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Feed-Forward Regulation of Gibberellin Deactivation in Pea

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Abstract

Multiple lines of evidence suggest that the genes involved in gibberellin (GA) biosynthesis are regulated by bioactive GA levels. With the recent cloning of GA 2-oxidase genes from pea, we investigated whether this homeostatic regulation extends to the genes controlling GA deactivation in this species, utilizing two well-characterized GA-deficient mutants, *ls* and *na* and a GA-accumulating mutant, *sln*. The pea GA 2-oxidases showed feed-forward effects at the mRNA level, while the endogenous levels of GA_{20}, GA_{29}, GA_1, and GA_8 showed no evidence of feed-forward regulation. Analyses of genomic Southern blots and expressed sequenced tag (EST) databases suggest that other GA 2-oxidases could possibly account for this lack of feed-forward on GA levels.

Key words: Gibberellin; Feed-back; Feed-forward; Deactivation; GA 2-oxidation

Introduction

The cloning of GA biosynthetic genes may help to determine the regulatory mechanisms controlling the *in situ* levels of bioactive GAs. This is important because developmentally and/or environmentally induced changes in GA levels play a crucial role in regulating processes such as seed germination (Yamaguchi and others 1998), bolting (Graebe 1987), and de-etiolation (Ait-Ali and others 1999). Further, normal levels of the bioactive 3β-hydroxylated GAs (for example, GA_1 and GA_4, Cowling and others 1998) are necessary for normal stem elongation and leaf expansion (Ross and others 1997). Thus normal growth of the plant probably depends on the interaction of developmental and environmental regulation of GA levels with homeostatic mechanisms (Kamiya and Garcia-Martinez 1999).

Several lines of evidence indicate that the levels of bioactive GAs are maintained by homeostatic mechanisms (Hedden 1999). Some of the earliest evidence came from measuring the levels of GA_1 precursors in GA biosynthesis mutants blocked early in the pathway (Hedden and Croker 1992). In GA-deficient plants, the immediate precursors of bioactive GA_1 were reduced much more than GA_1 itself (for example, Fujioka and others 1988). Furthermore, mutants with a constitutive GA response had reduced GA_1 levels (Croker and others 1990). These results suggested that the activities of the enzymes catalyzing the last two steps in GA_1 biosynthesis, GA 20-oxidase, and GA 3-oxidase are altered in a manner consistent with feed-back regulation. Indeed, feeding plants with radioactively labeled precursors confirmed that the activity of the GA 3-oxidase was increased in GA-deficient pea mutants (Ross and others 1999). When genes encoding GA 20-oxidases

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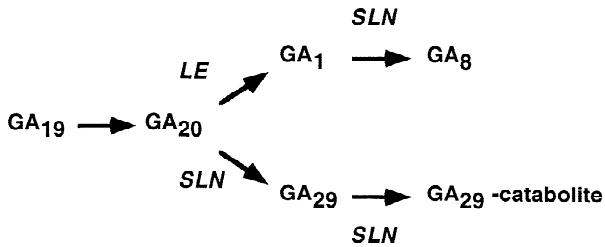

Figure 1. Important regulated steps in the biosynthesis and deactivation of the bioactive GA_1. Mendel's *LE* gene codes for the GA-3 oxidase, PsGA3ox1, and *SLN* codes for the GA-2 oxidase, PsGA2ox1.

and GA 3-oxidases were cloned, it was shown that this feed-back regulation extends to the mRNA level (Chiang and others 1995; Phillips and others 1995; Xu and others 1995; Martin and others 1996, 1997; Hedden and Kamiya 1997; Ross and others 1999). Thus, there is very clear evidence for feed-back regulation of the later steps of GA biosynthesis.

Besides biosynthesis, there are several other potential ways of regulating GA levels, including inactivation by conjugation or deactivation via 2-oxidation. Like the GA 3-oxidases and the GA 20-oxidases, the GA 2-oxidases of *Arabidopsis* show altered transcript levels in response to GA_3 treatment (Thomas and others 1999). GA_3 increased GA 2-oxidase transcript levels, indicating a feed-forward regulation that would act to maintain bioactive GA levels. Whether the GA 2-oxidase genes in other species respond in a similar feed-forward manner has not been determined. In the present paper we have investigated GA 2-oxidase gene expression in pea (*Pisum sativum* L.) mutants with reduced (*na* and *ls-1*) or elevated (*sln*) GA levels (Figure 1). The *SLN* gene encodes a GA 2-oxidase, *PsGA2ox1,* and is one of two GA 2-oxidase genes cloned from pea (Lester and others 1999). The mutation in *sln* plants results in a truncated, inactive GA 2-oxidase, leading to an accumulation of the GA_1 precursor GA_{20}. GA_{20} accumulates in seeds because the GA_{20} to GA_1 step catalyzed by Mendel's *LE* gene shuts down in developing seeds before the other biosynthetic enzymes. Normally this GA_{20} is catabolized by PsGA2ox1 to GA_{29} and then GA_{29}-catabolite. As a group, GA 2-oxidases can catabolize both bioactive GAs (GA_1 and GA_4) as well as their immediate precursors GA_{20} and GA_9 (Lester and others 1999; Martin and others 1999; Thomas and others 1999). Upon germination, the accumulated GA_{20} in *sln* seeds moves into the shoot where it is converted into active GA_1, resulting in the elongated growth habit of *sln* plants (Reid and others 1992; Ross and others 1995). With the recent cloning of two of the genes controlling GA deactivation in pea, we investigated whether these GA 2-oxidase genes respond to changes in bioactive GA levels. Further, we explored the size of the GA 2-oxidase gene family by Southern blot analysis and examination of the expressed sequenced tag (EST) databases to address the question of regulation of GA deactivation by other 2-oxidases.

Materials and Methods

Plant Materials and Growth Conditions

The garden pea lines used are held in the collection at Hobart, Tasmania. The *ls-1* line 181 is isogenic with line 107 (*LS*), derived from the WT cv. Torsdag, and the *na* and *NA* lines are also isogenic, as products of eight generations of single-plant selection, after a cross between closely related lines 1766 (*na*) and 1769 (*NA*). The *sln* plants used were as described in Lester and others (1999). All plants were grown (two per pot) in a heated glasshouse under an 18 h photoperiod. Node counts started from the cotyledons as zero. Where required, GA_1 (10 µg in 10 µl of ethanol) was applied to the third uppermost fully expanded leaf.

Northern and Southern Blot Analysis

Genomic DNA was isolated according to the protocol of Dellaporta and others (1983). For genomic Southern blots, 5 µg of genomic DNA from line 107 was digested and run on a 0.7% TAE gel, blotted to Genescreen Plus (Dupont/NEN) in 2xSSC, hybridized in the hybridization solution (7% sodium dodecylsulfate, 500 mM sodium phosphate, pH 7.2, 1 mM EDTA at 50°C), and then washed in either 0.1% SDS, 0.2x SSC at 65°C or 0.1% SDS, 1x SSC at 60°C. Total RNA was extracted and Northern blots were performed as described in Lester and others (1999).

Determination of GA Levels

Endogenous GAs were quantified using gas chromatography—mass spectrometry with internal standards, as described previously (Ross and others 1995).

Sequence Analysis

Sequences were aligned using ClustalX (Thompson and others 1997). Homology searches were performed using NCBI BLAST (Altschul and others 1997). Putative GA 2-oxidase ESTs were identified initially by high BLAST scores (normally greater than 90, in some cases lower), and confirmed by

searching the database to determine which gene the EST is most closely related to.

The phylogram was generated by PAUP 4.0 (Swofford 1999) using 285 amino acids (excluding gaps) with two GA-20 oxidases (PsGA20ox1 and AtGA20ox1) and two GA 3-oxidases (AtGA3ox1 and PsGA3ox1) as the outgroup. Sequences used in addition to PsGA2ox1 and PsGA2ox2 (Lester and others 1999), were *Marah* (MacMillan and others 1997); AtGA2ox1, AtGA2ox2, AtGA2ox3, PcGA2ox1 (Thomas and others 1999); AtGA20ox1 (AT2301, Phillips and others 1995); PsGA20ox1 (Martin and others 1996); AtGA3ox1 = AtGA4 (Chiang and others 1995); PsGA3ox1 = PsLE (Lester and others 1997) and sequences from GenBank for *Medicago* (BE204015 = BE205232 = AW584184), *Arabidopsis* (AAG00891), and *Oryza* (BAA96178.1).

Results and Discussion

Expression of GA 2-Oxidase Genes in Various Tissues and Genotypes

The first step towards investigating the regulation of the pea GA 2-oxidases was to verify in more detail the tissues in which they are expressed. Both genes, *PsGA2ox1* and *PsGA2ox2,* were expressed in tissues where GAs are thought to be active (Figure 2), such as expanding internodes (Ingram and others 1983). There was also expression in roots and cotyledons. The expression of *PsGA2ox2* was much lower in the leaves and the apical bud than that of *PsGA2ox1,* and was much lower than previously reported in Lester and others (1999). The pattern of expression in Figure 2 appears to be the case since it is reproducible and re-probing of the blot in Lester and others (1999) provided similar results. The expression of *PsGA2ox1* was similar to previous results and was confirmed by re-probing (Lester and others 1999).

Next we compared the expression of the two GA 2-oxidase genes and two GA biosynthesis genes (*PsGA3ox1* and *PsGA20ox1*) in wild-type and GA-deficient *ls* plants (Figure 3a). Both the GA biosynthetic genes, *PsGA3ox1* (equivalent to Mendel's *LE* gene, Lester and others 1997) and *GA20ox1* (Martin and others 1996) showed strong expression at the RNA level in the apical portion of GA-deficent *ls-1* plants, with lower levels in wild-type plants, reflecting feed-back regulation. In addition, the GA 3-oxidase also showed strong expression in the roots, with some feed-back regulation. The GA 20-oxidase mRNA was expressed at lower levels in internodes and roots, but also showed signs of feed-back regulation. Similarly, there was weak expression, with some feed-back regulation, of the GA 3-oxidase in

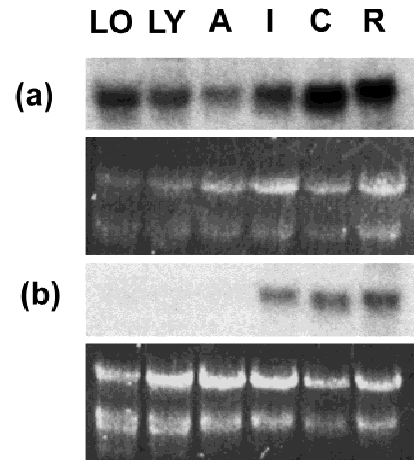

Figure 2. Northern blot analysis of *PsGA2ox1* **(a)** and *PsGA2ox2* **(b)** transcript levels in various parts of WT pea plants. LO, leaflets from the second uppermost fully expanded leaf; LY, leaflets from the uppermost fully expanded leaf; A, apical bud; I, internodes; C, cotyledons; R, roots. LO, LY, A and I were from 28-day-old plants (12–13 expanded leaves). C, from seeds harvested approximately 22 days after anthesis, and R from 15 day-old plants. Five micrograms of total RNA per lane were probed with the cDNAs for *PsGA2ox1* and *PsGA2ox2.* A photograph of the ethidium bromide-stained gel prior to blotting showing ribosomal RNA is shown below each autorodiagraph as a loading control.

leaflets. However, in some tissues these genes did not appear to show feed-back regulation; in leaflets, for the GA 20-oxidase, and internodes for the GA 3-oxidase.

The two GA 2-oxidases, *PsGA2ox1* and *PsGA2ox2,* on the other hand, showed the opposite effect: reduced mRNA levels in GA-deficient *ls-1* plants (Figure 3a). In all the tissues with significant levels of GA 2-oxidase expression, there was feed-forward regulation at the mRNA level. However, in the apical tissue (note: more specific apical tissue than used in Figure 2 or Figure 3b), where there was strong expression of the biosynthetic genes, there was little or no expression of the deactivating GA 2-oxidases. The reduced expression of *PsGA2ox1* in GA-deficient *na* plants can be reversed by the addition of exogenous GA_1 (Figure 3b). Thus, the GA 2-oxidases in pea appear to be feed-forward regulated at the mRNA level in a manner similar to the *Arabidopsis* GA 2-oxidases (Thomas and others 1999). The primary effect of the *sln* mutation is an increase in the levels of GA_{20} in maturing seeds. Because GA_{20} is not in itself directly a bioactive GA, and there is no GA 3-oxidase activity in cotyledons at this stage to convert it to a bioactive form, it is no surprise that *PsGA2ox1* (Figure 3c) and *PsGA2ox2* (data not

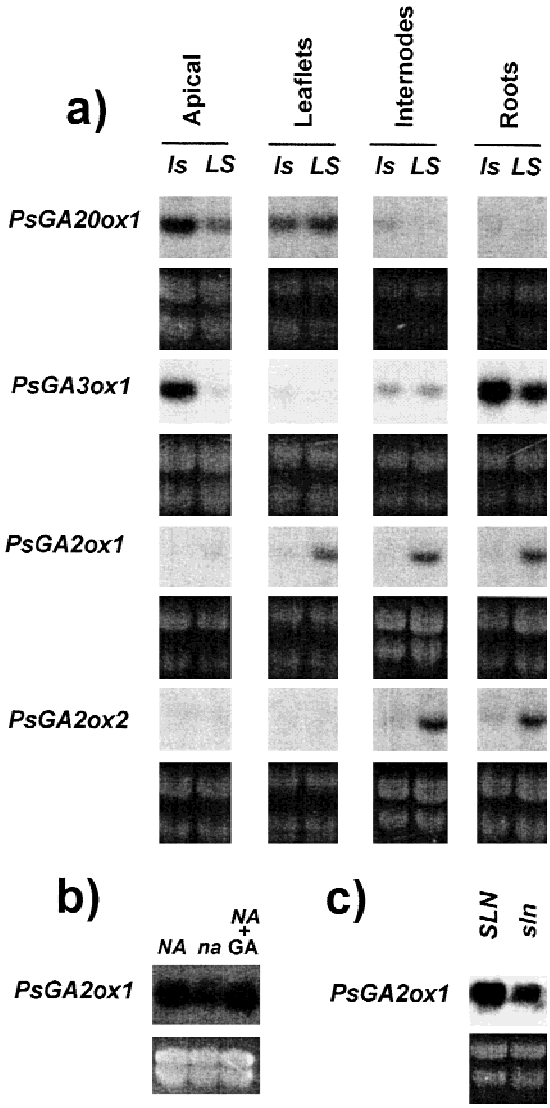

Figure 3. Gene expression in GA mutants and their wild types. **(a)** A comparison of the transcript levels for *PsGA2Oox1, PsGA3ox1, PsGA2ox1,* and *PsGA2ox2* in four different tissues: apical portions, leaflets, internodes, and roots. The exposure times for *PsGA2ox2* were approximately four times longer than for other genes. Leaflets were from the uppermost fully expanded leaf, and excluded petioles and stipules. Internodes were from the internode immediately below the uppermost fully expanded leaf. Apical portions were excised above the leaf above the uppermost fully expanded leaf. **(b)***PsGA2ox1* transcript levels in apical portions of 26-day-old *NA, na,* and GA_1-treated *na* plants. Note that the apical tissue used in this experiment (and Figure 2) contained more expanding leaf material than in Figure 3a. **(c)***PsGA2ox1* transcript levels in developing cotyledons approximately 22 days after anthesis from *SLN* and *sln* seeds. Five micrograms of total RNA were loaded in all cases. A photograph of the ethidium bromide-stained gel prior to blotting showing ribosomal RNA is shown below each autorodiagraph as a loading control.

shown) transcripts were not elevated in developing *sln* cotyledons (that is, no feed-forward, Figure 3c). In fact, the transcript level for *PsGA2ox1* was lower in *sln* cotyledons, which may be due to changes in mRNA stability in response to the frameshift-induced change in translation caused by the *sln* mutation.

Level of 2β-Hydroxylated GAs in a GA-Deficient Mutant

To investigate the relationship between feed-forward and GA levels, we quantified endogenous GAs in the GA-deficient (*ls-1*) mutant and its WT (Table 1). The *ls-1* mutation partially blocks GA biosynthesis at an early step and is a mutation in the CPS synthase gene (*ent*-copalyl diphosphate synthase, Ait-Ali and others 1997), resulting in reductions of all the GAs measured. In the *ls-1* mutant, compared with the WT, the relative amounts of the GAs were altered. Consistent with a feed-back mechanism affecting GA 3-oxidation and GA 20-oxidation, the level of the precursor GA_{19} was reduced more than GA_{20}, whereas the level of bioactive GA_1 showed a smaller reduction than both precursors. However, the reductions in GA_{29} and GA_8 were also less than for their respective substrates, GA_{20} and GA_1. This result is not consistent with the feed-forward theory, which would predict extremely low levels of GA_{29} and GA_8 in the *ls-1* mutant. However in the mutant, reductions in the formation of GA_{29}-catabolite and GA_8-catabolite (Figure 1) may have counteracted any tendency for a reduction in GA_{29} and GA_8 levels. Some data from *Arabidopsis* suggest that other mutants also contain GA_8 levels inconsistent with the feed-forward theory. For example, the GA 2-oxidase substrate and product levels in the GA-deficient *ga4* and *ga5* mutants show mixed results (Talon and others 1990). In the *ga5* mutant, the ratio of GA_1 to GA_8 changes relative to wild-type in a manner consistent with feed-forward regulation. However, in the *ga4* mutant, the ratio is the same as wild-type. Thus, measurements of endogenous GA levels in both pea and *Arabidopsis* provide little evidence for feed-forward regulation, unlike for feed-back regulation.

Are There Unidentified GA2-Oxidase Genes in Pea?

The lack of an apparent feed-forward effect on GA_8 levels may be due to an as yet uncloned member of the 2-oxidase gene family, whose expression is not subjected to feed-forward regulation. In *Arabidopsis,* three GA 2-oxidase genes have been identified

Table 1. Levels of Endogenous GAs in the Apical Portions of GA-Deficient *ls-1* and the Wild-type (*LS*) Plants

GA Level (ng · g^{-1} FW)					
Genotype	GA_{19}	GA_{20}	GA_{29}	GA_1	GA_8
LS	3.9 ± 0.3	9.1 ± 0.0	11.6 ± 0.4	11.7 ± 1.2	24.4 ± 0.2
ls-1	0.03 ± 0.03	0.19 ± 0.00	0.51 ± 0.05	0.27 ± 0.01	0.75 ± 0.03
Ratio *LS/ls-1*	130	48	23	43	32

Material was harvested immediately above the second uppermost fully expanded leaf. The results are shown as means ± standard error of two replicates.

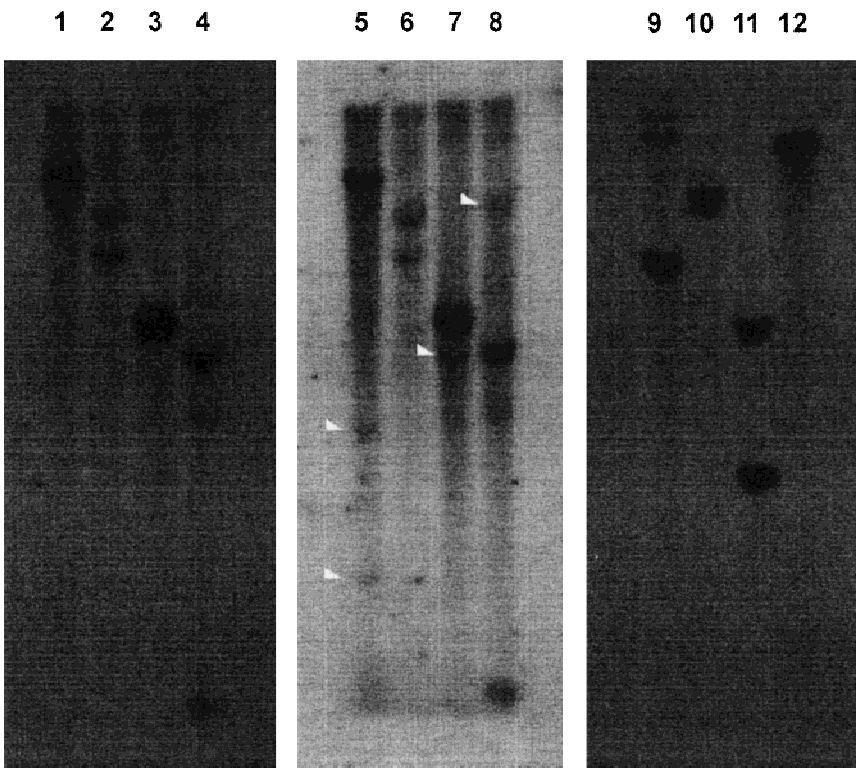

Figure 4. Genomic Southern blot analysis of the GA 2-oxidase gene family. Pea genomic DNA digested with *BamHI* (lanes 1,5,9), *BglII* (lanes 2,6,10), *EcoR1* (lanes 3,7,11), and *HindIII* (lanes 4,8,12) and probed with either *PsGA2ox1* (lanes 1–8) or *PsGA2ox2* (lanes 9–12) and then washed at high stringency (0.2x SSC at 65°C) (lanes 1–4, 9–12) or low stringency (1x SSC at 60°C) (lanes 5–8.). Arrowheads indicate bands appearing at low stringency when probed with *PsGA2ox1*.

(Thomas and others 1999). To assess the number of GA 2-oxidases in pea, we probed genomic Southern blots with both *PsGA2ox1* and *PsGA2ox2* (Figure 4). When probed at low stringency (1xSSC at 60°C) with PsGA2ox1 we see bands that are not present at high stringency, but no cross-hybridization between *PsGA2ox1* and *PsGA2ox2*. Experiments testing cross-hybridization of plasmids containing cDNAs for *PsGA2ox1* and *PsGA2ox2* show cross-hybridization at 2xSSC at 50°C, but the heterologous signal is very weak (data not shown). Thus, the bands that appear at low stringency suggest that there is at least one other sequence closely related to GA 2-oxidases that is as yet unidentified in pea. Whether this sequence(s) codes for a functional gene and is transcribed has not been determined. There may be pseudogenes, since pseudogenes have been found for other GA biosynthesis genes in pea (Ait-Ali and others 1997). Because we cannot show cross-hybridization between *PsGA2ox1* and *PsGA2ox2* on the genomic Southerns there appear to be at least three GA 2-oxidase sequences in pea, but the level of nonspecific hybridization prevents the use of lower stringency.

At the amino acid sequence level PsGA2ox1 is more closely related to the *Arabidopsis* GA 2-oxidases identified by Thomas and others (1999) than to PsGA2ox2 (Figure 5). Most of the cloned GA 2-oxidases fall into one group, including examples from bean (PcGA2ox1), and the three 2-oxidases from *Arabidopsis* (AtGA2ox1, AtGA2ox2, AtGA2ox3, Thomas and others 1999), *Marah* (a putative 2-oxi-

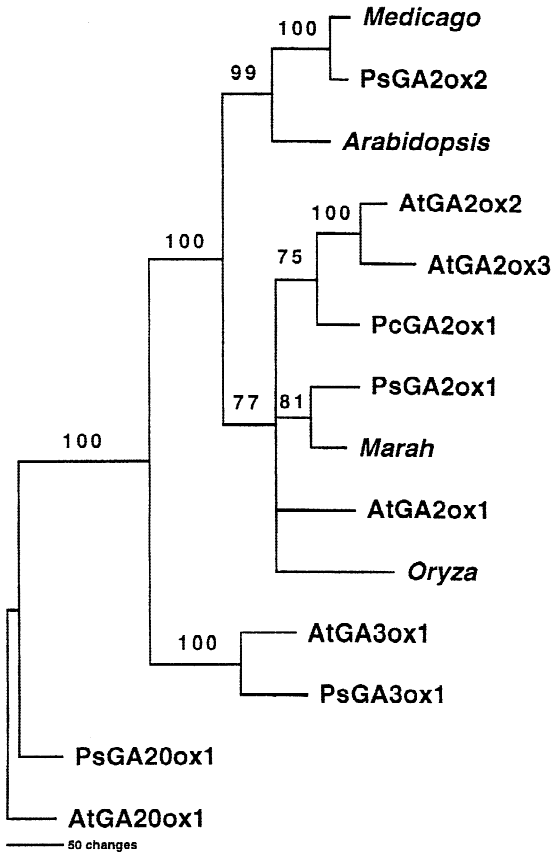

Figure 5. Inferred phylogenetic relationship of GA 2-oxidases and representatives of related enzymes. The GA 2-oxidases are part of the larger family of 2-oxoglutarate-dependent dioxygenases that includes other GA metabolizing enzymes, GA 3-oxidases, and GA 20-oxidases. Numbers shown represent the bootstrap support values (%). Only genes with full length sequences are shown. Putative GA2-oxidases are indicated by their generic name only (see Table 2).

Table 2. List of GA 2-Oxidase-Like Sequences from Genbank, Including Expressed Sequence Tags (ESTs) and Hypothetical Proteins Predicted from the *Arabidopsis* and Rice Genome Sequencing Projects

PsGA2ox1-like sequences	
Lotus	AV426634
	AV420834=AV420817
Medicago	AL382874=AL382875
	AW584268
Maize	BE510647 = BE510646
Marah	CAA70330†‡
Rice	BAA96178.1†‡
Soybean	AW277690 = AW309039 = AW705363
	AW 184969
	AW706973 = AW705614 = BE474583
	AW830753
Tomato	AI487548 = AI488712 = AW650160 =
	AW650238 = AI899222
	AW030225 = AW931003
	AW222239 = BE434782 = BE433301 =
	BE435345 = AW930043
	AI898755
PsGA2ox2-like sequences	
Arabidopsis	AI996723= AAG00891†‡
Lotus	AV426408
Medicago	BE204015=BE205232=AW584184‡
	AL381110=AW736315
Rice	C727618 = AQ157054†
Soybean	AW666013
Tomato	AW031637 = AW216742 = AI777086 =
	AW030757 = AW216362 =
	AI896334 = AW035295

†Sequences from the non-redundant section of genebank (protein) or the genome survey sequences section.
‡Sequences that appear to encode all 285 of the core amino acids used in the phylogenetic analysis in Figure 5.
= Indicates that the ESTs overlap and are essentially identical in the overlapping sequence. The relationship to either PsGA2ox1 *or* PsGA2ox2 *based on Figure 6 is shown. Proven GA 2-oxidases are not listed.*

dase, MacMillan and others 1997), and one of the pea GA 2-oxidases (PsGA2ox1, Lester and others 1999). In addition there are some uncharacterized ESTs likely to code for PsGA2ox1-like GA 2-oxidases (Table 2). Due to overlapping alignments, the EST data suggest that there are at least three different tomato genes (possibly four), three different soybean genes (possibly four), two different *Medicago* genes, and two different *Lotus* genes. Using BLASTX to assess similarity, these ESTs are most closely related to the *Marah* sequence, *PsGA2ox1*, or *PcGA2ox1*.

In contrast, PsGA2ox2 from pea is distinct among the proven GA 2-oxidases and is not closely related to the *Arabidopsis* or *Phaseolus* GA 2-oxidases previously cloned (Thomas and others 1999). The bootstrap values shown in Figure 5 provide some support for separating PsGA2ox2 into its own group. Further support comes from the ESTs used in Table 2. The sequences most closely related to PsGA2ox2 are some ESTs from rice, *Arabidopsis*, tomato, *Lotus*, soybean, and two in *Medicago*. The gene represented by *Medicago* (EST BE204015) in Figure 5 is very similar to PsGA2ox2 (data not shown); the gene represented by the other *Medicago* sequence (AL381110) clearly diverged much earlier (data not shown). Because the statistical methods underlying phylogeny programs like PAUP work best with sequences of equal length, PAUP is not well-suited to analyzing the relationships of the ESTs, which are of different lengths. A more robust method is to compare the relative BLAST scores of the ESTs against PsGA2ox1

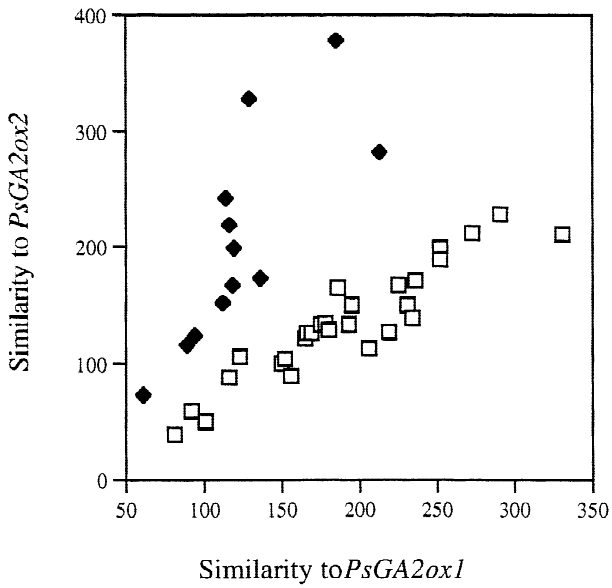

Figure 6. Graph of BLAST scores showing the similarity of ESTs to PsGA2ox1 and PsGA2ox2. The sequences most closely related to GA 2-oxidase genes fall into two groups, *PsGA2ox1*-like(□) and *PsGA2ox2*-like(◆). Table 2 gives the identity of the putative GA 2-oxidase sequences used.

and PsGA2ox2 (Figure 6). This analysis confirms that they fall into two groups. Thus, *PsGA2ox2* may represent a new class of GA 2-oxidase genes that includes previously unidentified genes from *Arabidopsis,* tomato, rice, and *Medicago.*

Conclusion

With the identification of more GA 2-oxidase genes, it becomes clear that *PsGA2ox2* and the genes represented by the sequences from *Arabidopsis,* rice, *Medicago,* and tomato represent a novel class of GA-2 oxidases (Figures 5 and 6). Although it is not yet clear how these *PsGA2ox2*-like genes differ from other 2-oxidases, it might be significant that PsGA2ox2 and PsGA2ox1 differ markedly in their ability to metabolize GA_{20} (Lester and others 1999). At this stage, there is no evidence from expression patterns for a consistent difference between *PsGA2ox2*-like genes and the other 2-oxidases. Although no systematic study of the expression patterns exists for the ESTs, based on the libraries they were cloned from, they are expressed in a range of tissues: roots, callus, ovaries, seedlings, etiolated apices, tomato fruit, seed coats, hypocotyls, symbiotic root nodules, tassels, and flowering-stage panicles. This overlaps with the expression pattern of *PsGA2ox2,* which is expressed in roots, internodes, and maturing seed. The expression of *PsGA2ox2* differs from that of *PsGA2ox1,* which is expressed at higher levels in leaves (Figure 2).

The gene expression studies reported here (Figure 3) showed reduction in expression of both *PsGA2ox1* and *PsGA2ox2* in two different GA_1-deficient mutants. Although these reductions were not reflected in the endogenous levels of the GAs monitored (Table 1), it appears that pea is a second species, after *Arabidopsis* (Thomas and others 1999), in which there is feed-forward regulation of GA 2-oxidase gene expression.

Acknowledgments

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