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Gibberellins regulate the abundance of RNAs with sequence similarity to proteinase inhibitors, dioxygenases and dehydrogenases

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Abstract. In an effort to understand the molecular mechanism of gibberellin (GA) action, we have cloned and performed an initial characterization of three cDNAs (GAD1, 2, and 3) which correspond to RNAs that become less abundant by 2 h after treatment of tomato (*Lycopersicon esculentum* Mill.) shoot tissue with gibberellic acid (GA_3). Treatment with either auxin or ethephon also decreases the abundance of all three of the GAD RNAs. The tomato ethylene-insensitive mutant, *Nr*, and the GA-deficient mutant, *gib1*, were used to show that GA or auxin regulation of GAD RNA abundance is not dependent on ethylene sensitivity, and that ethylene or auxin regulation is not dependent on normal levels of gibberellin biosynthesis. Treatment with abscisic acid (ABA) antagonizes the GA-induced suppression of the GAD1 and GAD2 RNAs. GAD1 is similar to type-II wound-inducible plant proteinase inhibitors. Like the well-characterized proteinase inhibitor II (pin II) of tomato, the GAD1 and GAD2 RNAs are wound inducible. Induction of pin II and GAD1 RNA in *gib1* was found to require less-severe wounding than was required using wild-type plants or plants doubly mutant for *gib1* and *sit* (the *sit* mutation causes ABA deficiency). The predicted GAD2 protein sequence is similar to 2-oxoglutarate-dependent dioxygenases while the predicted GAD3 protein sequence is similar to proteins belonging to the nonmetallo-short-chain alcohol-dehydrogenase family, especially the *TASSELSEED2* (*TS2*) gene of maize and bacterial hydroxysteroid dehydrogenases.

Key words: Dehydrogenase – Dioxygenase – Gene expression – Gibberellin – *Lycopersicon* – Proteinase inhibitor

Introduction

Little is known of the molecular mechanism of gibberellin (GA) action. Genetic analysis has identified two main classes of gene mutation which affect GA perception or signal tranduction. The first comprises GA-insensitive mutants which do not properly respond to GAs, and the second comprises slender-type mutants which have a phenotype similar to that of plants which have been repeatedly treated with GAs (Scott 1990). A complementary approach to understanding GA action is to isolate genes which are regulated by GAs and use these genes both as molecular markers for response to GA and also to isolate the molecules which are responsible for this regulation. Both approaches are necessary for a full understanding of the components of the signal transduction pathway from GA perception to gene activation.

Gibberellins are known to regulate gene expression in the aleurone layer of germinating cereal seeds (reviewed in Hooley 1994), in vegetative shoot tissue (Chory et al. 1987; Shi et al. 1992) and in flowers (Weiss et al. 1990; Jacobsen et al. 1994). Although most studies of GA-regulated genes focus on genes whose mRNAs are increased in abundance, studies of barley or wheat aleurone layers have also found RNAs which decrease in abundance following GA treatment, but with a relatively late time course (Baulcombe and Buffard 1983; Nolan and Ho 1988; Heck et al. 1993). In barley aleurone layers, three RNA species were found to decrease in abundance following treatment with Gibberellic acid (GA_3). One RNA which was detected with an alcohol-dehydrogenase clone from *Zea mays* was decreased in abundance within 8 h after GA treatment, while two additional mRNAs, one encoding a putative α-amylase/protease inhibitor and another encoding a storage globulin, were suppressed after 8–16 h of GA treatment (Nolan and Ho 1988; Heck et al. 1993).

Recent work indicates that treatment of GA-deficient (*gib1*) tomato shoots with GA_3 causes a rapid decrease in the abundance of a number of translatable mRNAs (Jacobsen et al. 1994). These results prompted us to screen for cDNA clones corresponding to RNAs that decrease in abundance following GA treatment of *gib1* shoots.

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Abbreviations: ABA = abscisic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; GA = gibberellin; GA_3 = gibberellic acid; pin II = proteinase inhibitor II

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This paper describes the cloning and initial characterization of three such cDNA clones.

Materials and methods

Isolation and sequencing of GAD clones. Twenty-four hours after *gib1* plants were sprayed with a GA_3 solution [5×10^{-5} M GA_3, 0.05% (v/v) Tween-20 (polyoxyethylene-sorbitan monolaurate)] or with a control solution (0.05% Tween-20), the entire shoot above the fourth internode (except for inflorescences) was harvested, frozen in liquid nitrogen, and stored at − 80 °C. Polyadenylated RNA was then isolated from these samples as previously described (Shi et al. 1992). The Poly(A)$^+$RNA from the control sample was used to construct a cDNA library using the Lambda-ZAP cDNA Synthesis kit, as described by the manufacturer (Stratagene Cloning Systems, La Jolla, Calif., USA). Three thousand plaques (total) were plated on four 10-cm Petri plates containing LB agar medium supplemented with 10 mM $MgSO_4$ (Sambrook et al. 1989). Duplicate phage lifts onto nitrocellulose filters (HATF 085 50; Millipore Corporation, Bedford, Mass., USA) were produced as described in Sambrook et al. (1989). The first duplicate filter from each plate was hybridized with a ^{32}P-labeled cDNA probe synthesized using poly(A)$^+$RNA from the GA_3 sample. The second duplicate filter was hybridized with a similar probe made using poly(A)$^+$RNA from the control sample. The prehybridization, hybridization and washing conditions were those described in Olszewski et al. (1989). Each of 46 plaques that hybridized more strongly to the control probe than to the GA_3 probe was removed from the agar with the small end of a Pasteur pipette and placed in 0.5 ml of a solution containing 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl (pH 7.5) and 0.01% gelatin, and stored at 4 °C. Of this solution, 5 µl was used as a template for polymerase chain reaction (PCR) using the oligonucleotide primers 5′-CCGGGCCCCCCCTCGAG and 5′-CGCTCTATAATACGACTCACTATAGGGCTGCAGGAATTCGGC. These primers are homologous to the lambda-ZAP vector and to the *Eco*R1 adaptor used in the Lambda-ZAP cDNA Synthesis kit. Each 50-µl PCR reaction included 1 × PCR buffer (Promega; Madison Wis., USA), 1.5 mM $MgCl_2$, 50 µM dNTPs (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), 30 pmol of each primer, and 1.25 units of Taq DNA polymerase (Promega). After 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2.5 min, 10 µl of each reaction was spotted onto each of two nylon membranes (Genescreen plus; NEN Research Products; Boston, Mass., USA) using a dot-blotting apparatus, according to the manufacturer (Schleicher and Schuell, Keene, N.H., USA). These duplicate dot blots were then probed with the control and GA_3 cDNA probes described above. This analysis identified 22 clones that hybridized more strongly to the control probe than to the GA_3 probe. Further analysis divided these clones into 9 groups. One group consisted of 14 clones which cross-hybridized to each other at high stringency. The clone from this group which contained the largest insert (GAD1) was selected for further analysis. The remaining 8 groups each consisted of a clone which did not cross-hybridize with other clones. To confirm the GA regulation of the RNA corresponding to each of these clones, RNA blot analysis (described below) was performed with poly(A)$^+$RNA from an independent experiment in which plants were treated with GA_3 or a control solution for 24 h. The RNA corresponding to GAD1, and RNAs corresponding to two of the unique clones (GAD2 and GAD3) were significantly decreased in abundance in the sample from GA_3-treated plants relative to the sample from control plants. RNAs corresponding to clones in the remaining 6 groups did not exhibit significant GA regulation when retested.

In-vivo excision of pBluescript plasmid sequences from the GAD1, GAD2 and GAD3 lambda ZAP clones was used to generate the pGAD1, pGAD2 and pGAD3 plasmids according to the manufacturer (Stratagene). These plasmids were then used for DNA sequencing of both strands of the GAD1, GAD2 and GAD3 cDNA inserts using the Sequenase Version 2.0 DNA sequencing Kit (United States Biochemical Corporation; Cleveland, Ohio, USA).

Analysis of RNA blots. This analysis was performed as described in Shi et al. (1992) except that the hybridization solution was similar to that of Church and Gilbert (1984). The hybridization solution contained 0.8 × SSPE (Sambrook et al. 1989), 7% sodium dodecyl sulfate, 50% formamide, 100 µg · ml^{-1} sheared salmon sperm DNA, 0.5% nonfat dry milk, and 125 µg · ml^{-1} poly(A)$^+$RNA (Sigma, St. Louis, Mo., USA). Blots were hybridized with antisense RNA probes (made according to Stratagene) synthesized from each of the GAD clones, a GAST1 cDNA clone (Shi et al. 1992) or from a potato proteinase inhibitor II (pin II) clone. The pin II clone was constructed by directionally subcloning the *Eco*R1 to *Sph*I restriction endonuclease fragment from plasmid pRT55 [kindly provided by Dr. Robert Thornburg; Iowa State University, Ames Iowa] into the *Eco*R1 and *Sma*I sites of pBluescript II KS- (Stratagene). This plasmid, pKSPINII contains the first exon, the intron, and a portion of the second exon of the potato pin IIK gene (Thornburg et al. 1987).The largest region of homology between pKSPINII and the tomato pin II mRNA (Graham et al. 1985) is 186 nucleotides of the second exon which shares 83% nucleic acid identity. Blots were hybridized at 50 °C (GAST1), 53 °C (pin II) and 58 °C (GAD1, 2 and 3). The most stringent wash was 0.2 × SSC, 1% SDS at 50 °C (GAST1), 65 °C (pin II) and 77 °C (GAD1, 2 and 3).

Each lane was loaded with 10 µg of total RNA based on spectrophotometric determination of RNA concentrations. Hybridization with a ^{32}P-end labeled polyuridine probe as described by Medberry et al. (1990) indicated that each lane contained equal amounts of poly(A)$^+$RNA (data not shown).

The sizes of the GAD RNAs were determined by comparing their mobility with that of a 0.24–9.5-kilobase (kb) RNA ladder (GIBCO BRL, Grand Island, N.Y., USA). Sizes were approximately 1.1, 1.5 and 1.2 kb for GAD1, GAD2, and GAD3, respectively (data not shown).

Plant culture and hormone treatments. Seeds of the *gib1*, *sit*, and *gib1*; *sit* double mutant (in the background *Lycopersicon esculentum* Mill. cv. Moneymaker) were obtained from M. Koornneef, Department of Genetics, Agricultural University, Wageningen, The Netherlands. The *flacca* mutation was in the Ailsa Craig background. Wild-type tomato *L. esculentum* cv, Moneymaker seeds were from Thompson and Morgan (Jackson, N.J., USA). The *gib1* seeds were germinated in 5×10^{-5} M GA_3 in Petri dishes and then transferred into 15-cm-diameter pots. Growth chamber conditions were 24 °C day, 18 °C night, and a 16-h photoperiod. Chambers contained cool-white fluorescent tubes and incandescent lamps which emitted 300 mol · s^{-1} · m^{-2} at plant level. The *Never ripe* mutant of tomato and the wild-type tomato cultivar Pearson were kindly provided by M. Lanahan (Monsanto Company, St. Louis, Mo., USA).

2,4-Dichlorophenoxyacetic acid (2,4-D), kinetin, GA_3, and abscisic acid (ABA) were from Sigma; 2,4-D was recrystallized from benzene. These hormones were dissolved in 95% ethanol and then diluted into water. Ethrel (39.5% ethephon) was from Union Carbide (Triangle Park, N.C., USA).

Results

Abundance of GAD RNA is suppressed by GA treatment. Three cDNA clones (GAD1, GAD2 and GAD3) which correspond to RNAs whose abundance was decreased by treatment of *gib1* tomato plants with GA_3 were isolated by differential screening of a cDNA library (see *Materials and methods*). Figure 1 shows a time course for the decrease in abundance of the GAD RNAs in *gib1* shoots following treatment with GA_3. Since the basal level of GAD RNA in the untreated control samples often varied (the cause of this variation is unknown), each GA_3-treated sample was accompanied by a control sample harvested at the same time. Decreases in the abundance of RNAs corresponding to the GAD clones were detectable

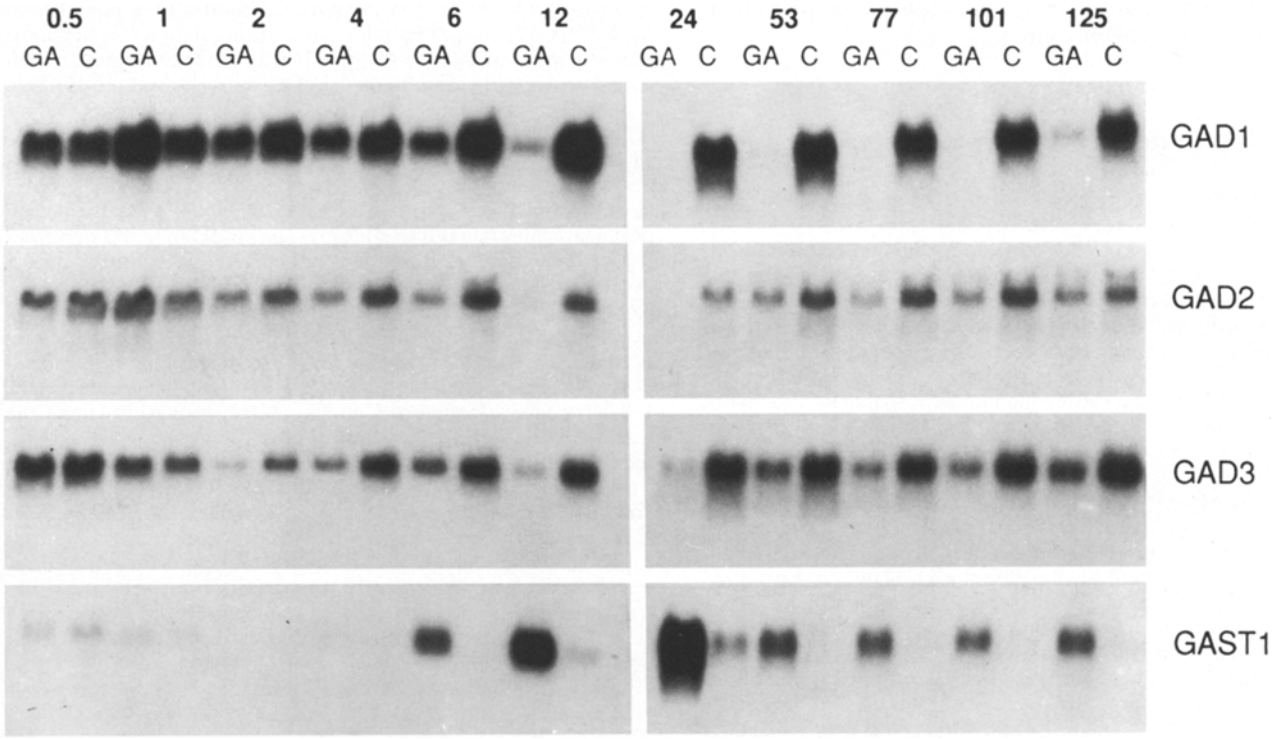

Fig. 1. Time course for the decrease in abundance of GAD RNAs. *gib1* tomato plants were sprayed to run-off with a GA_3 solution (5×10^{-5} M GA_3, 0.05% Tween-20) (*GA*) or with a control solution (0.05% Tween-20) (*C*). After the indicated number of hours, the entire shoot above the fourth internode (except for inflorescences) was harvested and total RNA from these tissues was isolated and used for RNA blot analysis. As indicated, blots were hybridized with antisense RNA probes made from the GAD1, GAD2, GAD3 and GAST1 cDNA clones

2 h after GA_3 treatment, and the maximum suppression of the level of these RNAs occurred by 12–24 h post treatment. The GAD1 RNA levels remained maximally suppressed for at least 5 d following GA_3 treatment (the longest time point tested), while the GAD2 and GAD3 RNA levels remained maximally suppressed only for 24 h (Fig. 1). Interestingly, control wild-type and *gib1* plants contained similar amounts of GAD RNAs (Figs. 2, 4). This suggests that the levels of GAD RNAs are insensitive to the absolute level of GA but instead are more highly correlated with changes in GA levels. As a control for GA response, RNA corresponding to a known GA up-regulated gene from tomato, GAST1, was also analyzed in these experiments (Fig. 1). The abundance of the GAST1 RNA was increased by treatment with GA_3 with approximately the same kinetics as the GAD RNAs were decreased (Fig. 1 and Shi et al. 1992).

The effect of other plant hormones on the expression of the GAD RNAs. Figure 2 shows that GAD RNA levels were decreased by treatment with the synthetic auxin, 2,4-D, and the plant growth regulator ethephon, a compound that releases ethylene (Warner and Leopold 1969), but not appreciably affected by treatment with kinetin. Ethephon and 2,4-D affected the GAD RNAs both in wild-type tomato and in the *gib1* mutant, suggesting that these hormones do not act through changes in GA levels (Fig. 2). Figure 2 also shows that GA_3 caused a decrease in abundance of the GAD RNAs both in *gib1* and in wild-type plants. GAST1 was again included as a control in these experiments. As shown by Shi et al. (1992), the GAST1 RNA abundance was not appreciably affected by treatment with ethephon, 2,4-D, or kinetin (Fig. 2).

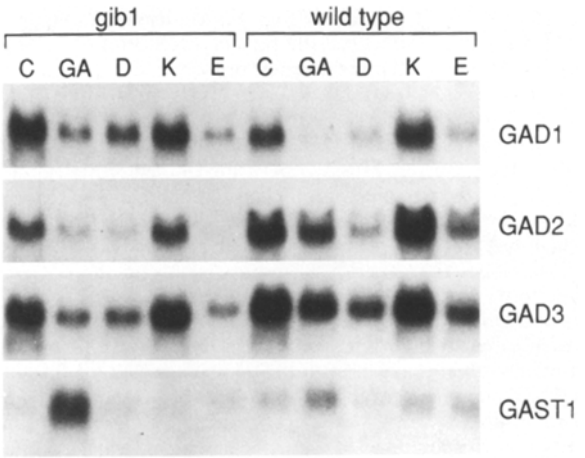

Fig. 2. The effect of plant growth regulators on the expression of GAD RNAs. Total RNA was isolated 12 h after *gib1* or wild-type tomato plants had been sprayed to run-off with a control solution (*C*) or with 50 μM solutions of GA_3 (*GA*), 2,4-D (*D*), or kinetin (*K*) or a 7 mM solution of ethephon (*E*). All solutions contained 10 mM KH_2PO_4 (pH 6.5) and 0.01% Tween 20. Samples were analyzed as described in Fig. 1

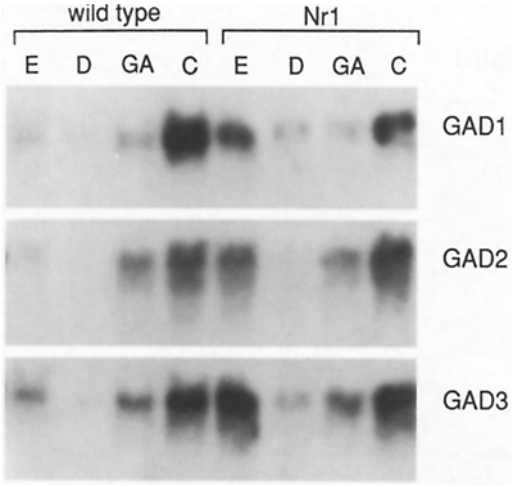

Fig. 3. The effect of the *Nr* mutation on the response of GAD RNA levels to treatment with ethephon, GA_3, or 2,4-D. Wild-type tomato cv. Pearson and *Nr* plants were treated with ethephon (*E*), 2,4-D (*D*), GA_3 (*GA*), or a control solution (*C*) as described in the legend to Fig. 2. Samples were analyzed as described in Fig. 1

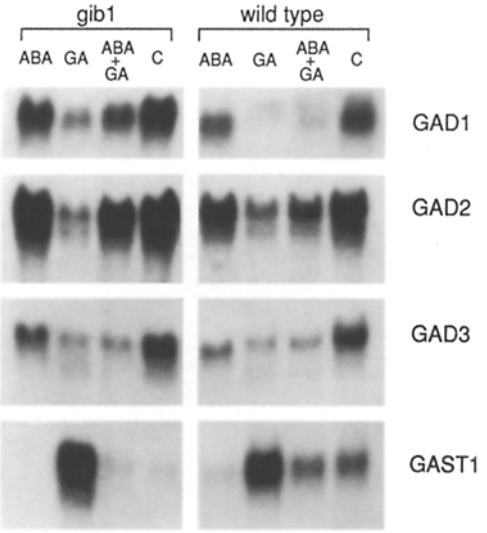

Fig. 4. The effect of ABA on the abundance of GAD RNAs. The petioles of detached leaves of wild-type or *gib1* tomato plants were incubated in solutions of 100 μM ABA (*ABA*), 50 μM GA_3 (*GA*), both ABA and GA_3 (*ABA + GA*) or in sterile water (*C*) for 12 h before harvesting. Samples were analyzed as described in Fig. 1

To test the interaction of ethephon, 2,4-D, and GA_3 with respect to their effect on GAD RNA levels, the effect of these plant growth regulators on the *Never ripe* (*Nr*) mutant of tomato was tested. This mutant, first described by Rick (1956), has been shown to be insensitive to treatment with ethylene with respect to a number of ethylene responses (Lanahan et al. 1994). Consistent with this conclusion, treatment of wild-type plants with either ethephon or 2,4-D caused severe leaf epinasty, but only treatment with 2,4-D caused epinasty in the *Nr* mutant (data not shown). Figure 3 shows that ethephon treatment decreased GAD RNA levels in the wild-type cultivar Pearson but not in *Nr* plants. In contrast, treatment with GA_3 or 2,4-D caused a decrease in the abundance of the GAD RNAs, both in wild type and in *Nr*. Thus, regulation of the level of GAD RNAs by GA_3 and 2,4-D is not affected by the *Nr* mutation.

Pena-Cortes et al. (1989) have shown that, although tomato plants do not respond well to treatment with ABA when the foliage is sprayed with ABA, leaves respond to ABA treatment if they are allowed to take up ABA solution through the cut petioles. Therefore, the effect of ABA on the expression of GAD RNAs was tested by placing the petioles of freshly cut tomato leaves in solutions containing ABA, ABA + GA_3, GA_3, or water alone. Figure 4 shows that treatment with 100 μM ABA had little effect on the level of the GAD1 and GAD2 RNAs, and that ABA slightly reduced the level of the GAD3 RNA. However, when ABA and GA_3 were applied in combination, ABA antagonized the action of GA_3, such that the levels of GAD1 and GAD2 RNAs were not reduced to the same extent. This effect was not observed, however, with RNA corresponding to GAD3. This effect of ABA on GAD1 and GAD2 RNAs was observed both in the *gib1* mutant and in the wild-type. GAST1 RNA was analyzed in these experiments as a control. As previously shown by Shi et al. (1992), the level of GAST1 RNA was substantially decreased by treatment with ABA, and ABA antagonized the GA_3-mediated increase in GAST1 RNA level (Fig. 4).

The effects of wounding on the expression of pin II and GAD RNAs in wild-type, GA-deficient, and ABA-deficient plants. As discussed below, GAD1 shows sequence similarity to pin II of tomato and potato. These proteinase inhibitors are known to be induced by wounding (Graham et al. 1986; Sanchez-Serrano et al. 1986). To test whether the GAD1 RNA is also wound inducible, blots containing RNA from wounded or unwounded wild-type plants were hybridized with probes corresponding to GAD1 and to a potato pin II gene (a probe derived from the pin IIK gene described in Thornburg et al. 1987, see *Materials and methods*). Figure 5 shows that GAD1 RNA was highly wound inducible, as is the RNA for the pin II gene (also see Graham et al. 1986). In addition, Fig. 5 shows that the level of GAD2 RNA was wound inducible, but to a lesser degree than GAD1 RNA, and that GAD3 RNA was not wound inducible.

Pena-Cortes et al. (1989) have shown that the response of the pin II RNA to wounding is severely reduced in ABA-deficient mutants. This result, coupled with the observations that GA regulates the expression of the wound-inducible genes GAD1 and GAD2, prompted us to compare the wound induction of pin II and GAD1 RNAs in wild-type, *gib1*, ABA-deficient (*sit* or *flacca*) mutant plants, and plants doubly mutant for *gib1* and *sit*. In different studies (Tal and Nevo 1973; Linforth et al. 1987), the *sit* and *flacca* mutants have been shown to contain 8–12% and 17–21% of the wild-type level of ABA, respectively. Under mild wounding conditions (see legend to Fig. 6), only *gib1* plants exhibited induction of pin II (Fig. 6A) or GAD1 (not shown). However, all genotypes

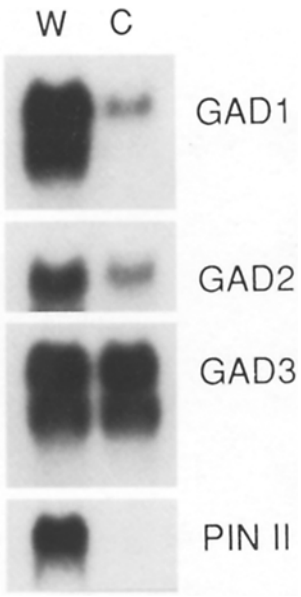

Fig. 5. The effect of wounding on the level of GAD RNAs. Wounding was performed by crushing leaves perpendicular to the midvein every 8 h with a hemostat (Graham et al. 1985; R. Thornburg, Iowa State University, Ames, Iowa, USA, personal communication). Wounds were made progressively closer to the leaf base at 5-mm intervals. Wounded leaves were harvested 24 h after the first wound was administered. RNA from wounded (*W*) or unwounded control leaves (*C*) was used for RNA blot analysis. RNA blots were probed with GAD1, 2 or 3 or a probe corresponding to a wound-inducible potato proteinase inhibitor II gene (*PIN II*). The signal below the band in the GAD2 panel (W lane) is from a previous probing of this filter with the GAD1 probe. GAD1 and GAD2 RNAs can easily be discriminated based on their difference in mobility. It is not known why the GAD3 probe occasionally detects a doublet (compare Fig. 5 with Figs. 1–4)

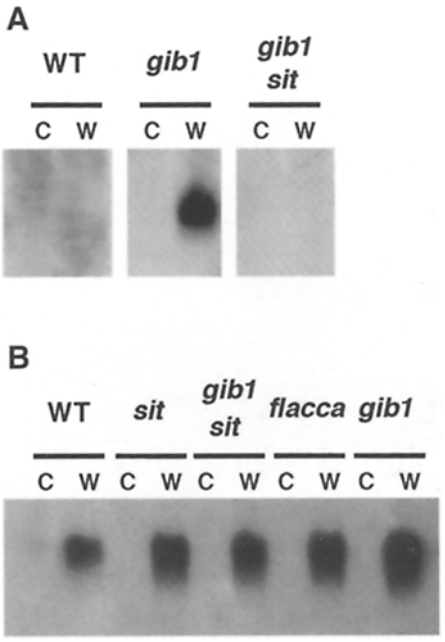

Fig. 6A, B. Wound induction of pin II RNA in GA- and ABA-deficient mutants. RNA from wounded (*W*) or unwounded control plants (*C*) from the indicated genotype was used for RNA blot analysis. *WT*, wild type plants; *gib1*, GA-deficient mutant; *sit* and *flacca*, ABA-deficient mutants. **A** Results from mild wounding conditions; plants were wounded once by crushing a leaf with a hemostat perpendicular to the midvien and half way across the blade, and wounded leaves were harvested 8 h later. **B** Results from severe wounding conditions (as described in Fig. 5)

showed an appreciable wounding response if the plants were wounded more severely (Fig. 6B). Thus, ABA-deficient plants are less sensitive to wounding than the wild-type (Pena-Cortes et al. 1989), while GA-deficient plants appear to be more sensitive.

Sequence analysis of GAD1. The 830-base pair (bp) GAD1 cDNA was found to be identical to a previously isolated cDNA (TR8) from the tomato cultivar VFN8 (Taylor et al. 1993). TR8 RNA was found to increase in abundance approximately 10-fold following auxin treatment of 12-d-old tomato seedlings, and was correlated with the induction of lateral root initiation. This induction was first detectable by 24 h post treatment, was maximal at 72 h post treatment, and was observed in roots and hypocotyls but not in cotyledons or epicotyls. This positive auxin regulation of GAD1 (TR8) in roots and hypocotyls contrasts with the negative regulation observed in this study. The two results are not inconsistent, however, since the present study only addresses GAD1 expression in mature leaves and stems.

The GAD1 (TR8) deduced polypeptide sequence shows similarity to a group of plant proteinase inhibitors. The GAD1 polypeptide shares approximately 47% identity with pin II from tomato (Graham et al. 1985) or potato (Keil et al. 1986; Thornburg et al. 1987), and approximately 57% identity with a stigma-specific proteinase inhibitor from *Nicotiana alata* (Atkinson et al. 1993). The putative GAD1 protein contains three 64-amino-acid domains which share between 89 and 97% amino-acid identity, and which each contains sequences thought to confer proteinase inhibitor specificity for trypsin (Taylor et al. 1993).

Sequence analysis of GAD2. The GAD2 cDNA is 858 bp in length including a 40-bp poly(A)$^+$ tail. Since the estimated size of the GAD2 RNA is 1500 nucleotides (see *Materials and methods*), this cDNA most likely does not correspond to a full-length RNA. The GAD2 cDNA has an open reading frame that begins at nucleotide 1 and extends to a stop codon at nucleotide 684. The deduced polypeptide encoded by this open reading frame is similar in amino-acid sequence to a group of 2-oxoglutarate-dependent dioxygenase enzymes. The highest similarity [44% amino-acid identity (calculated with the Fastdb program, IntelliGenetics, Inc.)] is with hyoscyamine 6-dioxygenase from *Hyoscyamus niger*, an enzyme involved in alkaloid biosynthesis (Matsuda et al. 1991). Figure 7 shows an alignment of the predicted GAD2 protein with amino-acid sequences of the five most closely related plant dioxygenase enzymes. Twenty-one amino acids are conserved in all six of the polypeptides, including two histidine residues and an aspartic acid residue (positions 100, 102, and 155 in the GAD2 sequence) which are thought to

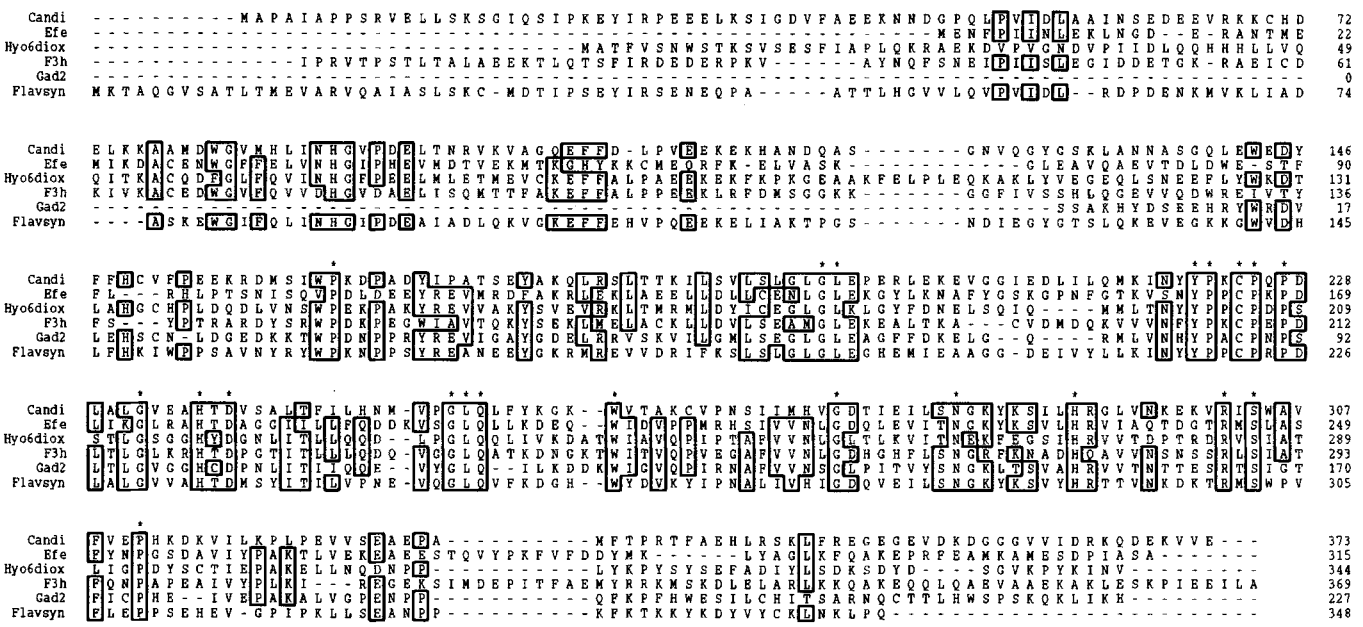

Fig. 7. Alignment of the predicted amino-acid sequence of the GAD2 cDNA with the predicted amino-acid sequence of the *CANDI* gene (*Candi*) of *Antirrhinum majus* (Martin et al. 1991; Weiss et al. 1993), ethylene-forming enzyme (*Efe*) from tomato (Kock et al. 1991), hyoscyamine 6-dioxygenase (*Hyo6diox*) from *Hyoscyamus niger* (Matsuda et al. 1991), flavanone 3-beta-hydroxylase (*F3h*) from petunia (Britsch et al. 1992), and flavonol synthase (*Flavsyn*) from petunia (Holton et al. 1993). Sequences were aligned using the pileup and prettyplot programs [Genetics Computer Group (1991), Madison, Wisconsin]. Identical residues are boxed if present in at least four of the six sequences. *Asterisks* denote invariant amino acids. Genebank accession number for GAD2 is U21800

be involved in the iron-binding site of these enzymes (Britsch et al. 1993).

Sequence analysis of GAD3. The 904-bp GAD3 cDNA has an open reading frame that begins at nucleotide 1 and extends to a stop codon at nucleotide 756. The deduced polypeptide encoded by this open reading frame is similar in amino-acid sequence to proteins belonging to the family of nonmetallo-short-chain alcohol dehydrogenases (Persson et al. 1991). The deduced GAD3 protein shares the highest level of amino-acid identity with the *TASSELSEED2* (*TS2*) gene of maize (39%) (DeLong et al. 1993) and with 20-beta-hydroxysteroid dehydrogenase from *Streptomyces exfoliatus* (38%) (Marekov et al. 1990). GAD3 also shows 33–34% amino-acid identity with dehydrogenase enzymes which operate on non-steroid substrates (Lampel et al. 1986; Sherman et al. 1989; Klein et al. 1992). Since the hydroxysteroid dehydrogenases do not contain conserved amino acids which are not present in the non-steroid dehydrogenases (Persson et al. 1991) it difficult to conclude that GAD3 acts on a steroid substrate. Figure 8 shows an alignment of GAD3 and TS2 with three bacterial hydroxysteroid dehydrogenases and three non-steroid dehydrogenases. Seventeen amino acids are conserved in all eight of the sequences, including residues which are thought be part of the NAD- or NADP-binding site (the three glycine residues at positions 9, 13, and 15 in the GAD3 sequence) and residues which are thought to participate in substrate binding and catalysis (serine at position 136, tyrosine at position 149, and lysine at position 153 in the GAD3 sequence) (Ghosh et al. 1991; Persson et al. 1991).

Discussion

This work describes the cloning and initial characterization of three cDNAs (GAD1, 2, and 3) which correspond to RNAs that become less abundant 2 h after treatment of *gib1* tomato shoot tissue with GA_3. The kinetics of GAD RNA suppression by GA_3 correlate closely with the kinetics of the changes in the abundance of in-vitro translation products previously reported in *gib1* shoots (Jacobsen et al. 1994). One class of in-vitro translation products was found to decrease in abundance by 6 h post GA treatment and remain decreased in abundance for at least 48 h (similar to the kinetics of GAD1 RNA), while a second class of in-vitro translation products was found to decrease in abundance by 6 h post GA treatment but become more abundant again by 24 or 48 h post treatment (similar to the kinetics of GAD2 and GAD3 RNAs).

In addition to being regulated by GA_3, the abundance of the GAD RNAs is also regulated by treatment with the synthetic auxin, 2,4-D, and the ethylene-releasing compound, ethephon. The regulation by 2,4-D and ethephon is likely to be gibberellin independent, since it occurs in both the wild type and in the *gib1* mutant. Similarly, the regulation by GA_3 and 2,4-D is observed both in the wild type and in the *Nr* mutant of tomato, which is insensitive to ethylene (Lanahan et al. 1994). This suggests that GA_3 and 2,4-D do not act by affecting ethylene levels or by affecting any steps in the ethylene signal transduction pathway occurring at or prior to the block caused by *Nr*. These results suggest that the three hormones GA, auxin and ethylene act independently to regulate the abundance of the GAD RNAs. Our results, however, do not preclude

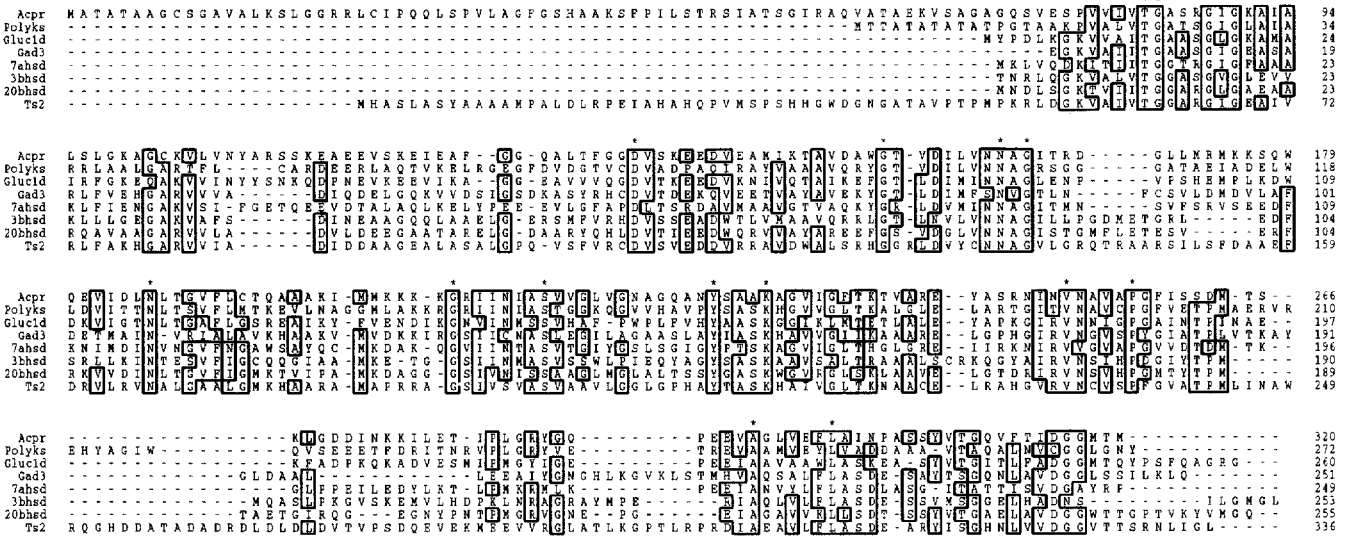

Fig. 8. Alignment of the predicted amino-acid sequence of the GAD3 cDNA with 3-oxoacyl-(acyl-carrier protein) reductase (*Acpr*) from *Cuphea lanceolata* (Klein et al. 1992), granaticin polyketide synthase (*Polyks*) from *Streptomyces violaceoruber* (Sherman et al. 1989), glucose 1-dehydrogenase (*Gluc1d*) from *Bacillus subtilis* (Lampel et al. 1986), 7-alpha-hydroxysteroid dehydrogenase (*7ahsd*) from *Eubacterium sp.* (Gopal-Srivastava et al. 1990), 3-beta hydroxysteroid dehydrogenase (*3bhsd*) from *Pseudomonas testosteroni* (Yin et al. 1991), 20-beta-hydroxysteroid dehydrogenase (*20bhsd*) from *Streptomyces exfoliatus* (Marekov et al. 1990), and the predicted amino-acid sequence of the *TASSELSEED2* (*Ts2*) gene of maize (DeLong et al. 1993). Identical residues are boxed if present in at least five of the eight sequences. *Asterisks* denote invariant amino acids. Genebank accession number for GAD3 is U21801

the possibility that the regulation of the GAD RNAs by GA and/or ethylene is accomplished through changes in auxin concentrations.

Treatment with the plant growth regulator ABA had only a minor effect on the level of the GAD RNAs when applied alone. However, when applied in combination with GA, ABA antagonized GA's affect on the abundance of the GAD1 and GAD2 RNAs. In a similar manner, ABA has been shown to antagonize the effect of GA_3 with respect to the suppression of a putative alcohol-dehydrogenase RNA and a putative alpha-amylase/protease inhibitor RNA in barley aleurone layers (Nolan and Ho 1988). Furthermore, the GA-suppressed barley embryo globulin 1 gene is known to be induced by ABA (Heck et al. 1993). Although these results imply a general role for ABA in antagonizing GA-induced suppression of RNA abundance, GA regulation of the GAD3 RNA was unaffected by ABA treatment (Fig. 4), suggesting that this interaction is not universal.

The nucleic-acid sequence of the GAD1 cDNA is identical to a previously identified cDNA, TR8 (Taylor et al. 1993). An increase in the abundance of RNA corresponding to TR8 was found 24 h after treatment of roots and hypocotyls with auxin, and correlated with the induction of lateral root formation (Taylor et al. 1993). Thus, in some tissues GAD1 RNA is up-regulated by auxin while in others it is down-regulated.

Analysis of RNA blots indicates that, like other proteinase inhibitor RNAs, the GAD1 RNA is highly wound inducible. Recent studies have determined that a number of compounds may be involved in the signal transduction pathway leading to the induction of pin II and other wound-inducible genes (Farmer and Ryan 1992). These include oligosaccharides, auxin, ABA, jasmonic acid, linolenic acid and an 18-amino-acid polypeptide, systemin. Hildmann et al. (1992) have shown that a number of wound-inducible genes are responsive to the hormone ABA and that these genes are not induced by wounding in a mutant which is deficient in ABA. This has led to the proposal that ABA may be central to the molecular mechanism by which wounding induces these genes (Hildmann et al. 1992; Pena-Cortes et al. 1989). Although previous work indicates that GA can block chitosan induction of the pin II gene of potato (Pena-Cortes et al. 1989), a role for GA in the regulation of other wound-inducible genes has not been suggested.

In contrast to previously identified proteinase inhibitors, GAD1 RNA is not significantly regulated by exogenously added ABA but is repressed by GA. In the same experiments in which ABA was shown to have no effect on the level of GAD1 RNA, ABA decreased the level of GAST1 RNA (Fig. 4) and also increased the level of RNA corresponding to the pin II gene (data not shown). GAD1 therefore exhibits regulation which is distinct from the wound-inducible genes characterized thus far, and may be useful in determining the general mechanism by which all wound-inducible genes are induced.

We have examined the expression of GAD1 and the pin II gene of tomato in the *gib1* mutant. These results indicate that in the *gib1* mutant, induction of both pin II RNA and GAD1 RNA is more sensitive to wounding than in wild-type plants (Fig. 6A and data not shown). Interestingly, this enhanced sensitivity is not observed in the *gib1*;

sit double mutant, indicating that normal ABA biosynthesis is required for this phenomenon. As mentioned above, Pena-Cortes et al. (1989) have shown that the response of the pin II RNA to wounding is severely reduced in the ABA-deficient *sit* mutant. Our studies using the *sit* and *flacca* mutants, indicate that pin II expression is induced at wild-type levels if plants are wounded severely (Fig. 6B). These results suggest that GA and ABA may act in an antagonistic fashion to determine the sensitivity of plants to wounding.

A model in which ABA and GA determine the sensitivity of plants to wounding is attractive because ABA and GA levels in the plant are affected by the amount of environmental stress experienced by the plant. Gibberellin decreases with increasing stress while ABA increases under these conditions (Bensen et al. 1990). Thus ABA and GA levels may act to regulate the magnitude of the response to fungal or herbivore attack such that stressed plants mount a very strong response while plants that are healthy, and perhaps more resistant to attack, mount a smaller response.

The sequence of the GAD2 cDNA suggests that the GAD2 gene encodes a 2-Oxoglutarate-dependent dioxygenase. 2-oxoglutarate-dependent dioxygenases perform many functions in the cell, including ethylene biosynthesis (Spanu et al. 1991), anthocyanin biosynthesis (Martin et al. 1991; Britsch et al. 1992; Holton et al. 1993; Weiss et al. 1993), alkaloid biosynthesis (Matsuda et al. 1991) and GA biosynthesis (reviewed in Graebe and Lange 1988). However, the predicted GAD2 protein only shares 30–44% amino-acid identity with enzymes known to be involved in ethylene, anthocyanin, or alkaloid biosynthesis (Fig. 7), and only about 30% identity with the recently isolated gibberellin 20 oxidase (Lange et al. 1994). It is therefore difficult to predict the specific function of GAD2, and hence to understand the relevance of its regulation by GA.

The predicted GAD3 amino-acid sequence is similar to that of proteins belonging to the family of nonmetallo-short-chain alcohol dehydrogenases. These NAD- or NADP-linked oxidoreductase enzymes are involved in many aspects of metabolism, including glucose, steroid, and fatty-acid metabolism (Persson et al. 1991). Thus, sequence comparisons do not predict a specific biochemical function for the putative GAD3 protein. Although it is interesting that Nolan and Ho (1988) found that a putative alcohol dehydrogenase mRNA was decreased in abundance following GA treatment of barley aleurone layers, the significance of the GA regulation of these dehydrogenases is unclear.

The long-term goal of these studies is to understand the molecular mechanism of GA action. Further studies of the structure and regulation of the GAD genes may help to elucidate the mechanisms by which GA coordinately regulates these three genes.

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