 ± 0.03	5.93 ± 0.11	6.72 ± 0.09
AG555	0.54 ± 0.09	0.81 ± 0.03	7.18 ± 0.05	7.89 ± 0.09
PP2	0.09 ± 0.02	8.36 ± 0.02	6.69 ± 0.10	7.03 ± 0.08
Staurosporine	0.10 ± 0.03	9.24 ± 0.09	6.03 ± 0.09	7.11 ± 0.07

Each value shown is the mean ± SE from 12 half-grains.

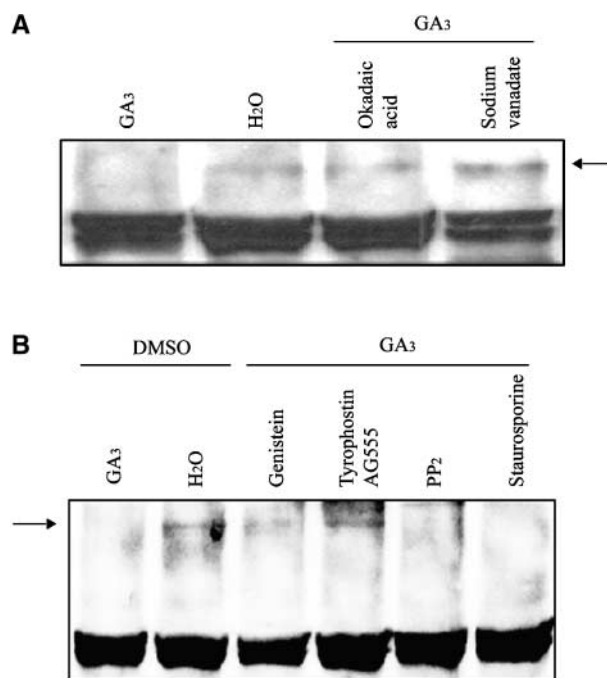
of *SLN1* plants must be attributable to a specific effect of that inhibitor on the activity of *SLN1*, either via a direct effect on *SLN1* itself or via an effect on steps in the GA signaling pathway leading to the destabilization of *SLN1*.

We identified two additional steps in GA signaling. First, we showed that protein phosphorylation inhibitors can block GA responses and the GA-mediated destabilization of *SLN1*, implying a protein phosphorylation step in GA signaling. The inhibitors that were effective at blocking GA responses and *SLN1* destabilization are Tyr kinase inhibitors, making it possible that phosphotyrosine is involved in GA signaling. It has been suggested previously that the DELLA proteins are structurally, and perhaps functionally, related to the STAT proteins (which signal via Tyr phosphorylation) (Peng et al., 1999; Richards et al., 2000). In addition, GRAS proteins contain a C-terminal sequence that is related to a consensus Tyr phosphorylation site (Bolte et al., 2000). Second, we showed that protein phosphatase inhibitors can block GA responses and the GA-mediated destabilization of *SLN1*, implying a protein dephosphorylation step in GA signaling. This step was identified previously because of the effect of OA on GA-induced α -amylase production by wheat aleurone cells (Kuo et al., 1996). Here, we have extended this finding by showing that the protein dephosphorylation step is necessary for the GA-mediated destabilization of *SLN1*.

We propose the following model to explain the phenomena reported in this article (Figure 5). First, GA interacts with an unknown plasma membrane-associated specific receptor (Hooley et al., 1991; Gilroy and Jones, 1994). This interaction stimulates a signal transduction cascade that may involve the phosphorylation or dephosphorylation of proteins on Ser, Thr, or Tyr (Kuo et al., 1996). Eventually, the signal reaches the nuclear *SLN1* protein. *SLN1* acts as a repressor of GA responses, inhibiting for example the transcription of the gene encoding the GAmby activator of the α -amylase response (Gómez-Cadenas et al., 2001). The GA signal alters *SLN1*, resulting in proteasome-dependent *SLN1* destabi-

lization and the release of GA responses from *SLN1*-mediated restraint.

It has become apparent that targeted proteasome-mediated protein degradation is crucial to many signaling pathways in plants. For example, the accumulation of AUX/IAA proteins is key to auxin signaling (Rouse et al., 1998). AUX/IAA accumulation is regulated via auxin-mediated targeting of AUX/IAAs for destruction via the proteasome (Gray et al., 2001). Other signaling pathways are regulated at the level of targeted protein destruction, either via the proteasome or the related COP9 signalosome complex, including jasmonic acid signaling (Xie et al., 1998), photomorphogenesis (Osterlund et al., 2000), floral development pathways (Samach et al., 1999), and disease resistance pathways (Austin et al., 2002; Azevedo

**Figure 4.** Effect of Protein Phosphatase and Protein Kinase Inhibitors on the GA-Induced Degradation of *SLN1*.

(A) Effect of protein phosphatase inhibitors on GA-induced *SLN1* degradation. Extracts were prepared from 5-day-old *SLN1* seedlings treated for 2 h with GA₃ with or without OA or SV. Water-treated *SLN1* seedlings were used as a positive control. The arrow indicates the *SLN1* protein. Additional nonspecific bands served as loading controls.

(B) Effect of protein kinase inhibitors on GA-induced *SLN1* degradation. Extracts were prepared from 5-day-old *SLN1* seedlings treated for 2 h with GA₃ and 1% DMSO with or without Tyrophostin B46 (AG555), PP2, genistein, or staurosporine. Extracts also were prepared from control *SLN1* seedlings treated with water and 1% DMSO. Total protein (15 μ g/lane) was loaded and probed with anti-GAI antibodies. The arrow indicates the *SLN1* protein. The bottom band in all lanes represents a nonspecific background protein as described for Figure 1.

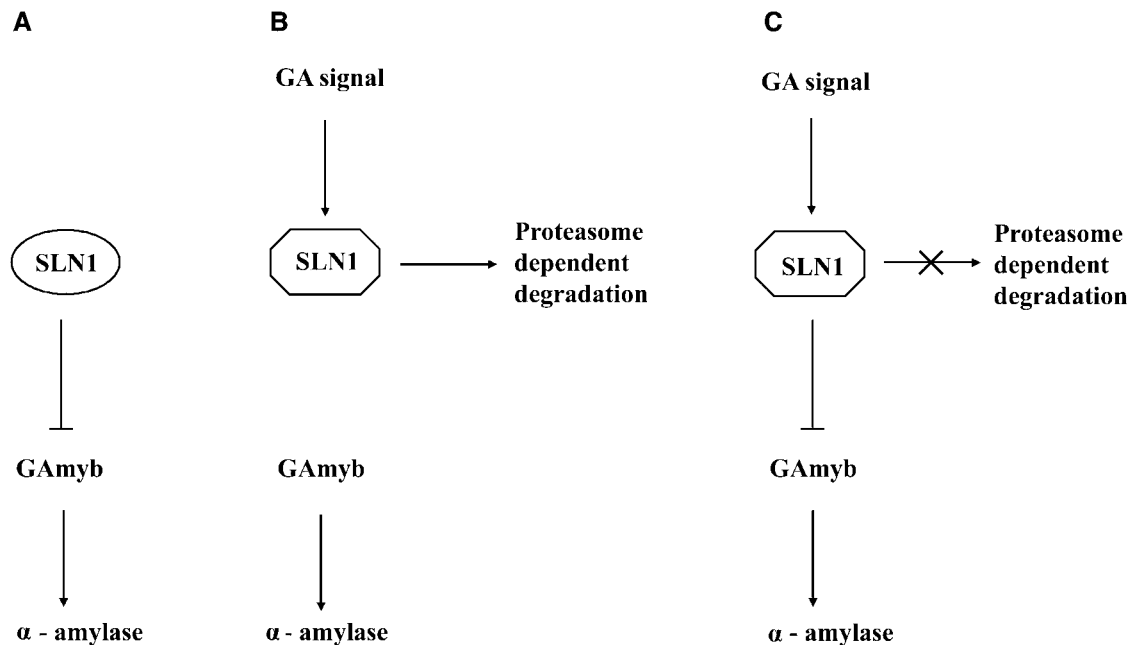


Figure 5. Scheme of SLN1 Function in GA Signaling.

Bioactive GA interacts with the membrane-associated GA receptor (not shown), thus activating signal transduction via second messengers, perhaps mediated by protein phosphorylation or dephosphorylation (GA signal). SLN1 is in the nucleus, in an active form that represses GA responses (e.g., SLN1 represses the accumulation of transcripts that encode GAmYb, as shown in **[A]**). The arrival of the GA signal causes the modification of active SLN1 into a form that is destroyed via a proteasome-dependent mechanism (**B**), resulting in the activation of GAmYb transcription and α -amylase production. Inhibition of the proteasome pathway (**C**) prevents the destruction of SLN1, which therefore persists and continues to repress GAmYb transcription and α -amylase production. Because protein kinase and protein phosphatase inhibitors also block the destruction of SLN1, it is possible that protein phosphorylation or dephosphorylation is required for the modification of SLN1 or before the destruction of the modified form can occur.

et al., 2002). Here, we have shown that GA signaling in barley operates via proteasome-dependent, GA-elicited destabilization of SLN1. The fact that targeted protein destruction is key to so many different plant signaling pathways may explain why they often operate negatively, via derepression.

METHODS

Plant Material and Growth Conditions

The experiments described here used the barley (*Hordeum vulgare*) cv Herta as the source of the wild-type *SLN1* allele. The *sln1-1* mutant allele was induced by diethyl sulfate treatment of Herta (Foster, 1977), and the segregating material used was the selfed progeny of a line backcrossed multiple times onto the Herta genetic background.

Seeds were surface-sterilized by washing first with 70% ethanol for 2 min, then with sodium hypochlorite for 30 min, and finally with sterile distilled water. Sterilized seeds then were grown at 20°C (16-h photoperiod) on moistened filter paper. In tests of the seedling growth response to gibberellin (GA), seeds were germinated in 100

μ M GA₃ (Sigma). In further tests of GA-promoted leaf extension growth, 3-day-old seedlings were incubated with water or 100 μ M GA₃ in the presence or absence of 100 μ M MG132. In tests of GA-induced SLN1 protein degradation, 5-day-old seedlings were treated with 100 μ M GA₃ (Sigma) or with water in the presence or absence of different pharmacological agents in the presence of 1% DMSO.

Cloning and Sequence Analysis of *SLN1* Alleles

Total RNA was isolated from *SLN1* seedlings using Trizol reagent (Gibco BRL). Genomic DNA was isolated from single *SLN1* or *sln1-1* seedlings according to the method of Edwards et al. (1991). Based on sequence conservation of the DELLA subfamily of GRAS regulator genes, sequence encoding the N-terminal end of SLN1 was obtained by 5' rapid amplification of cDNA ends (Gibco BRL) using primers A1 (5'-TCGAGCTGCTCCAGCTTCTG-3'), A2 (5'-ACGGTG-TCCGTGGCGAGGTG-3'), and A3 (5'-CGTTGAGCTCGGACAGCA-TG-3'). Sequence encoding the C-terminal end of SLN1 was obtained by 3' rapid amplification of cDNA ends (Gibco BRL) using primers B1 (5'-TCGAGAAGTCTCTGGGCACG-3'), B2 (5'-TGACCCTGGTTCGAGCAGGAG-3'), and B3 (5'-CTGCACTACTACTCC-ACCATG-3'). Primers A1, A2, A3, B1, B2, and B3 were derived from conserved sequences in *Rht-B1a* and *Rht-D1a* (Peng et al., 1999). PCR

products from *SLN1* and *sln1-1* were amplified using the Expand Long Template PCR System (Roche Molecular Biochemicals, East Sussex, UK) using primers BA1 (5'-GATGGGGATCCGAGATGAGCGCGAGTACCAGGACGGC-3') and BA2 (5'-CTTCGAATCCC-TATCACGGCGCGCGAGGGCGCCATGC-3'). The PCR products were digested with BamHI and EcoRI and then cloned into the pGEM-T vector (Promega). Sequencing was performed using the ABI Prism Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (PE-Applied Biosystems, Foster City, CA).

Production of Anti-GAI Polyclonal Antibodies

A full-length GAI cDNA was cloned into the BamHI and PstEI sites of pQE-30 vector (Qiagen, Crawley, UK) to fuse a His tag at the N terminus of the cloned sequence. The His-tagged GAI recombinant protein was overexpressed in the *Escherichia coli* host strain BL21(DE3) (Novagen, Madison, WI). Cells were grown at 30°C to an OD₆₀₀ of 1.0, induced by 0.8 mM isopropylthio-β-galactoside for 90 min, and then harvested. Cells were resuspended in a buffer containing 50 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 100 mg/mL lysozyme, and 0.1% Triton X-100 and sonicated on ice. After centrifugation at 10,000 rpm for 20 min at 4°C, inclusion bodies were resuspended in the binding buffer solution containing 6 M urea, 5 mM imidazole, 50 mM NaCl, and 20 mM Tris-HCl, pH 8.0. The GAI recombinant protein was affinity-purified with nickel-nitrilotriacetic acid agarose beads (Qiagen). The purified GAI recombinant protein solution then was dialyzed against PBS solution overnight at 4°C before being used to raise antibodies in a rabbit.

The antisera obtained, although prepared against *E. coli*-expressed Arabidopsis GAI, were capable of detecting the SLN1 protein. A band that approximated the size expected for SLN1 was detected in extracts from *SLN1* plants but not in extracts from *sln1-1* plants.

Protein Gel Blot Analysis

Barley seedlings were treated with or without 100 μM GA₃ in the presence or absence of inhibitors. Whole seedlings were harvested and frozen in liquid nitrogen, after which total plant proteins were extracted using Trizol reagent (Gibco BRL) and dissolved into buffer E (125 mM Tris-HCl, pH 8.8, 1% SDS, 10% glycerol, and 50 mM Na₂S₂O₅). The resulting mixture was centrifuged (in a microcentrifuge) for 10 min at 4°C. The supernatant was transferred to new tubes, and protein concentrations were determined by the Bradford assay (Sigma). Total proteins (15 μg) from each extract were fractionated by 10% SDS-PAGE and analyzed on immunoblots using a 500-fold dilution of the anti-GAI polyclonal antiserum and a 10,000-fold dilution of peroxidase-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech). The blots then were incubated with ECL protein gel blotting detection reagents (Amersham Pharmacia Biotech), and the signals were detected by chemiluminescence. For each blotted gel, a duplicate gel was run and then stained with Coomassie Brilliant Blue R250 to act as a loading control.

Inhibitor Studies

Three broad-range Ser protease inhibitors were used: Pefabloc SC (4-[2-aminoethyl]-benzenesulfonyl-flouride hydrochloride), phenylmeth-

ylsulfonyl fluoride, and aprotinin (all three from Roche Molecular Biochemicals).

MG115 is a potent, reversible proteasome inhibitor that specifically inhibits the chymotrypsin-like activity of the proteasome (Peptides International, Louisville, KY). MG132 (also from Peptides International) is a tripeptide aldehyde, a potent, reversible proteasome inhibitor (Callis and Vierstra, 2000). Proteasome inhibitor I is an inhibitor of the multicatalytic proteinase complex (20S proteasome) (A.G. Scientific, San Diego, CA). Proteasome inhibitor II is a potent proteasome inhibitor that inhibits the chymotrypsin-like activity of the multicatalytic proteinase complex (20S proteasome) (A.G. Scientific). Lactacystin is an irreversible proteasome inhibitor that specifically inhibits the 26S proteasome and blocks proteasome activity by targeting the catalytic β-subunit (A.G. Scientific).

Okadaic acid (Calbiochem) is an inhibitor of the Ser/Thr protein phosphatases PP1 and PP2B (Bialojan and Takai, 1988). Vanadium ions (purchased as sodium orthovanadate; Sigma) are widely used as general inhibitors of protein phosphatases (Lau et al., 1989). Staurosporine (Calbiochem) is a broad-range inhibitor of Ser/Thr protein kinases and a potent inhibitor of Tyr kinases (Tamaoki, 1991). Genistein (Calbiochem) is a specific inhibitor of Tyr-specific protein kinases (Akiyama et al., 1987). Tyrohostin B46 (AG555; Calbiochem) and PP2 (Calbiochem) also are Tyr kinase inhibitors (Gazit et al., 1991; Hanke et al., 1996).

For the inhibitor analyses, 5-day-old seedlings were transferred to 100 μM GA₃ (Sigma) for 30 min, 2 h, or 24 h in the presence of 1% DMSO (control) or 1% DMSO with inhibitor at the following concentrations: MG115 (100 μM), MG132 (100 μM), Pefabloc SC (0.5 mg/mL), okadaic acid (1 μM), sodium vanadate (3 mM), AG555 (10 μg/mL), staurosporine (50 μM), genistein (50 μg/mL), PP2 (10 μg/mL), and proteasome inhibitor I (100 μM). After treatment, the seedlings were harvested and extracted for immunoblot analysis as described above. Data shown are representative of the results of three independent experiments.

α-Amylase Assays

Barley seeds were surface-sterilized by washing with 70% ethanol for 2 min, with sodium hypochlorite for 30 min, and then with sterile distilled water. De-embryonated *SLN1* half-grains were used for the measurement of α-amylase activity. To identify seeds homozygous for *sln1-1*, the progeny seeds of self-pollinated *SLN1/sln1-1* heterozygotes were cut in half. The half-seeds containing the embryo were transferred to Murashige and Skoog (1962) medium and germinated in a cold room (4°C) to facilitate the identification of *sln1-1* mutant homozygotes (the stems and leaves of the barley *sln1-1* mutant grow faster than those of wild-type controls, and this effect is particularly pronounced at lower temperatures [Harrison et al., 1998]). De-embryonated half-grains corresponding to *sln1-1* seedlings then were used for the measurement of α-amylase activity. The de-embryonated half-grains were transferred aseptically to 5 mL of aqueous buffer (Fu et al., 2001) and incubated in 5 μM GA₃ or without GA₃ in the presence or absence of each pharmacological agent for 18 h (inhibitors were at the same concentration as described above). α-Amylase activity in the incubation medium was measured as described previously (Fu et al., 2001).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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