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Gibberellin Dose-Response Curves and the Characterization of Dwarf Mutants of Barley

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Dose-response curves relating gibberellin (GA) concentration to the maximal leaf-elongation rate (LER_{max}) defined three classes of recessive dwarf mutants in the barley (*Hordeum vulgare* L.) 'Himalaya.' The first class responded to low (10^{-8}–10^{-6} M) [GA_3] (as did the wild type). These *grd* (GA-responsive dwarf) mutants are likely to be GA-biosynthesis mutants. The second class of mutant, *gse* (GA sensitivity), differed principally in GA sensitivity, requiring approximately 100-fold higher [GA_3] for both leaf elongation and α-amylase production by aleurone. This novel class may have impaired recognition between the components that are involved in GA signaling. The third class of mutant showed no effect of GA_3 on the LER_{max}. When further dwarfed by treatment with a GA-biosynthesis inhibitor, mutants in this class did respond to GA_3, although the LER_{max} never exceeded that of the untreated dwarf. These mutants, called *elo* (elongation), appeared to be defective in the specific processes that are required for elongation rather than in GA signaling. When *sln1* (slender1) was introduced into these different genetic backgrounds, *sln* was epistatic to *grd* and *gse* but hypostatic to *elo*. Because the rapid leaf elongation typical of *sln* was observed in the *grd* and *gse* backgrounds, we inferred that rapid leaf elongation is the default state and suggest that GA action is mediated through the activity of the product of the *Sln* gene.

Dwarf mutants have proven to be valuable tools in hormone studies in a wide range of plant species. This has been particularly so for the GAs (for review, see Ross et al., 1997) and more recently for the brassinosteroids (Clouse and Sasse, 1998). The identification of hormone-biosynthetic mutants that are normalized by hormone application illustrates the importance of such hormones in determining plant stature. Therefore, when characterizing new dwarf mutants, the growth response after hormone application is an important first screen because it allows potential hormone biosynthetic mutants to be identified. However, a considerable proportion of new mutants may show either no or only partial growth response to applied hormone; and these are potentially altered in signal transduction or in processes affecting growth. For the GAs, several such classes of dwarf can be recognized (Ross et al., 1997). In addition, there are GA-signaling mutants that show either a constitutive or an enhanced GA response (Ross et al., 1997).

Current interpretations of this broad grouping of GA "response" mutants are imprecise, largely because plant growth is regulated by many factors in addition to GA. Therefore, failure to respond to applied GA does not necessarily mean a deficiency in GA signaling. For example, two pea mutants that were originally interpreted as GA-response mutants were later shown to be brassinosteroid deficient and nonresponsive (Nomura et al., 1997). It is clearly an advantage to use, when possible, an independent GA response that does not involve growth; in this respect cereal systems have proved valuable because they allow changes in leaf growth to be compared with α-amylase production by aleurone tissue (Gale and Marshall, 1973; Chandler, 1988; Lanahan and Ho, 1988).

Another problem arises because the final extent of a response may not be the most accurate measure of hormone responsiveness. Nissen (1988) analyzed data in the literature for several GA responses, including leaf elongation, and concluded that they were "almost uniformly subsensitive"; i.e. a greater-than-expected concentration range of applied GA was required for the response to go from 10% to 90% of maximal values. Weyers et al. (1987, 1995) have emphasized the importance of determining the initial or maximal rates of response to hormone application rather than the final extent, but despite the renewed interest in hormone-response mutants (largely because of studies using Arabidopsis), this approach has not been widely adopted. When a reduced rate of response to hormone application is observed, it is necessary to determine whether there has also been a change in the concentration range over which the response occurs. In this manner, a mutant that requires higher hormone concentrations than the wild type does to bring about a similar response can be distinguished from one with a reduced response capacity (Firn, 1986).

Hormone dose-response curves therefore provide data essential for characterizing GA-response mutants (Weyers et al., 1995; Swain and Olszewski, 1996). Where the responses being measured are complex (such as organ-elongation rates) and likely to integrate a number of "simpler" components, it is important that mutants be compared with the appropriate wild-type background. In the case of an induced mutation, backcrossing is required so that the possibility is reduced that independent mutational events contributed to the response being measured. In this study we describe a leaf-elongation assay for GA

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Abbreviations: L_1, first leaf or leaf one; LER_{max}, maximal leaf-elongation rate.

responsiveness that defines three classes of dwarf mutants in barley: *grd* (GA-responsive dwarf), *gse* (GA sensitivity), and *elo* (elongation). Genetic interactions of these mutants with the *sln* (slender) "constitutive GA response" mutant (Foster, 1977) suggest that GA signaling proceeds through the SLN gene product.

MATERIALS AND METHODS

Chemicals

Initial experiments used commercial preparations of GA_3 (>90%, Sigma), but to obtain saturation in dose-response experiments, very high (millimolar) concentrations were required. Considerable GA_1 was detected by GC-selected ion monitoring in the commercial GA_3 preparation, so pure preparations of GA_1 and GA_3 were kindly provided by L.N. Mander, Research School of Chemistry, Australian National University, Canberra, ACT. Samples of GA_{44}, GA_{19}, and GA_{20} were also kindly provided by L.N. Mander. All GA solutions were prepared by dissolving powder in 1 mM potassium-phosphate buffer, pH 5.5, and diluting in this solution when necessary. The stock solution of GA_3 was 9.76 mM, and excess acidity due to GA_3 was neutralized by the dropwise addition of 20 mM KOH until the pH returned to 5.5. GA solutions were stored at −20°C. Tetcyclacis (94.8%) was kindly provided by Dr W. Rademacher (BASF, Limburgerhof, Germany) and dissolved in ethanol at 3.5 mM.

Plant Material

The dwarf mutants of barley (*Hordeum vulgare*) 'Himalaya' were isolated after mutagenesis with sodium azide as described by Zwar and Chandler (1995). From about 200 independent dwarf mutants we selected several different types for detailed study, based on GA_3 application and genetic-complementation studies. Seven mutants are described here, all of which are recessive and all of which have been through three back-crossing generations before the establishment of homozygous seed stocks; at the seedling stage they ranged in height from approximately 20% to 50% of the wild-type parent. The first class (M117, M359, and M411) showed a large response to GA_3 application (microdrops or spray), and the three mutants represent three genetic loci. Phenotypically these mutants are similar, with leaves that are shorter and darker green and stem internodes that are shorter than the wild type. The second class (M121 and M488) showed only partial growth responses to GA_3, even at high concentrations. These two mutants are phenotypically similar to those described above, and represent two alleles at a single locus. The third class (M21 and M626) did not show any growth response to GA_3, and the two mutants are at different genetic loci.

The introduction of the *sln* mutation into the wild type and M117 was described by Smith et al. (1996). The same procedure was followed in crossing *sln1* into the other classes described here. In one class (M121 and M488) the *sln* phenotype is expressed as it is in the wild type and in M117. In the other class (M21 and M626) the *sln* phenotype is not observed during growth of the first two leaves, but homozygous (*sln1sln1*) plants could be identified later in their growth on the basis of abnormal stem elongation. Plants homozygous for *sln1* do not set seed, so comparisons were made between phenotypically normal (*Sln1*−) and slender (*sln1sln1*) segregants in the progeny of *Sln1sln1* heterozygotes in different genetic backgrounds.

Seedling Growth and Determination of LER_{max} and Final Blade Length

Grains were surface-sterilized as described previously (Chandler and Jacobsen, 1991) and placed embryo-side down between two sheets of autoclaved paper (3MM, Whatman) "envelopes" that were moistened with the appropriate solution and held vertically in a plastic frame placed in the solution. After the grains were stratified (4°C in the dark) for 48 h, they were placed under low-intensity fluorescent lighting at 20°C (d 0). After a further 3 d, the germinated grains in each envelope were culled for uniformity of shoot length (providing approximately 15 seedlings per sample), and the envelope was aligned with positional markers on a clear plastic sheet. The position of the tip of each leaf was marked on the plastic sheet, and the envelope was returned to the growth assembly. After an additional 1, 2, 3, and 4 d, the envelopes were again placed on the original sheets in their original position, and the new position of each leaf tip was marked. For each 24-h interval, the distance between marks was recorded and the mean length increment was determined. In most cases the maximal elongation occurred between d 4 and 5 after transfer from 4°C to 20°C. Occasionally, maximal growth occurred in either the previous or the following 24-h interval. For each genotype and treatment, the maximal value in each set of daily increments was expressed as a millimeter-per-day rate and abbreviated LER_{max}. In one experiment, seedlings of M117 and M411 were maintained until the growth of L_1 had ceased. The shoot was then dissected to determine the final blade length.

Construction of Dose-Response Curves

LER_{max} data were analyzed as a function of [GA] using individual seedling data and PEST software (Weyers et al., 1987). This program provides estimates of hormone-sensitivity parameters fitted to a modified Hill equation. The curves fitted to the data by the PEST software were plotted together with raw data points representing the means ± SE of LER_{max}.

α-Amylase Determinations

The embryonic axes of dry grains of the wild type and M488 were removed using a dissecting blade under a dissecting microscope. There was minimal damage to the scutellum. "De-axised" grains do not produce α-amylase unless incubated with GA_3 (Jones and Armstrong, 1971), and any α-amylase produced remains within the grain. The

de-axised grains were surface-sterilized, placed in paper envelopes, and incubated under conditions identical to those for intact grains (see above). α-Amylase activity was determined as previously described (Chandler and Jacobsen, 1991) on duplicate samples of five grains each.

RESULTS

Growth of L_1

Elongation of L_1 initially involved only the blade, but between d 5 and 7 both blade and sheath were elongating (Fig. 1). At later stages, elongation of the leaf involved only the sheath. The rate of leaf elongation was far from uniform, and LER_{max} occurred just before blade growth began to slow (just before sheath growth commenced; Fig. 1, inset). Maximal rates of sheath elongation did not exceed those of the blade (data not shown).

L_1 of the dwarf mutants was always smaller than that of the wild type, and LER_{max} values were considerably lower (7–15 mm d^{-1}, compared with 37 mm d^{-1} for the wild type; see below). However, the pattern of L_1 growth of the mutants was similar to that of the wild type; blade elongation preceded that of the sheath, and LER_{max} occurred just before the transition from blade growth to sheath growth (data not shown).

Effects of GA_3 on LER_{max}

Grains of the wild type and of the dwarf mutants were germinated in a range of concentrations of GA_3, and the LER_{max} was determined. The resulting dose-response curves (Fig. 2) show an LER_{max} for the wild type of about 37 mm d^{-1} at low [GA_3] (<10^{-8} M), increasing to about 67 mm d^{-1} at high [GA_3] (>10^{-6} M). Among the dwarf mutants, three response classes were identified that differed in the effect of GA_3 on LER_{max}: the first class (M117, M359, and M411) responded to GA_3 over the same concentration range (10^{-8}–10^{-6} M) as the wild type; the second class (M121 and M488) responded over a much higher and wider concentration range (10^{-6}–10^{-3} M); and the final class (M21 and M626) showed no response to [GA_3] as high as 10^{-3} M. Growth rates on the lowest concentrations of GA_3 were not significantly different from rates on control medium without GA_3 (compare Figs. 2, 6, and 7).

For the wild type and the first two classes, the mean values of different parameters were estimated from the fitted curves: LER_{max} at zero and saturating concentrations of GA_3 (R_{min} and R_{max}, respectively); the Hill interaction coefficient (p), which provids a measure of the "steepness" of the response to increasing concentration of GA_3; and

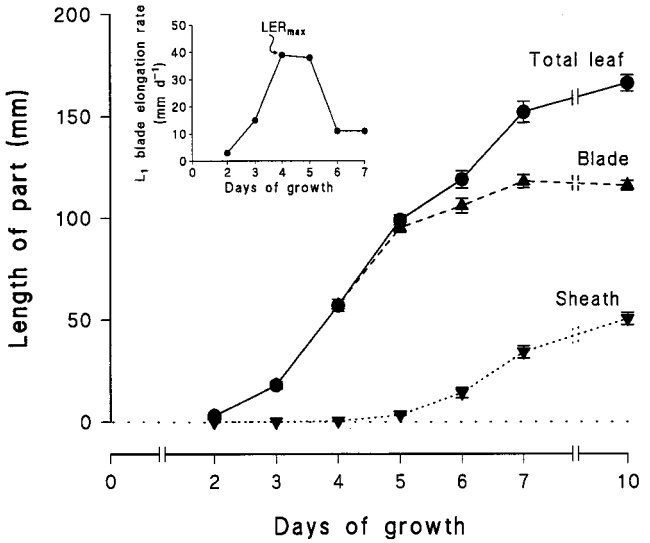

Figure 1. Growth of L_1 of wild-type barley. Grains were surface-sterilized, placed in moist paper envelopes, stratified, and incubated as described in "Materials and Methods." At the indicated times the mean L_1 lengths of 10 seedlings were determined, as well as the mean lengths of the blade and sheath. Where not visible, error (SE) bars lie within the symbols. Inset, Mean elongation rate of the blade of L_1 in the previous 24 h was plotted as a function of days of growth. The LER_{max} value for this set of data is indicated.

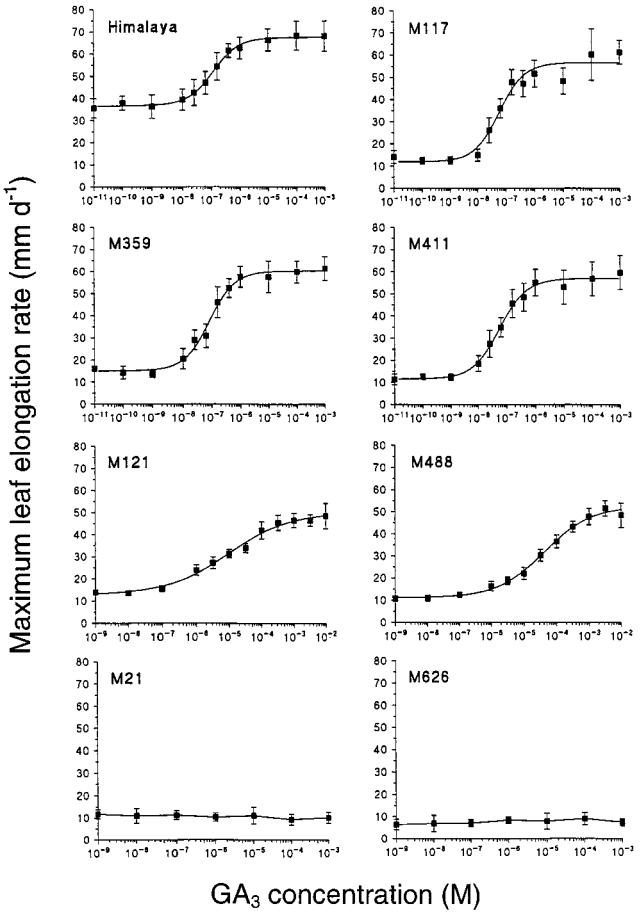

Figure 2. Dose-response curves relating LER_{max} of L_1 to [GA_3]. Grains of the indicated lines were surface-sterilized, placed in paper envelopes moistened with the appropriate [GA_3], stratified, incubated in low light, and LER_{max} (mean ± SE) of seedling L_1 was determined as described in "Materials and Methods." Curves in the top six panels were fitted using PEST software (Weyers et al., 1987). Note differences in the range of GA_3 concentrations in different panels.

$[H]_{50}$, the concentration of GA_3 at which 50% of the maximal response to GA_3 is attained (Table I). The values of these parameters highlight the similarity between the wild type and the first class of mutant in their response to GA_3, with *p* values close to unity (reflecting near Michaelis-Menten behavior), and the estimated concentration of GA_3 required for a half-maximal response falling within a narrow range of 56 to 120 nM. The R_{MIN} values of the three dwarf mutants in this class were lower than those of the wild type, which is consistent with their dwarf nature, but their R_{max} values were not quite as high as those of the wild type, possibly because the grains of the dwarf mutants were smaller by 5% to 20% on a dry-weight basis (data not shown). As a consequence, in mature grains the L_1 primordium of the dwarf would probably be smaller than that of the wild type, and its capacity to increase the elongation rate in response to exogenous GA could potentially be compromised.

The values shown in Table I contrast the behavior of the wild type and the first class of mutants (M117, M359, and M411) against that of the second class (M121 and M488). In particular, there was a much broader response in the latter mutants (*p* values considerably less than unity), and much higher (90- to 350-fold) concentrations of GA_3 were required for half-maximal response ($[H]_{50}$). R_{max} values for these mutants were again somewhat less than those for the wild type, and an argument similar to that above could be made based on grains that are approximately 25% smaller (dry weight) than those of the wild type. In addition, even at 10^{-2} M GA_3 (the highest concentration tested), the response may not have been saturated. The estimated values of the parameters in Table I for this second class of mutant might be less reliable than those estimated for the wild type and the first mutant class because saturation was barely attained. Nevertheless, the main differences (a broader transition and a displacement of the response to higher $[GA_3]$) are clearly discernible in the curves of Figure 2.

Similar experiments were carried out with GA_1, which is also an important bioactive GA for leaf elongation. A less extensive range of concentrations and mutants was studied, but the results (data not shown) were in close agreement with those described for GA_3. The principal difference was that the wild type and the first class of mutant had an $[H]_{50}$ value for GA_1 (500–1700 nM) that was approximately 10-fold higher than that estimated for GA_3. If we assume that GA_1 and GA_3 have equal intrinsic activity and similar rates of uptake, the difference in $[H]_{50}$ values may reflect more rapid catabolism of GA_1. The behavior of the three mutant classes in their responses to GA_1, including the estimates for *p* and the relative differences in $[H]_{50}$, paralleled the behavior observed for GA_3.

Comparing the Effects of GA_3 on LER_{max} and on Final Blade Length

Seedlings of two mutants (M117 and M411) were maintained on a range of GA_3 concentrations until growth of L_1 stopped, allowing the final length of the L_1 blade to be determined. To allow a direct comparison between the effects of GA_3 on LER_{max} and on final blade length, each response was normalized to an R_{MIN} value of 1 (Fig. 3). Final blade length responded to increasing concentration of GA_3, but the response was lower in magnitude than that of LER_{max} and occurred over a wider range of GA_3 concentrations. These effects may have resulted from differences in the duration of elongation.

Definition of Mutant Classes

Based on the dose-response curves (Fig. 2), three classes of dwarf mutants can be defined. The first, *grd*, responds to GA_3 over the same concentration range as the wild type. These mutants are proposed to have normal GA signaling and their dwarfism is associated with low levels of endogenous bioactive GAs (P.M. Chandler and J.R. Lenton, unpublished results). The three mutants in this class represent three different genetic loci: *grd1* (M117), *grd2* (M359), and *grd3* (M411). Mutants in the second class are primarily characterized by an alteration in GA sensitivity (the *gse* mutants). M121 and M488 represent alleles at the *gse1* locus, because no complementation is observed when they are intercrossed. The greatly reduced sensitivity to GA of these two mutants probably explains why they showed only poor growth responses to GA_3 in preliminary experiments (see "Materials and Methods"). Mutants in the third class show no elongation response to GA. On the basis of the results presented below, these mutants are proposed to be defective in the specific processes that are required for leaf elongation (the *elo* mutants), rather than in GA signaling. M21 and M626 represent different *elo* loci. For each of

Table I. *Parameters estimated from GA_3 dose-response curves*

Mean values (and their 95% confidence limits) of parameters (see text for explanation of symbols) were estimated for the fitted curves shown in Figure 2 using PEST software (Weyers et al., 1987).

Genotype	R_{MIN}	R_{MAX}	*p*	$[H]_{50}$
	mm d^{-1}			*nM*
Wild type	36.5 ± 3.1	67.5 ± 5.8	0.96 ± 0.08	120
grd Mutants				
M117 (*grd1*)	12.0 ± 1.1	56.5 ± 5.3	0.98 ± 0.09	58
M359 (*grd2*)	15.0 ± 1.4	60.3 ± 5.8	0.98 ± 0.09	84
M411 (*grd3*)	11.6 ± 1.0	56.9 ± 5.1	0.91 ± 0.08	56
gse Mutants				
M121 (*gse1*)	12.8 ± 1.0	50.0 ± 4.0	0.47 ± 0.04	9,500
M488 (*gse1*)	11.2 ± 0.9	52.5 ± 4.3	0.61 ± 0.05	43,000

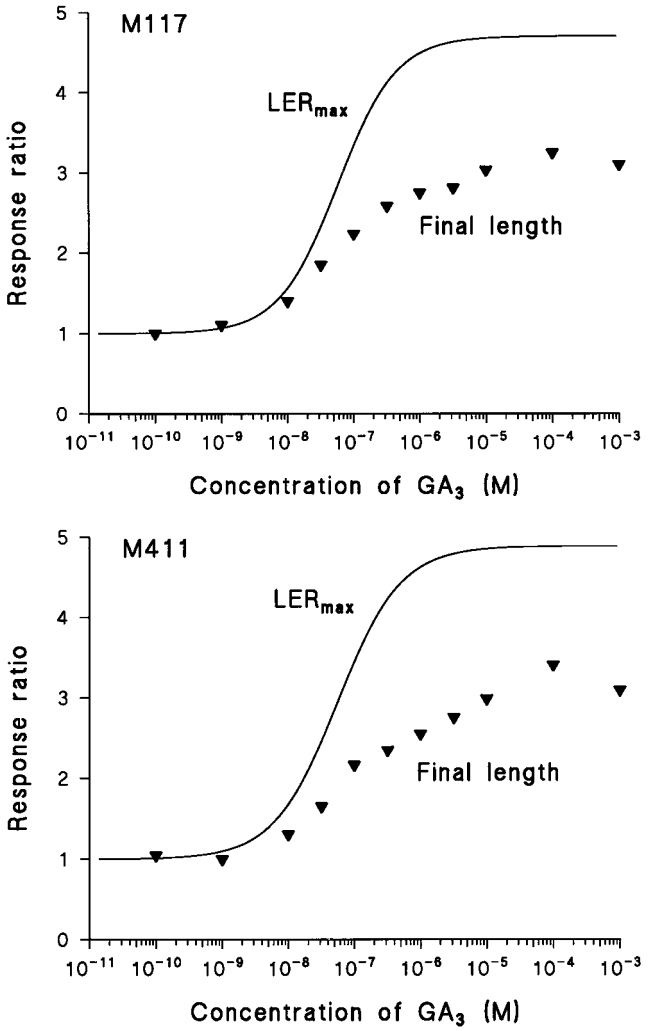

Figure 3. Dose-response curves comparing the effects of GA_3 on either LER_{max} or final blade length. Grains of the indicated lines were surface-sterilized, placed in paper envelopes moistened with the appropriate [GA_3], stratified, and incubated in low light, and LER_{max} (mean ± SE) or final blade length of seedling L_1 was determined as described in "Materials and Methods." To allow direct comparison, response ratios are plotted in which the LER_{max} or L_1 blade length in the absence of GA_3 is assigned a value of unity. Curves are the same (minus data points) as shown in Figure 2. Individual data points (▼) are for final blade length.

these three mutant classes, additional experiments aimed at a more detailed characterization were performed.

Response of *grd* Mutants to GA-Biosynthetic Intermediates

GA-biosynthetic intermediates may be active or inactive in promoting elongation in dwarf mutants, depending on the concentration at which they are applied, the severity of the dwarfing mutation, and the step in the GA biosynthetic pathway in which the mutant is blocked. The growth responses of L_1 of the three *grd* mutants to late intermediates of the early 13-hydroxylation pathway (Grosselindemann et al., 1992) were determined (Fig. 4). For each mutant, GA_1 treatment resulted in LER_{max} values that were greater than the wild-type values (approximately 37 mm d^{-1}). The [GA] used in these experiments (2×10^{-6} M) was only slightly greater than the $[H]_{50}$ value determined for GA_1 (approximately 1×10^{-6} M) so that LER_{max} would be highly responsive to the content of active GAs. GA_{20} was very effective in stimulating LER_{max} of the *grd1* and *grd3* mutants, and GA_{44} and GA_{19} were slightly less effective. This pattern differed markedly from that seen for the *grd2* mutant, in which each of the intermediates had very low activity in stimulating elongation. We inferred from this that the *grd1* and *grd3* mutants convert "inactive" GA precursors such as GA_{20} to growth-active GAs, whereas *grd2* mutants do not (or do so at a greatly reduced rate). This pattern would be consistent with *grd2* mutants having reduced levels of 3β-hydroxylation (converting GA_{20} to GA_1), whereas the other two loci are presumably blocked earlier in the pathway.

α-Amylase Production by *gse1* Grains in Response to GA_3

Mutants in the *gse1* locus were characterized by reduced sensitivity to GA_3 for leaf elongation; therefore, α-amylase production by aleurone tissue was also examined. α-Amylase activity in de-axised wild-type grains increased with time in the presence of 10^{-8} to 10^{-7} M GA_3 at approximately one-half the maximal rate observed with 10^{-6} and 10^{-5} M GA_3 (Fig. 5). In contrast, α-amylase activity of de-axised *gse1* grains showed no increase with time at GA_3 concentrations ≤ 10^{-6} M, intermediate rates of accumulation with 10^{-5} M GA_3, and high rates of accumulation at

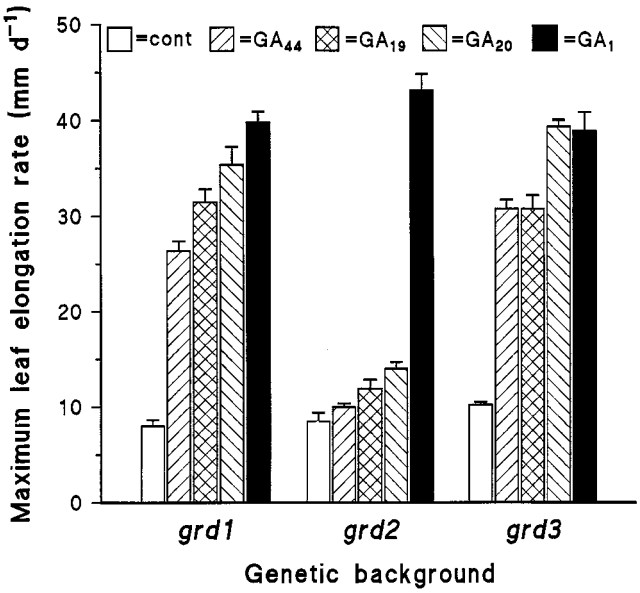

Figure 4. LER_{max} (means ± SE) of different *grd* mutants in the presence of GA-biosynthetic intermediates. Grains of M117 (*grd1*), M359 (*grd2*), and M411 (*grd3*) were surface-sterilized, placed in paper envelopes moistened with the indicated GA at 2 μM, stratified, and incubated in low light, and LER_{max} (means ± SE) of seedling L_1 was determined as described in "Materials and Methods." GA_{44}, GA_{19}, and GA_{20} are successive biosynthetic intermediates in the early 13-hydroxylation pathway leading to the formation of the bioactive GA_1.

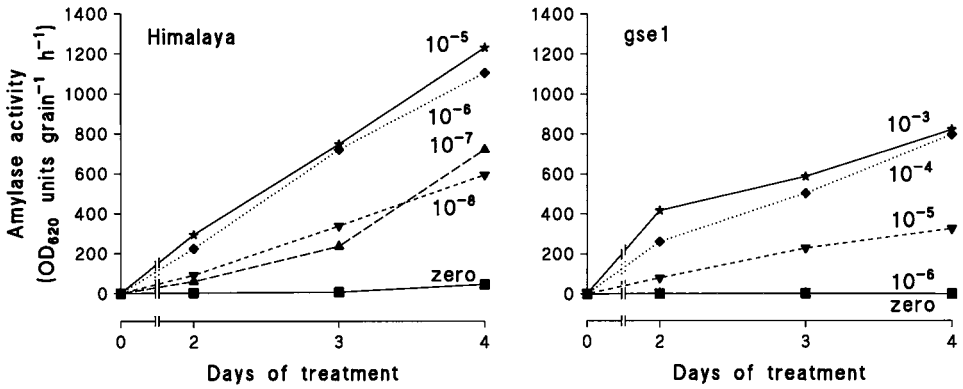

Figure 5. α-Amylase activity of de-axised grains of the wild type and a *gse1* mutant incubated with different [GA_3]. The embryonic axes of wild type and M488 (*gse1*) grains were removed, and the resulting de-axised grains were surface-sterilized, placed in paper envelopes moistened with the indicated [GA_3], stratified, and incubated in low light as described in "Materials and Methods." At the indicated times, duplicate samples (five grains each) were harvested and α-amylase was extracted and assayed. Each data point is the mean of duplicate samples.

10^{-4} to 10^{-3} M GA_3. This pattern parallels that observed for leaf elongation, in which responses equivalent to those of the wild type required at least 100-fold-higher concentrations of GA_3. The maximal rate of α-amylase accumulation in the mutant was less than that of the wild type, possibly because the grains were 25% smaller (on a dry-weight basis) than those of the wild type, and perhaps there was an equivalent reduction in aleurone cell number. We concluded that the *gse1* mutants are defective in a component of GA signaling that is required for two independent GA responses: leaf elongation and α-amylase production by aleurone.

Conditional Regulation of LER_{max} in *elo* Mutants by GA_3

The two *elo* mutants were characterized by low rates of leaf elongation even at very high concentrations of GA_3 (Fig. 2); however, the aleurone of both mutants showed near-normal responses to GA_3 for α-amylase production (data not shown). We considered the possibility that leaf-elongation rates were limited by defective components involved in leaf elongation rather than in GA signaling. Inhibitors of GA biosynthesis such as tetcyclacis induce dwarfing in barley, but this effect can be overcome by GA_3 (Zwar and Chandler, 1995). Grains were germinated of the two *elo* mutants and of the *grd1* mutant (as a control), and the seedlings were grown in control conditions in the presence of tetcyclacis alone or tetcyclacis plus GA_3.

Significant additional dwarfing was induced in all of the lines by tetcyclacis treatment, as revealed by the LER_{max} values (Fig. 6). When GA_3 was also present, the LER_{max} values returned to control levels for both *elo* mutants but greatly exceeded control levels for the *grd1* mutant (as expected). We inferred from this that the *elo* mutants are capable of responding to GA_3 provided leaf elongation occurred at a lower rate than in control conditions. In more detailed experiments with the *elo1* mutant, the concentration dependence for restoration of LER_{max} by GA_3 was examined. The results (data not shown) indicated that in the presence of tetcyclacis, concentrations of GA_3 as low as 10^{-7} M were able to restore LER_{max} to control levels, indicating that the *elo* mutants were capable of responding to low concentrations of GA_3. The failure of such mutants to respond to GA_3 in the dose-response experiment (Fig. 2) was presumably because they were already elongating at their maximal rate.

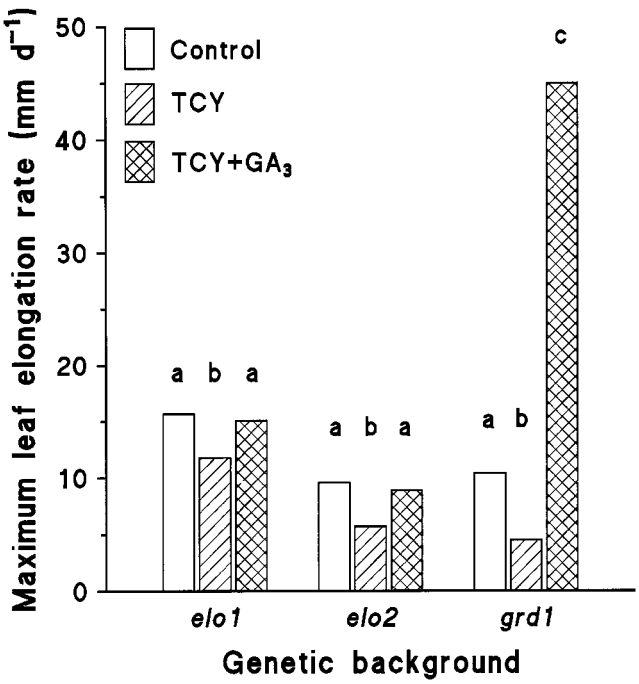

Figure 6. LER_{max} of L_1 of *elo* mutants and a *grd* mutant growing with or without tetcyclacis and GA_3. Grains of M21 (*elo1*), M626 (*elo2*), and M117 (*grd1*) were surface-sterilized, placed in moist paper envelopes containing, where appropriate, 2 μM tetcyclacis or 2 μM tetcyclacis plus 10 μM GA_3, stratified, and incubated under low light; and the mean LER_{max} of seedling L_1 was determined as described in "Materials and Methods." A replicate consisted of 10 seedlings, and there were three replicates for each genotype and treatment. Within a genotype, letters (a, b, or c) indicate significance ($P < 0.05$) for the differences between the means of each treatment.

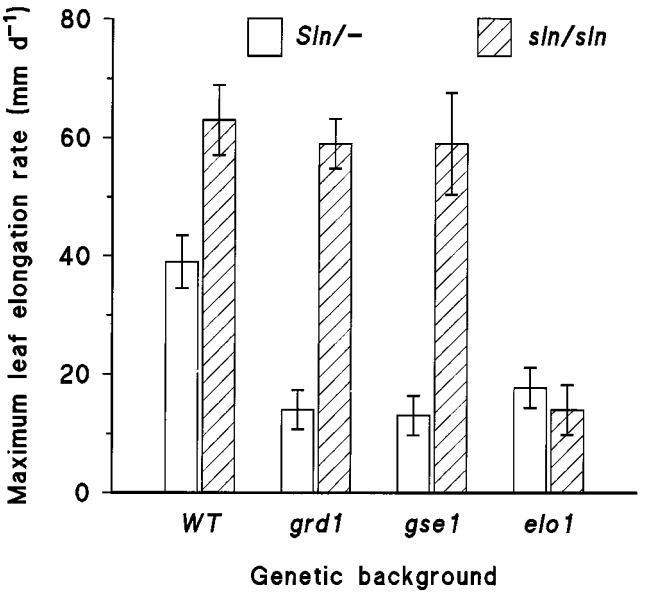

Figure 7. LER_{max} of L_1 of *Sln1*− and *sln1sln1* segregants in different genetic backgrounds. Grains from stocks in which the *sln1* allele was segregating in different genetic backgrounds (*WT,* wild type; *grd1,* M117; *gse1,* M121; and *elo1;* M21) were surface-sterilized, placed in moist paper envelopes, stratified, and incubated in low light, and the LER_{max} (means ± SE) of seedling L_1 was determined as described in "Materials and Methods." In an *elo1* background, slender (*sln1sln1*) seedlings cannot be distinguished at the first leaf stage from *Sln1*− seedlings, but after transplanting and further growth, the early stem elongation characteristic of *sln1sln1* plants, which still occurs in an elo1 genetic background, allowed the genotype to be determined.

Leaf Elongation of the *sln1* Mutant in Different Genetic Backgrounds

The *sln1* mutant of barley (Foster, 1977) exhibits rapid leaf elongation without added GA_3, yet has lower than normal levels of active GAs in its leaves (Croker et al., 1990). On this basis, and because it shows high rates of α-amylase production by aleurone incubated without added GA_3, *sln1* is regarded as a constitutive GA-response mutant (Chandler, 1988; Lanahan and Ho, 1988). We previously showed that *sln1* derivatives of M117 (*grd1*) elongated rapidly despite the dwarfing background (Smith et al., 1996). Similar results were found for *sln1* in *grd2* or *grd3* backgrounds (P.M. Chandler, unpublished data). To determine whether double mutants of *sln1* with either *gse1* or *elo* would also elongate rapidly without added GA_3, LER_{max} values were determined for segregating *Sln1*− and *sln1sln1* types in the different genetic backgrounds. The results (Fig. 7) show that *sln1* homozygotes elongate equally rapidly in the wild type, *grd1*, and *gse1* genetic backgrounds, indicating that *sln1* is epistatic to *gse1* and *grd1*. In contrast, the LER_{max} value of *sln1* homozygotes in an *elo1* (Fig. 7) or *elo2* genetic background (data not shown) did not differ significantly from the *Sln1*− segregants, indicating that *sln1* was hypostatic to these two *elo* loci, which is consistent with the proposal that these mutants were already elongating at their maximal rate. The effect of homozygosity at *sln1* on LER_{max} values for the wild type, *grd1*, and *gse1* was equivalent to that of saturating concentrations of GA_3 (compare Figs. 2 and 7).

DISCUSSION

Hormone dose-response studies on the effects of differing concentrations of GA on LER_{max} provided an effective basis for discriminating between different classes of dwarf mutants in barley. In earlier studies GA application to seedlings had readily distinguished fully responsive dwarfs (thought to be affected in GA biosynthesis) from mutants that showed no response to GA. Dwarf mutants that gave a partial response to GA were problematic, because they may have involved alterations either in the magnitude of response at saturating hormone concentrations or in the concentration range over which a response occurred. Dose-response experiments distinguished between these possibilities. The *gse* mutants fit the latter category, and thereby define a novel type of mutant that is involved in GA signaling. Growth responses to GA in a recently described pea mutant (*lgr*) have similar properties (Ross et al., 1997).

The current interest in hormone signal transduction requires that quantitative assays be used to characterize the mutants that are affected in such processes. The GA dose-response curves described here provide a framework for future characterization of the remaining barley dwarf mutants in our collection. Sensitivity parameters were estimated from the dose-response curves using PEST software (A'Brook, 1987; Weyers et al., 1987), which fits data to a modified Hill equation. It was significant that near Michaelis-Menten responses were observed even for growth rates of whole leaf blades (at least for the wild type and *grd* mutants), because there are presumably many steps between GA perception and the final leaf growth rate at which the initial magnitude of a response to GA could be modified.

In many previous studies there was a broader-than-expected GA concentration range over which responses occurred (Nissen, 1988). In contrast, the range of GA concentrations over which a response occurred in the wild type and the *grd* mutants was relatively narrow ($p \approx 1$; see Table I). This difference was probably because we used LER_{max} as a measure of hormone response (Weyers et al., 1987) rather than the extent of response, which was used in the earlier studies. In some cases such broad transitions may be genuine, perhaps reflecting attenuation so that a wide range in hormone content can be accommodated. However, until they are analyzed in terms of rate rather than final extent, the wide concentration range might also be misleading. When we monitored final blade length rather than LER_{max}, a broader transition was observed and the magnitude of the response was smaller (Fig. 3). The probable explanation for this difference is a shorter duration of the response at high concentrations of GA_3, so that the effect of GA_3 on growth rate was never exactly matched by the effect on final length.

A $[GA_3]$ of approximately 10^{-7} M stimulated LER_{max} in *grd* dwarfs to that of the wild type given only water. This concentration is close to the $[H]_{50}$ value estimated for GA_3

(Table I), a condition in which LER_{max} changes most rapidly as the concentration of applied GA_3 changes. In this range there was a 20% change in LER_{max} for a 2-fold change in [GA_3], illustrating the potential for relatively small changes in the content of endogenous bioactive GAs to have considerable effects on the leaf-elongation rate when, for example, plants respond to different environmental factors. It is difficult to compare the [GA_3] applied in a treatment (e.g. 10^{-7} M) with the endogenous contents of bioactive GAs, because we know neither the relative contributions of different GAs (GA_1, GA_3, and possibly other GAs) in determining leaf growth rate, nor the most appropriate part of the leaf (zones of cell division or elongation, or perhaps only the epidermis of such regions) in which to determine GA content.

Tonkinson et al. (1997) determined GA_1 and GA_3 contents in the elongation zones of the second leaves of wheat seedlings and, assuming uniform distribution, their values correspond to 2 to 7 nM. These estimates are considerably lower than the $[H]_{50}$ values above, but there are many factors that could account for such a discrepancy, including species and leaf differences and the assumption of uniform GA distribution. It is apparent that the maintenance of normal growth rates requires an adequate supply of and an ability to sense endogenous GAs, because mutants that affect either process are dwarfed. The relative importance of these two processes in explaining natural variation in growth rate is difficult to assess. Weyers et al. (1995), in discussing hormonal control in a general sense, argued that combined control should always be assumed unless there is evidence to the contrary. In this context it is interesting that the growth rate of the *grd1gse1* double mutants was considerably lower than either of the single mutants (P.M. Chandler, unpublished observations).

Interpretation of the *grd* mutants is relatively straightforward, because equivalent mutants have been isolated in a range of other plant species, and have generally involved mutations in the GA-biosynthetic pathway (for review, see Ross et al., 1997). For example, the growth responses of the *grd2* mutant (Fig. 4) are typical of 3β-hydroxylase mutants that have been isolated in several species. There are two GA-responsive dwarf mutants in barley that have been studied in some detail (Hentrich et al., 1985; Boother et al., 1991), and both of these are allelic with the *grd1* locus described here (P.M. Chandler, unpublished data).

An important advantage of barley and some other cereals is the availability at the seedling stage of two well-defined GA responses (leaf elongation and α-amylase production) that involve different components (the meristem-leaf-elongation zone and aleurone, respectively). This has been important in interpreting the *gse* mutant category. In both assays the *gse* mutants were capable of responding to GA_3, probably to the same extent as the wild type and *grd* mutants, but the *gse* mutants required approximately 100-fold higher concentrations of GA_3. These recessive mutants are unique in showing reduced sensitivity for two different GA responses. One interpretation is that they define receptors that have a lower affinity for GA than the receptors in the wild type. Loeb and Strickland (1987) showed that dose-response curves can reflect the activity of components involved in signal transduction, rather than initial receptor-hormone interactions; thus the *gse1* mutants may also involve changes in the downstream components of GA signaling. An alternative interpretation is that a "primary" GA receptor or signaling pathway is rendered nonfunctional in the *gse* mutants, and the activity of a redundant pathway(s) with different properties is revealed. An interesting feature of the dose-response curves of both *gse* mutants was the broader range of concentrations over which the response occurred (Fig. 2). The associated lower values of p (Table I) may result from negative cooperativity in the binding of interacting components (e.g. a ligand to its receptor), either as a result of mutational change or because a different signaling pathway was operating.

Two other interpretations of the *gse* phenotype are possible. The first, involving overproduction of an enzyme that inactivates GAs, is considered unlikely for several reasons: (a) we would expect the trait to show some degree of dominance if it resulted from increased levels of a catabolic enzyme; (b) the dose-response curves indicate that extremely high concentrations of GA_3 (by in vivo standards) are still subsaturating, yet during normal growth the *gse* mutants are not severely dwarfed, and an altered enzyme that was capable of inactivating such high concentrations of exogenous GA_3 might be expected to have an extreme effect on the endogenous GA content, resulting in a much more severe dwarf phenotype; and (c) determination of the endogenous GA content of developing grains of M488 exhibits a profile that is very similar to that of the wild type (P.M. Chandler, unpublished data), indicating that there are no major changes in GA metabolism. This includes stages when the *gse* phenotype of developing grains is being expressed, revealed by the failure of 10 μM GA_3 to induce germination of isolated immature grains.

The second alternative interpretation of the *gse* mutants is that their reduced sensitivity to GA may have resulted either from increased levels of endogenous ABA or from enhanced responses to ABA (Cutler et al., 1996), because in barley grains and seedlings, ABA antagonizes many of the effects of GA. This interpretation is difficult to exclude until more information is available, but two lines of investigation have failed to provide support: first, the quantitative hormone analysis of developing grains of M488 (see above) revealed ABA contents similar to those of the wild type, and second, there were similar relative reductions in the L_1 growth rate observed when *gse1*, *grd1*, and wild-type grains were germinated in the presence of 1 μM ABA (P.M. Chandler, unpublished data).

The two *elo* mutants showed no significant growth stimulation by GA_3, yet their ability to perceive and initially respond to GA_3 was probably not affected. For example, LER_{max} was responsive to GA_3 with approximately the normal concentration dependence when the mutants were further dwarfed either by chemical means (Fig. 6) or by making double mutants with a *grd* locus (P.M. Chandler, unpublished data). This observation and the epistasis of *elo* to *sln1* (Fig. 7) suggest that the mutations affect specific components required for leaf elongation rather than those involved in GA signaling. There was no restoration of normal growth when these mutants were germinated on

other growth-related hormones such as brassinolide, IAA, or kinetin (P.M. Chandler, unpublished observations).

The characterization of these dwarf mutants suggests that they are representative of three broad areas involving GA control of growth: GA biosynthesis (*grd*), GA signaling (*gse*), and the growth processes themselves (*elo*). In the simplest model, GA elicits a positive signaling pathway and growth is stimulated. According to this model, the low growth rates of the *grd* and *gse* mutants are due to the effects of reduced GA content and GA sensitivity, respectively, on GA signaling. The *sln1* mutant is recessive, and slender plants show "constitutive" GA responses. Thus, the product of the wild-type *Sln1* gene (SLN) presumably functions as a negative regulator of GA signaling (if *sln1* involves a loss of function). Is SLN a negative regulator that plays a direct role in GA signaling, or does it play an indirect role? Other signaling pathways in the plant could modulate flux through a positive GA-signaling pathway via SLN acting as a negative regulator of this pathway.

Slender derivatives of the *grd* mutant showed a typical slender phenotype rather than a dwarf phenotype. The same result was observed with slender derivatives of the *gse* mutant. Thus, in an *sln1* background, mutations such as *grd* and *gse* that result in reduced GA signaling had no effect on growth rate. If SLN is an indirect negative regulator of a positive GA-signaling pathway, we might still expect to see reduced growth rates in the double mutants because of reduced GA signaling. We favor the view that SLN is a negative regulator whose activity is directly involved in GA signaling. If the GA-signaling pathway is under negative control, the positive responses observed when, for example, GA is applied must involve reducing the extent of negative regulation mediated by SLN. In the same manner, 'Himalaya' barley grows at wild-type rates because with wild-type levels of GA signaling, it can substantially reduce the extent of negative regulation imposed by SLN. By contrast, the *grd* and *gse* mutants have lower levels of GA signaling and are less able to reduce the extent of negative regulation by SLN, and consequently their growth is slow.

This interpretation is similar to that reached for the product of the *GAI* gene in Arabidopsis, which, according to genetic evidence, also functions as a negative regulator involved in GA signaling and whose activity is proposed to be regulated by GA (Peng et al., 1997; Harberd et al., 1998). In Arabidopsis there are now three different proteins that, on the basis of mutant studies, are proposed to be negative regulators of GA signaling. GAI (Peng et al., 1997) and RGA (Silverstone et al., 1997) are closely related; based on sequence comparisons, they are putative VHIID transcription factors. SPY is a protein with a sequence closely related to *O*-linked GlcNAc transferases (Jacobsen et al., 1996). Only for SPY has the proposed role as a negative regulator of GA signaling been confirmed: transient expression of barley SPY (HvSPY) largely prevented GA-induced α-amylase promoter activity in aleurone (Robertson et al., 1998). Is barley SLN related to these other proteins? It is known that SLN does not correspond to HvSPY (Robertson et al., 1998), but there is no evidence yet concerning its relationship to GAI or RGA. Scott (1990) suggested that (semi)-dominant GA "insensitive" mutants (encoded by *gai*, *Rht3*, and *D8*) might involve the same gene that is affected in recessive constitutive GA-response mutants such as *sln1*: GAI would involve a gain of function (a negative regulator whose activity was no longer regulated by GA), whereas the mutant SLN would involve a loss of function. The cloning of GAI and RGA should allow their relationship to SLN to be investigated.

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