Analysis of the Developmental Roles of the Arabidopsis Gibberellin 20-Oxidases Demonstrates That GA20ox1, -2, and -3 Are the Dominant Paralogs

Andrew R.G. Plackett,a,1 Stephen J. Powers,b Nieves Fernandez-Garcia,a,2 Terezie Urbanova,a,3 Yumiko Takebayashi,c Mitsunori Seo,c Yusuke Jikumaru,c Reyes Benlloch,d,4 Ove Nilsson,d Omar Ruiz-Rivero,a,5 Andrew L. Phillips,a Zoe A. Wilson,e Stephen G. Thomas,a and Peter Heddena,6

a Plant Science Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom
b Biomathematics and Bioinformatics Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom
c RIKEN Plant Science Center, Yokohama, Kanagawa 230-0045, Japan
d Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S-90183 Umeå, Sweden
e School of Biosciences, University of Nottingham, Loughborough, Leicestershire LE12 5RD, United Kingdom

Gibberellin (GA) biosynthesis is necessary for normal plant development, with later GA biosynthetic stages being governed by multigene families. Arabidopsis thaliana contains five GA 20-oxidase (GA20ox) genes, and past work has demonstrated the importance of GA20ox1 and -2 for growth and fertility. Here, we show through systematic mutant analysis that GA20ox1, -2, and -3 are the dominant paralogs; their absence results in severe dwarfism and almost complete loss of fertility. In vitro analysis revealed that GA20ox4 has full GA20ox activity, but GA20ox5 catalyzes only the first two reactions of the sequence by which GA12 is converted to Ga3. GA20ox3 functions almost entirely redundantly with GA20ox1 and -2 at most developmental stages, including the floral transition, while GA20ox4 and -5 have very minor roles. These results are supported by analysis of the gene expression patterns in promoter:b-glucuronidase reporter lines. We demonstrate that fertility is highly sensitive to GA concentration, that GA20ox1, -2, and -3 have significant effects on floral organ growth and anther development, and that both GA deficiency and overdose impact on fertility. Loss of GA20ox activity causes anther developmental arrest, with the tapetum failing to degrade. Some phenotypic recovery of late flowers in GA-deficient mutants, including ga1-3, indicated the involvement of non-GA pathways in floral development.

INTRODUCTION

Gibberellins (GAs) are a class of tetracyclic diterpenoid carboxylic acids, members of which function as hormones in many land plant lineages (Hirano et al., 2007), regulating multiple aspects of plant growth and development, including reproduction (reviewed in Fleet and Sun, 2005). In addition to dwarfism during vegetative growth, severely GA-deficient or - insensitive mutants in both dicot and monocot model species, such as the Arabidopsis thaliana biosynthesis mutant ga1-3 (Koornneef and Van der Veen, 1980), and the rice (Oryza sativa) GA receptor mutant gid1 (Ueguchi-Tanaka et al., 2005), are infertile. Several functions for GA have been identified during stamen development (reviewed in Plackett et al., 2011), which is arrested prematurely in both the ga1-3 and gid1 mutants (Cheng et al., 2004; Aya et al., 2009). In particular, recent work in rice demonstrates a dependence on GA for tapetal secretory functions and programmed cell death (PCD) during stamen development (Aya et al., 2009).

The synthesis of bioactive GA involves a multistep pathway (Figure 1A) of enzymes that are conserved in higher plants (reviewed in Yamaguchi, 2008). While early GA-biosynthetic steps are encoded by single-copy genes, the GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) enzymes are encoded by multigene families, the presence of which is likewise conserved between multiple species (Rebers et al., 1999; Hedden et al., 2002; Sakamoto et al., 2004; Lange et al., 2005). The individual tissue expression patterns of the four A. thaliana GA3ox paralogs have been described (Mitchum et al., 2006; Hu et al., 2008), but
those of the *A. thaliana* GA20ox gene family are not fully resolved (Rieu et al., 2008). Transgenic studies found GA20ox activity, which converts C20-GA substrates through successive oxidative reactions to form C19-GA products (Figure 1A), to be limiting for the production of bioactive GA in *A. thaliana* (Huang et al., 1998; Coles et al., 1999); thus, GA20ox may represent an important regulatory node in this pathway. The *A. thaliana* genome carries five GA20ox paralogs based on sequence similarity (Hedden and Phillips, 2000), three of which (GA20ox1, -2, and -3) have been shown to possess GA20ox activity in vitro (Phillips et al., 1995). Previous analysis of GA20ox1 and -2 loss-of-function mutants identified partial functional redundancy between these paralogs (Rieu et al., 2008), and the genes were found to have partially overlapping expression patterns throughout development. In

**Figure 1. GA Biosynthesis and the GA20ox Gene Family.**

(A) Schematic of GA biosynthesis in *A. thaliana*. Biosynthetic enzymes are marked in gray.

(B) Phylogenetic analysis of selected GA20ox protein sequences encompassing all known paralogs from the species *A. thaliana* (Ath1-5, highlighted in black, correspond to GA20ox1-5, respectively), *A. lyrata* (Aly), *Brachypodium distachyon* (Bdi), *B. rapa* (Bra), *Cucumis sativa* (Csa), *Lycopersicon esculentum* (Les), *O. sativa* (Osa), *Prunus persica* (Ppe), and *Vitis vinifera* (Vvi). In addition, single GA20ox sequences from *C. maxima* (Cma) and *Marah macrocarpus* (Mma) are included. Scale represents 0.1 amino acid substitutions per site. See Supplemental Table 7 online for details of paralog numbering.

(C) Gene models of *A. thaliana* GA20ox1, -2, -3, -4, and -5, showing the position and nature of putative loss-of-function alleles. Horizontal boxes denote exons, and horizontal lines between exons denote introns. Vertical lines indicate the positions of mutant alleles, with positions given in base pairs from the translational start. Changes to protein sequence caused by nucleotide substitutions are given in gray.
contrast with ga1 mutants, which display severe dwarfism, delayed flowering, and complete sterility (Koornneef and Van der Veen, 1980; Goto and Pharis, 1999; Cheng et al., 2004), the ga20ox1 ga20ox2 double mutant displays a semidwarf, semifertile phenotype (Rieu et al., 2008), with early flowers failing to set seed. Self-rescue of seed set occurs in later flowers, although the underlying mechanism remains undetermined. The three remaining GA20ox paralogs are expressed in wild-type inflorescence tissue (Rieu et al., 2008), but their contribution to floral development remains unclear.

Here, we report the systematic analysis of the roles of the GA20ox paralogs during A. thaliana development, including the creation and analysis of a severely dwarfed, highly infertile ga20ox1 ga20ox2 ga20ox3 triple loss-of-function mutant. We show that GA20ox3 contributes to GA biosynthesis in multiple developmental processes, acting redundantly with GA20ox1 and -2, whereas loss of GA20ox4 or GA20ox5 was not found to have a significant further impact on phenotype. GA20ox4 demonstrated full GA20ox catalytic activity in vitro; however, GA20ox5 catalyzed only the first two steps of the reaction sequence. Mapping of GA20ox expression in floral tissues through promoter:GUS transgenic reporter lines showed their coexpression in the tapetum prior to its breakdown, consistent with the observation that tapetum breakdown did not occur in ga20ox1 ga20ox2 ga20ox3 triple mutants. Analysis of GA20ox expression during late floral development identified distinct patterns of expression and a complex regulatory relationship among paralogs.

RESULTS

Functional Analysis of GA20ox Paralogs

Phylogenetic analysis comparing known examples of GA20ox protein sequences from numerous species found that A. thaliana GA20ox1, -2, -3, and -4 are closely related to each other, with GA20ox1 and GA20ox2 falling within the same clade, GA20ox3 and GA20ox4 into a closely related clade (Figure 1B), and both clades containing GA 20-oxidases only from species within the Brassicales. A. thaliana GA20ox5, by contrast, represents a divergent sequence that is more distantly related, although closely related sequences are found in Arabidopsis lyrata and Brassica rapa. A. thaliana mutant collections were screened for new GA20ox loss-of-function alleles, resulting in three candidates for GA20ox3, two for GA20ox4, and one for GA20ox5 (Figure 1C). The disruption of GA20ox expression by T-DNA insertions in the two alleles ga20ox3-3 and ga20ox5-2 was confirmed by RT-PCR (see Supplemental Figures 1A and 1B online), and the remaining single nucleotide polymorphism (SNP)-based alleles were screened by in vitro assays to determine their effects on GA20ox activity. These SNPs were replicated through site-directed mutagenesis of GA20ox cDNA clones, the activity of the mutant enzymes being compared with that of their respective wild-type proteins in bacterial lysates incubated with the radiolabeled substrate [14C]GA12. The mutations associated with ga20ox3-1 (a premature stop codon) and ga20ox4-2 (a 1-bp frame shift) resulted in complete loss of GA20ox activity in vitro (see Supplemental Figures 1C and 1D online). By contrast, although catalytic activity was reduced in ga20ox3-2, in which Leu-208 is replaced by Phe (Figure 1C), [14C]GA12, the C19 end product of the reaction sequence (Phillips et al., 1995), was detected after 24 h of incubation (see Supplemental Figure 1C online), ga20ox4-1 achieved partial conversion of [14C]GA12 to the intermediate [14C]GA9 over the same period, compared with the complete conversion to [14C]GA9 by wild-type GA20ox4 (see Supplemental Figure 1D online). Of the putative loss-of-function alleles identified, ga20ox3-1, ga20ox3-3, ga20ox4-2, and ga20ox5-2 were confirmed as knockouts in vitro and were used to evaluate the contribution of the genes to A. thaliana development.

The catalytic activity of wild-type GA20ox5 was also assayed in vitro, comparing its efficacy to that of GA20ox1. It was found that over 24 h, GA20ox1 catalyzed partial conversion of [14C]GA12 to [14C]GA9, while GA20ox5 instead accumulated the intermediate [14C]GA24 and also [14C]GA25 (see Supplemental Figure 1E online), the identities of which were confirmed by gas chromatography–mass spectrometry (GC-MS). As a direct test of the ability of GA20ox5 to convert GA24 to GA9, lysates containing either GA20ox1 or GA20ox5 were incubated with [14C]GA24. GA20ox1 converted [14C]GA24 completely to [14C]GA9, but lysates containing GA20ox5 produced no [14C]GA9 during incubation (see Supplemental Figure 1F online), indicating that GA20ox5 lacks full GA20ox catalytic activity.

Loss of GA20ox1, -2, and -3 Blocks Germination, Causes Severe Dwarfism, and Delays Flowering

The presence of either the ga20ox3-1 or ga20ox3-3 loss-of-function allele in the ga20ox1 ga20ox2 mutant background (previously described in Rieu et al., 2008) results in vegetative dwarfism that is more severe than that of ga20ox1 ga20ox2 (Figures 2A and 2B). These two triple mutants were identified through a nongerminating phenotype similar to that seen in ga1-3, subsequently confirmed in a germination assay (Table 1) in which both ga20ox1 ga20ox2 ga20ox3-1 and ga1-3 seed failed to germinate under permissive conditions. By contrast, all single and double mutant combinations of ga20ox1, ga20ox2, and ga20ox3-1 demonstrated germination frequencies similar to the wild type (close to 100%). The germination defect in ga20ox1 ga20ox2 ga20ox3-1 and ga1-3 was rescued by exogenous GA treatment (see Supplemental Table 1 online).

The relationships among GA20ox1, -2, and -3 during development were investigated by quantitative phenotyping of vegetative and reproductive characters of all single, double, and triple mutant combinations of ga20ox1, ga20ox2, and ga20ox3-1 (Table 1). Analysis of vegetative characters in mature plants and seedlings indicates that GA20ox3 plays a role in the growth of most vegetative tissues, including leaves, roots, hypocotyls, and internodes. For most phenotypic characters, GA20ox3 appears to be fully redundant with GA20ox1 and -2, with ga20ox1 ga20ox3-1 and ga20ox2 ga20ox3-1 double mutants phenocopying ga20ox1 and ga20ox2, respectively (Figure 3A). Exceptions included differences between ga20ox1 and ga20ox1 ga20ox3-1 in hypocotyl growth (P < 0.01) and between ga20ox2 and ga20ox2 ga20ox3-1 in seedling root growth and
vegetative internode elongation (P < 0.01), with the double mutants displaying reduced growth in each case.

For many phenotypic characters (hypocotyl growth, primary inflorescence length, and number and length of elongated vegetative internodes), ga20ox1 ga20ox2 ga20ox3-1 is not significantly different from the severely GA-deficient ga1-3, implying that all GA20ox activity in these tissues can be ascribed to GA20ox1, -2, and -3. Similarly, loss of AtGA20ox1, -2, and -3 is sufficient to replicate the floral phenotype of ga1-3, and additional loss of AtGA20ox4 or -5 from the ga20ox1 ga20ox2 ga20ox3-1 background does not have significant effects on floral or vegetative phenotypes (Figure 2C, Table 2). Exogenous GA treatment increases vegetative growth in all of these mutants to approximately that of the wild type (see Supplemental Tables 1 and 2 online).

It has been previously demonstrated that ga20ox1 ga20ox2 is delayed in flowering, but not as severely as ga1-3 (Rieu et al., 2008). Under inductive long-day (LD) conditions, the ga20ox1 ga20ox2 ga20ox3-1 mutant was found to have a longer period of vegetative development, before making the transition to flowering (as evidenced by the number of rosette leaves) compared with ga20ox1 ga20ox2 (P < 0.01; Figure 3A). The ga20ox2 ga20ox3-1 double mutant is also delayed in flowering (P < 0.01), though not as severely as ga20ox1 ga20ox2. GA treatment restored flowering time of all GA-deficient genotypes to that of

Figure 2. Loss of GA20ox1, -2, and -3 Function Affects Vegetative and Floral Phenotypes.

(A) Phenotypic comparison of single, double, and triple mutant combinations of ga20ox1, ga20ox2, and ga20ox3-1 against the wild type and ga1-3 at 44 d old.

(B) Phenotypic comparison of ga20ox triple and quadruple mutants against ga1-3 at 55 d old.

(C) Comparison of early floral phenotypes (inflorescence positions 1 to 5) in open flowers of ga20ox mutant combinations against the wild type and ga1-3 (two sepals removed).

[See online article for color version of this figure.]
the wild type, with the exception of ga20ox1 ga20ox2, the flowering time of which was nevertheless accelerated.

Flowering in ga20ox1 ga20ox2 ga20ox3-1 occurred significantly earlier than in ga1-3 (P < 0.01), suggesting possible functions for GA20ox4 or -5 in promoting flowering under LD. A comparison of flowering between ga20ox1 ga20ox2 ga20ox3-1, ga20ox1 ga20ox2 ga20ox3-3, and the two quadruple mutants found that loss of GA20ox4, but not of GA20ox5, causes a significant additional delay to flowering in the ga20ox1 ga20ox2 ga20ox3-1 background (P < 0.01; Figure 3B). However, flowering of ga20ox1 ga20ox2 ga20ox3-3 was not significantly different (P > 0.05) from ga20ox1 ga20ox2 ga20ox3-1 ga20ox4-2 (Figure 3B). Flowering of ga1-3 remained significantly delayed compared with either quadruple mutant (P < 0.01; Figure 3D). Under noninductive short-day (SD) conditions, neither ga20ox1 ga20ox2 ga20ox3-1 nor ga1-3 flowered during the experimental period (>6 months; Figure 3C). In SDs, flowering of the ga20ox2 mutant was significantly delayed compared with the wild type (P < 0.05), with ga20ox1 ga20ox2 demonstrating a further delay (P < 0.05). Loss of GA20ox3 had no significant effect (P > 0.05) when either GA20ox1 or -2 was functional. Interestingly, in SDs, a clear difference in the rate of rosette growth was observed between ga20ox1 ga20ox2 ga20ox3-1 (Figures 3D and 3E) and ga1-3 (Figures 3F and 3G), with the triple mutant rosettes growing more rapidly than ga1-3, although eventually each achieved a similar final size.

The contribution of GA20ox1, -2, and -3 to GA biosynthesis was tested by quantifying the products and substrates of GA20ox activity in planta through combined liquid chromatography–mass spectrometry (Table 3), with development between genotypes synchronized at the floral transition under LD. The predominant form of bioactive GA synthesized in A. thaliana is GA4 (Talon et al., 1990), and it was found that the concentration of GA4 was significantly lower in ga20ox1 ga20ox2 ga20ox3-1 than in ga20ox1 ga20ox2 (P < 0.01), in fact lower than in ga1-3 (P < 0.01), in which some GA4 was detected. There was a good, but not perfect, correlation between GA4 concentration and growth parameters, such as total height and mean length of the elongated vegetative internodes (V.I.) below the first flower were counted.

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**Table 1. Phenotypic Parameters for A. thaliana Single, Double, and Triple ga20ox1, -2, and -3 Mutants**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Germination Efficiency (%)</th>
<th>7 d Hypocotyl (mm)</th>
<th>7 d Root (mm)</th>
<th>Rosette Diameter (mm)</th>
<th>Total Primary Inflorescence Height (mm)</th>
<th>No. of Elongated V.I.</th>
<th>Mean V.I. Length (mm)</th>
<th>Mean No. of Siliques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0)</td>
<td>98.88</td>
<td>1.14 [0.0530]</td>
<td>37.86</td>
<td>120.80 [10.968]</td>
<td>548.30 [23.363]</td>
<td>2.917</td>
<td>36.44 [0.038]</td>
<td>61.33</td>
</tr>
<tr>
<td>ga20ox2</td>
<td>99.47</td>
<td>0.89 [0.0556]</td>
<td>33.78</td>
<td>134.70 [11.574]</td>
<td>527.50 [23.036]</td>
<td>2.344</td>
<td>31.17 [5.564]</td>
<td>52.8</td>
</tr>
<tr>
<td>ga20ox1 ga20ox2</td>
<td>98.65</td>
<td>0.95 [0.0768]</td>
<td>35.92</td>
<td>135.50 [11.602]</td>
<td>557.30 [22.036]</td>
<td>2.917</td>
<td>36.44 [6.038]</td>
<td>61.33</td>
</tr>
<tr>
<td>ga20ox1 ga20ox3-1</td>
<td>99.45</td>
<td>0.91 [0.0471]</td>
<td>32.60</td>
<td>116.40 [10.854]</td>
<td>549.30 [23.343]</td>
<td>2.404</td>
<td>10.50 [3.194]</td>
<td>47.93</td>
</tr>
<tr>
<td>ga20ox1 ga20ox2 ga20ox3-1</td>
<td>0.00</td>
<td>0.67 [0.1816]</td>
<td>20.08</td>
<td>65.20 [8.070]</td>
<td>13.80 [2.067]</td>
<td>0.167</td>
<td>0.38 [0.0469]</td>
<td>8.83</td>
</tr>
</tbody>
</table>

See Methods for design and statistical analysis. Transformed values used for statistical analysis are given in brackets. Superscript letters indicate significant difference (P < 0.01) from the wild type. Genotypes marked with different letters are significantly different from each other. Elongated vegetative internodes (V.I.) below the first flower were counted.

**Loss of GA20ox1, -2, and -3 Causes Male and Female Sterility**

Both ga20ox1 ga20ox2 ga20ox3-1 and ga1-3 were found to be infertile under LD growth conditions, failing to set seed and...
produce elongated siliques in the absence of exogenous GA treatment (Figures 4A and 4B). As previously found by Rieu et al. (2008), ga20ox2 and ga20ox2 ga20ox2 siliques were shorter than in the wild type (P < 0.01; Figure 4A), with ga20ox2 ga20ox2 exhibiting a greater reduction than ga20ox2 (P < 0.01). However, loss of GA20ox3 did not significantly affect wild-type, ga20ox2, or ga20ox2 silique length (P > 0.05). ga20ox1 ga20ox2 demonstrated a small reduction in seed number (per silique) compared with the wild type (P < 0.01; Figure 4B), consistent with previous results (Rieu et al., 2008), but seed numbers in the remaining single and double ga20ox mutants were not significantly different from the wild type. This discrepancy between silique growth and seed number manifests as an altered seed packing phenotype in siliques lacking GA20ox2 (Figure 4C), with two alternating files of...
developing seeds present rather than a single file. A similar phenotype has been observed in siliques of mutants lacking GA3ox1 (Hu et al., 2008) and is likely to be a consequence of reduced GA-dependent elongation of siliques, instead of reduced fertilization. Silique growth and seed number were increased in ga20ox1 ga20ox2 ga20ox3-1 and ga1-3 by GA treatment (P < 0.01), as was silique growth in ga20ox1 ga20ox2. However, GA treatment caused a small but significant reduction in the number of seeds in wild-type, ga20ox2, and ga20ox2 ga20ox3-1 siliques (P < 0.01; Figure 4B). This result agrees with a previous report of seed set in wild-type A. thaliana being reduced by exogenous GA (Jacobsen and Olszewski, 1993).

Reciprocal crossing between wild-type and ga20ox mutant plants was performed to determine the effects of loss of individual GA20ox paralogs on male and female fertility (Table 4). Crosses using ga20ox1 ga20ox1 ga20ox2 ga20ox3-1 or ga1-3 pollen on wild-type pistils produced virtually no seed, although technical difficulties were encountered in transferring pollen from anthers of these mutants. Similarly, ga20ox1 ga20ox2 ga20ox3-1 and ga1-3 flowers failed to set seed when pollinated by wild-type donors. These results suggest that ga20ox1 ga20ox2 ga20ox3-1 is both male and female infertile. However, when the effect of loss of GA20ox activity on postpollination pollen performance was tested in vivo through determining the segregation of the ga20ox alleles after self-pollination of a line that was heterozygous for ga20ox1, ga20ox2, and ga20ox3-1, no fitness penalty was associated with ga20ox1 ga20ox2 ga20ox3-1 pollen (see Supplemental Table 3 online), indicating near normal germination and pollen tube growth of this pollen. It is possible that the mutant pollen can acquire GA from neighboring tissues, although this result contrasts with that obtained by Chhn et al. (2007), who found very poor transmission of mutant alleles for GA biosynthesis genes in rice.

Wild-type pistils pollinated by all single or double ga20ox mutants produced similar numbers of seeds per silique as self-pollinated wild-type flowers (P > 0.05), indicating that male fertility is not significantly affected in these mutants. The numbers of seeds produced by ga20ox1 and ga20ox1 ga20ox3-1 pistils pollinated from wild-type donors were significantly different from the wild type (P < 0.05), with fewer seeds produced. However, the number of seeds set by ga20ox1 ga20ox2 pistils pollinated from wild-type donors was not significantly different from the wild type (P > 0.05), suggesting that female

Table 2. Seedling Root Lengths and Final Rosette Diameters for Triple and Quadruple GA20ox Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>7 d Root Length (mm)</th>
<th>Rosette Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0)</td>
<td>23.83</td>
<td>113.80 [10.6630]</td>
</tr>
<tr>
<td>ga20ox1 ga20ox2 ga20ox3-1</td>
<td>14.50</td>
<td>75.38 [8.6690]</td>
</tr>
<tr>
<td>ga20ox1 ga20ox2 ga20ox3-3</td>
<td>16.84</td>
<td>66.00 [8.1280]</td>
</tr>
<tr>
<td>ga20ox1 ga20ox2 ga20ox3-1 ga20ox4-2</td>
<td>14.22</td>
<td>66.25 [8.4560]</td>
</tr>
<tr>
<td>ga20ox1 ga20ox2 ga20ox3-1 ga20ox5-1</td>
<td>17.06</td>
<td>71.62 [8.4560]</td>
</tr>
<tr>
<td>ga1-3 (Col-0)</td>
<td>13.98</td>
<td>74.88 [8.6320]</td>
</tr>
<tr>
<td>1% LSD</td>
<td>3.59</td>
<td>[6.6197]</td>
</tr>
<tr>
<td>df</td>
<td>152</td>
<td>77</td>
</tr>
</tbody>
</table>

Transformed values used for statistical analysis are given in square brackets.

*aSignificantly different from the wild type (P < 0.01).

Table 3. GA Concentrations (ng per g Dry Weight) in Whole Shoots at Flowering

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GA4 (Bioactive)</th>
<th>GA9</th>
<th>GA12</th>
<th>GA13 (Bioactive)</th>
<th>GA20</th>
<th>GA33</th>
</tr>
</thead>
<tbody>
<tr>
<td>ga20ox1 ga20ox2</td>
<td>0.63* [0.7897]</td>
<td>0.47 [-1.137]</td>
<td>15.94* [3.990]</td>
<td>31.93 [3.467]</td>
<td>n.d.</td>
<td>-2.303</td>
</tr>
<tr>
<td>ga20ox1 ga20ox3-1</td>
<td>0.55* [0.7272]</td>
<td>0.70 [-1.148]</td>
<td>45.16* [6.720]</td>
<td>30.66 [3.425]</td>
<td>0.16 [-1.585]</td>
<td>n.d.</td>
</tr>
<tr>
<td>ga20ox1 ga20ox2 ga20ox3-1</td>
<td>0.15* [0.3911]</td>
<td>n.d.</td>
<td>-2.547</td>
<td>3.12* [1.759]</td>
<td>34.44 [3.534]</td>
<td>0.48 [-0.896]</td>
</tr>
<tr>
<td>ga1-3 (Col-0)</td>
<td>0.24* [0.4859]</td>
<td>n.d.</td>
<td>-2.501</td>
<td>0.32* [0.402]</td>
<td>n.d.</td>
<td>-2.303</td>
</tr>
<tr>
<td>df</td>
<td>20</td>
<td>12</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>–</td>
</tr>
</tbody>
</table>

Measurements represent the means of two to four biological replicates, with transformed data shown in square brackets (square root for GA4 and GA20, natural log for the others). All GA species quantified in each genotype were measured from the same tissue samples. Following linear mixed-model analysis, individual LSDs (data not shown) were used for pairwise comparisons of means on the transformed scale. Samples marked “n.d.” contained too low a concentration for detection (effectively zero for the purposes of analysis). Superscript letters denote significant difference from the wild type (P < 0.05). Genotypes marked with different letters are significantly different (P < 0.05) from one another.
Figure 4. Loss of GA20ox1, -2, and -3 Function Affects Silique Growth and Seed Set.

The paired phenotypic characters, silique length (A) and seed number (B), were measured from the same population of siliques from plants grown under control (black bars) and GA-treated (white bars) LD growth conditions. Bars represent the mean of 36 measurements, with error bars representing ±SE.
development is not impaired in this mutant and that the reduced seed set observed in Figure 4B is a function of self-pollination.

Male Sterility of ga20ox1 ga20ox2 ga20ox3-1 Is Caused by Anther Developmental Arrest

Given the similarity of the ga20ox1 ga20ox2 ga20ox3 and ga1-3 floral phenotypes (Figure 2C) and the reported arrest of ga1-3 anthers prior to pollen mitosis (Cheng et al., 2004), ga20ox1 ga20ox2 ga20ox3-1 stamen development was investigated for evidence of developmental arrest. The elongation of pistils and stamens between primordium initiation and flower opening was analyzed by nonlinear modeling (see Supplemental Table 4 online for means of estimated model parameters), comparing wild-type, ga20ox1 ga20ox2, ga20ox1 ga20ox2 ga20ox3-1, and ga1-3 flowers. Inflorescence development was synchronized between genotypes by harvesting floral clusters at the opening of the 10th flower. Pistil elongation differed significantly (P < 0.05) between all genotypes (Figure 5A; see Supplemental Table 4 online), with the greatest differences manifesting toward the end of development. Stamen elongation remained similar in all genotypes until late development (Figure 5B), when wild-type stamens exhibited a rapid acceleration of growth, corresponding to floral stages 11 to 12 (tapetal degeneration and pollen mitosis) (Cheng et al., 2004), respectively. Comparison at the 10th flower. Pistil elongation differed significantly (P < 0.05) between genotypes by harvesting floral clusters at the opening of the 10th flower. Pistil, stamen, and petal growth were all visibly increased in these later flowers, resulting in open flowers being visible on dwarf plants. Stamen development was substantially recovered in these flowers, with dehiscent anthers occasionally observed. 4’6-diamidino-2-phenylindole staining of anthers from inflorescence position 15 found some tunincule pollen in all genotypes (see Supplemental Figure 2A online). Pollen development in earlier inflorescence positions was not examined in detail, but previous work on ga1-3 found pollen development to be arrested prior to pollen mitosis II (Cheng et al., 2004). Viability staining of anthers from inflorescence positions 1 to 5 suggested a potential difference in pollen development between ga20ox1 ga20ox2 ga20ox3-1 and ga1-3, with pollen from the triple mutant staining as viable, while no viable pollen was observed in ga1-3 (see Supplemental Figure 2C online).

Expression Mapping Identifies Expression of Multiple GA20ox Paralogs within the Tapetum Cell Layer

To map floral tissue expression patterns of the AtGA20ox family in detail, transgenic promoter:GUS transcriptional fusion (TC) reporter lines were generated for GA20ox1, -2, -3, and -4, in which GUS expression was driven by 1.5 to 3 kb of 5’ promoter sequences from each paralog (Figure 6A; see Methods). These were analyzed alongside two previously published translational fusion (TL) GUS reporter lines incorporating promoter and exonic and intronic sequences of GA20ox1 (Hay et al., 2002) and GA20ox2 (Frigerio et al., 2006), respectively. Comparison at the seedling stage found differences in GUS expression between TC

Table 4. Seed Yields from Reciprocal Crosses between the Wild Type and ga20ox Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seeds per Silique</th>
<th>× Col-0</th>
<th>× Col-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0)</td>
<td>64.38</td>
<td>63.50</td>
<td></td>
</tr>
<tr>
<td>ga20ox1</td>
<td>66.06</td>
<td>55.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>ga20ox2</td>
<td>66.12</td>
<td>59.00</td>
<td></td>
</tr>
<tr>
<td>ga20ox3-1</td>
<td>67.50</td>
<td>57.38</td>
<td></td>
</tr>
<tr>
<td>ga20ox1 ga20ox2</td>
<td>64.12</td>
<td>57.33</td>
<td></td>
</tr>
<tr>
<td>ga20ox1 ga20ox3-1</td>
<td>62.25</td>
<td>48.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>ga20ox2 ga20ox3-1</td>
<td>68.50</td>
<td>57.50</td>
<td></td>
</tr>
<tr>
<td>ga20ox1 ga20ox2 ga20ox3-1</td>
<td>3.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>ga1-3 (Col-0)</td>
<td>1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5% LSD</td>
<td>6.286</td>
<td>6.448</td>
<td></td>
</tr>
<tr>
<td>df</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Values shown represent the mean of three independent crosses. Letters denote genotypes that are significantly different from the wild type (P < 0.05). Genotypes marked with different letters are significantly different from the wild type (P < 0.05). Genotypes marked with different letters are significantly different from the wild type (P < 0.05).

Figure 4. (continued).

Square root transformed silique lengths were used in statistical analysis so that lowercase letters denote significant difference (a to d, P < 0.01; e, P < 0.05) between genotypes and the wild type under control (black) and GA-treated conditions (gray), respectively, for data on the transformed scale. The transformed means are given in Supplemental Table 8 online. On the transformed scale, for these comparisons, LSD (1%) = 0.16952 on 158 df<sup>(A)</sup>, and LSD(1%) = 9.138 on 158 df<sup>(B)</sup>. Asterisks denote a significant difference (P < 0.01) between control and GA-treated conditions for that genotype. For these comparisons, LSD (1%) = 0.17387 on 168 df<sup>(A)</sup>, and LSD(1%) = 9.117 on 174 df<sup>(B)</sup>. Seed packing phenotypes under control and GA-treated growth conditions are given in <sup>(C)</sup>. Bars = 1 mm scale.
Figure 5. Loss of GA20ox1, -2, and -3 Function Affects Male Reproductive Development.

Floral organ growth in target genotypes was modeled for pistils (A) and stamens (B) between primordium initiation (0.0) and flower opening (1.0) where the developmental scale for inflorescence development was derived by synchronizing between genotypes by harvesting floral clusters at the opening of the 10th flower and then using, for each plant, the ratio of cumulative flower count to the total number of flowers. The curves show the predicted growth given the means of plant-specific estimated model parameters (see Supplemental Table 4 online for model parameters, details of the models, and means of estimated). Anther development from microspore release to dehiscence was compared between the wild type (C to F), ga20ox1 ga20ox2 (G to J), and ga20ox1 ga20ox2 ga20ox3-1 (K to N). dT, degenerating tapetum; E, endothecium cell layer; M, microspore; P, mature pollen; T, tapetum cell layer. Bars = 50 µm.
and TL variants (see Supplemental Figure 3 online), particularly for GA20ox2. Despite these differences, GUS expression in meristematic tissues of both TC and TL forms of the GA20ox1 and -2 reporter lines showed the expected responses to GA and paclobutrazol treatment (see Supplemental Figure 3 online). No clear GA responsiveness was observed in pGA20ox3-TC-GUS, while no GUS staining was observed in pGA20ox4-TC-GUS seedlings.

Different patterns of GUS staining were also observed between TC and TL variants during floral development (see Supplemental Figure 4A online). To validate the respective reporter lines, GUS staining patterns were compared against quantitative RT-PCR (qRT-PCR)-based transcript analysis at floral organ–level resolution (Figures 6B and 6C). This analysis was restricted to floral stage 12 (immediately prior to flower opening) both for reasons of feasibility and the clear differences in GUS expression patterns observed in reporter lines at this stage (Figure 6B). qRT-PCR analysis indicated that GA20ox1 is strongly expressed in the stamen filament and receptacle tissues, while GA20ox2 is strongly expressed in the pistil (Figure 6C). Expression of GA20ox3, -4, and -5 was very low in all tissues.

pGA20ox1-TC-GUS staining most closely matched qRT-PCR results for GA20ox1, with expression concentrated in the stamen filament and receptacle (Figure 6B). By contrast, pGA20ox1-TL-GUS expression was restricted to the anther. Similarly, GUS staining in pGA20ox2-TC-GUS, but not in pGA20ox2-TL-GUS, correlated with the qRT-PCR results for pistils. However, pGA20ox2-TC-GUS also predicted GA20ox2 expression in the stamen filament prior to flower opening, but qRT-PCR indicated expression only in the anther, as predicted by pGA20ox2-TL-GUS. The very low relative expression of GA20ox3 and -4 makes comparison with their respective reporter lines uninformative. pGA20ox3-TC-GUS predicted low-level expression in the anther-filament junction, sepal and pistil vasculature, and receptacle tissues, while pGA20ox4-TC-GUS expression was observed in mature pollen and sepal tissues (Figure 6B). From this comparison, it was concluded that the TC reporter lines more accurately represented GA20ox expression in floral tissues. Furthermore, analysis of pGA20ox-TC-GUS seed 24 h postgermination showed expression of GA20ox1, -2, and -3 but not GA20ox4 (see Supplemental Figure 4B online). This result is consistent with the nongermminating phenotype of ga20ox1 ga20ox2 ga20ox3 mutants and previous expression analyses during germination (Ogawa et al., 2003; Rieu et al., 2008), and the extremely strong staining of imbibed pGA20ox3-TC-GUS seed correlated with the very high levels of expression of GA20ox3 recorded during germination (Ogawa et al., 2003).

Previous expression analysis of the GA20ox family (Rieu et al., 2008) indicated that expression of GA20ox1, -2, and -3 was

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**Figure 6.** Functional Analysis of GA20ox:GUS Reporter Lines.

(A) Outline of GA20ox transcriptional (TC) and translational (TL) GUS reporter constructs. White boxes represent promoter sequence, black boxes AtGA20ox exonic sequence, and blue boxes UidA coding sequence. Breaks indicate intronic sequence.

(B) GA20ox:GUS expression at floral stage 12 (flower opening).

(C) Normalized expression of GA20ox paralogs in individual floral organs at floral stage 12. Bars represent the mean of three biological replicates (see Methods); error bars represent 1 se.
feedback regulated and reported transcriptional upregulation of GA20ox3 in ga20ox1 ga20ox2 inflorescence tissues. To test whether this occurs more specifically in floral tissues, expression of GA20ox3, -4, and -5 was compared by qRT-PCR between wild-type and ga20ox1 ga20ox2 floral organs at floral stage 12 (Table 5). Expression of GA20ox3 was significantly higher in ga20ox1 ga20ox2 pistils and stamen filament compared with the wild type (P < 0.05) but not in other tissues. No significant difference in GA20ox4 expression was found between the wild type and ga20ox1 ga20ox2, but a significant difference in GA20ox5 expression was found between the wild type and ga20ox1 ga20ox2 only in stamen filaments (P < 0.05). Previously, Rieu et al. (2008) found no statistically significant transcriptional response of GA20ox4 or -5 to exogenous GA treatment and so concluded that neither of these paralogs was subject to GA homeostasis, although Frigerio et al. (2006) reported GA regulation of GA20ox4 expression. These results could explain the difference in growth observed between ga20ox1 ga20ox2 and ga20ox1 ga20ox2 ga20ox3 flowers (Figure 2C), with GA20ox3 being upregulated in cruciferous reproductive tissues in the absence of GA20ox1 and -2.

Analysis of TC reporters throughout floral development identified early, anther-specific GUS staining in all lines (Figure 7A). GUS staining in pGA3ox3-3-L-GUS was reported at a similar stage of floral development and was found to be restricted to the tapetum (Hu et al., 2008) (see Supplemental Figure 4A online).

Microscopy analysis of anther-specific GUS staining showed expression of pGA20ox1-1-GUS and pGA20ox2-1-GUS in the tapetum and pollen mother cells (PMCs) around the time of meiosis (anther stage 5–6; Sanders et al., 1999) (Figure 7B). After meiosis, pGA20ox1-1-GUS was expressed in the tapetum, developing microspores and weakly in the endothecium layer, becoming focused within the tapetum during stages 9 and 10, prior to its degeneration (stage 11). Staining of pGA20ox2-1-GUS anther tissues declined after meiosis but remained present in the tapetum through to stages 9 and 10. pGA20ox3-1-GUS and pGA20ox4-1-GUS expression was only observed after meiosis and persisted until stages 9 and 10, again restricted to the tapetum. No GUS staining was observed in anther tissues during tapetum degeneration, but at anther dehiscence (stage 13), staining of mature pollen grains was observed in pGA20ox1-1-GUS, pGA20ox3-1-GUS, and pGA20ox4-1-GUS, as well as in the surrounding anther wall of pGA20ox4-1-GUS. These results support tapetal expression of GA20ox1, -2, and -3, consistent with the defects in tapetum degeneration observed in ga20ox1 ga20ox2 ga20ox3-1 anthers.

**DISCUSSION**

GA20ox Activity throughout *A. thaliana* Development under LD Conditions Can Be Ascribed to GA20ox1, -2, and -3

Multiple copies of GA20ox genes have been identified in species throughout the angiosperms, based on sequence similarity, although the number of copies in each species can vary and many of these genes have not been tested for GA20ox activity. Phenotypic analysis of *A. thaliana* mutants has now demonstrated that of the five paralogs identified, loss of GA20ox1, -2, and -3 is sufficient to substantially replicate both the vegetative and reproductive phenotypes of the extremely GA-deficient mutant ga1-3. Of these three GA20ox genes, loss of GA20ox3 has very little phenotypic effect if either GA20ox1 or -2 is present, suggesting that its functions in plant development are more limited and are redundant with those of GA20ox1 and -2. However, in a number of circumstances (root growth, internode elongation, and flowering), loss of GA20ox2 and -3 sufficiently reduces GA biosynthesis to have a significant effect on phenotype even in the presence of GA20ox1. GA20ox1, -2, -3, and -4 are each capable of converting the C22 substrate GA12 to the C19 product GA9 (Figure 1A) in vitro, suggesting that differences in their function are due to patterns of expression rather than enzymatic activity. By contrast, under the same reaction conditions, GA20ox5 accumulated the C23 intermediate GA26 and the by-product GA25 and could not convert GA24 into GA9. In the absence of other, fully functional GA20ox paralogs GA20ox5 would thus be incapable of synthesizing GA20 in planta. One other GA20ox enzyme, *Cucurbita maxima* GA20ox1, has been identified with similar catalytic properties (Lange et al., 1994), but sequence comparison and phylogenetic analysis indicate that these two enzymes are not closely related (Figure 1B), with *A. thaliana* GA20ox5 being found within a large clade containing members from both monocot and dicot species.

**Table 5. Comparison of Gene Expression for GA20ox3, -4, and -5 in Col-0 and ga20ox1 ga20ox2 Floral Tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GA20ox3</th>
<th>GA20ox4</th>
<th>GA20ox5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type (Col-0)</td>
<td>ga20ox1</td>
<td>ga20ox2</td>
</tr>
<tr>
<td>Sepal</td>
<td>0.0008 [10.722]</td>
<td>0.0020 [8.977]</td>
<td>0.0003 [12.470]</td>
</tr>
<tr>
<td>Petal</td>
<td>0.0015 [9.402]</td>
<td>0.0043 [8.117]</td>
<td>0.0012 [8.870]</td>
</tr>
<tr>
<td>Anther</td>
<td>0.0036 [8.142]</td>
<td>0.0079 [7.148]</td>
<td>0.0030 [8.380]</td>
</tr>
<tr>
<td>Filament</td>
<td>0.0012 [9.752]</td>
<td>0.0108* [7.330]</td>
<td>0.0003 [11.900]</td>
</tr>
<tr>
<td>Pistil</td>
<td>0.0012 [9.740]</td>
<td>0.0074* [6.844]</td>
<td>0.0009 [10.670]</td>
</tr>
</tbody>
</table>

Expression has been normalized against three reference genes expressed in the same tissues (see Methods). Values shown are the mean of three biological replicates. Transformed data are shown in brackets. Pairwise comparisons were made using individual LSDs (data not shown). Asterisks indicate a significant difference in expression (P < 0.05) between the wild type and ga20ox1 ga20ox2 within that tissue for the specified target gene.
Figure 7. GA20ox Expression during Anther Development.

(A) Comparison of floral GUS staining patterns at anther stage 9-10 between GA20ox:GUS TC and TL lines and the tapetum marker pGA3ox3-TL-GUS (Hu et al., 2008). Bars = 1 mm.

(B) Comparison of anther tissue expression patterns between GA20ox:GUS TC lines during pollen development. dT, degenerating tapetum; E, endothecium; Mi, microspore; P, mature pollen; T, tapetum. Bars = 50 μm.
Phylogenetic analysis indicates that *A. thaliana GA20ox1*, -2, -3, and -4 are related pairwise, with GA20ox1 being closely related to GA20ox2 and GA20ox3 to -4. Each GA20ox paralog is more closely related to GA20ox genes from other Brassica species (B. rapa and A. lyrata) than to other *A. thaliana* paralogs, suggesting that their duplication occurred prior to the divergence of these species. The codominance of GA20ox1 and -2 in regulating *A. thaliana* development may reflect the duplication of an ancestral GA20ox with these functions. The closest *A. thaliana* relative in our analysis, *A. lyrata*, carries recognizable orthologs for these species. The codominance of GA20ox1 and -2 in regulating *A. thaliana* development may reflect the duplication of an ancestral GA20ox with these functions. The closest *A. thaliana* relative in our analysis, *A. lyrata*, carries recognizable orthologs for these species. The codominance of GA20ox1 and -2 in regulating *A. thaliana* development may reflect the duplication of an ancestral GA20ox with these functions.

Although no distinct physiological functions for GA20ox4 or -5 could be identified through comparison of GA-deficient phenotypes under favorable LD growth conditions, a difference in the rate of growth between *ga20ox1 ga20ox2 ga20ox3* and *ga1-3* was apparently unmasked under SDs with 6-month-old *ga1-3* rosettes looking considerably smaller. However, the final rosette size of these two mutants under SDs did not differ substantially. Whether this is a GA-dependent effect has yet to be verified. Expression of *GA20ox1*, -2, and -3 has been previously shown to be independently regulated by environmental and endogenous factors in certain developmental contexts (*Yamauchi et al., 2004; Hisamatsu et al., 2005; Rieu et al., 2008*), and the importance of GA signaling to developmental processes can alter depending on environmental conditions, best exemplified by the floral transition and photoperiod (reviewed in Boss et al., 2004). With the exception of flowering, all phenotypic characterization described here was performed under LD conditions. It is not known whether expression of *GA20ox4* or -5 is altered by endogenous or environmental conditions, but phenotypic analysis under different or suboptimal environmental conditions might reveal functions for *GA20ox4* or -5.

**GA20ox1**, -2, and -3 Promote Reproductive Development and Fertility in *A. thaliana*

Loss of *GA20ox1*, -2, and -3 caused male and female sterility, similar to that seen in *ga1-3, ga20ox1 ga20ox2 ga20ox3* stamens demonstrated developmental arrest, indicated both by a cessation of growth and a failure in tapetum degeneration in rice, it has been demonstrated that GA deficiency blocks entry of the tapetum into PCD (*Aya et al., 2009*), and our results suggest that GA is similarly involved in regulating tapetum degeneration in *A. thaliana*. While a number of GA signaling targets involved in tapetum PCD are now known in rice (*Li et al., 2006; Aya et al., 2009*), the mechanism through which a stamen developmental block is effected at this stage in both rice and *A. thaliana* remains unknown.

Determining the status of pollen development in the arrested anthers of *ga20ox1 ga20ox2 ga20ox3* is complicated by the gradual recovery of stamen development in progressively later flowers (discussed below). Analysis of self-fertilized siliques and reciprocal-crossing experiments did not find evidence for severe defects in postpollination gametophyte development in *ga20ox* single or double mutant combinations. The failure of *ga20ox1 ga20ox2 ga20ox3* pollen to develop successfully on wild-type pistils might be due to gametophytic developmental defects but might also relate to sporophytic problems of releasing pollen from arrested anthers, even when opened manually. Pollen germination and development postpollination in rice require de novo GA biosynthesis within the gametophyte (*Chhun et al., 2007*), and GUS staining was observed in mature pollen of transgenic lines reporting expression of *GA20ox1*, -2, and -4. Surprisingly, no fitness penalty was attached to *ga20ox1 ga20ox2 ga20ox3* or *ga20ox1 ga20ox2 ga20ox3* in *ga20ox4* gametophytes in segregation distortion analyses. One possible technical explanation is that, due to the heterozygous nature of the parent in these experiments, the presence of bioactive GA in neighboring pollen grains or anthers, as well as in pistil and stamens tissue, might have masked GA-deficient phenotypes in the pollen. However, similar analytical methods in rice identified fitness penalties in pollen development for mutations at earlier stages in GA biosynthesis (*Chhun et al., 2007*). It is possible that, in contrast with the early GA biosynthesis genes, one or more *GA20ox* genes is expressed in pollen prior to meiosis and rescues mutant pollen development by migration of protein and/or mRNA into the subsequent microspores, as was suggested for components of the GA-signaling pathway in rice (*Chhun et al., 2007*). Indeed, on the basis of microarray data, *Tang et al. (2010) found expression of rice *GA20ox1* and *GA20ox3* in PMCs, while *Hirano et al. (2008) showed rice GA20ox4 expression in pollen at meiosis.* Although comparable transcript analyses are not available for *A. thaliana*, we see weak GUS expression in PMCs for *GA20ox1* and *GA20ox2* (Figure 7B). However, as discussed above, pollen rescue in our experiment might be simply the consequence of the relatively high mobility of late-stage GAs, which has been hypothesized from the results of grafting and GA transport experiments (*Proebsting et al., 1992; Eriksson et al., 2006*).

**Expression Mapping Reveals the Complex Regulatory Relationship of GA20ox1**, -2, and -3 in Some Tissues

The results presented here indicate that members of the GA20ox and GA3ox gene families (*Mitchum et al., 2006; Hu et al., 2008*) are coexpressed in the same floral organs (pistil, stamen, sepal, and receptacle) and within the same anther tissue layers during stamen development (PMCs, tapetum, mature pollen, and the dehiscent anther wall). By contrast, CPS expression in anthers is restricted to the vasculature and developing microspores until late in development (*Silverstone et al., 1997*). Within the floral context, GA3ox activity is provided by GA3ox1 in all tissues except the anther, where it is not expressed (*Mitchum et al., 2006*), while, conversely, expression of GA3ox2, -3, and -4 is restricted to the developing anther (*Hu et al., 2008*). Within the developing flower, GUS staining and qRT-PCR analysis indicate that expression of *GA20ox1* and -2 occurs mostly in different reproductive organs, with *GA20ox2* expressed predominantly in the pistil and *GA20ox1* in the stamen, which correlates with floral organ growth in *ga20ox1* and *ga20ox2* mutants. The reduced growth of all organs in *ga20ox1 ga20ox2* flowers, in contrast with the restricted floral expression patterns of *GA20ox1* and -2,
might be explained by overlapping expression in some tissues or the mobility of GA$_{4}$ or GA$_{4}$ between tissues. The tissue segregation for expression of the different GA20ox paralogs is not total, however: During anther development, GA20ox1, -2, -3, and -4 are all reported in the tapetum prior to its degeneration, a result supported by the tapetum degeneration defects found only in the ga20ox1 ga20ox2 ga20ox3-1 mutant. The expression patterns of GA20ox1, -2, -3, and -4 during anther development can be broadly divided along phylogenetic lines, with the patterns of GA20ox1 and -2 more closely resembling one another than GA20ox3 and -4 and vice versa. In addition to their expression in the tapetum prior to degeneration, GA20ox1 and -2 were reported in both the PMCs and tapetum during meiosis. Analysis of the GA-insensitive rice mutant gid1-4 identified a GA-dependent block in development during meiosis (Aya et al., 2009). Anther development of the equivalent A. thaliana gid1 triple mutant (Griffiths et al., 2006) has not yet been studied, but GA3ox2, -3, and -4 are expressed in all anther layers at meiosis (Hu et al., 2008), suggesting a similar function for GA signaling in A. thaliana. The differences in anther development between GA-insensitive (gid1) and GA-deficient (cps) mutants in rice (Aya et al., 2009) have not yet been explained.

Comparison of GA20ox:GUS expression patterns with qRT-PCR results at floral stage 12 validated the expression pattern of pGA20ox1-TC-GUS, but discrepancies were identified between GUS and qRT-PCR data in the remaining lines. Whereas the GUS reporters predict overlapping expression in particular floral organs, GA20ox transcript accumulation was instead compartmentalized, each organ dominated by expression of a single paralog. GA20ox1, -2, and -3 are all subject to negative feedback regulation (Rieu et al., 2008), and we demonstrate that GA20ox3 is upregulated in ga20ox1 ga20ox2 pistils and stamen filaments at this stage of floral development. Importantly, experiments by Rieu et al. (2008) suggest that GA20ox1, -2, and -3 do not respond independently to GA homeostasis; instead, a hierarchy exists: GA20ox1 expression is not affected by the loss of GA20ox2, but GA20ox2 is upregulated in some ga20ox1 tissues. GA20ox3 expression was not altered in the ga20ox1 x ga20ox2 single mutant backgrounds but was upregulated in ga20ox1 ga20ox2. Whether this hierarchical regulation occurs entirely through GA homeostasis (potentially caused by the relative impact of the loss of each paralog on the concentration of bioactive GA) or through a separate mechanism by which one GA20ox paralog directly or indirectly regulates the expression of another is unclear, but application of this hierarchy might contribute to the expression patterns observed. It is highly unlikely that this compartmentalization occurs in all tissues, given the very high levels of GA20ox3 expression observed alongside GA20ox1 and -2 in, for example, germinating seed (Ogawa et al., 2003, Rieu et al., 2008) and the observed coexpression in the tapetum.

We observed substantial differences in floral expression between the transcriptional and translational GUS reporter lines for GA20ox1 and GA20ox2 (see Supplemental Figure 4A online). By contrast, Mitchum et al. (2006) reported similar expression patterns for transcriptional and translational GUS fusions for GA3ox1 and GA3ox2, although only the latter construct for GA3ox2 reported expression in anthers. They observed generally weaker staining with the translational constructs, which they suggested may be due to inhibition of GUS activity in the fusion protein. Expression of the GA20ox-GUSTLs was restricted to the anthers, whereas we obtained much broader GUS expression with the TCs, except, as noted for GA3ox2:GUS, there was limited expression in anthers. It is possible that the introns contain regulatory elements, as reported for a GA20ox gene in tobacco (Nicotiana tabacum (Sakamoto et al., 2001)), or there might be posttranslational regulation, as suggested by Lee and Zeevaart (2007) for a GA20ox gene in spinach (Spinacia oleracea).

### A Regulatory Pathway Potentially Downstream or Independent of GA Signaling Enhances Organ Growth on Later Flowers

We identified unexpected changes to the growth of floral organs in wild-type and GA-deficient mutants as flowering progressed, leading to eventual recovery of stamen growth and development in mutants, such as ga20ox1 ga20ox2 ga20ox3-1 and ga1-3. While every effort was made to prevent contamination of these plants by bioactive GA, the changes in ga1-3 could potentially be ascribed to contamination by endogenous GA from neighboring plants, since this intermediate has been shown to be transmissible as an airborne volatile (Otsuka et al., 2004). Very small quantities of bioactive GA have been previously identified in ga1-3 tissues (King et al., 2001; Silverstone et al., 2001), as shown also in this study, although whether due to contamination or residual endogenous GA might be posttranslational regulation, as suggested by Lee and Zeevaart (2007) for a GA20ox gene in spinach (Spinacia oleracea).
METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as the wild type in all experiments; ga20ox1 and ga20ox2 mutant alleles were previously published (Rieu et al., 2008). Two mutant alleles of GA20ox3 and one of GA20ox4 were identified in the Col-0 background by the Seattle TILLING Project (http://tilling.fhcrc.org/; Colbert et al., 2001; Till et al., 2003); Nottingham Arabidopsis Stock Centre accessions CS86016 (herein referred to as ga20ox3-1) and CS87002 (ga20ox3-2) contain point mutations resulting in a truncated protein and substitution of a conserved amino acid (L208F), respectively (Figure 1C), and accession CS87368 (ga20ox4-1) contains a substitution of a conserved amino acid (P233S). A natural 1-bp deletion in GA20ox4 (ga20ox4-2) was identified from the Burren-0 ecotype (Ossowski et al., 2008). T-DNA insertions in and enhancer trap collections (Martienssen, 1998). Mutations were verified by sequencing. Alleles from other ecotypes were introgressed into Col-0 by six successive backcrosses prior to combining with other ga20ox mutant alleles. The ga1-3 allele, originally in the Landsberg erecta ecotype (Koornneef and Van der Veen, 1980), was previously introgressed into Col-0 (Tyler et al., 2004). All experiments described in this article use ga1-3 (Col-0).

Combinatorial mutant lines were established through crossing and subsequent selective breeding using PCR-based genotyping to track mutant alleles (see Supplemental Table 5 online). Wild-type and mutant-subsequent selective breeding using PCR-based genotyping to track mutant alleles. The ga1-3 mutation, originally in the Landsberg erecta ecotype (Koornneef and Van der Veen, 1980), was previously introgressed into Col-0 (Tyler et al., 2004). All experiments described in this article use ga1-3 (Col-0).

Phenotypic Characterization

Seed used in germination assays (n = 4, ~100 seed per replicate) was either imbibed in 50 μM GA₄ (GA pretreatment) or deionized water (control), sown on agar plates, and incubated under LD. Seed germination was scored by counting seeds with radicle protrusion after 6 d. Plants for hypocotyl (n = 18) and root length (n = 16) measurements were grown vertically on Murashige and Skoog media under continuous light, substituting Gelrite for agar, with measurements taken after 7 d. GA treatments comprised media containing 10 μM (hypocotyl) or 1 μM GA₄ (root). Characterization of vegetative characters (n = 12 for ga20ox1, -2, and -3; n = 8 for ga20ox4 and -5) was performed as described by Rieu et al. (2008), with measurements taken at 49 d unless specified otherwise. Floral organ lengths (n = 10) were measured from scaled photographs of dissected flower buds.

Histochemical GUS Staining

GUS staining was performed on 24 h germinating seed and primary inflorescence floral clusters, harvested at the opening of the 10th flower. GUS staining was performed with 1 mg mL⁻¹ X-GlC (Melford) in the presence of 0.5 μM potassium ferricyanide, the optimum concentration of which was determined empirically, and then decolorized by incubation in 70% ethanol.

Microscopy

Inflorescence tissues were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) and embedded in Technovit 7100 histochemical resin (TAAB Laboratory Supplies) following the manufacturer’s protocol. Sections (7 μm) were cut with a Leica-Reichert Jung 2053 Biocut rotary microtome (Leica Microsystems); non-GUS-stained inflorescence sections were stained with Toluidine Blue to improve visualization. Pollen developmental staging was assessed using whole anther squashes. Pollen nuclei were stained with 1 μg mL⁻¹ 4',6-diamidino-2-phenylindole solution and visualized under UV illumination. Sections and whole anthers were observed with a Zeiss Axioshot light microscope (Carl Zeiss) with attached Retiga EXi camera system (QImaging). Pollen viability was assessed using Alexander stain (Alexander 1969).

In Vitro Protein Activity Assay

A. thaliana GA20ox coding sequences were amplified from Col-0 seedling cDNA and cloned into the pET-32a expression vector (Novagen, Merck Biosciences) as Sall-NotI (GA20ox1 and GA20ox5), BamHI-NotI (GA20ox3), or Ncol-XhoI (GA20ox4) restriction fragments. GA20ox protein expression was induced in the Escherichia coli competent cell line BL21-CodonPlus(DE3)-RIL (Stratagene), and protein activity was assayed in bacterial lysates as described previously (MacMillan et al., 1997) through incubation for 24 h at 30°C with 14C-labeled GA12 or GA24 (obtained from L. Mander, Australian National University, Canberra, Australia) and dioxygenase cofactors. Products were separated by HPLC with online radiomonitoring (Radiodetector detector LB509; Berthold...
GA Analysis

Whole rosettes were harvested for GA analysis (n = 4, pools of 20 to 25), with development between genotypes synchronized at the point of floral transition. Finely ground freeze-dried material (~200 mg) was extracted with 80% acetonitrile containing 1% acetic acid (10 mL) for 1 h at room temperature after adding 400 pg [2H2]GAs (Olchemim) as internal standards. After centrifugation at 3000g for 20 min and collection of the supernatant, the residue was reextracted by mixing with the same solvent, followed by centrifugation. The combined supernatants were washed with an equal volume of n-hexane, which was discarded, after which the acetonitrile was removed under N2 and the aqueous residue loaded onto an Oasis HLB cartridge (60 mg, 3 cc; Waters) and prewashed/equilibrated consecutively with 3 mL of acetonitrile, methanol, and 1% aqueous acetic acid. After washing with 1% aqueous acetic acid (3 mL), GAs were eluted with 80% acetonitrile containing 1% acetic acid (2 × 3 mL). The dried eluate was dissolved in 1 mL CHCl3:ethyl acetate:acetic acid (50:50:1 v/v/v) and passed through a SepPak silica cartridge (100 mg; Waters), prewashed with CHCl3 (3 × 1 mL), and equilibrated with CHCl3:ethyl acetate:acetic acid (50:50:1; 1 mL). After washing the cartridge with a further 2 × 1 mL CHCl3:ethyl acetate:acetic acid (50:50:1), the combined eluate (3 mL) was dried and the residue dissolved in methanol (1 mL) and loaded onto a BONDELUT DEA cartridge (100 mg; Agilent), prewashed, and equilibrated with methanol (1 mL). After washing the loaded cartridge with methanol (1 mL), GAs were eluted with 1% acetic acid-methanol (2 × 1 mL), and the dried eluate dissolved in 1% aqueous acetic acid (30 μL), 15 μL of which was subjected to liquid chromatography–tandem MS (MS/MS) in negative ion mode using an 1200 LC module coupled to a 6410A triple quadrupole mass spectrometer (Agilent). LC separations were performed on a ZORBAX SB Phenyl column (2.1 × 50 mm, 1.7-μm particle size; Agilent) at a flow rate of 200 μL/min of 0.01% aqueous acetic acid as solvent A and 0.05% acetic acid-acetonitrile as solvent B. After retaining 3% B for 2 min, linear gradients were run from 3 to 20% B over 3 min, and then to 35% over 13 min and to 55% B over 7 min. After 5 min at 98% B, the initial condition was restored and allowed to equilibrate for 5 min. The MS/MS conditions and retention times are shown in Supplemental Table 6 online. The levels of GAs were determined using MassHunter B03.01 (Agilent).

Phylogenetic Analysis

Alignments of protein sequences were generated using Geneious (Biomatters), and all gaps and unaligned residue positions were removed. Phylogenetic analysis was performed by Bayesian inference using MrBayes within the software package TOPALi v2 (Mime et al., 2009), using the substitution model [JTT +I +G], selected as the best fit to the data by model test within TOPALI. MrBayes was run for 100,000 generations with a 30% burn-in to achieve convergence of the two Markov Chain Monte Carlo sampling runs. The phylogram was generated in MEGA5 (Tamura et al., 2011) and shows the posterior probabilities of the clades and branch lengths measured in expected substitutions per site. Very similar trees were also produced when the phylogenetic analysis was repeated using maximum likelihood and neighbor-joining inferences and tested by bootstrapping within MEGA5.

Accession Numbers

Sequence data from this article and their sources are given in Supplemental Table 7 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Function of GA20ox Paralogs and Mutant Alleles.

Supplemental Figure 2. Floral Phenotypic Recovery in GA-Deficient Mutants.

Supplemental Figure 3. GA Responsiveness of AtGA20ox:GUS Reporter Lines.

Supplemental Figure 4. AtGA20ox:GUS Expression Analysis.

Supplemental Table 1. Comparison of Phenotypic Parameters of Col-0 and ga20ox Mutants after GA Treatment.

Supplemental Table 2. Vegetative Phenotypes of ga20ox Triple and Quadruple Mutants after GA Treatment.

Supplemental Table 3. Mutant Segregation Distortion Analysis.

Supplemental Table 4. Modeling of Floral Organ Growth.

Supplemental Table 5. PCR Conditions and Primer Sequences for AtGA20ox Genotyping.

Supplemental Table 6. LC-MS Parameters.
Supplemental Table 7. Key, Source, and Identifier of the Sequences Compared in Figure 1B.

Supplemental Table 8. ga20ox Mutant Silique Length Transformed Data Set [\(\text{SQRT}(X + 0.1)\)].

Supplemental Data Set 1. Text File of Alignment Used for Phylogenetic Analysis in Figure 1.

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AUTHOR CONTRIBUTIONS

Tuomi, J.M., Voorbraak, F., Jones, D.L., and Ruijter, J.M. (2010). Bias in the C<sub>q</sub> value observed with hydrolysis probe based quantitative
PCR can be corrected with the estimated PCR efficiency value. Methods 50: 313–322.


Analysis of the Developmental Roles of the Arabidopsis Gibberellin 20-Oxidases Demonstrates That GA20ox1, -2, and -3 Are the Dominant Paralogs


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