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Vacuolar H^+-ATPase Is Expressed in Response to Gibberellin during Tomato Seed Germination[1]

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Completion of germination (radicle emergence) by gibberellin (GA)-deficient (*gib-1*) mutant tomato (*Lycopersicon esculentum* Mill.) seeds is dependent upon exogenous GA, because weakening of the endosperm tissue enclosing the radicle tip requires GA. To investigate genes that may be involved in endosperm weakening or embryo growth, differential cDNA display was used to identify mRNAs differentially expressed in *gib-1* seeds imbibed in the presence or absence of GA_{4+7}. Among these was a GA-responsive mRNA encoding the 16-kD hydrophobic subunit c of the V_0 membrane sector of vacuolar H^+-translocating ATPases (V-ATPase), which we termed *LVA-P1*. *LVA-P1* mRNA expression in *gib-1* seeds was dependent on GA and was particularly abundant in the micropylar region prior to radicle emergence. Both GA dependence and tissue localization of *LVA-P1* mRNA expression were confirmed directly in individual *gib-1* seeds using tissue printing. *LVA-P1* mRNA was also expressed in wild-type seeds during development and germination, independent of exogenous GA. Specific antisera detected protein subunits A and B of the cytoplasmic V_1 sector of the V-ATPase holoenzyme complex in *gib-1* seeds only in the presence of GA, and expression was localized to the micropylar region. The results suggest that V-ATPase plays a role in GA-regulated germination of tomato seeds.

Tomato (*Lycopersicon esculentum* Mill.) seeds are a useful model system to investigate the physiological and molecular basis of germination (Bewley, 1997a; Hilhorst et al., 1998). The tomato embryo is completely enclosed in a hard, thick-walled lateral endosperm surrounded by the testa. The mechanical restraint of the thinner-walled micropylar endosperm tissue opposite the radicle tip (termed the endosperm cap) is the primary determinant of when or whether radicle emergence occurs (Groot and Karssen, 1987, 1992; Dahal and Bradford, 1990; Ni and Bradford, 1993). The expansive force exerted by the embryo is also important for radicle emergence, but under well-hydrated conditions the embryo is generally capable of expansion if the endosperm cap is removed (Dahal and Bradford, 1990; Groot and Karssen, 1992; Nonogaki et al., 1992). The physical weakening of the endosperm cap tissue to allow radicle emergence is dependent upon gibberellin (GA) (Groot and Karssen, 1987). Endosperm cap weakening is accompanied by an increase in the activity of cell wall hydrolytic enzymes, including endo-β-mannanase (Groot et al., 1988; Nomaguchi et al., 1995; Nonogaki and Morohashi, 1996; Voigt and Bewley, 1996; Dahal et al., 1997; Nonogaki et al., 1998), cellulase (Sánchez et al., 1985; Leviatov et al., 1995), polygalacturonase (Sitrit et al., 1999), arabinosidase, β-1,3-glucanase, and chitinase (Bradford et al., 2000). In addition, the loss of lipid and protein bodies and cellular vacuolization occurs initially in the radicle tip and endosperm cap tissues prior to radicle emergence (Mella et al., 1995; Nonogaki et al., 1998). Thus, enzymes related to protein and lipid reserve mobilization are likely to be expressed in these tissues as well (e.g. Comai et al., 1992).

The transition from seed development to germination is accompanied by a corresponding change in gene expression patterns (Kermode, 1995; Bewley, 1997b; Holdsworth et al., 1999). While some apparent housekeeping genes are expressed throughout, the majority of genes expressed during germination are distinct from those expressed during development (Hughes and Galau, 1989; Kermode, 1990; Berry and Bewley, 1991). For example, differentially expressed genes potentially related to seed germination or dormancy have been identified in wheat (*Triticum aestivum*; Morris et al., 1991), cheatgrass (*Bromus secalinus*; Goldmark et al., 1992), wild oat (*Avena fatua*; Johnson et al., 1995; Li and Foley, 1995; Jones et al., 1997), beech (*Fagus sylvatica* L.; Nicolás et al., 1997), and Arabidopsis (Haslekås et al., 1998). Much is known about germination-specific genes associated with reserve mobilization during seedling growth (Jacobsen et al., 1995; Kermode, 1995), but less information is available on genes functionally related to the initial processes leading to endosperm weakening or embryo growth that result in the completion of germination (Bewley, 1997b; Bradford et al., 2000).

To identify molecular and biochemical events occurring early in germination prior to radicle emergence, we have used differential cDNA display (Liang and Pardee, 1992;

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Liang et al., 1993) to analyze mRNAs isolated from wild-type and GA-deficient mutant tomato seeds (Koornneef et al., 1981) imbibed in water or in solutions containing GA_{4+7}. Endosperm weakening and radicle emergence of *gib-1* seeds is strictly dependent upon exogenous GA (Groot and Karssen, 1987; Ni and Bradford, 1993), so we anticipated that genes functionally related to these processes would be differentially expressed in response to GA_{4+7}. We report here on the characterization of a GA-responsive and tissue-specific transcript encoding the membrane-spanning subunit c of the vacuolar H^+-translocating ATPase (V-ATPase) that is expressed in tomato seeds prior to radicle emergence. Other subunits of the V-ATPase holoenzyme complex are also induced by GA in *gib-1* seeds specifically in the micropylar tissues. Possible roles for V-ATPase activity in the early stages of tomato seed germination are discussed.

MATERIALS AND METHODS

Plant Materials and Seed Germination Conditions

Tomato (*Lycopersicon esculentum* Mill.) seeds were harvested from field-grown wild-type cv Moneymaker (MM) plants or from homozygous GA-deficient (*gib-1*) mutant plants grown in a greenhouse (seeds originally obtained from Dr. Cees Karssen, Wageningen Agricultural University, The Netherlands). Plant culture and seed extraction were as described previously (Ni and Bradford, 1993). For germination, approximately 200 seeds were incubated at 25°C in the dark in 9- × 100-mm-diameter Petri dishes on top of two layers of blotter paper moistened with 12 mL of either distilled, deionized water or 100 μM GA_{4+7} (Abbott Laboratories, Chicago).

Differential cDNA Display Analysis

Using differential cDNA display analysis (DCD) (Liang and Pardee, 1992), mRNA from the radicle tips or endosperm caps of *gib-1* mutant seeds imbibed in water (which do not germinate) were compared with mRNA from the same tissues of *gib-1* seeds imbibed in 100 μM GA_{4+7} (in which radicle emergence begins at 36 to 40 h and is completed by most seeds within 60 h). Seeds in both the presence and absence of GA_{4+7} were sampled at 40 h, excluding any seeds from which the radicle had emerged. The micropylar regions were excised from 100 seeds in each treatment and separated into endosperm caps and radicle tips (see Fig. 2 for diagram). Tissues were frozen immediately in liquid nitrogen (LN_2) and stored at −80°C. Frozen radicle tips or endosperm caps were pulverized in LN_2 and RNA was extracted and purified by the phenol/SDS method (Ausubel et al., 1987). Prior to use, aliquots of RNA were incubated with DNase I for 1 h at 37°C in digestion buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 20 units of RNasin, and 10 mM NaCl) followed by extraction with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1). The RNA was precipitated in ethanol and dissolved in the original volume of 2 mM EDTA.

Nine 3′ anchor primers were synthesized as 5′-T_{12}MM-3′, where M is A, G, or C. Anchor primers were then combined at equal concentration to give three sets: T_{12}MA, T_{12}MG, and T_{12}MC. These three anchor pools were used in reverse transcription reactions (Sambrook et al., 1989) and then in conjunction with eight specific 10-mers (A_{01}-A_{08}, Operon Technologies, Alameda, CA) in the differential display PCR reactions (Liang and Pardee, 1992). Two microliters from the RT reaction were used in each of the subsequent 20 μL differential display reactions (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 μM dNTPs, 1 μM of each anchor primer, 0.2 μM 10-mer primer, 1 unit of AmpliTaq (Perkin-Elmer Cetus, Foster City, CA), 4 μCi [α-^{35}S]-dATP (1200 Ci/mmol). Cycling conditions were 30 s at 94°C, 2 min annealing at 40°C, and 30 s extension at 72°C for 40 cycles in a thermal cycler (model 480, Perkin-Elmer Cetus). PCR reactions were loaded onto a 40-cm × 40-cm × 0.4-mm 6% (w/v) native polyacrylamide gel and electrophoresed at 40 W.

Selected cDNA fragment bands exhibiting differential amplification were cut from the DCD gels and recovered by boiling for 30 min in 50 μL of modified Tris-EDTA (TE) (10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA). The cDNAs were precipitated in ethanol in the presence of 10 μg of linear acrylamide as a carrier (Gaillard and Strauss, 1990) and dissolved in 20 μL of TE. Five microliters of each isolated cDNA was re-amplified using the same conditions as the first amplification except that the (now unlabeled) dNTP concentration was increased to 20 μM.

Fragments re-amplified from DCD gels were tested for differential expression using the reverse-northern technique. Four identical blots of electrophoresed DNA fragments were probed with labeled cDNA products from reverse transcription reactions using 10 μg of total RNA from endosperm caps or radicle tips of seeds imbibed for 40 h in water or 100 μM GA_{4+7} as template and labeled using 200 μM dNTP and 100 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol) per 50-μL reaction.

PCR fragments selected on the basis of the reverse-northern results were ligated into the TA cloning vector pCRII (Invitrogen, San Diego) and the resulting plasmids electroporated (Cooley et al., 1991) into competent *Escherichia coli* JM109 cells (Stratagene, La Jolla, CA) using an electroporator (Gene Pulser, Bio-Rad, Hercules, CA). The cloned DCD fragments were sequenced at the University of California, Davis, Advanced Plant Genetics Facility on a DNA sequencer (ABI Prism 377, Perkin-Elmer).

Northern Analyses

Total RNA was extracted as described above from intact wild-type MM or *gib-1* mutant seeds, isolated endosperm caps, or radicle tips, and the rest of the seed incubated in water or in 100 μM GA_{4+7} for the indicated times at 25°C. Additionally, total RNA was isolated from the flowers, leaves, and roots of mature MM tomato plants. Total RNA (2–20 μg per lane) was electrophoresed (Sambrook et al., 1989), transferred onto positively charged nylon membranes, and UV crosslinked at 120,000 μJ cm^{-2} on a Stratalinker (FB-UVXL-1000, Fisher Scientific, Santa Clara,

CA). Hybridization was detected using DIG-labeled RNA probes (Boehringer Mannheim, Indianapolis) synthesized by either Sp6 (Ambion, Austin, TX) or T7 (Pharmacia Biotech, Alameda, CA) RNA polymerase. Detection of DIG-labeled probes was performed according to instructions in the Genius System (Boehringer Mannheim, 1995) using disodium 3-(4-methoxyspiro[1, 2-dioxetane-3, 2′-{5′-chloro}tricyclo{3.3.1.1^{3,7}}decan]-4-yl) phenyl phosphate as substrate.

cDNA Library Screening

Transcripts hybridizing to PCR fragment *G21*, which were differentially expressed in seeds in response to GA, were shown by northern analysis to be expressed in tomato roots as well as in seeds. Therefore, 5×10^5 recombinants from a tomato root cDNA library in the plasmid pCGN1703 (Ewing et al., 1990) were screened with *G21* labeled in a random-priming reaction with [α-^{32}P]dATP. cDNA from hybridizing recombinants was recovered from the vector by restriction digestion with *Sma*I, and subcloned into the *Sma*I site in the polycloning region of pBSII KS (Stratagene). The full-length cDNA hybridizing to *G21*, subsequently termed *LVA-P1*, was sequenced at the Center for Engineering Plants for Resistance Against Pathogens (CEPRAP), University of California, Davis.

Protein Extraction and Western Blotting

Two grams of *gib-1* mutant tomato seeds imbibed in water or 100 μM GA_{4+7} for 36 h were homogenized for 1 min (Ultra-Turrax T25, Janke and Kunkel IkA Labortechnik, Staufel, Germany) at high speed in 5 mL of extraction buffer (70 mM Tris, pH 8.0, 250 mM Suc, 3 mM EDTA, 0.5% [w/v] PVP-40, 0.1% [w/v] bovine serum albumin [BSA], and 4 mM dithiothreitol). The slurry was filtered through cheesecloth, the filtrate was centrifuged at 12,000*g* for 15 min at 4°C, and the supernatant was again centrifuged at 113,000*g* for 30 min at 4°C. The resulting pellet was resuspended in buffer (10 mM Tris/2-(N-morpholino)-ethanesulfonic acid [MES] pH 7.0, 250 mM Suc, and 1 mM dithiothreitol) and electrophoresed on 12% [w/v] SDS-PAGE (20 μg of total protein per lane). The proteins were transferred to nitrocellulose and detected as described below for western tissue prints.

Northern and Western Tissue Printing Protocols

For tissue printing (Reid et al., 1992), tomato seeds were sliced longitudinally with a fresh razor blade. Each half-seed was pressed firmly in identical positions on separate nitrocellulose membranes for exactly 60 s (northerns) or 20 s (westerns) and then removed with forceps, providing two mirror-image prints of the same seed. The entire procedure was performed using powder-free gloves.

For northern prints, the membranes were UV crosslinked and treated with 10 units of RNase-free DNase I (Pharmacia Biotech) in 10 mL of digestion buffer for 1 h at 37°C in a roller tube. Subsequent steps in prehybridization, hybridization, washing, and detection were as described for the DIG-labeled northern analyses (see above) except that the signal was detected using disodium 4-chloro-3-(4-methoxyspiro{1, 2-dioxetane-3, 2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CDP-Star, Boehringer Mannheim) in conjunction with enhancer for chemiluminescent detection on nitrocellulose membranes (Tropix, Bedford, MA). One membrane was hybridized with an antisense probe to detect the target mRNA, while its mirror-image print was hybridized with a sense probe to control for nonspecific binding. Both membranes were then stripped and rehybridized with antisense and sense cDNAs complementary to a constitutively expressed mRNA coding for a ribosomal protein (*G46*) as a control for RNA bound to the membrane.

For western prints, the printed membranes were blocked using 1% (w/v) BSA in 1× Tris-buffered saline (TBS), washed four times for 5 min each in 1× TBS-Tween (Sambrook et al., 1989), and then incubated with primary antibody diluted 1:4,000 in 1% (w/v) BSA in 1× TBS. Rabbit polyclonal antibodies specific for either subunit A (68 kD) or subunit B (57 kD) of V-ATPase from mung bean (*Vigna radiata*) were provided by Dr. Masayoshi Maeshima (Matsuura-Endo et al., 1992). The membranes were subsequently washed in 1× TBS-Tween and incubated with a 1:10,000 dilution of goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody. After washing, the membranes were developed using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim).

RESULTS

Cloning of a V-ATPase Subunit c Gene from Germinating Tomato Seeds

Pools of total RNA from *gib-1* tomato radicle tips and endosperm caps were isolated separately after imbibition in either water or 100 μM GA_{4+7} for 40 h, or just prior to the initiation of radicle emergence in the presence of GA_{4+7} (radicle emergence does not occur in water). cDNA fragments identified by DCD as being differentially expressed were confirmed by northern analysis. One such fragment (*G21*) identified a transcript that increased in abundance in both endosperm caps and radicle tips in the presence of GA_{4+7} and was present in untreated roots (data not shown). This fragment was used to recover a homologous full-length cDNA from a tomato root cDNA library. The deduced amino acid sequence is highly homologous to that of the 16-kD hydrophobic subunit c that forms the membrane-spanning, proton conductance pathway of plant vacuolar H^+-translocating ATPases (Fig. 1; Stevens and Forgac, 1997; Sze et al., 1999).

The tomato cDNA sequence showed the following percentage amino acid identities (nucleotide identities in parentheses) to other V-ATPase c subunits: 98.2% (84%) to Arabidopsis (accession no. L44581; Perera et al., 1995); 98.2% (85%) to *Gossypium hirsutum* (U13669; Hassenfratz et al., 1995); and 95.8% (79%) to *Avena sativa* (M73232; Lai et al., 1991). We have therefore named this cDNA *LVA-P1* (*Lycopersicon* Vacuolar ATPase-Proteolipid 1), in analogy

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TTCCCCCCTGAACCCCCCGGTGAGGGAGGAGAAAGAGATCTATTCAACTCCAAAACTCAA   60
AATTCTCAGATCCAAATCCTAACGAAAATGTCGAACTTTGCCGGAGATGAAACTGCTCCC  120
                          M  S  N  F  A  G  D  E  T  A  P      11

TTCTTCGGCTTCCTTGGCGCCGCCGCTGCCCTCGTCTTCTCATGTATGGGGGCAGCTTAT  180
F  F  G  F  L  G  A  A  A  A  L  V  F  S  C  M  G  A  A  Y    31

GGAACAGCAAAGAGTGGTGTTGGAGTGGCGTCAATGGGAGTGATGAGGCCAGAGTTGGTG  240
G  T  A  K  S  G  V  G  V  A  S  M  G  V  M  R  P  E  L  V    51

ATGAAGTCCATTGTGCCAGTGGTTATGGCTGGTGTGTTAGGTATTTATGGCTTGATTATT  300
M  K  S  I  V  P  V  V  M  A  G  V  L  G  I  Y  G  L  I  I    71

GCTGTGATCATCAGTACTGGGATTAACCCCAAAACAAAGTCGTATTACCTATTTGATGGC  360
A  V  I  I  S  T  G  I  N  P  K  T  K  S  Y  Y  L  F  D  G    91

TATGCTCATCTCTCATCTGGTCTTGCTTGTGGTCTTGCTGGTCTTTCTGCTGGAATGGCT  420
Y  A  H  L  S  S  G  L  A  C  G  L  A  G  L  S  A  G  M  A   111

ATTGGTATTGTTGGAGATGCTGGTGTTAGGGCTAATGCACAACAACCCAAGCTTTTTGTC  480
I  G  I  V  G  D  A  G  V  R  A  N  A  Q  Q  P  K  L  F  V   131

GGAATGATCCTCATTCTCATTTTCGCTGAAGCCTTGGCTCTTTATGGGCTTATTGTTGGC  540
G  M  I  L  I  L  I  F  A  E  A  L  A  L  Y  G  L  I  V  G   151

ATTATCTTGTCTTCCCGAGCTGGGCAGTCTAGAGCCGAGTGAAGTTAACTCCATTCTTAC  600
I  I  L  S  S  R  A  G  Q  S  R  A  E  *                     164

CGCACTGTGTGTGGTTTCTGAAGACCAAGACAGCTAAAGCCTAAAGTCAGAGATCTAATA  660
TGTGTATTGTTATTCATGACACCACAGCTGCCACTTTTCGTGTTATGATCTGTTTGTAGA  720
GTAGGAATTCTCTTTTTTCTACTTAATAATAGCTTAAAGAGCTGTGCAATTGGTCTGTAT  780
TTTTGTGTATTTGCACTCATTATTTGTGGACAGTTGAGAACTATTTATTTTCTAAGATTG  840
TGCACGTATGAACCACTCTTCATCTATATACCACCATGGTTATTCTCAAAAAAAAAAAAA  900
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Figure 1. Nucleotide and amino acid sequences of *LVA-P1* cDNA. Nucleotide and amino acid (bold) numbers are indicated on the right. The open reading frame beginning with the ATG at nucleotides 88 to 90 is in shown in bold, with the deduced amino acid symbols indicated below each codon. The predicted amino acid sequence is >98% identical to vacuolar H^+-ATPase subunit c sequences from other dicot species (see text). The asterisk indicates the stop codon. The underlined 3′ region is the *G21* fragment isolated by differential cDNA display and used to obtain the full-length *LVA-P1* cDNA. The nucleotide sequence has been entered into the GenBank database under accession no. AF010228.

with *AVA-P1* and related genes in Arabidopsis (Perera et al., 1995). The highly conserved amino acid sequence among species (including over 60% identity to corresponding mouse [M64298] and yeast [L07105] genes) and the >98% amino acid sequence identity of *LVA-P1* to other dicot V-ATPase subunit c genes leave little doubt that *LVA-P1* is a tomato homolog of this gene.

LVA-P1 Expression Patterns during Germination

To determine the expression pattern of *LVA-P1*, total RNA was extracted from endosperm caps, radicle tips, and the rest of *gib-1* seeds (includes the lateral endosperm and most of the embryo) after imbibition for 1, 12, 24, and 40 h in water or in 100 μM GA_{4+7}, and hybridized with full-length *LVA-P1* cDNA (Fig. 2). In the presence of GA_{4+7}, *LVA-P1* transcript accumulated preferentially in the micropylar region of the seed (endosperm cap and radicle tip) within 12 h of imbibition. Subsequently, *LVA-P1* mRNA abundance in the endosperm caps declined by 40 h but remained high in the radicle tips. Approximately 30% of the seeds had completed radicle emergence by 40 h, although RNA was extracted only from ungerminated seeds. Transcripts hybridizing to *LVA-P1* were also detected in wild type MM flowers, leaves, and roots (Fig. 2).

Previous results have documented that a wide range of enzyme activities can exist among individual seeds, even in homozygous inbred tomato lines (Still and Bradford, 1997; Still et al., 1997). Thus, mRNA extracted from pooled seed samples may not accurately reflect individual seed responses to GA. We therefore utilized tissue printing to assay mRNA abundance on a single-seed basis. Individual

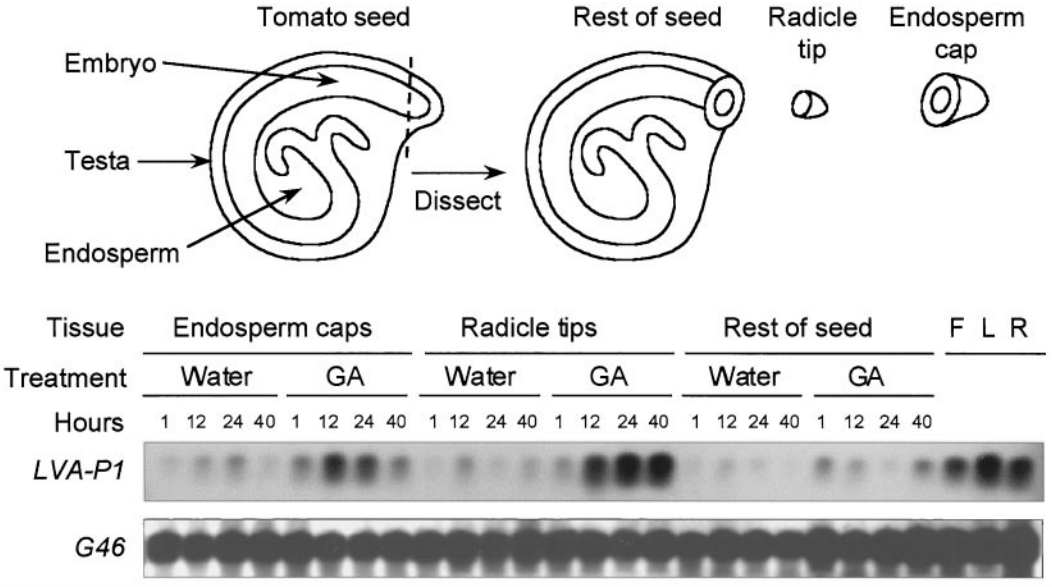

Figure 2. Northern blot of total RNA from GA-deficient *gib-1* mutant tomato seeds and from flowers (F), leaves (L), and roots (R) of isogenic wild-type MM plants. After 1, 12, 24, and 40 h of imbibition in either water or 100 μM GA_{4+7}, the micropylar region was dissected from ungerminated seeds as indicated by the dashed line in the diagram and further separated into endosperm caps, radicle tips, and the rest of the seed (remaining embryo, endosperm, and testa). Gel blots (3 μg of total RNA per lane) were hybridized with DIG-labeled RNA antisense probes to *LVA-P1* or *G46* (constitutively expressed RNA loading control).

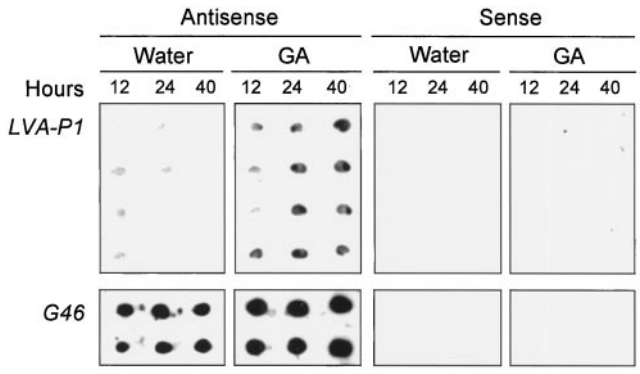

Figure 3. Northern tissue prints revealing mRNA abundance in individual tomato seeds. Tomato seeds of the *gib-1* mutant were imbibed for 12, 24, or 40 h on either water or 100 μM GA_{4+7}. Individual seeds were sliced along their median longitudinal plane, and both cut surfaces were printed on separate nitrocellulose membranes, then hybridized to either antisense or sense DIG-labeled riboprobes for *LVA-P1*. The lack of hybridization with the sense probes for *LVA-P1* and *G46* (right two membranes) indicates little nonspecific hybridization to the prints. The hybridization of the antisense *LVA-P1* riboprobe (top left membranes) is consistent with that found with northern blots of extracted RNA (Fig. 2), including the greater expression at the micropylar end of the seed (only four seeds of each treatment are shown at each time of a larger number printed). Subsequent hybridization with the antisense probe for the constitutive *G46* mRNA shows good transfer of RNA to the membranes (bottom left).

seeds were bisected after various times of imbibition and each mirror-image half was printed in an ordered array on separate nitrocellulose membranes. The membranes were treated with DNase, then hybridized with riboprobes made from either the antisense or the sense strand of the cDNA. This tissue printing method was specific, with little or no hybridization detected with riboprobes made from the sense strands of *LVA-P1* or *G46* (Fig. 3). Hybridization of the antisense riboprobe to the constitutive *G46* mRNA, however, showed that approximately equal amounts of RNA were bound to the membrane by each seed (Fig. 3). Hybridization of the antisense *LVA-P1* riboprobe revealed an increase in *LVA-P1* mRNA abundance only in the presence of GA_{4+7}. Furthermore, in most seeds, *LVA-P1* mRNA was most abundant in the micropylar region (Fig. 3 and additional data not shown), in agreement with the pattern inferred from the northern blots of pooled samples (Fig. 2).

As *LVA-P1* expression in *gib-1* seeds was dependent upon GA_{4+7}, transcript abundance was investigated in wild-type MM seeds, which do not require additional GA for germination. *LVA-P1* mRNA was present during seed development but declined in seeds from fruits at the mature green and breaker stages of development, before increasing again in seeds from ripe fruit (0 h of imbibition) (Fig. 4). Following imbibition, whole seeds were sampled every 12 h and separated into germinated and ungerminated seeds at 48 and 60 h. *LVA-P1* mRNA content changed relatively little prior to radicle emergence and remained abundant in germinated seeds (Fig. 4). As was observed for *gib-1* seeds in the presence of GA_{4+7} (Fig. 2), *LVA-P1* mRNA was most abundant in the micropylar tissues (data not shown). Imbibition of MM seeds in 100 μM GA_{4+7} had no additional effect on mRNA abundance (data not shown), indicating that the endogenous GA content of wild-type seeds is sufficient to saturate the response.

GA-Dependent Expression of V-ATPase Protein Subunits in *gib-1* Seeds

As GA stimulated the expression of *LVA-P1* mRNA in *gib-1* seeds (Figs. 2 and 3), we tested whether other protein subunits associated with the V-ATPase complex were induced as well. The membrane-spanning hydrophobic subunit c coded by *LVA-P1* is difficult to extract and detect with antibodies, so western blots of proteins from *gib-1* seeds imbibed in either water or GA_{4+7} were performed using antibodies specific to the major 68-kD catalytic nucleotide-binding subunit A and the 57-kD noncatalytic nucleotide-binding subunit B of mung bean V-ATPase (Matsuura-Endo et al., 1992; Maeshima et al., 1994). The V-ATPase complex requires the membrane-spanning subunit c for assembly of the V_0 membrane sector, to which the cytoplasmic V_1 sector containing subunits A and B is attached (Sze et al., 1999). Thus, detection of both A and B subunits is likely to be a good indicator of the presence of the holoenzyme, including the subunit c protein. Protein bands of the expected size increased in intensity in extracts from *gib-1* seeds that had been imbibed in GA_{4+7} (Fig. 5). No other proteins were detected, so the antibody for the 57-kD subunit B was used with tissue prints to determine the localization of the V-ATPase within the seed. In agreement with the expression pattern of *LVA-P1* mRNA (Figs.

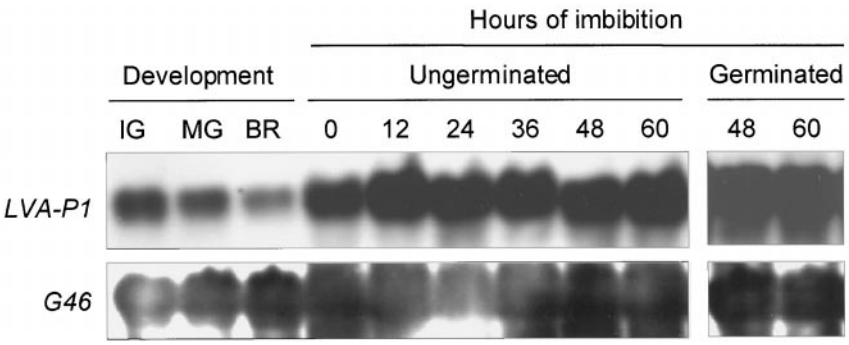

Figure 4. Northern blots of wild-type MM tomato seed RNA (20 μg per lane) probed with DIG-labeled antisense RNA probes to *LVA-P1* and *G46*. Seeds were obtained from tomato fruits classified as immature green (IG), mature green (MG), breaker (B), and red ripe (0-h imbibed). Seeds from ripe fruit were imbibed for 12, 24, 36, 48, or 60 h on water. After 36 h, ungerminated and germinated seeds were analyzed separately.

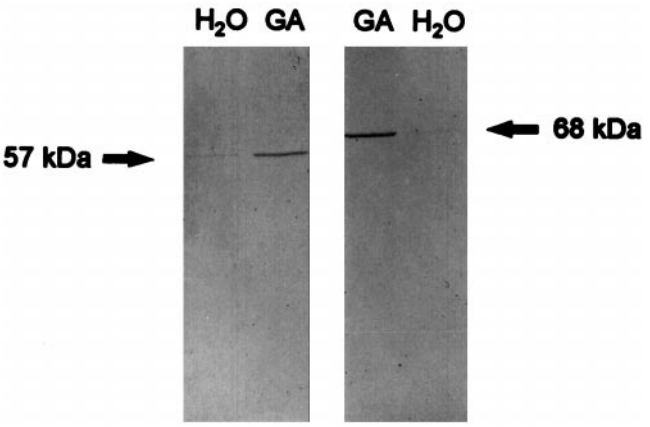

Figure 5. Western blots of proteins extracted from *gib-1* mutant tomato seeds after 36 h of imbibition in either water or 100 μM GA_{4+7}. Proteins (10 μg) were loaded in each lane of a 12% (w/v) SDS-PAGE gel. After transfer to nitrocellulose, the membrane was blotted and challenged with primary antibody to either the 57-kD subunit B (left lanes) or the 68-kD subunit A (right lanes) of mung bean vacuolar H^+-ATPase.

2, 3), the subunit B protein was most abundant in the micropylar region of GA-treated *gib-1* seeds, particularly in the endosperm cap (Fig. 6B), while seeds imbibed in water exhibited only background staining (Fig. 6A).

DISCUSSION

In many species, GA is required to stimulate germination, presumably via the induction of germination-specific genes (Hilhorst and Karssen, 1992; Jacobsen et al., 1995). In tomato and other Solanaceae, GA, apparently derived from the embryo, triggers weakening of the micropylar endosperm cap and promotes radicle emergence (Watkins and Cantliffe, 1983; Groot and Karssen, 1987; Ni and Bradford, 1993; Sánchez and de Miguel, 1997). We used DCD to identify transcripts that are differentially expressed in endosperm caps or radicle tips of GA-deficient *gib-1* mutant tomato seeds in the presence or absence of GA. By using only radicle tips and endosperm caps, we expected to increase the likelihood of identifying GA-responsive genes expressed in the micropylar region controlling radicle emergence, rather than genes involved in other GA-dependent processes in the rest of the seed. This strategy was successful, allowing us to identify a number of GA-regulated genes that are expressed in tomato seeds prior to radicle emergence (Bradford et al., 2000).

One mRNA detected by DCD as being enhanced by GA in micropylar tissues of *gib-1* seeds encodes the 16-kD subunit c of the V-ATPase, termed *LVA-P1* (Fig. 1). This gene is highly conserved among plant, fungal, and animal species (Stevens and Forgac, 1997; Sze et al., 1999), and *LVA-P1* shares greater than 98% predicted amino acid sequence identity with the corresponding genes of other dicot species. Subunit c proteins are highly hydrophobic and form the transmembrane channel of the V_0 sector of the V-ATPase responsible for proton translocation across the membrane (Stevens and Forgac, 1997; Sze et al., 1999). Both northern blots of extracted RNA (Fig. 2) and northern tissue prints of individual seeds (Fig. 3) showed that *LVA-P1* mRNA abundance is low in mature *gib-1* seeds, is up-regulated by GA during imbibition and is localized primarily in the micropylar tissues (endosperm cap and radicle tip). *LVA-P1* mRNA was also present in wild-type seeds during development and decreased as seeds approached maturity (Fig. 4). However, unlike *gib-1* seeds, *LVA-P1* mRNA content was high in mature dry wild-type seeds and remained high during imbibition and germination (Fig. 4). The endogenous GA content of wild-type seeds is apparently sufficient to maintain expression of this gene, and additional GA_{4+7} had no further effect. However, both the low abundance of *LVA-P1* mRNA in mature *gib-1* seeds and the increase following exposure to GA showed that *LVA-P1* expression is dependent upon endogenous GA. To our knowledge, this is the first demonstration that GA regulates expression of a V-ATPase subunit gene.

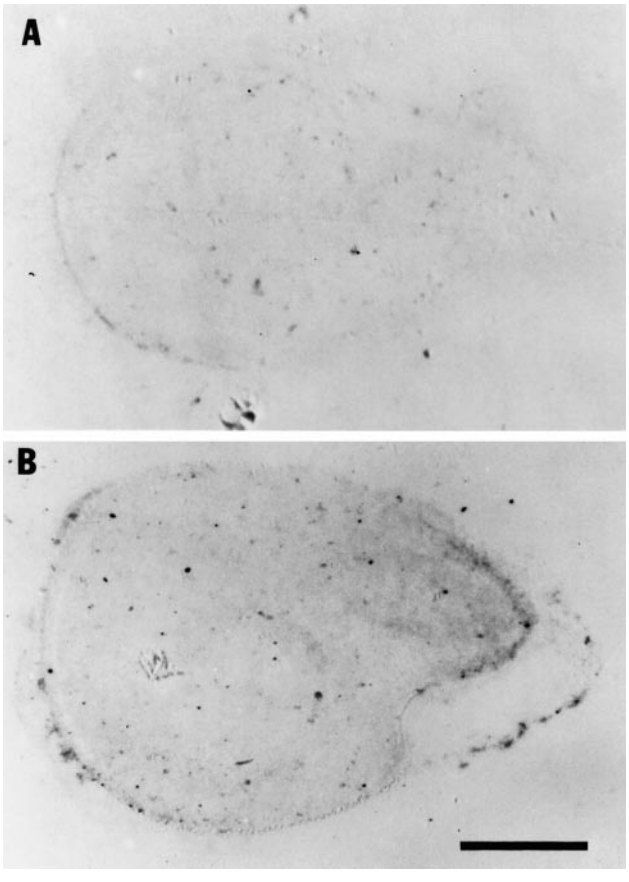

Figure 6. Western tissue prints of *gib-1* mutant tomato seeds imbibed for 36 h on either water (A) or 100 μM GA_{4+7} (B). Seeds were bisected longitudinally, printed on nitrocellulose paper, and processed with antiserum specific for the 57-kD subunit B of the mung bean vacuolar H^+-ATPase. Only diffuse background staining is evident in the water-imbibed seeds (A), while seeds imbibed in GA_{4+7} show more intense staining localized at the micropylar end of the seed, particularly in the endosperm cap tissue (B). Bar in B represents 1 mm.

Other environmental, developmental, and hormonal factors are known to influence V-ATPase subunit c gene expression. In *Mesembryanthemum crystallinum*, salt stress resulted in tissue-specific and age-dependent increases in V-ATPase subunit c mRNA levels (Löw et al., 1996; Tsiantis et al., 1996) and in V-ATPase activity (Barkla et al., 1999). ABA also caused an increase in subunit c mRNA abundance (Tsiantis et al., 1996) and in V-ATPase activity (Barkla et al., 1999) in *M. crystallinum*. In Arabidopsis and cotton, V-ATPase c subunits are encoded by multiple genes that are differentially regulated in diverse tissues (Hasenfratz et al., 1995; Perera et al., 1995). We do not know the number of subunit c genes in tomato, but we detected mRNA hybridizing to *LVA-P1* in seeds, flowers, leaves, and roots (Fig. 2). Given the high sequence homology within this gene family, we would likely have detected mRNA from any expressed subunit c genes.

Expression of other V-ATPase subunits is also sensitive to environmental or hormonal conditions. The V_1 sector of the V-ATPase, which binds to the V_0 sector on the cytoplasmic side of the membrane, contains the nucleotide-binding subunits A and B, along with at least six other proteins (Sze et al., 1999). Salt stress caused an increase in subunit A mRNA in both tobacco cells (Narasimhan et al., 1991) and tomato leaves (Binzel and Dunlap, 1995). While ABA could at least partially mimic this effect in tobacco (Narasimhan et al., 1991), ABA did not appear to be involved in the response to salt in tomato (Binzel and Dunlap, 1995). Löw et al. (1996) found that mRNA abundance of the A and c subunits were differentially affected by tissue age and salt stress, while the abundance of subunit B mRNA was unaffected by the same factors.

Since all subunits of the V-ATPase complex are not always coordinately expressed, we wanted to know whether expression of protein subunits of the catalytic V_1 sector of the V-ATPase was also regulated by GA in *gib-1* seeds. Antibodies to the A (68 kD) and B (57 kD) subunits of mung bean V-ATPase specifically identified protein bands of the same size extracted from *gib-1* tomato seeds imbibed in GA_{4+7}, but not from seeds imbibed in water (Fig. 5). This was further confirmed using tissue prints, where antibody to subunit B highlighted the increased abundance of this protein, primarily in the endosperm cap and radicle tip region of the *gib-1* tomato seed in the presence of GA (Fig. 6). Thus, expression of at least three of the subunits of the V-ATPase complex is up-regulated specifically in the micropylar tissues of *gib-1* tomato seeds in response to GA. This confirms at the gene and protein expression levels previous anatomical, physiological, and biochemical evidence indicating that the micropylar endosperm cap tissue is differentiated from the remaining lateral endosperm (e.g. Haigh, 1988; Mella et al., 1995; Toorop et al., 1996; Still et al., 1997; Hilhorst et al., 1998; Nonogaki et al., 1998; Bradford et al., 2000).

We are aware of at least seven genes in addition to *LVA-P1* that are expressed in the micropylar tissues of tomato seeds in response to GA, including endo-β-mannanase, cellulase, arabinosidase, β-1,3-glucanase, chitinase, expansin, and a GA-stimulated transcript (Bradford et al., 2000), and no doubt many more remain to be identified. Evidence also exists for variation in GA sensitivity among individual barley aleurone cells and protoplasts (Jacobsen and Knox, 1973; Hillmer et al., 1993). As in tomato seeds, the responsiveness of barley aleurone cells to GA was greater at the micropylar (proximal) end of the seed than at the distal end (Ritchie et al., 1999). Variation in cell and tissue sensitivity to GA, in combination with changes in GA concentration and separation of sites of synthesis and action, are all likely to be involved in coordinating complex developmental transitions such as seed germination (Bradford and Trewavas, 1994).

The GA dependence of *LVA-P1* and its pattern of expression in the micropylar tissues suggest that V-ATPase plays a role in early events leading to radicle protrusion. The primary function of the V-ATPase is to establish and maintain an acidic pH in the vacuole and other endomembrane compartments (Sze et al., 1999). Seeds contain an array of hydrolases having acidic pH optima involved in the breakdown of protein reserves and in the transformation of protein bodies into vacuoles (Nishimura and Beevers, 1978). Both enzyme activity and expression of V-ATPase subunits increased in association with the mobilization of proteins from the storage vacuoles of pumpkin (*Cucurbita* sp.) cotyledons following germination (Maeshima et al., 1994).

Even prior to radicle emergence, the mobilization of protein reserves, protein body breakdown, and vacuolization are initiated in the micropylar endosperm in tomato (Nonogaki et al., 1998) and other seeds of the Solanaceae (Mella et al., 1995; Sánchez and de Miguel, 1997). Barba et al. (1997) showed by in vivo ^{31}P-NMR that between 1 and 2 d of imbibition, the phytin-containing protein bodies of vacuolar origin in the maize (*Zea mays* L.) embryo acidified from pH 5.5 to as low as pH 4.1. Swanson and Jones (1996) demonstrated that GA_{4+7} induces vacuolar acidification in barley aleurone cells, but did not detect significant differences in V-ATPase protein content among control, GA-treated, and ABA-treated aleurone cells. They suggested that other mechanisms, such as cytosolic pH or redox state, might regulate the activity of the V-ATPase. While expression of V-ATPase in tomato seeds clearly is dependent upon at least a minimal level of GA, additional post-transcriptional mechanisms are undoubtedly involved in regulating V-ATPase activity (Stevens and Forgac, 1997). V-ATPase is also associated with the ER, Golgi, and other endomembrane systems involved in protein trafficking and secretion (Sze et al., 1999). It could therefore play a part in the secretion of cell wall hydrolases that modify and weaken the endosperm cap, allowing radicle emergence (e.g. Nonogaki et al., 1998; Toorop et al., 1998).

Using the GA-deficient *gib-1* tomato mutant and differential cDNA display, we have demonstrated that a gene (*LVA-P1*) encoding subunit c of the V-ATPase V_0 transmembrane sector is specifically transcribed in the endosperm cap and radicle tip tissues in response to GA. The A- and B-subunit proteins of the V_1 cytoplasmic sector of the V-ATPase are also up-regulated in response to GA in the same tissues. In wild-type seeds, *LVA-P1* mRNA expression did not require exogenous GA, but was still localized primarily in the micropylar tissues prior to radicle

emergence. It is likely, therefore, that V-ATPase activity increases in the endosperm cap tissue prior to radicle emergence in association with acidification of protein bodies during reserve mobilization or secretion of cell wall hydrolases required for tissue weakening.

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