Mutants at the *Slender1* Locus of Barley cv Himalaya. Molecular and Physiological Characterization

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A dominant dwarf mutant of barley (*Hordeum vulgare*) that resembles dominant gibberellin (GA) "-insensitive" or "-nonresponsive" mutants in other species is described. α-Amylase production by endosperm half-grains of the mutant required GA_3 at concentrations about 100 times that of the *WT*. The mutant showed only a slight growth response to GA_3, even at very high concentrations. However, when additionally dwarfed, growth rate responded to GA_3 over the normal concentration range, although only back to the original (dwarf) elongation rate. Genetic studies indicated that the dominant dwarf locus was either closely linked or identical to the *Sln1* (Slender1) locus. A barley sequence related to Arabidopsis *GAI/RGA* was isolated, and shown to represent the *Sln1* locus by the analysis of *sln1* mutants. The dominant dwarf mutant was also altered in this sequence, indicating that it too is an allele at *Sln1*. Thus, mutations at *Sln1* generate plants of radically different phenotypes; either dwarfs that are largely dominant and GA "-insensitive/-nonresponsive," or the recessive slender types in which GA responses appear to be constitutive. Immunoblotting studies showed that in growing leaves, SLN1 protein localized almost exclusively to the leaf elongation zone. In mutants at the *Sln1* locus, there were differences in both the abundance and distribution of SLN1 protein, and large changes in the amounts of bioactive GAs, and of their metabolic precursors and catabolites. These results suggest that there are dynamic interactions between SLN1 protein and GA content in determining leaf elongation rate.

There are two distinctive categories of GA-signaling mutants that have been characterized across a range of plant species. The first exhibits a (partially) dominant GA "-insensitive" or "-nonresponsive" phenotype, and includes representatives from wheat (*Triticum aestivum*; *Rht*; Gale and Marshall 1973), maize (*Zea mays*; *D8*; Phinney, 1956), and Arabidopsis (*gai*; Koornneef et al., 1985). The cloning of the Arabidopsis *GAI* gene identified the protein involved in GA signaling, and revealed that the *gai* mutant had a 17-amino acid deletion near the NH_2 terminus (Peng et al., 1997). The analysis of *GAI*-related sequences in *Rht* wheat and *D8* maize revealed sequence alterations near the NH_2 terminus of a protein that was conserved between the three species, and presumably orthologous (Peng et al., 1999). These dominant dwarf mutants are commonly referred to as GA "insensitive" because they fail to grow more rapidly in response to applied GA. However, when they are further dwarfed either by genetic means (Koornneef et al., 1985) or by application of a GA biosynthesis inhibitor (Winkler and Freeling, 1994), a growth response to applied GA is observed. This growth response is restricted in magnitude because growth rate is restored only back to that of the original dwarf, and its GA concentration dependence has not been reported. It is probably more accurate to describe the dominant dwarf category of mutant as having a limited or reduced GA-signaling output.

The second distinctive group of GA-signaling mutants, the so-called slender mutants, are characterized by extremely rapid growth, and appear to have constitutive GA responses. Slender mutants have been characterized in barley (*Hordeum vulgare*; Foster 1977), pea (*Pisum sativum*; where it is a double gene combination, Potts et al., 1985), and rice (*Oryza sativa*; Ikeda et al., 2001), and they exhibit rapid growth even in GA-deficient backgrounds (Potts et al., 1985; Chandler and Robertson, 1999), or when treated with inhibitors of GA biosynthesis (Croker et al., 1990; Ikeda et al., 2001). This observation suggests that growth of slender plants is either independent of bioactive GA, or requires much lower than normal concentrations of bioactive GAs. The slender mutants of barley and rice also show apparent GA independence for a different GA response, α-amylase production by aleurone (Chandler, 1988; Lanahan and Ho, 1988; Ikeda et al., 2001). The slender phenotype is recessive, and assuming that it represents a loss of function, the *WT* slender gene product (encoded by the *Sln1* locus in barley) would be a negative regulator or "repressor" of GA-regulated responses, through which GA signaling proceeds (Chandler and Robertson, 1999).

There has been considerable progress in formulating models to explain how the proteins encoded by *GAI*, and by the closely related *RGA* locus (Silver-

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stone et al., 1997), function in GA signaling in Arabidopsis (Richards et al., 2001; Silverstone et al., 2001). The GAI/RGA proteins are thought to "repress" GA-regulated responses, but the degree of repression is modulated by GA signaling. In the wild type, a high content of endogenous bioactive GA will promote GA signaling, derepress GAI/RGA action, and growth will be rapid. In a GA-deficient mutant, the low content of bioactive GA will result in a low amount of GA signaling, repression by GAI/RGA will remain high, and growth will be slow (see the "Discussion" for further details).

There are substantial similarities between the model to explain the slender phenotype (Chandler and Robertson, 1999) and that which has emerged from studies on *GAI* and *RGA* in Arabidopsis—both invoke "repressors" or negative regulators of GA responses that are modulated by GA signaling. It has been speculated that these two mutant categories might result from mutations at the same locus, despite their radically different phenotypes (Scott, 1990), but there has been no single species in which both mutant types were described, so genetic tests for allelism were not possible. In this paper, we characterize a dominant dwarf mutant of barley, and show by crossing and progeny analysis that this mutant locus is very closely linked to the *Sln1* locus. We isolated a barley clone related to *GAI/RGA*, and showed on the basis of sequence alterations in slender mutants that the clone represents the *Sln1* locus. Furthermore, the dominant dwarf mutant is also altered in this sequence, indicating that it is a mutant at the *Sln1* locus. Similar conclusions have recently been reached for the *SLR1* locus in rice (Ikeda et al., 2001), although their GA-insensitive dwarf phenotype resulted from expression of a SLR1 transgene containing a 17-amino acid deletion similar to that in the *gai* mutant of Arabidopsis under the control of an actin promoter. We have also examined the expression of SLN1 protein in relation to leaf elongation, and studied the effects of both types of mutation at *Sln1* on GA and abscisic acid (ABA) contents of growing leaf blades.

RESULTS

M640, a Dominant Dwarf Mutant with Altered GA Responses

The tall barley cv Himalaya was treated with sodium azide and the M_2 generation screened for dwarf mutants (Zwar and Chandler, 1995). One such line, designated M640, had a dwarf phenotype that showed a high degree of dominance, and there was little response to applied GA_3. The effect of GA_3 on growth rate of the first leaf blade was determined for homozygous and heterozygous BC3 (three backcross generations) stocks of M640, for barley cv Himalaya, as well as for two other types of dwarf mutant—*grd2*, a putative GA biosynthetic mutant, and *gse1*, which responds to GA_3 only at concentrations 100 to 1,000-fold higher than normal (Chandler and Robertson, 1999). The results (Table I) indicate that both homozygous and heterozygous stocks of M640 show a greatly reduced response to GA_3 when compared either with the *WT*, or with a typical GA-responsive dwarf such as *grd2*. For lines with normal sensitivity to GA, an applied concentration of 10 μM GA_3 is saturating for leaf growth (Chandler and Robertson, 1999). At this concentration, the homozygous and heterozygous M640 lines showed about 10% of the response of *grd2*. The M640 heterozygotes grew slightly faster than the homozygous line, indicating a high degree (about 85%) of dominance of the dwarfing phenotype. The growth response to 1,000 μM GA_3 was examined because *gse1* mutants respond to much higher concentrations of GA_3 than do lines with normal GA sensitivity. There was only a slight response of M640 heterozygotes and homozygotes to the 100-fold increase in GA_3 concentration, in contrast to the considerable response shown by *gse1*. Therefore, M640 shows a very limited response to applied GA_3, even at very high concentrations.

A GA deficiency mutation (*grd3*) was crossed into the M640 background, and a segregating population established that is homozygous at the dominant dwarfing locus, but segregating at the GA deficiency locus, producing 3:1 single dwarf:double dwarf seedlings. Results presented below indicate that M640 is mutant at the *Sln1* locus, with the allele being designated *Sln1d*. Therefore, the genotypes of these single dwarf and double dwarf lines are *Sln1d,Grd3* and *Sln1d,grd3*, respectively. Grains were germinated in the presence of GA_3 at different concentrations, and the maximal growth rates of L1 blade determined. At very low concentrations of GA_3 (0 and 10^{-8} M), the *Sln1d,grd3* seedlings were easily identified as extremely dwarfed segregants representing approximately one quarter of the population (Fig. 1). They were also readily identified at GA_3 concentrations from 10^{-8} to 10^{-6} M, and their (LER_{max}, the maximum daily rate of elongation attained by the L1 blade) increased throughout this range. At concentrations higher than 10^{-6} M, it was no longer possible to reliably identify *Sln1d,grd3* from *Sln1d,Grd3*, so

Table I. *Effect of GA_3 on the maximal rate of elongation of the first leaf blade of different barley lines*

Maximal elongation rates (mean ± SE) of the first leaf blade are shown in mm d^{-1}.

Line	Concentration of GA_3		
	Control	10	1,000
		μM	
Himalaya (*WT*)	35.4 ± 0.8	58.0 ± 0.9	60.1 ± 1.2
gse1	9.8 ± 0.5	23.9 ± 0.5	48.1 ± 0.8
grd2	10.3 ± 0.4	50.4 ± 1.6	52.2 ± 1.2
M640 (homozygous)	10.1 ± 0.3	14.4 ± 0.5	16.1 ± 0.6
M640 (heterozygous)	13.0 ± 0.2	17.7 ± 0.4	20.3 ± 0.6

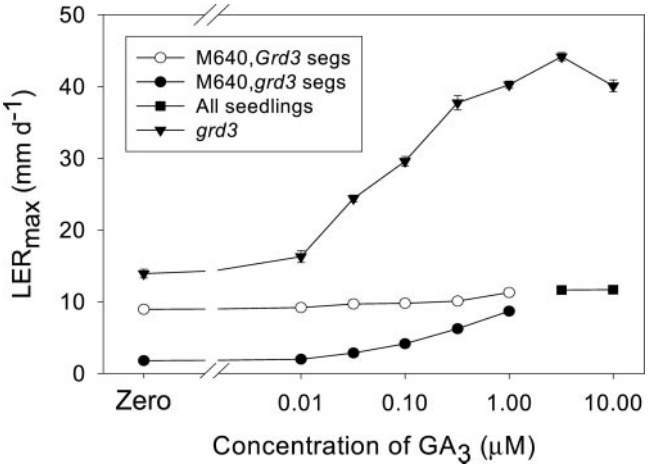

Figure 1. LERs of M640 segregants that are heterozygous (*Sln1d,Grd3*) or homozygous (*Sln1d,grd3*) for a GA deficiency allele, and of *grd3*, growing in different concentrations of GA_3. Grains were germinated and seedlings grown in the presence of GA_3 at the indicated concentrations. Maximal leaf elongation rate (LER_{max}; mean ± SE) was determined for the *grd3* mutant, and for *Sln1d,Grd3* and *Sln1d,grd3* seedlings as previously described (Chandler and Robertson, 1999). At GA_3 concentrations higher than 1 μM, it was no longer possible to reliably identify *Sln1d grd3* seedlings in the segregating population, so LER data are for the whole population. Where not visible, error bars are within the symbol.

LER_{max} values are for the total population. The *Sln1d,grd3* seedlings responded to applied GA_3 over the concentration range 10^{-8} to 3.2×10^{-6} M. There was only a slight effect on growth rate of *Sln1d,Grd3* seedlings over the same concentration range, consistent with the data in Table I. The *grd3* mutants responded to GA_3 over the concentration range 10^{-8} to 10^{-6} M, as previously reported (Chandler and Robertson, 1999). So, in *Sln1d,grd3*, LER_{max} values increase as the concentration of GA_3 increases, and the concentration range over which this occurs is similar to that over which *grd* mutants respond. This suggests that the dominant dwarf is not completely unresponsive to GA, but its GA response is saturated by endogenous GA.

The production of α-amylase by M640 aleurone was investigated so that its response to applied GA_3 could be characterized. Endosperm half-grains of barley cv Himalaya and homozygous M640 were incubated in a range of GA_3 concentrations and α-amylase activity determined at different times of incubation. The results (Fig. 2A) indicate that production of α-amylase by Himalaya half-grains is highly dependent on applied GA_3. At 10^{-9} M GA_3, α-amylase production is just over one-half that which is observed at 10^{-8} M and higher concentrations. M640 half-grains showed little response to GA_3 either at 10^{-9} or 10^{-8} M. However, they showed considerable α-amylase production at 10^{-7} M, and at 10^{-6} M α-amylase production was near *WT*. Thus, for α-amylase production, M640 appears to be about 100-fold less sensitive to GA_3 than barley cv Himalaya.

We conclude that the degree of GA "insensitivity" or "nonresponsiveness" seen in M640 depends on which response is being monitored. Aleurone appears to have a response to GA_3 that is normal in magnitude, but with a reduction in apparent GA sensitivity of about 100-fold. In contrast, LER shows only a slight response to GA_3, which probably occurs over the normal concentration range, suggesting that M640 has a limited GA-signaling output.

The Mutation in M640 Is Either Closely Linked to, or an Allele of, *Sln1*

M640 homozygotes were crossed with a line (M54) segregating for the *sln1a* allele at the *Sln1* locus. Twenty-three F_1 grains were obtained and the dwarf F_1 plants were allowed to self. Nine of 23 F_2 families segregated dwarf:tall seedlings in a 3:1 ratio, representing F_1 plants that had received a *Sln1* (*WT*) allele from the M54 parent. Fourteen of 23 F_2 families segregated dwarf:slender seedlings in a near 3:1 ratio, indicating that these had received the *sln1a* mutant allele from M54. Importantly, no *WT* plants were observed in >2,500 seedlings, suggesting either close linkage or identity between the dominant dwarf locus and *Sln1*. If these loci were independently segregating, we would expect to have observed >470 *WT* plants in an F_2 family of this size.

M770, a New Slender Mutant of Barley cv Himalaya

The Himalaya line segregating for the *sln1a* allele, M54, was constructed by repeated backcrossing of the original slender mutant (barley cv Herta; Foster, 1977) with Himalaya as the recurrent parent. M54 is BC6 material but is still likely to contain considerable regions of barley cv Herta sequence. During the course of these studies, a slender mutant arose in a sodium azide-treated barley cv Himalaya M_2 population, and this seedling was rescued by crossing with barley cv Himalaya pollen. The resultant line, M770, was backcrossed further to barley cv Himalaya, and crossed with M54 for allelism tests. The mutation in line M770 was allelic to *sln1a* (P.M. Chandler, unpublished data), and this new allele has been designated *sln1c*. The leaf elongation rate of *sln1c* segregants is identical to that reported previously (Chandler and Robertson, 1999) for *sln1a* (data not shown). The endosperm half-grains of *sln1c* segregants produce α-amylase in the absence of applied GA_3 at the same rate as barley cv Himalaya grains treated with GA_3 (Fig. 2B).

A GAI-Related Sequence from Barley cv Himalaya Defines the Sln1 Locus and the Mutant Locus in M640

A rice expressed sequence tag that was related to Arabidopsis *GAI/RGA* was used to screen a barley

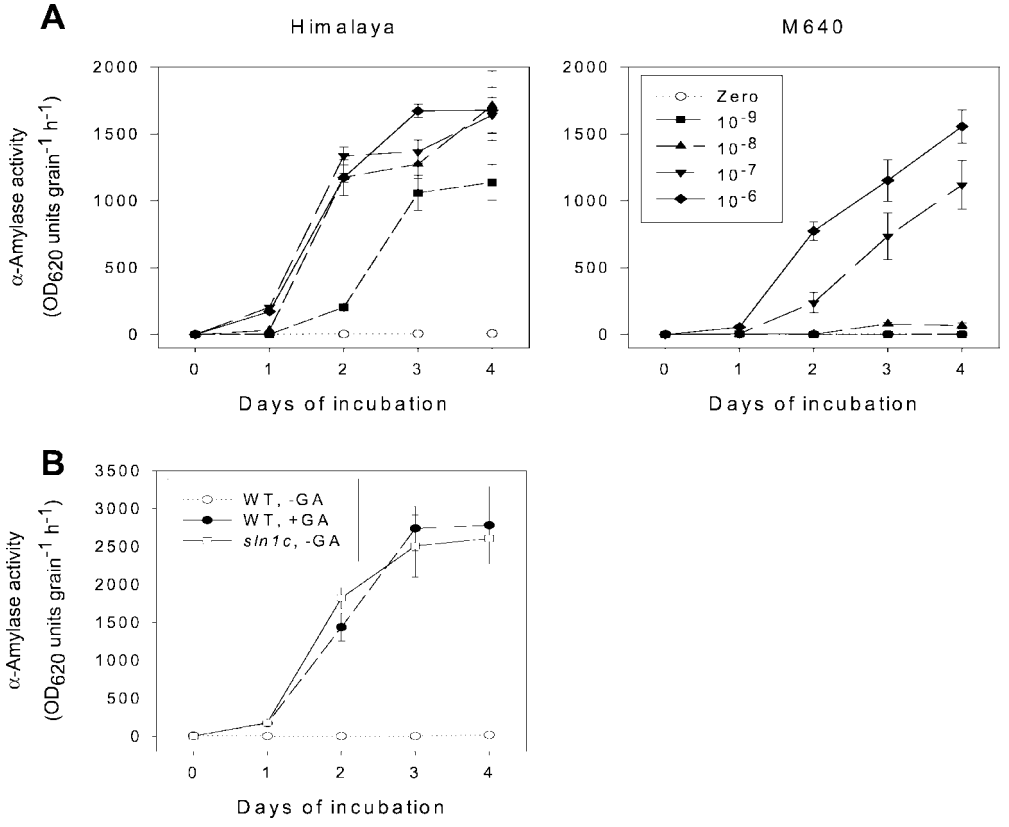

Figure 2. α-Amylase production by endosperm half-grains. A, α-Amylase production by half-grains of barley cv Himalaya and M640 in response to GA_3. Half-grains were incubated in GA_3 solutions of the indicated concentrations (M), and samples were frozen at the indicated time of incubation before homogenization, extraction, and assay of α-amylase activity. The legend in the right applies to both graphs, and the data represent means ± SE of triplicate samples. Where not visible, error bars are within the symbol. B, α-Amylase production by half-grains of barley cv Himalaya and slender segregants of M770. Half-grains corresponding to *sln1c* homozygous segregants in the M770 stock were identified by scoring growth (slender or normal) of the corresponding embryo half-grain. Slender half-grains were incubated without addition of GA_3, whereas barley cv Himalaya half-grains were incubated with or without GA_3 at 10^{-6} M. Samples were frozen at the indicated time of incubation before homogenization, extraction, and assay of α-amylase activity. The data represent means ± SE of triplicate samples. Where not visible, error bars are within the symbol.

aleurone cDNA library. A number of partial-length cDNAs were isolated and these were used to isolate a full-length clone from a Morex barley genomic library. The corresponding sequences were determined for barley cv Himalaya, for independent slender mutants and M640, and for individual plants segregating for the mutant alleles. The results for barley cv Himalaya (Fig. 3) reveal an ORF of 618 amino acid residues, and a predicted protein molecular mass of 65.2 kD. The deduced amino acid sequence has 97% identity with *rht-D1a* from wheat, 88% identity with *d8* from maize (Peng et al., 1999), and 89% identity with *OsGAI* from rice (Ogawa et al., 2000). To determine its relationship to the *Sln1* locus, the gene was sequenced in three independent slender mutants. No mutation was found in the ORF of *sln1a*. It is possible that this mutant is altered in either the expression or translation of its mRNA. The *sln1b* allele was associated with a single nucleotide frameshift mutation at amino acid position 93, which resulted in an early termination codon being created at position 252. The *sln1c* allele was associated with the creation of an early termination codon, resulting in a protein of predicted mass 63.2 kD that lacks the COOH-terminal 17 amino acid residues. These sequence alterations in *sln1b* and *sln1c*, and for plants segregating at *sln1c*, establish that the sequence corresponds to the *Sln1* locus. Finally, an alteration in this sequence was also observed in M640, the dominant dwarf, and in lines that were segregating for this allele. This alteration is a nonconservative amino acid substitution (G to E) in a residue that is conserved in sequences from wheat, maize, rice, and Arabidopsis *GAI* and *RGA*. It occurs very close to the DELLA motif (see legend to Fig. 3) already implicated in GA signaling in mutants such as *gai*, *Rht*, and *D8* (Peng et al., 1999). It is of interest that the mutation is a single amino acid substitution, in contrast to the deletions or premature stop codons that have been more typically observed in this region. We conclude that M640 is a mutant at the *Sln1* locus, and this new allele is designated *Sln1d*.

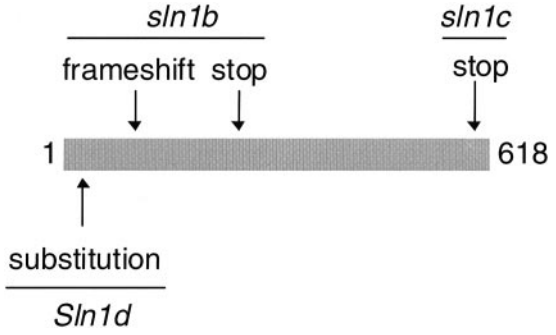

Figure 3. Representation of mutants in the SLN1 sequence. The barley cv Himalaya (*WT*) open reading frame (ORF) is 618 amino acid residues in length. Slender mutants: *sln1b* has a frameshift mutation in amino acid residue 93 (Thr, ACC to A-C), resulting in an early termination codon at residue 252, and *sln1c* has a G to A substitution in amino acid residue 602 (Trp, TGG to TGA), resulting in an early termination codon. Dominant dwarf: *Sln1d* has a G to A substitution in amino acid residue 46 (Gly, GGG to GAG), causing a Gly to Glu change in the DELLA region, namely ^{39}DELLAALG^{46} → ^{39}DELLAALE^{46}

SLN1 mRNA and Protein Are Preferentially Expressed in Elongating Regions of the Leaf

The sites of expression of *SLN1* mRNA and protein have been determined with particular reference to growing leaves because GA has large effects on LER, and there are dramatic differences in phenotype between different *Sln1* mutants (Fig. 4). Elongating blades of L2 were harvested midway through growth, when their growth rate had reached a high and sustained value. They were divided into five segments, with the lower segment contained within the EZ, and segments further along the blade corresponding to progressively more mature regions. Figure 5 shows a preferential localization of *SLN1* mRNA in the basal regions of the blade, relative to total RNA. The content of *SLN1* mRNA in leaves of *Sln1d* was slightly lower than in barley cv Himalaya and *sln1c*.

Antibodies raised to the first 170 amino acid residues of the SLN1 ORF detect SLN1 protein in aleurone (Gubler et al., 2002), and these were used to localize SLN1 protein in extracts of the growing L3 blade. Preliminary experiments showed that SLN1 protein was localized almost exclusively to the basal EZ for the elongating blades of L1, L2, and L3. More detailed fractionation of the L3 blade showed that SLN1 protein in barley cv Himalaya is preferentially localized to the basal third of the EZ, but its presence can still be detected toward the end of the EZ (Fig. 6A). Thus, SLN1 protein is apparently restricted to regions where growth is occurring in the leaf blade, and this seems to be more marked for SLN1 protein than for *SLN1* mRNA (Figs. 5 and 6A).

The Content of SLN1 Protein Is Altered in Mutants at Sln1

The content of SLN1 protein was assessed in the EZ and in the next segment of growing L3 blades of barley cv Himalaya and of homozygous mutants at *sln1b*, *sln1c*, and *Sln1d* (Fig. 6B). As expected, essentially all of the SLN1 protein was localized in the EZ segment of barley cv Himalaya. There was no SLN1 protein detectable in the EZ of *sln1b*, which contains a frameshift mutation that results in an early termination (Fig. 3). The slender mutant in a barley cv Himalaya background, *sln1c*, had higher than normal amounts of SLN1 protein, and its distribution extended into the next segment of the leaf. The mobility of SLN1 in *sln1c* is slightly faster than in barley cv Himalaya because it lacks 17 amino acids at the COOH terminus. The *Sln1d* mutant had very little SLN1 protein in either segment. A similar experiment was carried out using the elongating blade of L1, rather than L3, and this produced a very similar pattern of results. Together, these results indicate that there are major effects on the amount and distribution of SLN1 protein in each of the three mutants examined.

Mutants at *Sln1* Are Altered in Their Content of Endogenous GAs in Growing Leaves

Previous studies have indicated that dominant dwarf mutants have higher, and slender mutants

Figure 4. Above-ground parts of seedlings of *sln1c*, *WT*, and *Sln1d* 2 weeks after sowing.

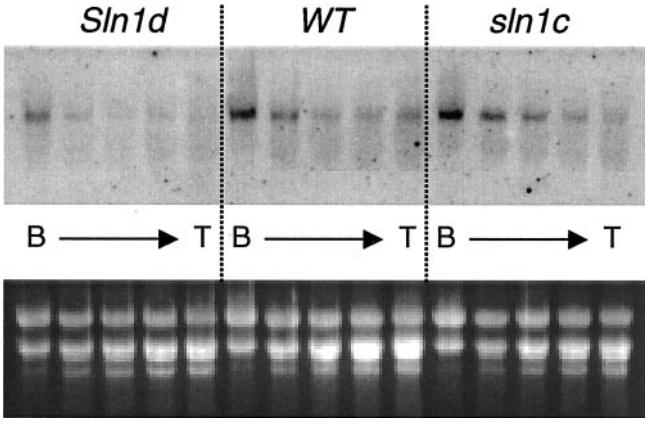

Figure 5. Distribution of *SLN1* mRNA along the growing blade of L2 of barley cv Himalaya, *sln1c*, and *Sln1d*. Blades were harvested at approximately 50% final length, cut into five segments of equal length, frozen and RNA extracted, electrophoresed, blotted, and the filter hybridized with a *SLN1* probe. The upper panel shows the hybridization profile, and the lower panel the ethidium bromide-stained gel before transfer. Lanes from left to right: *Sln1d*, base (B) to tip (T), five segments; Himalaya, base to tip (five segments); and *sln1c*, base to tip (five segments). The basal segment in each case is contained within the EZ.

lower, amounts of active GAs than *WT* (Fujioka et al., 1988; Croker et al., 1990; Talon et al., 1990). The availability of both a slender mutant and a dominant dwarf mutant in a common genetic background allows detailed studies to be made on the effects of these very different phenotypes on the content of endogenous GAs and ABA. Plants were grown in controlled conditions, and the second leaf blade harvested midway through growth, when LER was maximal. Regions corresponding to the EZ (at the base of the blade), and a region of the same length just distal to the EZ were harvested, and hormones analyzed. Three lines were examined, namely *Sln1d*, and both *WT* and *sln1c* segregants of M770.

The results for the *WT* segregants of M770, assumed to be equivalent to barley cv Himalaya, reveal that GA contents are generally higher in the growing part of the leaf (EZ) than in the next segment, which has ceased elongation (Table II). For instance, GA_{19}, GA_1, and GA_8 are all present at higher contents in the EZ, although GA_{20} (and ABA) are notable exceptions. *Sln1d* plants and *sln1c* segregants of M770 differed from the *WT* in hormone content in several important respects. First, the amount of bioactive GA_1 was much higher in *Sln1d*, and much lower in *sln1c*, than in the *WT*. A similar pattern was observed for GA_8, the 2-hydroxylated (inactive) catabolite of GA_1, and for GA_{34}, the corresponding catabolite of the bioactive GA_4. (GA_4 was not determined in this experiment because GC-SIM spectra revealed an interfering ion.) *Sln1d* plants showed much greater effects on the accumulation of GA_{34} than of GA_8, and this has been confirmed in independent experiments. The second main difference involved the content of GA_{19} and the earlier intermediates GA_{53} and GA_{44}. These GAs were present in lower amounts than *WT* in *Sln1d*, but in *sln1c* they were close to *WT* or varied nonuniformly. Finally, *sln1c* had a much lower content of GA_{20} than either the *WT* or *Sln1d*, which were similar to each other.

DISCUSSION

A new mutant in barley cv Himalaya resembles the (partially) dominant dwarf mutants that have been described previously in wheat (*RhtB1b* and *RhtD1b*), maize (*D8* and *D9*), and Arabidopsis (*gai*). Based on previous studies of the slender (*sln1*) mutant of barley, and our emerging understanding of GA signaling in plants, we investigated the possibility that this new mutant might represent a novel allele at *Sln1*. Genetic studies were consistent with this possibility. A *GAI-/RGA*-related sequence was isolated from barley and shown to correspond to the *Sln1* locus because two independent slender mutants were altered in this sequence. The dominant dwarf mutant was also altered in this sequence, having a nonconserva-

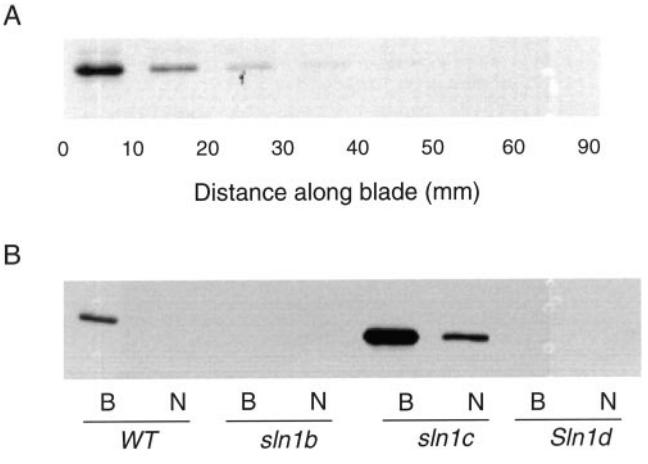

Figure 6. Distribution of SLN1 protein in growing leaf blades. A, Distribution of SLN1 protein along the growing L3 blade of barley cv Himalaya. Blades of L3 were harvested when 90 mm in length (approximately 50% final length), and cut into six 10-mm segments from the base, and then a single 30-mm segment remaining at the tip. Protein was extracted from each segment, electrophoresed, blotted, and the filter developed with antibodies prepared against SLN1. Lanes from left to right represent protein from the six 10-mm segments, and then the single 30-mm segment (base of blade to tip of blade). The EZ is 30 mm, represented by the first three lanes. B, Contents of SLN1 protein in EZ and next segment of growing L3 blades of barley cv Himalaya and mutants at *Sln1*. Blades of L3 were harvested at approximately 50% final length. One segment equal in length to the EZ was cut from the base, and then another segment of equal length adjoining the first ("next" segment). Segment lengths were 30, 50, and 14 mm for barley cv Himalaya, slender, and dominant dwarf types, respectively. Protein was extracted from each segment, electrophoresed, blotted, and the filter incubated with antibodies prepared against SLN1. Lanes from left to right represent protein from the basal (B) and next (N) segments of Himalaya, *sln1b*, *sln1c*, and *Sln1d*.

Table II. *Hormone determinations on the second leaf blade of WT, slender, and dominant dwarf lines*

The elongating second leaf blade was cut into a basal elongation zone (EZ) and a segment of equal length immediately distal along the blade (next). ABA and GAs were extracted from these segments, purified, and quantified by gas chromatography-selected ion monitoring (GC-SIM).

Line	Hormone Content							
	ABA	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_1	GA_8	GA_{34}
	ng (g dry wt)$^{-1}$							
Sln1 (WT), EZ	15.9	8.2	30.2	57.1	3.1	5.2	128	9.2
Sln1 (WT), next	24.5	2.7	16.6	24.9	5.1	1.6	57.8	3.2
sln1c (Slender), EZ	33.3	15.3	10.8	44.0	0.81	0.18	5.42	0.77
sln1c (Slender), next	29.8	8.0	7.7	20.9	0.69	0.20	1.54	0.74
Sln1d (Dominant dwarf), EZ	23.5	3.1	3.8	6.18	3.45	28.6	158	110
Sln1d (Dominant dwarf), next	33.1	4.05	<dl	7.14	4.76	11.7	129	31.4

tive amino acid substitution in the DELLA region, already implicated in GA signaling (Peng et al., 1999). Mutation at the *Sln1* locus therefore can generate plants of radically different phenotype; either dwarfs that are largely dominant and GA "-insensitive/-nonresponsive," or the recessive slender types in which GA responses appear to be constitutive (see Fig. 4). A recent study (Ikeda et al., 2001) shows that a similar situation exists for the related *SLR1* locus in rice; slender types were isolated after mutagenesis, and dominant dwarf types were isolated after transformation with *SLR1* sequences that incorporated deletions similar to those found in the Arabidopsis *gai* mutant (Peng et al., 1997). The lack of slender mutants in species such as wheat, maize and Arabidopsis is presumably a consequence of genome redundancy (to different extents), so that phenotypes resulting from a loss of function may not be efficiently recovered.

Current models to explain how these proteins act in GA signaling propose that they function as "repressors" of GA-regulated responses whose activity is modulated by GA signaling (Richards et al., 2001; Silverstone et al., 2001). These two activities appear to involve different parts of the protein, with the DELLA region (near the NH_2 terminus) involved in modulation (Peng et al., 1999; *Sln1d*) and COOH-terminal regions involved in repression (Ikeda et al., 2001; *sln1b* and *sln1c*). Modulation of repression by GAI/RGA and SLN1 may involve altered subcellular localization of the protein, and protein turnover. Green fluorescent protein-RGA fusion proteins localize to the nucleus in Arabidopsis and barley, but fluorescence disappears rapidly after GA treatment (Silverstone et al., 2001; Gubler et al., 2002). Similarly, western blotting shows that after GA treatment there is a loss of green fluorescent protein-RGA protein detected within 2 h in Arabidopsis (Silverstone et al., 2001), and of SLN1 protein within minutes in barley aleurone (Gubler et al., 2002). Although GAI/RGA and their orthologues in other species are proposed to be repressors of GA-regulated responses, these proteins are members of a gene family (GRAS, Pysh et al., 1999) believed to function in transcriptional co-activation. A GAI/RGA-related protein from rice has been shown to have transcriptional co-activation activity (Ogawa et al., 2000). The "repressor" activity of these proteins may therefore be indirect, and require transcriptional activation of downstream components that are negative regulators of GA signaling.

Analysis of the *Sln1d* mutant phenotype revealed intriguing differences between the effects of this mutation on GA responses in aleurone and in elongating leaves. In an aleurone assay, the mutant behaved as a "sensitivity" mutant, i.e. the response was approximately normal in magnitude, but occurred at concentrations of GA_3 that were about 100-fold higher than usual. In elongating leaves, the mutant (when dwarfed further by introduction of a GA deficiency mutation) responded to GA_3 over an approximately normal concentration range, but the magnitude of the response was greatly reduced. This behavior is distinct from the previously described *gse1* mutant of barley, where the responses of both aleurone and leaf elongation showed parallel reductions of 100- to 1,000-fold in GA sensitivity (Chandler and Robertson, 1999).

These different facets of the *Sln1d* phenotype are difficult to reconcile with our current knowledge of GA signaling. It is possible that the single amino acid substitution responsible for the *Sln1d* phenotype affects GA signaling in a manner that is response specific, in contrast to the effects of the 17 amino acid deletion in the *gai* mutant of Arabidopsis At present we have only a single dominant dwarf allele at *Sln1* in barley cv Himalaya, and do not know whether this phenotype will be general for mutation in the DELLA region, or specific to this particular allele.

In the elongating blade of L3, SLN1 protein was localized almost exclusively to the EZ where growth occurs. It occurred in highest amounts in the basal part of the EZ, declining progressively toward the distal end of the EZ, and was largely absent from the next segment along the blade that has ceased elongation. From an analysis of leaf growth rates and epidermal cell lengths, the "next" segment along the leaf is composed of cells that 24 h previously formed the distal 70% of the EZ. Therefore, it follows that SLN1 protein is lost from cells as they stop elongating, although whether there is a causal relationship

between these two events is unknown. It may seem surprising that the highest amounts of SLN1 protein (a repressor of GA signaling) are observed in the EZ, where GA-regulated growth occurs. However, it is presumably the balance of positive factors (GA content and signaling) and negative factors (SLN1 protein) that finally determines LER. The marked heterogeneity in the distribution of SLN1 protein between growing and nongrowing regions of the leaf has not been reported for GAI/RGA. Leaves of dicotyledonous plants lack the clearly defined growth zones present in cereals, but by comparing old and growing leaves, or by in situ approaches, it will be possible to determine whether the same applies in Arabidopsis.

There were major alterations in the abundance of SLN1 protein in leaves of slender and dwarf mutants. The *sln1b* and *Sln1d* mutants had greatly reduced contents of SLN1 protein, whereas the *sln1c* mutant had much more, extending into nonelongating regions of the leaf. The relationship between the abundance of SLN1 protein (or its orthologues in other species) and phenotype is far from clear. Fu et al. (2001) recently reported that overexpression of wild-type GAI protein of Arabidopsis in rice can lead to dwarfing. Similarly, Ikeda et al. (2001) showed varying degrees of dwarfing in transgenic rice plants expressing a truncated SLR1 gene under the control of an actin promoter. Neither of these studies monitored protein expression. It is also known that treatment with GA leads to rapid reductions in the amount of SLN1 protein in barley aleurone (Gubler et al., 2002) and leaves (data not shown), and of RGA protein from Arabidopsis (Silverstone et al., 2001). These effects presumably result from alterations in the rate of protein turnover. The interpretation of differences in SLN1 protein content between *WT* and mutants therefore is likely to be complicated. First, the active component of SLN1 protein might be only a minor fraction of the total immunoreactive protein (Gubler et al., 2002). Second, it is possible that mutations in the SLN1 protein result in differences in protein stability. Despite these considerations, it is noteworthy that *Sln1d*, which shows a very clear dwarf phenotype, has very little SLN1 protein detectable in its leaf EZ, in contrast to *WT* and *sln1c*. We can only speculate that the mutant must have a small amount of SLN1 protein that is either more active than the *WT* protein or less subject to turnover. Also noteworthy is the large difference in SLN1 protein content and distribution between *sln1b* and *sln1c*, despite their near-identical phenotypes.

The value of leaf sectioning experiments was also revealed in studies on the effects of different mutations at *Sln1* on the contents of endogenous GAs. It is obvious from comparing the data for growing and nongrowing regions of the leaf blade (Table II) that the distribution of most GAs is far from uniform along the leaf. It is likely to be the content of GA in the EZ that is most relevant to leaf growth, and that provides the best comparison between genotypes that differ so markedly in growth (Tonkinson et al., 1997). The EZ of *Sln1d* had a much (nearly 10-fold) lower content of GA_{44} and GA_{19} than WT, but much higher contents of GA_1 and GA_{34} (6- and 12-fold, respectively). There was only a slight effect on the content of GA_8, despite the fact that GA_8 and GA_{34} are equivalent catabolites in the two major GA metabolic streams (early 13 hydroxylation, and nonearly hydroxylation). It is possible that the much larger pool size of GA_8 compared with GA_{34} provides some buffering to change, but note that both of these GAs show corresponding reductions in the slender mutant (see below). Overall, this pattern is similar to results reported for equivalent dwarfs such as *D8* maize (Fujioka et al., 1988), *gai* Arabidopsis (Talon et al., 1990), and *Rht3* wheat (Tonkinson et al., 1997), which all show reduced amounts of GA_{19} and elevated amounts of GA_1 relative to the *WT*. In contrast, the slender (*sln1c*) segregants had much (12–30-fold) lower contents of GA_1, GA_8, and GA_{34} than normal segregants in the same stock. An earlier study of GAs in the *sln1a* mutant of barley also reported reduced amounts of GA_1 and GA_8 relative to the *WT* (Croker et al., 1990), although the magnitude of the reduction was less than observed here, probably because of differences in the type of leaf material used for analysis. Compared with the results in barley, it is of interest that a recent study of GAs in a slender mutant of rice found only a 2- to 3-fold reduction (Ikeda et al., 2001).

In barley, leaf growth rate depends on the content of bioactive GA and on the activity of SLN1 protein. These two components of GA signaling are preferentially localized to the leaf EZ, where they appear to interact. A high content of active GA causes reduced amounts of SLN1 protein, and GA signaling output leads to feedback regulation of GA biosynthesis. Future studies will be aimed at further elucidation of the mechanisms involved.

MATERIALS AND METHODS

Plant Material

All lines are derived from the tall barley (*Hordeum vulgare* cv Himalaya). Lines segregating for slender mutant phenotypes were M54, M58, and M770. M54 shows segregation for the original allele (*sln-1*; Foster, 1977) after six backcrossing generations with barley cv Himalaya as the recurrent parent. M58 shows segregation for Foster's *sln-2* allele after four backcrossing generations to barley cv Himalaya. These alleles have been shown by intercrossing to be at the same locus (P.M. Chandler, unpublished data), and have been renamed *sln1a* and *sln1b*, respectively. M770 (this paper) is segregating for a new slender allele (*sln1c*) that occurred in a barley cv Himalaya background. The dominant dwarf mutant M640 (this paper) also carries a novel allele at the *Sln1* locus, designated *Sln1d* (note the uppercase "*S* " because the mutant phenotype is domi-

nant). A GA deficiency mutation from M411 (*grd3*; Chandler and Robertson, 1999) has been crossed into the M640 background. The double homozygote is too severely dwarfed to produce grains, but a segregating line (M86) was constructed that is homozygous at *Sln1d* and segregating at the *Grd3* locus. M488 (*gse1*) and M489 (*grd2*) have been described previously (Chandler and Robertson, 1999). Seeds of all lines are available upon request from P.M. Chandler.

Plant Growth

The effect of GA_3 on the maximal rate of L1 blade elongation was determined as previously described (Chandler and Robertson, 1999). Plants to be harvested for RNA, protein, and hormone analysis were grown in perlite/vermiculite and watered with nutrient solution in an artificially lit cabinet at 50% relative humidity with 16-h (18°C) day (400 μmol m^{-2} s^{-1} photosynthetically active radiation), and 8-h (13°C) night.

α-Amylase Production by Endosperm Half-Grains

Endosperm half-grains were prepared, surface sterilized, and placed in sterile McCartney bottles (five half-grains per bottle) containing filter-sterilized solution (0.6 mL of 10 mM $CaCl_2$ with cefotaxime [150 μg mL^{-1}], nystatin [50 units mL^{-1}], and GA_3 at the indicated concentration). After incubation with gentle shaking at 22°C for 0, 1, 2, 3, and 4 d, the samples were frozen until assay. To each bottle, 1.5 mL of a solution of 10 mM $CaCl_2$ was added, the half-grains were homogenized, and an aliquot of 1 mL was clarified by centrifugation (20,000*g* for 5 min). The supernatant was analyzed for α-amylase activity using Phadebas powder (Pharmacia Diagnostics AB, Uppsala) as previously described (Chandler, 1988).

Determination of Leaf EZs

The lengths of the blade EZs for L2 and L3 were determined by measuring abaxial between-vein cell lengths on cleared leaves as described (Wenzel et al., 2000).

Leaf Sectioning for RNA Blots and Hybridization

Second leaf blades of barley cv Himalaya, *sln1c*, and *Sln1d* were harvested when they had attained about one-half of their final length, cut into five equal sections from the base of the blade to the tip, and frozen on dry ice and stored at −80°C. RNA was extracted, electrophoresed, blotted, and hybridized with a 3′ *Bgl*II-*Hin*dIII fragment of the SLN1 cDNA clone.

Isolation of SLN1 Clone and Sequence Analysis

A rice EST (D39460), that was later shown to represent *SLR1*, was used to screen a barley cv Himalaya aleurone cDNA library (Stratagene, La Jolla, CA). A partial clone related to the probe was used to isolate the full-length gene from a Morex genomic library (kindly donated by Dr. Tim Close, University of California, Riverside). A 4.1-kb *Xba*I/*Hin*dIII fragment was sequenced (GenBank accession no. AF460219), and shown to represent barley *Sln1* by the analysis of mutants. DNA prepared from leaves was the template for PCR amplification of different regions of the *Sln1* gene. Amplified fragments were electrophoresed in agarose gels, excised, purified, and sequenced in both strands. Segregation of the appropriate mutant and *WT* sequences with phenotype was shown for nine slender and 11 normal seedlings from a plant heterozygous for *sln1c*, and seven normal and seven dwarf seedlings from a *Sln1d* heterozygote.

Preparation of Antibodies and Immunoblot Analyses

Equivalent proportions of protein extracts from growing leaves were electrophoresed, blotted, and reacted with antibodies to SLN1 protein as described (Gubler et al., 2002).

Leaf Harvests and Hormone Analysis

Blades of L2 of *Sln1d* and *WT* and *sln1c* segregants of M770 were harvested when approximately 50% final length. Two segments were cut from each blade and immediately frozen in liquid nitrogen: a basal segment (corresponding to the EZ), and a segment of equal length ("next" segment) immediately distal to the EZ. The lengths of these segments were 7 mm (*Sln1d*), 22 mm (*WT* segregants), and 36 mm (*sln1c* segregants), and there were approximately 100 blades of each genotype harvested. GAs and ABA were extracted and partially purified as described (Green et al., 1997), except for the omission of the NH_2 cartridge chromatography step, and for higher resolution pooling of fractions from the reverse phase C_{18} HPLC: 7 through 11 (GA_8 and GA_{29}), 14 through 18 (GA_1), 21 through 24 (ABA and GA_{20}), 25 through 28 (GA_{19}, GA_{34}, and GA_{44}), and 29 through 32 (GA_4 and GA_{53}). Procedures for derivatization of GAs and ABA, and for analysis by GC-SIM were described previously (Green et al., 1997). Endogenous contents of GAs and ABA were calculated with reference to known amounts of deuterated internal standards, and calibration curves for each compound.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial research purposes.

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