

# Gibberellin Signaling in Barley Aleurone Cells. Control of SLN1 and GAMYB Expression

Frank Gubler\*, Peter Michael Chandler, Rosemary G. White, Danny J. Llewellyn, and John V. Jacobsen

Commonwealth Scientific and Industrial Research Organization, Plant Industry, G.P.O. Box 1600, Canberra, Australian Capital Territory 2601, Australia

We have previously identified GAMYB, a gibberellin (GA)-regulated transcriptional activator of  $\alpha$ -amylase gene expression, in aleurone cells of barley (*Hordeum vulgare*). To examine the regulation of GAMYB expression, we describe the use of nuclear run-on experiments to show that GA causes a 2-fold increase in the rate of GAMYB transcription and that the effect of GA can be blocked by abscisic acid (ABA). To identify GA-signaling components that regulate GAMYB expression, we examined the role of SLN1, a negative regulator of GA signaling in barley. SLN1, which is the product of the *Sln1* (*Slender1*) locus, is necessary for repression of GAMYB in barley aleurone cells. The activity of SLN1 in aleurone cells is regulated posttranslationally. SLN1 protein levels decline rapidly in response to GA before any increase in GAMYB levels. Green fluorescent protein-SLN1 fusion protein was targeted to the nucleus of aleurone protoplasts and disappeared in response to GA. Evidence from a dominant dwarf mutant at *Sln1*, and from the *gse1* mutant (that affects GA "sensitivity"), indicates that GA acts by regulating SLN1 degradation and not translation. Mutation of the DELLA region of SLN1 results in increased protein stability in GA-treated layers, indicating that the DELLA region plays an important role in GA-induced degradation of SLN1. Unlike GA, ABA had no effect on SLN1 stability, confirming that ABA acts downstream of SLN1 to block GA signaling.

Cereal mutants impaired in GA signaling have proved to be useful tools in identifying GA signal transduction components that regulate gene expression in aleurone cells. The dwarf rice mutant, *d1*, has a mutation in a gene encoding a heterotrimeric  $G\alpha$  protein that impairs GA signaling in aleurone cells and internodes (Ashikari et al., 1999; Fujisawa et al., 1999; Ueguchi-Tanaka et al., 2000). GAMYB,  $\alpha$ -amylase, and  $Ca^{2+}$ -ATPase expression was greatly reduced in GA-treated aleurone layers from *d1* grain compared with wild type. The role of  $G\alpha$  protein in GA signaling is further supported by the demonstration that Mas 7, a stimulator of  $G\alpha$  protein function, induced  $\alpha$ -amylase expression in oat (*Avena sativa*) protoplasts (Jones et al., 1998).

Analyses of another class of GA response mutants in wheat (*Triticum aestivum*), rice (*Oryza sativa*), and barley (*Hordeum vulgare*) have identified a role for GAI/RGA-like proteins in GA signaling in cereal aleurone cells (Peng et al., 1999; Ikeda et al., 2001; Chandler et al., 2002). In Arabidopsis, RGA (Silverstone et al., 1998) and GAI (Peng et al., 1997) act as negative regulators of GA signaling and similar roles are proposed for Rht-B1/Rht-D1 in wheat (Peng et al., 1999), SLR1 in rice (Ikeda et al., 2001), and SLN1 in barley (Chandler et al., 2002). These proteins belong to the plant-specific GRAS family of regulatory proteins

and are characterized by conserved amino acid sequence domains; a central VHIID and carboxy-terminal RVER, homopolymeric Ser and Thr, Leu heptad repeats, nuclear localization signals, and a highly conserved amino-terminal DELLA domain. Recent new evidence indicates that GA derepression of RGA function occurs through promotion of RGA degradation (Silverstone et al., 2001). Further work is needed to test whether other members of this family also exhibit GA-dependent protein degradation.

Mutations in the DELLA region of GAI in Arabidopsis (Peng et al., 1997), *Rht-B1/Rht-D1* in wheat (Peng et al., 1999), and *Sln1* in barley (Chandler et al., 2002) show dominance and result in reduced GA responses, producing a dwarf plant phenotype. Aleurone cells expressing these mutant alleles in wheat (Gale and Marshall, 1973; Ho et al., 1981) and barley (Chandler et al., 2002) show a reduced sensitivity to GA. The DELLA domain has been proposed to be a GA-signaling domain, which controls repressor stability (Peng et al., 1997; Dill et al., 2001). Mutations outside the DELLA region in the rice *SLR1* and barley *Sln1* genes result in constitutive expression of  $\alpha$ -amylase genes, indicating a loss of SLR1 and SLN1 function. The double mutant *d1/slr1* in rice shows a phenotype identical to *slr1*, indicating that SLR1 is epistatic to D1. Other negative regulators of GA signaling such as SPY (Jacobsen et al., 1996) and SHI (Fridborg et al., 1999) have been identified in Arabidopsis by mutational analysis, but as yet no corresponding mutants have been identified in cereals. A gene orthologous to SPY has been isolated from barley and there is supporting evidence of a role for

\* Corresponding author; e-mail frank.gubler@csiro.au; fax 61-0262465000.

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HvSPY in GA signaling in barley aleurone (Robertson et al., 1998).

The recent isolation of the *Sln1* gene in barley (Chandler et al., 2002) provides us with an opportunity to study its role in GA-regulated gene expression in the well-characterized aleurone experimental system. Recent evidence indicates that early GA response genes such as GAMYB (Gubler et al., 1995) are likely targets for SLN1 repression (Gómez-Cadenas et al., 2001). Expression of a GAMYB promoter  $\beta$ -glucuronidase (GUS) construct was reported to be higher in *sln1a* mutant aleurone cells than in wild type. In this paper, we show that treatment of isolated barley aleurone layers with GA results in an increase in GAMYB transcription rates and that SLN1 is required for repression of GAMYB. To understand how SLN1 is acting as a negative regulator of GAMYB and  $\alpha$ -amylase gene expression, we analyzed the effect of GA on SLN1 mRNA and protein levels, and found that SLN1 protein levels fell rapidly in response to GA, before the increase in GAMYB protein levels. We propose that GA signaling acts through SLN1 by modulating SLN1 degradation, similar to that recently found for RGA in Arabidopsis (Silverstone et al., 2001). Further evidence is presented that indicates that the DELLA region and the COOH terminus of SLN1 are important in GA-regulated breakdown of SLN1.

## RESULTS

### GA and Abscisic Acid (ABA) Regulate GAMYB Transcription

GA had been shown to induce a rapid increase in GAMYB mRNA levels in barley aleurone layers (Gubler et al., 1995). Recent transient expression experiments using a GAMYB promoter-GUS construct suggest that GA regulates GAMYB transcription in aleurone cells (Gómez-Cadenas et al., 2001). To confirm whether the increase in mRNA levels in response to GA was a result, at least in part, of an increase in the rate of transcription, nuclear run-on experiments were performed with isolated nuclei from aleurone layers treated with and without GA<sub>3</sub> for 2 h (Fig. 1, A and B). The in vitro-synthesized RNA was hybridized to a 3' fragment of the GAMYB cDNA and, as a control, a cDNA encoding a rRNA gene. In response to GA<sub>3</sub>, accumulation of GAMYB transcripts increased over 2-fold compared with control treatments. There was no significant increase in the rate of transcription of the rRNA gene.

ABA blocks the expression of many GA-induced genes in aleurone cells. Figure 1C shows that a 50-fold molar excess of ABA partly blocks the GA<sub>3</sub>-induced increase in GAMYB mRNA in aleurone layers. These effects on GAMYB steady-state mRNAs were mirrored in the amount of mRNA of one of its downstream targets,  $\alpha$ -amylase (Fig. 1C). Quantitation of GAMYB mRNA showed that amounts of tran-

script were reduced by 50% in aleurone layers treated with GA<sub>3</sub> and ABA compared with layers incubated with GA<sub>3</sub> alone (data not shown). To determine whether ABA also acts at the level of GAMYB gene transcription, GAMYB transcripts were quantified in nuclei from layers treated with GA<sub>3</sub> and ABA. Figure 1, A and B, demonstrate that ABA inhibits the GA-induced increase in rate of GAMYB transcript synthesis and that GAMYB transcript accumulation is partly blocked by ABA.

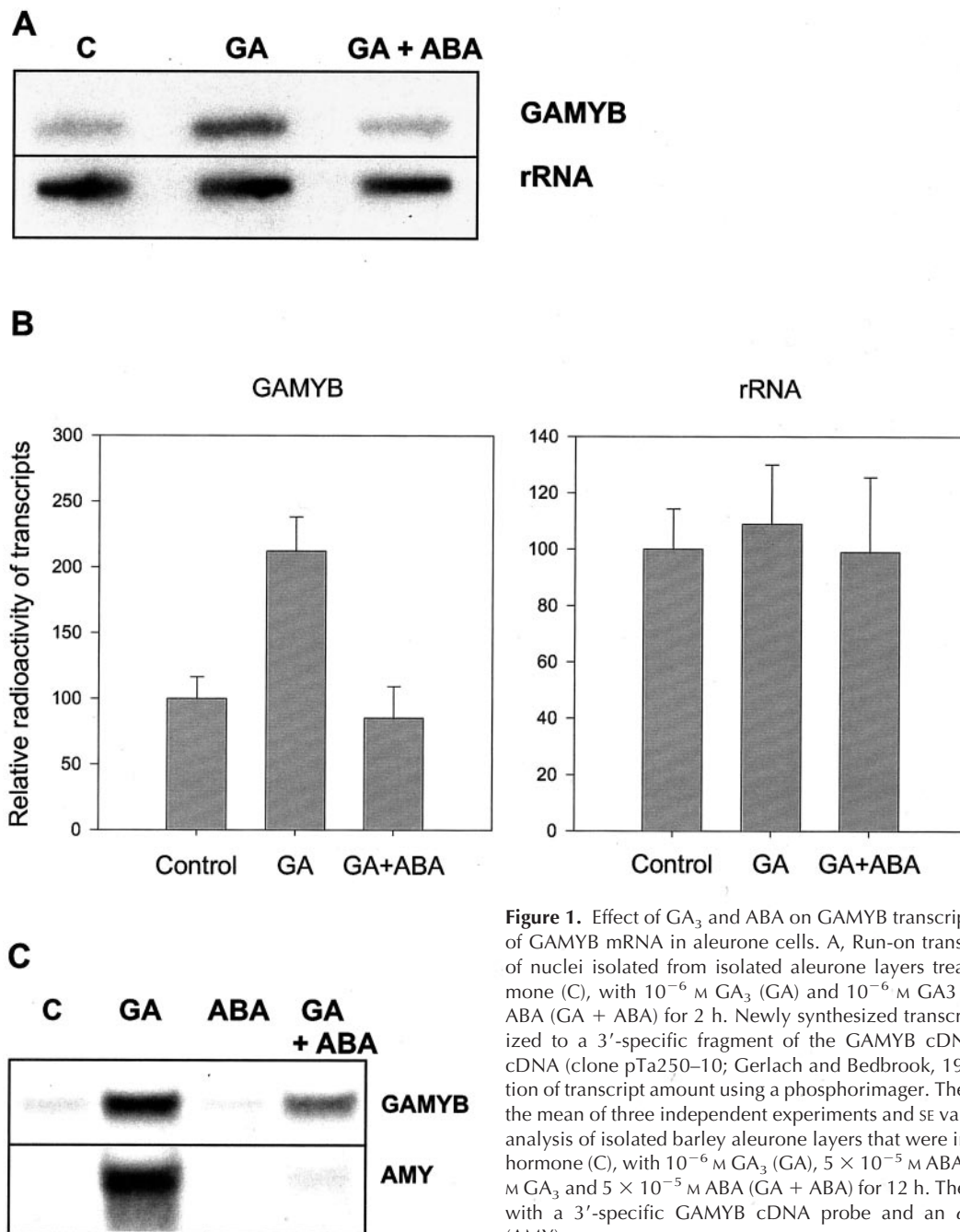
### SLN1 Is a Negative Regulator of GAMYB Gene Expression

SLN1 is a negative regulator of GA responses in aleurone cells. The dominant dwarf allele *Sln1d* results in aleurone cells that are less sensitive to applied GA<sub>3</sub> (Chandler et al., 2002). Applications of 10<sup>-9</sup> M GA<sub>3</sub> induce high levels of  $\alpha$ -amylase in wild-type barley cv Himalaya aleurone cells, but fail to induce  $\alpha$ -amylase expression in *Sln1d* aleurone cells. At higher concentrations of GA<sub>3</sub> (10<sup>-6</sup> M), both mutant and wild-type cells showed a large response to GA<sub>3</sub>. To test if GAMYB expression was also affected by the *Sln1d* allele, RNA was isolated from wild-type and mutant aleurone layers treated with no hormone, 10<sup>-9</sup> M GA<sub>3</sub>, or 10<sup>-6</sup> M GA<sub>3</sub>. Figure 2 shows that in wild-type aleurone cells, GAMYB mRNA levels increased in response to 10<sup>-9</sup> and 10<sup>-6</sup> M GA<sub>3</sub>, but in *Sln1d* cells, GAMYB showed only a response at 10<sup>-6</sup> M GA<sub>3</sub>. This mutation reduced the sensitivity of GAMYB expression to GA<sub>3</sub>.

### GA Regulates SLN1 Protein Levels

The above results suggest that GA may act on GAMYB expression via the negative regulator SLN1. To investigate GA-regulated derepression of SLN1 activity in aleurone cells, we investigated the possibility that GA regulates SLN1 transcription or steady-state amounts of SLN1 mRNA. RNA was isolated from aleurone layers treated for up to 12 h with and without GA<sub>3</sub> and probed for SLN1 and  $\alpha$ -amylase mRNA. Figure 3 shows that SLN1 mRNA levels did not change in response to GA<sub>3</sub>, in contrast to  $\alpha$ -amylase mRNA, which increased strongly.

To investigate whether GA<sub>3</sub> regulates SLN1 protein levels in aleurone cells, antibodies were raised to His-tagged fusion protein containing SLN1 amino acid residues 1 through 170. When total protein isolated from wild-type aleurone layers were probed with anti-SLN1 antibodies, a polypeptide of approximately 70 kD was recognized, a size close to the predicted molecular mass of 65.2 kD for SLN1 (Fig. 4). The 70-kD band was not readily detectable in aleurone layers isolated from homozygous *sln1b* grains confirming the specificity of the anti-SLN1 antibodies. The *sln1b* gene has a mutation that causes a frameshift in the *Sln1* open reading frame, resulting



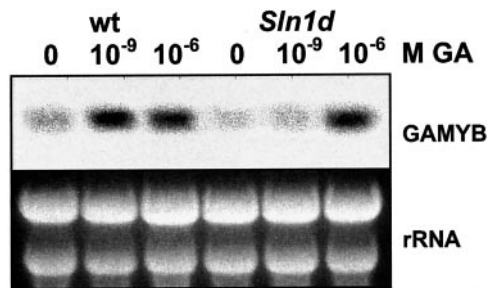
**Figure 1.** Effect of GA<sub>3</sub> and ABA on GAMYB transcription and amount of GAMYB mRNA in aleurone cells. A, Run-on transcription analyses of nuclei isolated from isolated aleurone layers treated without hormone (C), with 10<sup>-6</sup> M GA<sub>3</sub> (GA) and 10<sup>-6</sup> M GA<sub>3</sub> and 5 × 10<sup>-5</sup> M ABA (GA + ABA) for 2 h. Newly synthesized transcripts were hybridized to a 3'-specific fragment of the GAMYB cDNA and an rRNA cDNA (clone pTa250-10; Gerlach and Bedbrook, 1979). B, Quantitation of transcript amount using a phosphorimager. The values represent the mean of three independent experiments and se values. C, RNA-blot analysis of isolated barley aleurone layers that were incubated without hormone (C), with 10<sup>-6</sup> M GA<sub>3</sub> (GA), 5 × 10<sup>-5</sup> M ABA (ABA), and 10<sup>-6</sup> M GA<sub>3</sub> and 5 × 10<sup>-5</sup> M ABA (GA + ABA) for 12 h. The blot was probed with a 3'-specific GAMYB cDNA probe and an α-amylase cDNA (AMY).

in a protein of a predicted molecular mass of 27.8 kD (Chandler et al., 2002).

The antibodies to SLN1 were used in western blots to monitor amounts of SLN1 protein in aleurone cells treated with GA<sub>3</sub> (Fig. 5A). In the absence of GA<sub>3</sub>, amounts of SLN1 protein increased slowly over the 12-h incubation period. GA<sub>3</sub> treatment resulted in a decrease in the amount of SLN1 protein within the first 30 min of incubation and remained low over the next 12 h. Figure 5B shows that the decline in the amount of SLN1 protein was very rapid in GA<sub>3</sub>-treated aleurone cells. The decrease began within 5

min of GA<sub>3</sub> application and was complete by 10 min. As a control, the amounts of GAMYB protein were also monitored using antibodies raised against the COOH-terminal domain of GAMYB. Figure 5A shows that GAMYB protein increased in GA<sub>3</sub>-treated layers within 2 h of application and continued to rise up to 6 h, but declined between 6 and 12 h of GA<sub>3</sub> treatment. In the absence of GA<sub>3</sub>, only low amounts of GAMYB were detected. Figure 5C shows that ABA had no effect on the GA<sub>3</sub>-induced decrease in SLN1 protein.

To examine the subcellular localization of SLN1, aleurone protoplasts were transfected with a green

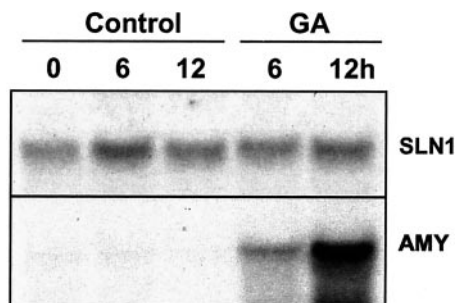


**Figure 2.** The dominant dwarf allele (*Sln1d*) of the SLN1 gene affects responsiveness of GAMYB gene expression to GA<sub>3</sub> in aleurone cells. RNA-blot analysis of wild-type (wt) and mutant *Sln1d* aleurone layers after incubation without hormone, and 10<sup>-9</sup> and 10<sup>-6</sup> M GA<sub>3</sub> for 12 h. The blots were hybridized with a 3'-specific GAMYB cDNA probe. RNA loading was monitored by ethidium bromide staining.

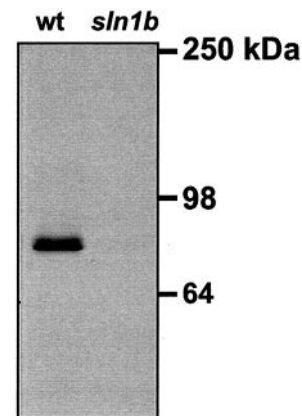
fluorescent protein (GFP)-SLN1 reporter construct. A 4.1-kb SLN1 genomic clone containing the open reading frame, promoter, and terminator sequences was isolated from a barley genomic library. To localize expression of the gene, a GFP gene was inserted in frame at the N terminus of the SLN1 open reading frame. In transfected aleurone protoplasts, GFP-SLN1 accumulated almost exclusively in the nucleus, with weak expression also detected in the cytoplasm (Fig. 6). Addition of GA caused an 80% reduction in the number of protoplasts with detectable GFP in the nucleus within 5 h. No increase in cytoplasmic GFP fluorescence was detected in GA-treated protoplasts.

#### Response of Mutant SLN1 Proteins to GA

The response of mutant SLN1 proteins to GA was tested in aleurone layers carrying mutations in the *Sln1* open reading frame. The dominant dwarf *Sln1d* has a mutation near the DELLA box, which reduces  $\alpha$ -amylase and GAMYB sensitivity to GA<sub>3</sub>. Figure 7A shows that homozygous *Sln1d* aleurone has two distinct bands recognized by the anti-SLN1 antibodies, a



**Figure 3.** SLN1 mRNA accumulation is not regulated by GA<sub>3</sub> in aleurone cells. RNA-blot analysis of SLN1 expression in isolated aleurone layers incubated for up to 12 h without hormone (Control) and 10<sup>-6</sup> M GA<sub>3</sub> (GA). The blots were hybridized with the SLN1 cDNA and an  $\alpha$ -amylase cDNA. RNA loading was monitored by ethidium bromide staining.



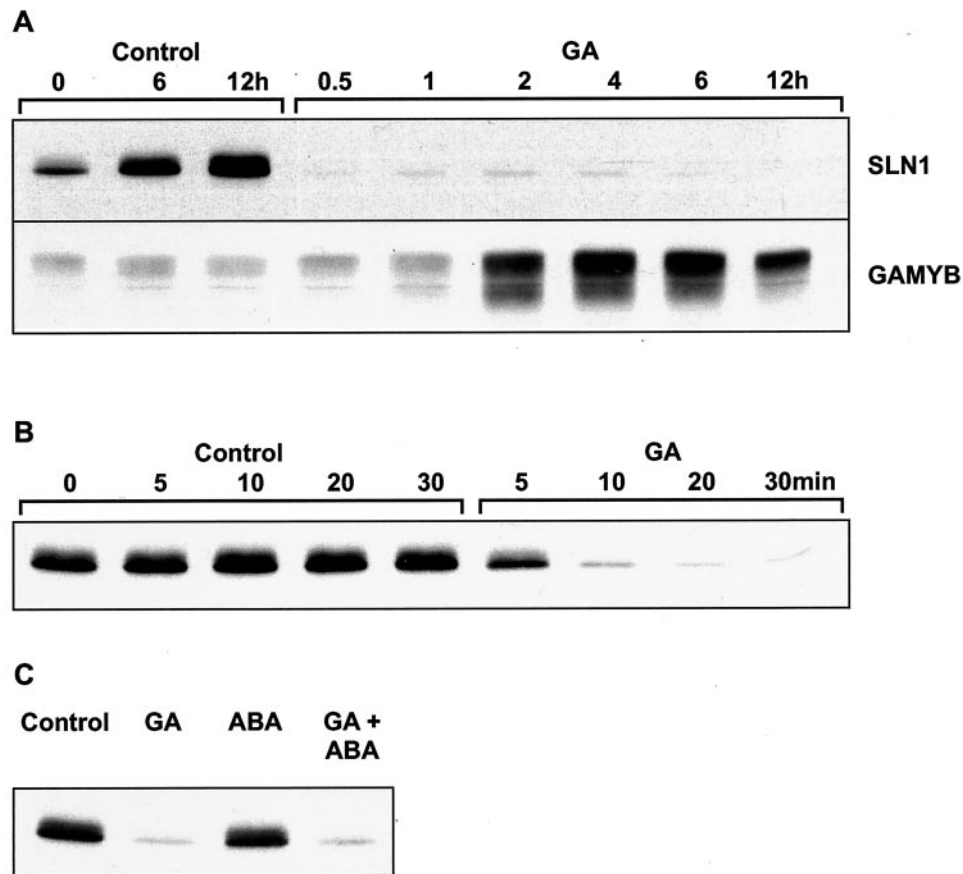
**Figure 4.** Anti-SLN1 antibodies specifically recognize the product of the SLN1 gene in aleurone cells. Total protein was extracted from wild-type (wt) and *sln1b* mutant aleurone layers and run on an SDS-PAGE gel. SLN1 protein was detected on protein blots by using anti-SLN1 antibodies.

band of similar mobility to that found in the wild type (form I) and a prominent band of slightly lower mobility (form II). This form II band is also present in wild type aleurone (e.g. Figs. 4 and 5A), but is often poorly resolved because of the prominence of the lower band. Application of GA<sub>3</sub> at 10<sup>-6</sup> M for 10 min caused a small reduction in total SLN1 protein in *Sln1d* aleurone layers compared with the large reduction in wild-type layers (Fig. 7A). Longer incubations with GA<sub>3</sub> (6 h) resulted in large decreases in SLN1 protein in *Sln1d* layers (data not shown).

SLN1 expression was also examined in *sln1c* aleurone layers (Fig. 7B). The *sln1c* mutation introduces a premature stop codon 18 amino acids before the stop codon and results in loss of SLN1 function. In homozygous *sln1c* aleurone layers,  $\alpha$ -amylase genes are expressed in the absence of GA<sub>3</sub>. Figure 7B shows that in *sln1c* aleurone layers, the anti-SLN1 antibodies detected a truncated polypeptide of lower molecular mass than the full-length polypeptide. The COOH-terminal deletion mutant failed to show any response to GA<sub>3</sub> in *sln1c* aleurone layers in contrast to the full-length protein, which decreased in abundance in wild-type aleurone layers treated with 10<sup>-6</sup> M GA<sub>3</sub>.

#### GSE1 Is a Regulator of SLN1 Response to GA

The barley *gse1* mutant is characterized by reduced sensitivity to GA and genetic analysis indicates that the gene is hypostatic to *Sln1* (Chandler and Robertson, 1999).  $\alpha$ -Amylase expression in de-embryonated mutant *gse1* grains failed to show an increase at GA<sub>3</sub> concentrations less than or equal to 10<sup>-6</sup> M GA<sub>3</sub> compared with wild-type grains, which showed a strong response at 10<sup>-7</sup> M. To test the response of SLN1 protein to GA<sub>3</sub> in a *gse1* background, aleurone layers from wild-type and *gse1* grain were treated



**Figure 5.** GA<sub>3</sub> reduces the amount of SLN1 protein in aleurone cells. A, Time course analyses of SLN1 and GAMYB proteins in aleurone layers incubated for up to 12 h without hormone (Control) and 10<sup>-6</sup> M GA<sub>3</sub> (GA). Total protein was extracted from aleurone layers and run on SDS-PAGE gels. SLN1 protein and GAMYB protein were detected on blots using antibodies to SLN1 and GAMYB. B, Short time course analysis of SLN1 protein expression in aleurone layers treated without hormone (Control) and 10<sup>-6</sup> M GA<sub>3</sub> (GA) for up to 30 min. SLN1 protein was detected as described above. C, Effect of GA<sub>3</sub> and ABA on the amount of SLN1 protein in aleurone layers. Aleurone layers were incubated without hormone (Control), and with 10<sup>-6</sup> M GA<sub>3</sub> (GA), 5 × 10<sup>-5</sup> M ABA (ABA), and 10<sup>-6</sup> M GA<sub>3</sub> and 5 × 10<sup>-5</sup> M ABA (GA + ABA) for 30 min. SLN1 protein was detected as described above.

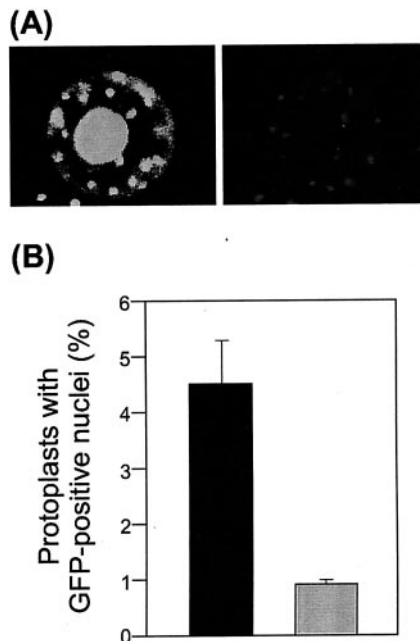
with 0, 10<sup>-7</sup>, and 10<sup>-4</sup> M GA<sub>3</sub> for 30 min (Fig. 8A). SLN1 protein amount was very much elevated in *gse1* aleurone layers incubated without GA compared with similarly treated wild-type layers. In addition, the anti-SLN1 antibodies recognized two equally prominent bands (forms I and II) in *gse1* aleurone layers, which contrasts with wild-type layers, where the faster mobility polypeptide is more prominent.

To determine the effect of *gse1* on the stability of SLN1 protein, we tested the effect of the protein synthesis inhibitor cycloheximide on SLN1 protein over a 6-h period in wild-type and mutant aleurone cells. Within 1 h of cycloheximide treatment of wild-type aleurone layers, the amount of SLN1 protein had declined and remained low up to 6 h of treatment (Fig. 8B). These results indicate that SLN1 protein in aleurone cells has a short half-life (<1 h) in wild-type aleurone layers. In *gse1* mutant aleurone layers, SLN1 protein levels declined more slowly after addition of cycloheximide.

## DISCUSSION

Current genetic evidence indicates that SLN1 and related proteins such as Rht, SLR1, GAI, and RGA are negative regulators of GA signaling in plants (Richards et al., 2001). Our data bring new insights into the molecular mechanisms of GA control of SLN1 repressor function in barley aleurone cells. Within 10 min of GA application, the amount of SLN1 protein decreased and remained low up to 12 h. This result indicates that GA relieves SLN1 "repression" of gene expression by modulating the amount of SLN1 protein. Furthermore, we show that the DELLA domain and the COOH terminus are important in regulating the stability of SLN1 protein.

The failure to detect any significant changes in SLN1 mRNA in aleurone cells in response to GA rules out the possibility that the rapid decline in SLN1 protein is a result of GA-induced changes in SLN1 transcription and/or mRNA stability. This is



**Figure 6.** GFP-SLN1 fusion protein responds to GA<sub>3</sub> in transfected aleurone protoplasts. A, Scanning laser confocal microscope images of aleurone protoplasts transfected with GFP-SLN1 construct. Left, GFP fluorescence; right, autofluorescence. B, GFP-positive nuclei were expressed as a percentage of total numbers of cells after treatment with 10<sup>-6</sup> M GA<sub>3</sub> (gray histogram) or without GA<sub>3</sub> (control; black histogram) for 5 h. Error bars represent the SE values of the means.

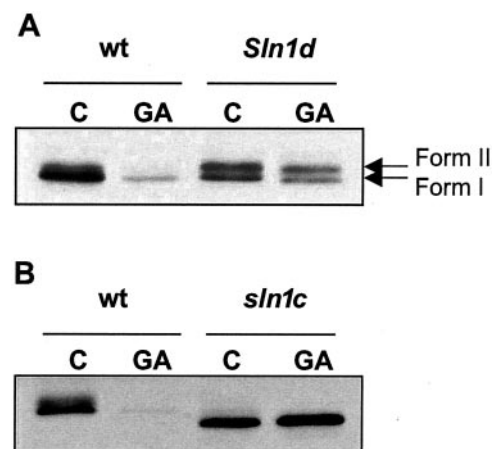
further supported by studies in rice (Ogawa et al., 2000) and Arabidopsis (Silverstone et al., 1998), which show that GA causes only small increases in mRNA of *Sln1*-like genes in young seedlings. On the basis of this evidence, it seems most likely that GA may regulate the amounts of SLN1 protein either by blocking translation and/or by promoting SLN1 protein degradation. Evidence from the *sln1c* and *Sln1d* mutants strongly indicates that GA acts through the latter process. The *sln1c* mutation has a premature stop codon 18 amino acids before the SLN1 COOH terminus that causes loss of repression of  $\alpha$ -amylase genes (Chandler et al., 2002). Using anti-SLN1 antibodies, we were able to show that in contrast to wild type, SLN1 protein in the *sln1c* mutant did not change in response to GA. This result is consistent with GA promoting SLN1 degradation in aleurone cells. If GA blocked translation, one would expect SLN1 protein to decline in the mutant at a similar rate to the wild-type protein in response to GA. Enhanced SLN1 expression in leaf blades in the *sln1c* mutant is consistent with this model and suggests that GA also regulates SLN1 protein stability in leaves (Chandler et al., 2002). The reduced response of SLN1 protein in mutant *Sln1d* layers to GA is also consistent with GA regulating the stability of SLN1, rather than its translation.

Further evidence for GA regulation of SLN1 stability was obtained by comparing SLN1 protein half-life

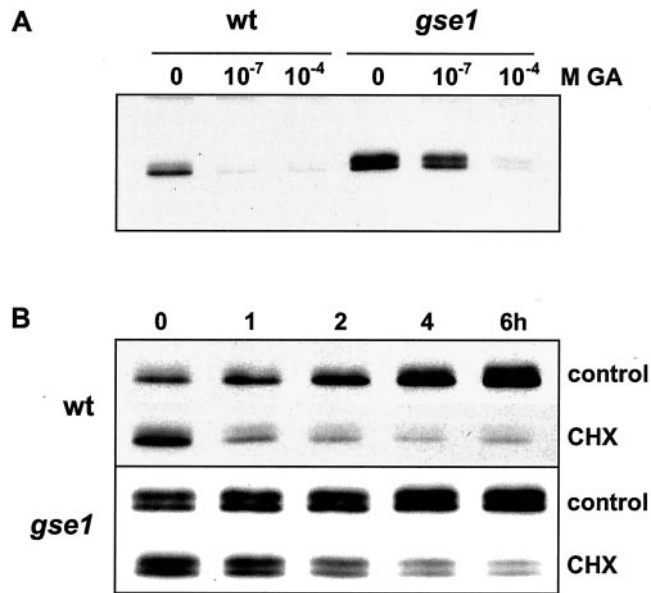
in wild-type and *gse1* mutant aleurone layers. SLN1 protein was more stable in cycloheximide-treated *gse1* mutant aleurone layers compared with wild-type aleurone layers. The increase in protein stability is likely to be because of the partial block in GA signaling caused by the mutant *gse1* allele. The *gse1* mutant aleurone layers exhibit reduced sensitivity to GA and genetic analysis reveals that the mutation is hypostatic to *Sln1* (Chandler and Robertson, 1999).

The mechanism of GA derepression of SLN1 activity in barley aleurone cells is similar to that found in Arabidopsis for RGA (Silverstone et al., 2001). Addition of GA to GA-deficient Arabidopsis plants caused a reduction in the amount of RGA protein within 2 h. Transgenic Arabidopsis expressing GFP-RGA also responded to GA. GFP fluorescence was detected in nuclei of roots of transgenic plants, but quickly disappeared after GA application. A similar result was found in our study with transiently expressed GFP-SLN1 in aleurone protoplasts. These results together indicate that GA-regulated protein degradation might be a common mechanism for regulating the activity of SLN1 and RGA proteins in cereals and dicots. It is now important to determine the GA-regulated mechanisms involved in the degradation of SLN1 and RGA. Ubiquitination has been shown to be important in a number of plant signaling pathways (Callis and Vierstra, 2000; Karniol and Chamovitz, 2000) and is likely to play a role in degradation of RGA (Silverstone et al., 2001) and also SLN1.

Mutations near the NH<sub>2</sub> terminus in the DELLA region of GAI, Rht, and SLN1 result in a semidominant dwarf phenotype with reduced GA responses (Peng et al., 1997, 1999; Chandler et al., 2002). The *Sln1d* allele of barley has an amino acid substitution in the DELLA region that causes a 100-fold decrease



**Figure 7.** Effects of GA on SLN1 protein levels in *Sln1d* and *sln1c* aleurone layers. A, Wild-type (wt) and mutant *Sln1d* aleurone layers were treated with and without GA<sub>3</sub> for 10 min. Protein blots of total aleurone protein were probed with anti-SLN1 antibodies. B, Wild-type (wt) and mutant *sln1c* aleurone layers were incubated with and without 10<sup>-6</sup> M GA<sub>3</sub> for 30 min. Protein blots of total aleurone protein were probed with anti-SLN1 antibodies.



**Figure 8.** Effects of GA on SLN1 protein levels in aleurone of wild-type and *gse1* grains. A, Wild-type (wt) and mutant *gse1* aleurone layers were incubated with GA<sub>3</sub> or without GA<sub>3</sub> for 30 min. Protein blots of total aleurone protein were probed with anti-SLN1 antibodies. B, Total protein was extracted from wild-type and *gse1* aleurone layers that were incubated without (Control) and with the protein biosynthesis inhibitor, cycloheximide (CHX). SLN1 protein levels were detected on protein blots using anti-SLN1 antibodies.

in GA sensitivity for  $\alpha$ -amylase production in aleurone cells (Chandler et al., 2002). In the absence of GA, the amounts of SLN1 protein in *Slm1d* aleurone layers were similar to those in wild-type layers except that form II was more prominent in the mutant background. Our results indicate that the increased GA insensitivity was associated with increased SLN1 protein stability in the mutant background. After 10 min of exposure to 10<sup>-6</sup> M GA<sub>3</sub>, amounts of SLN1 protein were higher in mutant *Slm1d* layers compared with wild type. Similarly, in transgenic Arabidopsis plants, a 17-amino acid deletion within the DELLA domain of RGA has a strong effect on the stability of the mutant protein (Dill et al., 2001). The *rga- $\Delta$ 17* protein is resistant to applied GA in contrast to the wild-type protein, which is rapidly degraded in response to GA. Both these results support the hypothesis that the mutations in the DELLA region result in proteins that act as constitutive repressors of GA signaling (Peng et al., 1997).

The increased prominence of a slower mobility form of SLN1 (form II) in both *Slm1d* and *gse1* mutant aleurone layers is interesting. In both instances, form II is as abundant as the higher mobility form of SLN1 (form I) in contrast to wild type, where form I predominates. We speculate that form II may be the active form of SLN1, perhaps activated by posttranslational modifications. Immunoblot analysis of transgenic Arabidopsis plants expressing *rga- $\Delta$ 17* protein, also detect an extra protein with slower mobility,

indicating that posttranslational modifications may also be important in regulating RGA function (Dill et al., 2001). Posttranslational modifications, which regulate function of SLN1, require an intact COOH terminus. Higher mobility forms of SLN1 were not detected in the loss of function mutant *slm1c*, which has a premature stop codon 18 amino acids before the COOH terminus.

We provide genetic evidence that SLN1 is necessary for repression of GAMYB, a GA-regulated transcription factor implicated in activation of  $\alpha$ -amylase genes (Gubler et al., 1995, 1999). Our data support recent evidence indicating the SLN1 functions upstream of *GAMYB* gene transcription (Gomez-Cadenas et al., 2001). Using nuclear run-on experiments, we have demonstrated that the rate of *GAMYB* transcription approximately doubles within 2 h after addition of GA. This is consistent with transient expression experiments that show increases in *GAMYB* promoter:GUS reporter activity after addition of GA (Gomez-Cadenas et al., 2001). The authors also reported that the GUS reporter activity in bombarded *slm1a* aleurone layers was comparable with GA-treated wild-type aleurone layers, indicating that *GAMYB* expression is regulated by SLN1. In contrast, we used the dominant gain-of-function mutant, *Slm1d*, to show that GA responsiveness of *GAMYB* gene expression was reduced in mutant aleurone layers compared with wild type.

Very little is known about immediate downstream targets of SLN1 repression. The considerable lag time between SLN1 disappearance and *GAMYB* expression indicates that *GAMYB* may not be an immediate downstream target. Recent evidence indicates that SLR1 may function as a transcriptional activator (Ogawa et al., 2000). GAL4-SLR1 fusion protein activated expression of a reporter gene in bombarded spinach (*Spinacia oleracea*) leaf cells, suggesting that SLR1 has a transcriptional activation domain. If this is also the case for SLN1, then SLN1 and other related proteins may be acting as transcriptional activators of a repressor that inhibits expression of GA-regulated target genes such as *GAMYB*. The lag time between SLN1 disappearance and *GAMYB* gene expression in GA-treated aleurone layers may be explained by such a model. In addition, there is evidence that cGMP may play an intermediary role between SLN1 and *GAMYB* (Gomez-Cadenas et al., 2001). The transient rise in cGMP levels correlates closely with the start of the rise in *GAMYB* protein levels (Penson et al., 1996). An inhibitor of guanylyl cyclase, LY83583, has been shown to block the rise in cGMP and in *GAMYB* mRNA.

Finally, our data extend recent observations that indicate that ABA-signaling pathways interacts with the GA-signaling pathway downstream of SLN1 and upstream of *GAMYB* transcription (Gomez-Cadenas et al., 2001). We show that ABA has no effect on GA-enhanced SLN1 degradation, thus suggesting

that ABA is acting downstream of SLN1. This is consistent with data that show that *sln1a* aleurone cells (loss-of-function mutant) are still responsive to ABA (Lanahan and Ho, 1988). Addition of ABA to *sln1a* aleurone cells blocks the constitutive expression of  $\alpha$ -amylase. Data from transient expression experiments with a *GAMYB* promoter:GUS construct (Gómez-Cadenas et al., 2001), together with our data from nuclear run experiments, show that ABA blocks GA-induced increases in *GAMYB* transcription, suggesting that ABA acts upstream of *GAMYB*. PKABA1, a protein kinase, has been proposed to mediate the down-regulation of *GAMYB* expression by ABA (Gómez-Cadenas et al., 2001), but as yet PKABA1 phosphorylation targets have not been identified. Further studies are required to identify potential candidates that interconnect GA- and ABA-signaling pathways in aleurone cells.

## MATERIALS AND METHODS

### Plant Materials

All lines are derived from the tall barley (*Hordeum vulgare* cv Himalaya) and are described by Chandler et al. (2002).

### Hormonal Treatment of Aleurone Layers

Aleurone layers were prepared from barley cv Himalaya grains as described previously (Chrispeels and Varner, 1967). The isolated layers were incubated in flasks containing 2 mL of 10 mM CaCl<sub>2</sub>, 150  $\mu$ g mL<sup>-1</sup> cefotaxime, 50 units mL<sup>-1</sup> nystatin, and no hormone (control), 10<sup>-6</sup> M GA<sub>3</sub> (GA), 5  $\times$  10<sup>-5</sup> M ABA, or 10<sup>-6</sup> M GA<sub>3</sub> and 5  $\times$  10<sup>-5</sup> M ABA (GA + ABA) at 25°C for various times. In some experiments, aleurone layers were incubated with 30  $\mu$ M cycloheximide, 10 mM CaCl<sub>2</sub>, and antibiotics.

### RNA Analyses

After incubation, 20 aleurone layers were frozen in liquid nitrogen and homogenized to a fine powder in a mortar and pestle. The powder was added to an equal volume of 10 mM Tris, pH 8.0, containing 1 mM EDTA and 100 mM NaCl and phenol:chloroform:isoamyl alcohol (25:24:1 [v/v]) and vortexed vigorously. After centrifuging, the supernatant was re-extracted with phenol:chloroform:isoamyl alcohol. RNA was precipitated from the supernatant by addition of an equal volume of 4 M LiCl. After washing the RNA pellet with 70% (v/v) ethanol, the pellet was dissolved in water and stored at -20°C.

Aleurone RNA (10  $\mu$ g per lane) was analyzed by electrophoresis in a formaldehyde-agarose gel and transferred to nylon membrane by capillary blotting with 20 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate). Random-labeled DNA probes to barley *GAMYB* cDNA (1,242–1,925 bp; Gubler et al., 1995), barley *SLN1* gene (233–1,957 bp), and barley  $\alpha$ -amylase cDNA (pHv19 cDNA; Chandler et al., 1984) were hybridized overnight in 6 $\times$  SSC, 5 $\times$  Den-

hardt's solution, 0.1% (w/v) SDS, and 100  $\mu$ g mL<sup>-1</sup> salmon sperm DNA at 65°C. After the final wash in 0.1 $\times$  SSC and 0.1% (w/v) SDS at 65°C, the blots were prepared for autoradiography.

### Preparation of Antibodies and Immunoblot Analyses

A PCR product encoding amino acid position 300 through 553 of *GAMYB* was ligated into pEt32b (Novagen, Madison, WI) and the recombinant plasmid was transformed into *Escherichia coli* BL21(DE3). After induction with isopropyl  $\beta$ -D-thiogalactoside, the cells were lysed in 6 M urea and the His-tagged *GAMYB* fusion protein was purified on a metal affinity column (CLONTECH Laboratories, Palo Alto, CA). The purified fusion protein was used to raise polyclonal antibodies in rabbits according to standard procedures. A similar approach was used to raise antibodies to *SLN1*. A His-tagged *SLN1* fusion protein (corresponding to amino acids 1–170) was expressed in BL21(DE3) cells transformed with a recombinant pET19b construct.

To reduce nonspecific staining during protein blotting, the IgG fractions of both sera were purified on a Protein A-Sepharose column (Amersham-Pharmacia Biotech, Uppsala) followed by antigen-CNBr-Sepharose columns (Amersham-Pharmacia Biotech) according to manufacturer's instructions. One hundred millimolar Gly, pH 2.5, was used to elute antibodies from the antigen columns and immediately neutralized with 1 M Tris-HCl, pH 8.5.

Aleurone protein for immunoblot analysis was extracted by grinding the tissue in a mortar with Laemmli buffer. The homogenate was heated to 100°C and then centrifuged to remove the insoluble debris. After SDS-PAGE and electroblotting, *GAMYB* and *SLN1* protein was detected with 0.5 to 1.0  $\mu$ g mL<sup>-1</sup> affinity-purified antibodies in Tris-buffered saline buffer containing 0.5% (v/v) Tween 20 and 0.2% (w/v) I-Block (Tropix, Bedford, MA). Donkey anti-rabbit antibody conjugated to horseradish peroxidase (Amersham-Pharmacia Biotech) diluted 1:10,000 (w/v) in the same buffer was used to detect the primary antibodies. Detection of the secondary antibody complex was by chemiluminescence (NEN Life Science Products, Boston).

### Nuclear Run-on Experiments

For nuclear run-on experiments, isolated aleurone layers (200 layers per treatment) were incubated for 2 h at 25°C and then frozen in liquid nitrogen. The frozen layers were homogenized in liquid nitrogen and the powder stirred into 50 mL of 25 mM Tris, pH 8.5, containing 0.44 M Suc, 2.5% (w/v) Ficoll, 5% (w/v) Dextran T40, 5 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 1 mM spermine, and 0.5% (v/v) Triton X-100. After filtering the homogenate with Miracloth (Calbiochem, San Diego), nuclei were recovered by centrifugation at 5,000 rpm. The nuclei pellet was resuspended in the Tris buffer minus spermine and purified on a Percoll gradient. The purified nuclei were washed twice in 50 mM Tris, pH 8.5, containing 5 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, and 20% (v/v) glycerol, and finally resuspended



in the above buffer containing 50% (v/v) glycerol and stored at  $-80^{\circ}\text{C}$ . Nuclear run-on transcription assays and hybridizations were performed as described by Cox and Goldberg (1988).

### Transient Expression and Confocal Microscopy

A 4.1-kb genomic fragment containing the SLN1 gene was isolated from a Morex barley genomic library (Chandler et al., 2002). The genomic clone included 1.7 kb of 5' sequences upstream of the translation start and 0.5 kb of 3' sequences downstream from the translation stop. An amino terminal GFP fusion was constructed by firstly introducing a *SrfI* site between amino acids  $^1\text{Met}$  and  $^2\text{Arg}$  by PCR. A sGFP(S65T) gene (Chui et al., 1996) was introduced into the *SrfI* site resulting in an open reading frame containing a GFP-SLN1 fusion.

Aleurone protoplasts were prepared from barley cv Himalaya aleurone layers and transfected with GFP-SLN1 construct as described previously (Gubler and Jacobsen, 1992). Five micromolar ABA was added to the solutions used for protoplast isolation to prevent endogenous GA activating the GA response pathway (Bethke et al., 1999). After isolation, the protoplasts were incubated overnight at  $24^{\circ}\text{C}$  in flasks containing  $5\ \mu\text{M}$  ABA. After 16 h,  $\text{GA}_3$  was added to flasks (final concentration  $10^{-6}\ \text{M}$   $\text{GA}_3$ ) or an equivalent volume of water was added and incubated for a further 5 h. A TCS SP2 scanning laser confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to examine  $10\text{-}\mu\text{L}$  aliquots of transfected protoplasts. After excitation at 488 nm, GFP fluorescence from 500 to 600 nm and autofluorescence from 600 to 720 nm were collected simultaneously in separate channels. After image collection, the total number of live cells and the total number of cells expressing GFP were counted, and the percentage of live cells expressing GFP was calculated.

### Distribution of Materials

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

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