Overexpression of a Novel Class of Gibberellin 2-Oxidases Decreases Gibberellin Levels and Creates Dwarf Plants

Fritz M. Schomburg, Colleen M. Bizzell, Dong Ju Lee, Jan A. D. Zeevaart, and Richard M. Amasino

Degradation of active C_{19}-gibberellins (GAs) by dioxygenases through 2β-hydroxylation yields inactive GA products. We identified two genes in Arabidopsis (AtGA2ox7 and AtGA2ox8), using an activation-tagging mutant screen, that encode 2β-hydroxylases. GA levels in both activation-tagged lines were reduced significantly, and the lines displayed dwarf phenotypes typical of mutants with a GA deficiency. Increased expression of either AtGA2ox7 or AtGA2ox8 also caused a dwarf phenotype in tobacco, indicating that the substrates for these enzymes are conserved. AtGA2ox7 and AtGA2ox8 are more similar to each other than to other proteins encoded in the Arabidopsis genome, indicating that they may constitute a separate class of GA-modifying enzymes. Indeed, enzymatic assays demonstrated that AtGA2ox7 and AtGA2ox8 both perform the same GA modification: 2β-hydroxylation of C_{20}-GAs but not of C_{19}-GAs. Lines containing increased expression of AtGA2ox8 exhibited a GA dose–response curve for stem elongation similar to that of the biosynthetic mutant ga1-11. Double loss-of-function Atg2ox7 Atg2ox8 mutants had twofold to fourfold higher levels of active GAs and displayed phenotypes associated with excess GAs, such as early bolting in short days, resistance to the GA biosynthesis inhibitor ancymidol, and decreased mRNA levels of AtGA20ox1, a gene in the GA biosynthetic pathway.

INTRODUCTION

The gibberellins (GAs) are a class of plant hormones that are involved in a variety of growth and developmental processes, including seed germination, leaf expansion, stem elongation, floral induction, fruit maturation, and apical dominance (Harberd et al., 1998). GAs are substituted tetracyclic diterpene carboxylic acids that require many biosynthetic steps to create (for review, see Hedden and Phillips, 2000). To date, 126 different GAs have been identified in plants, fungi, and bacteria (http://www.plant-hormones.info/gibberellins.htm); however, most of these are precursors or degradation products. Examples of bioactive GAs synthesized by higher plants are GA_{1}, GA_{3}, GA_{4}, and GA_{7} (Hedden and Phillips, 2000).

The GA biosynthetic pathway can be classified into three stages (Olszewski et al., 2002). In the first stage, geranylgeranyl diphosphate is cyclized to ent-kaurene by copalyl diphosphate synthase and ent-kaurene synthase. In the second stage, ent-kaurene is oxidized by ent-kaurene oxidase to ent-kaurenoic acid, which in turn is oxidized by ent-kaurenoic acid oxidase in three steps to GA_{12}. All reactions in stage 2 are catalyzed by cytochrome P450 monoxygenases (Helliwell et al., 1998, 2001). In the third stage, GA_{12} is modified by oxidative reactions involving 2-oxoglutarate–dependent dioxygenases. In the first reaction of the third stage, C-20 is oxidized and removed. In Arabidopsis, this is followed by 3β-hydroxylation to give bioactive GA_{1} and GA_{4} (Hedden and Phillips, 2000).

Mutants in GA biosynthesis have been identified in a variety of species. In Arabidopsis, the most prominent phenotypes of GA biosynthesis mutants are reduced internode length and small dark green leaves (Koornneef and van der Veen, 1980). In such mutants, normal growth can be restored by the application of active GAs. The ga1-3 mutation is thought to represent the most complete block to GA biosynthesis because ga1-3 mutants display the most severe GA-deficient phenotypes (Wilson et al., 1992) and the GA1 gene encodes the only copy of copalyl diphosphate synthase in Arabidopsis (Sun and Kamiya, 1994). Mutations in genes involved in the later stages of GA biosynthesis in Arabidopsis, such as ga4 and ga5, cause a less severe or semi-dwarf phenotype (Talon et al., 1990a). GA5 enzymatic activity is encoded by a small gene family of GA 20-oxidases (Phillips et al., 1995). Thus, functional redundancy is likely to be the reason that mutations in genes involved in the later stages of GA biosynthesis result in a phenotype that is less severe than that of ga1.
In contrast to biosynthesis, less is known about the pathways and regulation of GA degradation. The first step in the degradation of biologically active GAs involves reactions similar to those seen in the final steps of GA biosynthesis. For example, the last step of GA biosynthesis involves a 3β-hydroxylase (also referred to as 3-oxidase) that introduces a hydroxyl group at the C-3 of GA precursors to form active GAs (Talon and Zeevaart, 1992; Chiang et al., 1995). The first step of GA degradation involves GA 2-oxidases that hydroxylate the C-2 of active GAs (Martin et al., 1999; Thomas et al., 1999; Sakamoto et al., 2001). Thus, the site of similar hydroxylation reactions determines the activity of GA molecules.

GA 2-oxidase genes have been cloned from several species, including three from Arabidopsis (Thomas et al., 1999). These GA 2-oxidases use C₁₉-GAs as their substrates. Expression studies of the three Arabidopsis 2-oxidases revealed that two of them were most abundant in the inflorescence and developing siliques, whereas the other one could not be detected in any tissue (Thomas et al., 1999). This expression pattern is consistent with a role of GA 2-oxidases in reducing GA levels in seeds to promote dormancy. Further evidence for this role comes from studies of the SLENNDER gene of pea, which also encodes a GA 2-oxidase (Martin et al., 1999). The hyperelongation of the slender mutant phenotype is most apparent in seedlings. This mutation results in high levels of GA precursors in seeds, which are converted to active GAs upon germination. This excess GA causes the slender seedling phenotype (Martin et al., 1999).

Here, we describe the identification of novel GA 2-oxidases in Arabidopsis that hydroxylate C₁₉-GA precursors but not C₁₇-GAs. We demonstrate that increased expression of these 2-oxidases results in decreased levels of active GAs and corresponding dwarf phenotypes in Arabidopsis and tobacco. Loss of these oxidases in Arabidopsis results in increased levels of active GAs and in phenotypes associated with increased GA levels.

RESULTS

Activation Tagging of Loci That Confer a Dominant Dwarf Phenotype

A mutant screen was performed using T-DNA from the pSKI015 activation-tagging vector as the mutagen. This vector contains four repeats of the enhancer region of the constitutively expressed 35S promoter of Cauliflower mosaic virus (CaMV) in the T-DNA, and introduction of this T-DNA into the genome can cause increased gene expression near the site of integration in an orientation-independent manner (Weigel et al., 2000). Dominant phenotypes are observed in the first mutagenized (T1) generation. In this screen, we identified two dwarf mutants (Figure 1A) from ~60,000 independent T1 lines of Arabidopsis (accession Wassilewskija) grown in long-day photoperiods.

Both mutant phenotypes appeared to be caused by T-DNA insertion, because in the subsequent (T2) generation, the T-DNA was present in all mutant plants and wild-type plants lacked the T-DNA. In both mutants, the dwarf trait was dominant: the T2 generation segregated 3:1 (dwarf to wild-type plants). The dominance of the dwarf trait indicated that the mutations likely resulted from increased gene expression from the 35S enhancers in the T-DNA.

Identification of the Dominant Dwarfing Genes

To identify the genes responsible for the mutant phenotypes, the sites of the T-DNA insertions were characterized. The junction between the T-DNA and Arabidopsis genomic DNA was determined by thermal asymmetrical interlaced (TAIL)-PCR (see Methods). In both mutants, the T-DNA insertion of the right border (which contains the 35S enhancer regions) was positioned at the 5’ end of the predicted genes, as illustrated in Figure 2. The mutant lines are referred to here as AtGA2ox7 and AtGA2ox8, and the genes predicted to be activated by the T-DNA are designated AtGA2ox7 and AtGA2ox8. The designations AtGA2ox7 and AtGA2ox8 were chosen because AtGA2ox1 through AtGA2ox3 were described by Thomas et al. (1999) and genes designated AtGA2ox4 through AtGA2ox6 were noted by Hedden and Phillips (2000). The genes identified in this study (AtGA2ox7 and AtGA2ox8) are distinct from AtGA2ox1 through AtGA2ox6 (P. Hedden and A.L. Phillips, personal communication).

Reverse transcription–based PCR (RT-PCR) showed that the mRNA levels of AtGA2ox7 and AtGA2ox8 were increased substantially in the respective mutant lines (Figure 3). To verify that the increased expression of these genes was capable of causing a dwarf phenotype, we attempted to independently recreate the phenotype with constructs designed to increase the expression of these genes. Accordingly, constructs in which the constitutively expressed CaMV 35S promoter was joined to genomic clones of AtGA2ox7 and AtGA2ox8 (Figure 2) were introduced into wild-type Arabidopsis. The introduction of these constructs was able to recapitulate the dwarf phenotype (Figure 1B, Table 1), confirming the identity of the genes responsible for the dwarf phenotypes.

We determined the cDNA sequences of both genes by RT-PCR and created constructs in which the cDNAs were driven by the CaMV 35S promoter (Figure 2). Increased expression of either cDNA was sufficient to produce dwarf plants (data not shown), demonstrating that biologically active cDNAs for both genes had been identified. The protein sequences predicted by these cDNAs are shown in Figure 4.

The Activation-Tagged Genes Display Similarity to Gibberellin Dioxygenases

Alignments of the AtGA2ox7 and AtGA2ox8 predicted proteins indicate that they share similarity to the dioxygenase
Figure 1. Phenotypes of Arabidopsis (Accession Wassilewskija) and Tobacco (var Wisconsin 38) with Increased Expression Levels of AtGA2ox7 and AtGA2ox8.

(A) AtGA2ox7<sub>ACT</sub> and AtGA2ox8<sub>ACT</sub> activation-tagged dwarf mutants are shown at 24 and 48 days after germination (DAG), Ws, Wassilewskija. 
(B) Arabidopsis containing the 35S::AtGA2ox7 or 35S::AtGA2ox8 genomic construct. 
(C) Tobacco containing the 35S::AtGA2ox7 or 35S::AtGA2ox8 cDNA construct. W38, Wisconsin 38. 
(D) The range of dwarf phenotypes obtained in transgenic lines containing 35S::AtGA2ox7. 
(E) and (F) Responses of AtGA2ox7<sub>ACT</sub> and AtGA2ox8<sub>ACT</sub> mutants to GA<sub>4</sub> at 12 and 14 days, respectively, after GA application. Plants were transferred from short days to long days at the start of GA treatment.
family of GA-modifying enzymes (Table 2). AtGA2ox7 and AtGA2ox8 are more similar to each other than to any other predicted protein in the Arabidopsis genome, indicating that these genes may constitute a separate branch of GA metabolism (Table 2, Figure 4). The closest match in the Arabidopsis genome is AtGA20ox1, which is 32% identical to both AtGA2ox7 and AtGA2ox8. The overall relatedness of the GA-modifying dioxygenase enzymes is not surprising, because these enzymes are likely to have conserved motifs that bind GAs and other common cofactors. However, a unique region of similarity between AtGA2ox7 and AtGA2ox8 (at positions 115 to 143 of AtGA2ox8; Figure 4) may define the specificity of the reactions performed by these enzymes.

Overexpression of AtGA2ox7 and AtGA2ox8 in Tobacco Results in Dwarf Phenotypes

To determine whether the overexpression of AtGA2ox7 or AtGA2ox8 would cause a GA-deficient phenotype in a different plant species, the 35S promoter–AtGA2ox7 and –AtGA2ox8 cDNA fusion constructs were transformed into the Wisconsin 38 variety of tobacco. Overexpression of both AtGA2ox7 and AtGA2ox8 caused dwarfing (Figures 1C and 1D), indicating that the substrates for these enzymes are required for normal elongation growth in tobacco. A range of dwarf phenotypes was observed with the overexpression of both genes; however, AtGA2ox7 overexpression generally caused a more severe dwarf phenotype than did AtGA2ox8 overexpression (similar to the effects of overexpression in Arabidopsis). Transgenic lines were obtained with phenotypes that ranged from nearly wild type to severely dwarf compact rosettes that had no discernible internodes (Figures 1C and 1D). This is likely the result of copy number and/or transgene position effect variation in expression. Because the expression of AtGA2ox7 and AtGA2ox8 can generate plants that display a range of dwarf phenotypes in Arabidopsis and tobacco, which are species in the two major clades of eudicots (Soltis et al., 1999), these genes may prove broadly useful in agriculture to control plant stature without the use of chemical treatments to inhibit GA biosynthesis.

AtGA2ox7 and AtGA2ox8 Proteins Render C20-GA Precursors Inactive by Hydroxylation of the 2 Position

To investigate the reactions catalyzed by AtGA2ox7 and AtGA2ox8, we produced the proteins in Escherichia coli for enzyme assays. Specifically, the cDNAs were cloned into the pET-28a vector, which permits inducible protein production in E. coli, and total cellular lysates of induced cells were used for enzyme assays. Substrate and product(s) were separated by reverse-phase HPLC with online radioactivity
A Novel Class of Gibberellin 2-Oxidases

Detection. The results for AtGA2ox7 are presented in Table 3; similar results were obtained for AtGA2ox8 (data not shown). When 14C-GA12 (retention time = 26.6 min) was used as a substrate, a product with a retention time of 26.6 min was formed with both enzymes. This product was identified by gas chromatography–mass spectrometry as 14C-GA110. In the case of 14C-GA53 (retention time = 29.7 min) as a substrate, the product was identified as 14C-GA97 (retention time = 12.6 min). Thus, both proteins are able to catalyze the 2β-hydroxylation of the C20-GA precursors GA12 and GA53. By contrast, the C19-GAs GA1, GA4, GA9, and GA20, with a γ-lactone ring were not 2β-hydroxylated by these enzymes. Thus, AtGA2ox7 and AtGA2ox8 represent a new class of 2-oxidases that specifically inactivate C20-GA precursors. All previously characterized GA 2-oxidases 2β-hydroxylate predominantly C19-GAs (Martin et al., 1999; Thomas et al., 1999; Sakamoto et al., 2001). Several putative GAs have been detected in extracts of Arabidopsis (Talon et al., 1990b). Two of these compounds have been identified as GA97 and GA110 (J.A.D. Zeevaart, unpublished results), indicating that AtGA2ox7 and AtGA2ox8 do function in vivo.

**Increased Expression of AtGA2ox7 or AtGA2ox8 Affects GA Levels but Not GA Responsiveness**

The levels of endogenous GAs were much reduced in the AtGA2ox7ACT and AtGA2ox8ACT plants compared with those in wild-type plants (Table 4). The level of bioactive GA4 was below the limit of detection in both mutants. Thus, the dwarf phenotype is attributable to GA deficiency. This result is further supported by the observation that the application of GA restores normal stem growth and flowering time in AtGA2ox7ACT and AtGA2ox8ACT plants (Figures 1E and 1F), and a comparison of AtGA2ox8ACT with the biosynthetic mutant ga1-11 revealed the same dose–response relationship for stem elongation in both lines (Figure 5).

**Altered Expression of AtGA2ox7 and AtGA2ox8 Affects Flowering Behavior**

Decreased GA levels severely delay floral induction in Arabidopsis in noninductive short days and also cause a slight

| Table 1. Phenotypic Characterization of the Overexpression Lines of AtGA2ox7 and AtGA2ox8 |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Trait                         | Wild Type       | AtGA2ox7ACT     | AtGA2ox8ACT     | 35S::AtGA2ox7   | 35S::AtGA2ox8   |
| Flowering behavior (number of leaves) | 8.1 (1.0)       | 15.0 (1.1)      | 13.8 (1.5)      | 16.2 (1.6)      | 14.9 (1.7)      |
| Height (cm)                   | 48 (1.1)        | 7.5 (1.0)       | 9.6 (1.3)       | 8.2 (1.3)       | 9.6 (1.3)       |
| Number of inflorescence branches | 41 (5.3)        | 87 (28)         | 84 (25)         | >100            | >100            |
| Internode length (mm)         | 9.1 (0.13)      | 1.9 (0.27)      | 2.4 (0.37)      | 2.5 (0.49)      | 2.7 (0.57)      |

Plants were grown in long-day photoperiods. Numbers in parentheses indicate 1 SD. Flowering time was scored upon the emergence of floral buds.

a Number of rosette leaves formed by the primary meristem before the transition to flowering.

b Total number of inflorescences and inflorescence branches produced.

c Internode length between siliques on the primary inflorescence measured as (n−1)/L, where n is the total number of siliques and L is the length of the inflorescence containing siliques.
delay in inductive long days (Wilson et al., 1992). Because AtGA2ox7ACT and AtGA2ox8ACT have reduced levels of active GAs, it was not surprising to find that these mutants behaved similarly to known GA-deficient mutants: AtGA2ox7ACT and AtGA2ox8ACT plants flowered slightly later in long days (producing 15 and 14 leaves on the primary stem versus 8 leaves for the wild type; Table 1) and flowered much later in short days (producing >80 leaves versus an average of 30 leaves for the wild type). The application of GA3 was sufficient to rescue this late-flowering phenotype (data not shown), indicating that the decreased level of GA was responsible for the delay in flowering.

To determine whether flowering was affected by loss-of-function mutations in AtGA2ox7 and AtGA2ox8, mutant lines that contained T-DNAs inserted in the coding region of AtGA2ox7 and AtGA2ox8 were obtained from the Arabidopsis Knockout Facility at the University of Wisconsin (Krysan et al., 1999). The Atga2ox7 mutant line contained a T-DNA insertion at the 3’ end of the second exon, and the Atga2ox8 mutant line contained a T-DNA insertion at the beginning of the first intron (Figure 2). A double mutant that contained both lesions (Atga2ox7 Atga2ox8) was obtained from an F2 population derived from a cross of the two single mutants. The flowering behavior of the loss-of-function mutants was evaluated in long and short days. There were no significant differences in the flowering behavior of the loss-of-function mutants versus the wild type in long days, but in short days, Atga2ox8 and Atga2ox7 Atga2ox8 mutants formed fewer rosette leaves before bolting (Figure 6A). However, the number of cauline leaves formed in short days was greater in the Atga2ox8 and Atga2ox7 Atga2ox8 lines compared with the wild type. Therefore, the total number of leaves produced on the primary stem was not significantly different from that produced in the wild type (Figure 6A). Rather, the ratio of cauline leaves to rosette leaves increased in both Atga2ox8 and Atga2ox7 Atga2ox8 mutants (Figure 6B), indicating that the reduced rosette leaf number of these lines in short days likely resulted from the relocation of leaves that would have been present in the wild-type rosette to the inflorescence stem.

Loss-of-Function Mutants Display GA Excess Phenotypes

Increased expression of AtGA2ox7 and AtGA2ox8 causes a decrease in GA levels; therefore, loss-of-function mutations...
in these genes might lead to increased GA levels. To test for phenotypic changes consistent with increased GA levels in the Atga2ox7 and Atga2ox8 mutants, hypocotyl lengths in several light conditions and the ability of seeds to germinate in the presence of the GA biosynthesis inhibitor ancymidol were evaluated. To control for environmentally caused variability in seed dormancy and seedling traits, seeds were harvested from several wild-type, single mutant, and Atga2ox7 Atga2ox8 double mutant plants that were derived from a single F2 population grown in the same flat. Seeds from several individual plants of each genotype were used for each assay, and each assay was performed at least twice.

Although germination was inhibited completely at 16 μM ancymidol in all lines, both Atga2ox8 and Atga2ox7 Atga2ox8 were more resistant to 4 and 8 μM ancymidol concentrations than were Atga2ox7 and the wild type (Figure 7). Thus, one role of AtGA2ox8 may be to inhibit seed germination. The Atga2ox7 Atga2ox8 double mutant did not display an increased propensity for germination on ancymidol plates compared with Atga2ox8 mutants, indicating that AtGA2ox8 and AtGA2ox7 functions do not overlap in seed dormancy.

Atga2ox8 and Atga2ox7 Atga2ox8 mutants displayed longer hypocotyls compared with wild-type seedlings in medium-light (50 μmol·m⁻²·s⁻¹; Figure 8) and low-light (15 μmol·m⁻²·s⁻¹; data not shown) conditions. Although the hypocotyl length of Atga2ox7 mutants was not significantly different from that of the wild type, the hypocotyls of the Atga2ox7 Atga2ox8 double mutants were longer than those of the Atga2ox8 single mutant. Thus, AtGA2ox7 and AtGA2ox8 both may function in the control of hypocotyl elongation.

Loss-of-Function Mutants Possess Higher Levels of Active GAs and Decreased GA Biosynthetic Activity

Gas chromatography–selected ion monitoring analyses showed that Atga2ox7 Atga2ox8 mutants contain increased

**Table 3. Identification of Products Formed by the Incubation of Recombinant GA 2-Oxidase (AtGA2ox7) with GA12 or GA53**

<table>
<thead>
<tr>
<th>Substrate Product</th>
<th>Mass Spectra of Productsb [mass-to-charge ratio (% relative abundance)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C$<em>{4}$-GA$</em>{12}$</td>
<td>M$^{+}$ 456 (11), 448 (8), 441 (11), 433 (8), 424 (51), 416 (44), 396 (78), 388 (66), 379 (7), 373 (7), 322 (13), 316 (10), 306 (100), 298 (92), 291 (71), 283 (64), 262 (27), 261 (28), 258 (23), 257 (26), 245 (98), 239 (74), 229 (37), 223 (24), 199 (16), 197 (12), 147 (37), 145 (41)</td>
</tr>
<tr>
<td>$^{14}$C$<em>{4}$-GA$</em>{53}$</td>
<td>M$^{+}$ 544 (45), 536 (22), 529 (12), 521 (5), 510 (5), 504 (4), 483 (13), 477 (6), 452 (3), 446 (2), 393 (8), 387 (4), 377 (5), 371 (4), 331 (5), 327 (5), 301 (5), 297 (3), 243 (19), 239 (17), 210 (73), 209 (100), 208 (31), 207 (56)</td>
</tr>
</tbody>
</table>

*a* As the methyl ester trimethylsilyl ethers.

**Table 4. GA Content of Wild-Type Arabidopsis (Accession Wassilewskija [Ws]) and AtGA2ox7ACT and AtGA2ox8ACT Lines**

<table>
<thead>
<tr>
<th>GAs</th>
<th>Ws</th>
<th>AtGA2ox7ACT</th>
<th>AtGA2ox8ACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-13-hydroxylated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA$_{24}$</td>
<td>51.8</td>
<td>0.06</td>
<td>0.23</td>
</tr>
<tr>
<td>GA$_{9}$</td>
<td>1.01</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>GA$_{4}$</td>
<td>1.84</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>13-Hydroxylated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA$_{53}$</td>
<td>6.43</td>
<td>0.39</td>
<td>0.30</td>
</tr>
<tr>
<td>GA$_{44}$</td>
<td>0.79</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GA$_{19}$</td>
<td>9.29</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>GA$_{20}$</td>
<td>0.19</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GA$_{1}$</td>
<td>0.12</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

All values are ng/g dry weight. ND, not detectable.

**Figure 5. Stem Elongation Dose–Response Curves for the GA Biosynthetic Mutant ga1-11 and AtGA2ox8$_{ACT}$ Lines.**

GA$_{9}$ was applied twice (at 0 and 5 days) to the shoot apex of flowering plants to achieve the final amount noted on the x axis. Stems were measured at 20 days after the first GA$_{9}$ application. Both genotypes flowered at the same time. Error bars indicate ±SD.
Previous studies have shown that GA application results in feedback inhibition of the GA biosynthetic pathway gene GA5 (Phillips et al., 1995; Xu et al., 1999). Therefore, we investigated whether Atga2ox7 Atga2ox8 mutants displayed altered levels of GA5 mRNA. Indeed, two independent experiments demonstrated that GA5 expression levels were lower in Atga2ox7 Atga2ox8 double mutants than in the wild type (Figure 9). Thus, the loss of AtGA2ox7 and AtGA2ox8 activity may lead to increased levels of active GAs, which in turn may result in feedback inhibition of the expression of GA biosynthetic genes.

**DISCUSSION**

This study describes the identification, using an activation-tagging mutant screen (Weigel et al., 2000), of two Arabidopsis loci that cause a dominant dwarf phenotype upon activation. Increased expression of either of these two loci results in a GA deficiency phenotype that is associated with a reduction in the levels of both GA precursors and active GAs. The genes (AtGA2ox7 and AtGA2ox8) responsible for the dwarf phenotypes exhibit similarity to dioxygenases involved in GA metabolism. However, AtGA2ox7 and AtGA2ox8 proteins are more similar to each other than to other genes in the Arabidopsis genome. Thus, AtGA2ox7 and AtGA2ox8 form a class within the GA dioxygenase superfamily that represents a novel aspect of GA metabolism.

Studies of the enzymatic activity of AtGA2ox7 and AtGA2ox8 demonstrate that these proteins catalyze a novel reaction: both proteins act as 2-oxidases that hydroxylate carbon 2 of C20-GA precursors (e.g., GA12) but not C19-GAs. The 2β-hydroxylation of C20-GA precursors should render them unable to be converted to active GAs and thus decrease the levels of active GAs and cause a dwarf phenotype. The specificity of these enzymes for C20-GAs but not C19-GAs predicts that plants that are dwarfed by the increased expression of AtGA2ox7 or AtGA2ox8 should be...
fully GA responsive. Indeed, the dose–response relationship for elongation versus the amount of applied GA is nearly identical in AtGA2ox8<sub>ACT</sub> and the GA biosynthetic mutant ga1-11.

Because overexpression of AtGA2ox7 and AtGA2ox8 results in decreased levels of active GAs and dwarf phenotypes, it was of interest to determine whether the loss of these genes would cause increased GA levels. Gas chromatography–mass spectrometry analysis of the shoots of mature plants revealed that the double loss-of-function mutant Atga2ox7 Atga2ox8 contained twofold to fourfold higher levels of GA<sub>4</sub> and GA<sub>9</sub> than the wild type. The Atga2ox7 Atga2ox8 double mutant and the Atga2ox8 single mutant also displayed phenotypes typical of plants treated with GAs: flowering in short days after producing fewer rosette leaves but more cauline leaves than the wild type, longer hypocotyls than the wild type, and reduced sensitivity to the inhibition of germination by the GA biosynthesis inhibitor ancymidol, presumably as a result of increased levels of active GAs in the seeds. It is interesting that the Atga2ox8 single mutant exhibits seedling phenotypes but the Atga2ox7 single mutant does not. Consistent with this observation, the Atga2ox8 mRNA but not the Atga2ox7 mRNA was detectable by RT-PCR in seedlings (data not shown). Although these data indicate that the loss of AtGA2ox7 and AtGA2ox8 activity results in phenotypes associated with increased GA levels, these phenotypes are relatively weak compared with, for example, a constitutive GA signaling mutant such as spindly (Jacobsen and Olszewski, 1993).

The relatively weak phenotypes associated with the Atga2ox7 and Atga2ox8 single and double loss-of-function mutants might be the result of feedback compensation in the GA biosynthetic pathway. In the Atga2ox7 Atga2ox8 double mutant, GA<sub>5</sub> mRNA levels were reduced. GA5 encodes an enzyme involved in GA biosynthesis, and GA5 expression is subject to feedback regulation (Xu et al., 1999). The reduction in GA5 expression in the Atga2ox7 Atga2ox8 double mutant indicates that loss of the activities of these genes is sufficient to affect this feedback system and to reduce GA biosynthetic activity. In the double mutant, the levels of GA<sub>3</sub> and GA<sub>8</sub> also were lower, indicating that this feedback also might affect earlier steps in the biosynthetic pathway. The lower level of precursors in the double mutant also should contribute to the decreased severity of the double mutant phenotype.
The weak phenotype of the double mutant also could result if AtGA2ox7 and AtGA2ox8 are expressed primarily in certain tissues or cell types, at specific stages of development, or in response to specific inductive conditions. Thus, the mutant analyses may not have been performed under the proper conditions to reveal additional phenotypes, and measurements of whole-plant GA levels would not reveal differences in GA levels in specific cell types. The transcripts of AtGA2ox7 and AtGA2ox8 were not detectable by RNA gel blot analysis, and only AtGA2ox8 was detected by RT-PCR in seedling tissue after >38 cycles. AtGA2ox7 was not detected in any tissue examined, and AtGA2ox8 was not detected in any adult tissue. Another possible explanation for the weak phenotype of the double mutant could be the existence of additional C20-GA 2-oxidases in the genome. However, the Arabidopsis genome does not contain other genes predicted to encode proteins that have high homology with AtGA2ox7 or AtGA2ox8.

The unique activities of AtGA2ox7 and AtGA2ox8 may provide a strategy for the development of new dwarf or semidwarf varieties of crop and ornamental plant species. A major component of the increased yield of the “green revolution” varieties of crop plants is the introduction of dwarfed varieties (Peng et al., 1999; Spielmeyer et al., 2002). The creation of such varieties has relied upon natural genetic variation within the crop species. The possibility of introducing AtGA2ox7 or AtGA2ox8, other GA-metabolizing enzymes (Thomas et al., 1999), or GA-INSENSITIVE (Fu et al., 2001) to create dominant dwarf varieties without the need to identify mutants or native genes involved in GA metabolism or perception from each plant species should facilitate the production of new crop varieties. AtGA2ox7 and AtGA2ox8 may be particularly useful in this regard because the substrates of AtGA2ox7 and AtGA2ox8 are common intermediates in the GA biosynthetic pathway of most flowering plants (Hedden and Phillips, 2000); thus, the expression of these genes should cause dwarfing in a wide range of species. Indeed, we have shown that these Arabidopsis genes are capable of causing extreme dwarfing in tobacco. Furthermore, because AtGA2ox7 and AtGA2ox8 do not modify (or hydroxylate) C19-GAs, active C19-GAs can be applied to AtGA2ox7- or AtGA2ox8-derived dwarf plants to rescue the dwarf phenotypes. By altering the expression levels and/or spatial expression patterns of AtGA2ox7 and AtGA2ox8, it should be possible to produce plants with a range of dwarf phenotypes.

### METHODS

#### Plant Growth Conditions

*Arabidopsis thaliana* plants were grown under cool-white fluorescent light (100 μmol·m⁻²·s⁻¹; Sylvania, Danvers, MA) at 22 ± 1°C in Fafard Germination Mix (Fafard Co., Agawam, MA). Plants were fertilized with 2 g/L Dyna-Grow 7-9-5 fertilizer (Dyna-Grow Corp., San Pablo, CA) at 2-week intervals. Daylengths were 8 h of light and 16 h of darkness for short days and 16 h of light and 8 h of darkness for long days. Germination assays were performed on Murashige and Skoog (1962) medium, pH 5.8, that was supplemented with 5.5 g/L agar, 0.5 g/L Mes (Sigma), and 1% Suc. Ancymidol (Sigma) was added to autoclaved medium from a filter-sterilized ×100 stock solution.

Plants to be used for gibberellin (GA) quantification were grown in trays as described (Talon et al., 1990a). Short-day conditions consisted of 9 h of light (23°C) from fluorescent and incandescent lamps at 150 μmol·m⁻²·s⁻¹ and 15 h darkness at 20°C. For long days, the 9-h main light period was extended to 15 h with light from incandescent lamps at 25 μmol·m⁻²·s⁻¹. Plants were grown in short days until flower primordia began to appear. At that time, the plants were ex-
posed to 3 or 4 long days before harvest. Plants with stems, if present, were harvested and frozen in liquid N₂. All plant material was lyophilized.

Isolation of AtGA2ox7 and AtGA2ox8 Knockout Alleles

The BASTA T-DNA (accession Wassilewskija) population of the Wisconsin T-DNA collection was screened for insertion (i.e., knockout) alleles of AtGA2ox7 and AtGA2ox8 at the Arabidopsis Knockout Facility (http://www.biotech.wisc.edu/Arabidopsis/default.htm) according to Krysan et al. (1999). Gene-specific primers for AtGA2ox7 (5'-CTAAGTGGTGAGGGTGCTCAAC-3' and 5'-TCGAGATAGGA-GATACCGAAGAAG-3') and AtGA2ox8 (5'-TGTGTCTCTCTTCAG-3' and 5'-AGGAACCTAGGAGGCCAAC-3') were used in combination with the left border T-DNA primer JL202 (Krysan et al., 1999) to identify knockout mutants. AtGA2ox7 is in BAC F8A12.18, and AtGA2ox8 is in BAC F7J7.140.

Identification of DNA Flanking the Site of T-DNA Insertion

Identification of the T-DNA insertion site in the AtGA2ox7/ACT and AtGA2ox8/ACT lines in Arabidopsis was determined by thermal asymmetrical interlaced (TAIL)-PCR. Genomic DNA for use in TAIL-PCR was prepared as described (Liu et al., 1995). PCR reactions were conducted using hot-start addition of Takara ExTaq enzyme (Panvera, Madison, WI). Two rounds of PCR amplifications were used to isolate DNA flanking the T-DNA insertion site. Fifteen picoliters of the left border T-DNA primer JL202 was used with 150 pmoI of the partially degenerate primer AD-2 (5'-NGTGCAAGWWGGNAWGA-3') for the first PCR reaction. Conditions for the first reaction were as follows: (1) 96°C for 5 min; (2) 94°C for 10 s; (3) 65°C for 30 s; (4) 72°C for 1 min; (5) repeat four additional cycles of steps 2 through 4; (6) 94°C for 10 s; (7) 25°C for 3 min; (8) ramp to 72°C over 3 min; (9) 72°C for 3 min; (10) 94°C for 10 s; (11) 65°C for 30 s; (12) 72°C for 1 min; (13) repeat one additional cycle of steps 10 through 13; (14) 94°C for 10 s; (15) 44°C for 1 min; (16) 72°C for 1 min; (17) repeat 14 additional cycles of steps 10 through 16; (18) 72°C for 3 min; and (19) 4°C until needed.

DNA produced in the first PCR was diluted 1:50, and 1 µL of this dilution was used for the second round of PCR. Similar to the first reaction, 15 pmoI of the left border primer was used with 15 pmoI of AD-2 for amplification. The PCR cycle conditions were as follows: (1) 96°C for 5 min; (2) 94°C for 10 s; (3) 61°C for 30 s; (4) 72°C for 1 min; (5) repeat one additional cycle of steps 2 through 4; (6) 94°C for 10 s; (7) 44°C for 1 min; (8) 72°C for 1 min; (9) repeat 17 additional cycles of steps 2 through 8; (10) 72°C for 4 min; and (11) 4°C until needed. The resulting PCR products were sequenced with the JL270 primer.

Generation of Overexpression Constructs of AtGA2ox7 and AtGA2ox8

The overexpression constructs of AtGA2ox7 and AtGA2ox8 were created by PCR amplification of genomic DNA from the start to the stop codon of the putative open reading frame of the dioxygenases. The primers used to generate overexpression of AtGA2ox8 were 5'-AAAGGATCCATGCAGGGCAAGCTTCATCAAACGAAATATAC-3' and 5'-AAAGGATCCCTTCAGGAACCTAGGAGGCCAAC-3' (restriction sites are shown in boldface, and the sequence corresponding to AtGA2ox8 is underlined), which generated a 3838-bp fragment that was digested with BamHI and SacI and ligated into the BamHI and SacI sites of pRAM1. pRAM1 was created by ligating the HindIII-EcoRI fragment of pBI121 containing the 35S promoter and nopaline synthase terminator sequences into the HindIII and EcoRI sites of pPZP211 (Harberd et al., 1998). The same AtGA2ox8 primer pair produced a 1017-bp cDNA fragment from a cDNA preparation (see below) that was used to create a cDNA overexpression construct in pRAM1. Similarly, AtGA2ox7 genomic and cDNA overexpression constructs were produced with the primer pair 5'-AAAGGATCCATGGCTCTCTCAACCTCCCT-3' and 5'-AAAGGATCCCTCAAAATGAAGAAACCTGGGACAAG-3', which produced 2171-bp genomic and 1011-bp cDNA fragments. These were cloned into pRAM1 as described above. The constructs were transferred into Agrobacterium tumefaciens strain ABI and transformed into Arabidopsis by the floral dip method (Clough and Bent, 1998).

mRNA Detection by Reverse Transcriptase–Mediated PCR

RNA was prepared from tissue that had been immediately frozen in liquid N₂. RNA was isolated with TRI reagent (Sigma) according to instructions for subsequent reverse transcription reactions. Five micrograms of total RNA was annealed to 500 ng of random decamer oligonucleotides (Integrated DNA Technologies, Coralville, IA). Superscript II reverse transcriptase (Gibco Life Technologies, Gaithersburg, MD) was used to generate cDNA. Takara ExTaq was used for PCR amplification of cDNA. PCR conditions were 26 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 2 min.

Production of AtGA2ox7 and AtGA2ox8 Proteins

AtGA2ox7 and AtGA2ox8 cDNAs were obtained by reverse transcription–based PCR as described above. Primers were designed to allow simple cloning of the cDNAs into pET-28a (Promega, Madison, WI). The AtGA2ox8 cDNA was amplified with 5'-AAAGGATCCATGCCCATGCAAGGCATATAATAC-3' and 5'-AAAGGATCCATTAGGAAGGCCAAC-3' and subsequently ligated into pET-28a in the BamHI and SacI sites of the polylinker. The AtGA2ox7 cDNA was amplified with 5'-AAAGGATCCATGGCTCTCTCAACCTCCCT-3' and 5'-AAAGGATCCATGGAACACCTGGGACAAG-3' and also cloned into pET-28a using the BamHI and SacI restriction sites. The full-length cDNA clones pET-AtGA2ox7 and pET-AtGA2ox8 were transformed into Escherichia coli strain BL21pLysS. A starter culture (25 ml) was added to 0.5 L of Luria-Bertani medium containing 100 mg/L kanamycin and incubated at 37°C with vigorous shaking. When the OD₆₀₀ had reached 0.6, isopropylthio-β-D-galactoside was added to a final concentration of 3 mM and the culture was incubated for another 2 h at 30°C. The cells were harvested and suspended in lysis buffer (100 mM Tris-HCl, pH 7.5, and 10 mg/mL lysozyme) and shaken at room temperature for 10 min. After brief sonication, the suspension was frozen in liquid N₂ and then thawed in an ice bath for 15 min. The lysates were centrifuged at 13,000 rpm for 20 min, and the supernatant was stored at −80°C until use for enzyme assays.

RNA Gel Blot Analysis

Total RNA was isolated from leaves and stems using the TRIzol reagent (Invitrogen, Carlsbad, CA). Forty micrograms of total RNA was fractionated on a 1.2% agarose gel in the presence of formaldehyde (Sambrook et al., 1989), transferred to nitrocellulose, and probed
with $^{32}$P-dCTP–labeled cDNA using the Random Primers DNA Labeling System (Gibco BRL). Hybridization was performed at 42°C using a 50% formamide system (Sambrook et al., 1989). Membranes were washed twice for 10 min in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at room temperature and then washed again for 10 min in 0.2× SSC with 0.1% SDS at 65°C (high stringency). At low stringency, the membrane was washed once for 10 min in 2× SSC at room temperature and then washed again for 10 min in 2× SSC at 63°C. The relative amounts of mRNA were determined with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each experiment was repeated at least twice with similar results.

Enzyme Assays and Product Analysis
Enzyme assays with recombinant proteins were performed with ~30,000 dpm of $^{14}$C-labeled GAs. The reaction mixture consisted of 80 μL of enzyme, 10 μL of 100 mM Tris-HCl, pH 7.5, and 10 μL of cofactors to give a final concentration of 5 mM ascorbate, 5 mM 2-oxoglutarate, and 0.5 mM FeSO$_4$. Cofactors were replenished after 1 h. The mixture was incubated for up to 6 h with gentle shaking. The products were separated by reverse-phase HPLC with online radioactivity detection (Zeveaart et al., 1993). For product identification by gas chromatography–mass spectrometry (see below), larger scale enzyme assays were used.

Gibberellin Extraction and Quantification
The procedures for extraction, purification, and quantification of endogenous GAs with deuterated GAs as internal standards were as described (Talon et al., 1990a; Zeveaart et al., 1993), except that the charcoal column step was omitted. The gas chromatograph was equipped with a DB-5MS capillary column (30 m × 0.32 mm × 0.25 μm film; J&W Scientific, Folsom, CA) which was operated in splitless mode. The oven temperature was kept at 100°C for 1 min after sample injection and then programmed from 100°C to 230°C at 40°C/min, from 230 to 280°C at 8°C/min, and finally to 300°C at 20°C/min.

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

Accession Numbers
The accession numbers for the genes mentioned in this article are as follows: AC079284 and AL021960 (AtGA2ox7 and AtGA2ox8); AJ132435, AJ132436, and AJ132437 (AtGA2ox1 to AtGA2ox3); X83379, X83380, and X83381 (AtGA2ox0 to AtGA2ox3); and L37126 and T51691 (AtGA3ox1 and AtGA3ox2).

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