Plant, Cell and Environment (2007) **30**, 388–398 doi: 10.1111/j.1365-3040.2006.01624.x

An α-amylase (At4g25000) in *Arabidopsis* leaves is secreted and induced by biotic and abiotic stress

ELIZABETH A. DOYLE[1], ASHLEIGH M. LANE[1], JAMES M. SIDES[1], MARY BETH MUDGETT[2] & JONATHAN D. MONROE[1]

[1]*Department of Biology, MSC 7801, James Madison University, Harrisonburg, VA 22807 and* [2]*Department of Biological Sciences, Stanford University, Stanford, CA 94305*

ABSTRACT

Leaves are reported to contain a secreted α-amylase that accumulates during senescence or after biotic or abiotic stress; however, a gene encoding this enzyme has not been described. Because a secreted amylase is isolated from plastidic starch, the function of this enzyme is difficult to predict, but circumstantial evidence suggests that it may degrade starch after cell death. The *Arabidopsis thaliana* genome contains three α-amylase genes, one of which, *AMY1* (At4g25000), has a putative signal sequence suggesting that the protein may be secreted. Two independent T-DNA insertion mutants in *AMY1* lacked an amylase band on starch zymograms, which was previously named 'A1'. Washed leaf protoplasts contained reduced A1 activity suggesting that the enzyme is secreted. Native AMY1, fused to a weakly fluorescent form of GFP, was sensitive to proteinase K infiltrated into leaf apoplastic spaces, while a cytosolic form of GFP was unaffected until cell breakage, confirming that the AMY1 protein is secreted. Amylase A1 was transcriptionally induced in senescing leaves and in leaves exposed to heat stress, treated with abscisic acid or infected with *Pseudomonas syringae* pv. tomato expressing *avrRpm1*. The A1 amylase was also extremely heat resistant and its expression was up-regulated in *cpr5-2*, an activated defence response mutant.

Key-words: abscisic acid; secreted.

INTRODUCTION

In photosynthetic tissues, transitory starch accumulates in chloroplasts during the daylight and is degraded at night to provide reduced carbon compounds when photosynthesis is not possible (Caspar, Huber & Somerville 1985). Recent studies have provided evidence that the major pathway for starch degradation in chloroplasts at night involves phosphorylation of glucans on the granule surface by glucan, water dikinase (Lorbeth *et al*. 1998; Yu *et al*. 2001; Ritte *et al*. 2002) and phosphoglucan-water dikinase (Baunsgaard *et al*. 2005; Kötting *et al*. 2005) followed by hydrolysis of α-1, 4 linkages by β-amylase to form maltose (Scheidig *et al*. 2002; Nittylä *et al*. 2004; Weise, Weber & Sharkey 2004). For more detailed reviews of starch metabolism, see Lloyd, Kossmann & Ritte (2005) and Smith, Zeeman & Smith (2005). It is ironic that α-amylases play no major role in this pathway, despite long-held assumptions to the contrary and their prominent role in endosperm starch degradation during cereal seed germination (Beck & Ziegler 1989). Disruption of any or all of the three predicted α-amylase genes in *Arabidopsis* has no effect on transitory starch metabolism (Yu *et al*. 2005), although Delatte *et al*. (2006) recently identified a minor role for a chloroplastic α-amylase in *Arabidopsis*. However, one important difference between leaf and endosperm tissues is that leaf cells are alive during transitory starch degradation, whereas cereal endosperm is dead at germination.

While chloroplastic enzymes carry out the conversion of starch to maltose, it has long been noted that much of the starch hydrolysing activity in leaves is located outside the chloroplast (Stitt, Bulpin & ap Rees 1978; Okita *et al*. 1979; Kakefuda, Duke & Hostak 1986; Lin, Spilatro & Preiss 1988). In fact, the predominant α-amylase in vegetative tissues of pea (*Pisum sativum*) is secreted from the cell (Beers & Duke 1988). What function this secreted α-amylase activity plays in starch degradation is unknown, but a role in starch degradation after cell death was suggested by its expression pattern. The secreted pea α-amylase is induced by a variety of stresses including heat (Commuri & Duke 1997), prolonged darkness (Saeed & Duke 1990), inhibitors of chloroplast function (Saeed & Duke 1988) and by senescence (Saeed & Duke 1990). In tobacco, a secreted α-amylase is induced by tobacco mosaic virus (TMV) infection (Heitz *et al*. 1991). Heitz *et al*. (1991) hypothesized that this apoplastic α-amylase may act on plastidic starch after cell death resulting from the hypersensitive response (HR) with the resulting sugars being made available to neighbouring cells. Indeed, Weintraub & Ragetti (1964) observed that after infection by TMV, starch levels in tobacco cells declined rapidly after membrane deterioration. We sought to identify a homolog of the secreted α-amylase in *Arabidopsis* and to use T-DNA insertion mutants to test the hypothesis that it functions in dead cells.

Of the three *Arabidopsis* α-amylases, only one, *AMY1* (At4g25000), contains a predicted signal sequence

Correspondence: Jonathan D. Monroe. Fax: 540 568 3333; e-mail: monroejd@jmu.edu

© 2007 James Madison University
Journal compilation © 2007 Blackwell Publishing Ltd

indicating that it could be translated on the rough endoplasmic reticulum and then be secreted from cells. We sought to identify the product of this gene, to characterize its subcellular location and patterns of expression, and to investigate a possible phenotype of knockout mutants.

MATERIALS AND METHODS

Plant culture

Arabidopsis thaliana, ecotype Columbia (Col-0), plants were grown at 24 °C with 12 h of illumination (300–350 μmol m^{-2} s^{-1}) on a growth cart (Grower's Supply Co., Ann Arbor, MI, USA). The growth medium was Sunshine Mix #1 or Sunshine Mix #3 (Sun Gro Horticulture Inc., Bellevue, WA, USA), supplemented with macronutrients and micronutrients as described by Lehle Seeds (Round Rock, TX, USA). The *cpr5-2 Arabidopsis* mutant line (stock number CS3770), as well as the T-DNA insertion mutants *amy1-1* (SALK_094382) and *amy1-2* (SALK_014462), was obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH, USA) and was grown under the same conditions described earlier. Sites of the T-DNA insertions were verified by PCR and by sequencing, which was performed at the Center for Molecular Neurobiology at Ohio State University (Columbus, OH, USA).

For abscisic acid (ABA) treatments of detached leaves, leaves were floated abaxial side up on sterile water supplemented with ABA or on the same concentration of ethanol used to dissolve the ABA (1% v/v), and maintained in the same growth conditions in which the plants were grown. For ABA treatments of whole plants, plants were sprayed for two consecutive days with 100 μM ABA or with the same concentration of ethanol used to dissolve the ABA (1% v/v).

Generation of transgenic plants

To clone the *AMY1* gene into pCAMBIA1302 (Roberts *et al*. 1998), an internal *Nco* I was first eliminated using PCR mutagenesis. Internal primers homologous to the *AMY1* gene and overlapping the *Nco* I site were created. Two PCR reactions were prepared, one with AMY1 F (5′-TACCATGGCTACATCTCTCCATA-3′) and Nco R (5′-CAAATCTCCAACCGTGGAATC-3′) primers and one with AMY1 R (5′-TAACTAGTCTTCTTCTCCCAG-3′) and Nco F (5′-GAAATCGGATTCCACGGTTG-3′) primers. The *Arabidopsis* cDNA template was prepared from senescent leaf tissue using total RNA from the Col-0 ecotype of *Arabidopsis*. The cycling conditions for the AMY1 F and Nco R primers were 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, for 30 cycles. When primers AMY1 R and Nco F were used, the annealing temperature was lowered to 52 °C. Following gel purification, the two PCR fragments were combined in one PCR reaction. For the first 10 cycles of PCR, no additional primers were added. After 10 cycles, primers AMY1 F, AMY1 R (0.2 mM each), 0.2 mM dNTPs, and 1 Unit of Taq polymerase were added. The PCR cycling conditions were as follows: 94 °C for 30 s, 45 °C for 30 s, 72 °C for 2 min, for 10 cycles and then 94 °C for 30 s. The resulting product was cloned into pCR II-TOPO (Invitrogen, Carlsbad, CA, USA). A double restriction digest was performed on the resulting plasmid using the restriction enzymes *Spe* I and *Nco* I (New England Biolabs, Ipsvoich, MA, USA). The *AMY1* insert was gel purified and ligated with pCAMBIA1302 vector that had also been digested with *Nco* I and *Spe* I, using a quick ligation kit (New England BioLabs). The presence of an insert was confirmed with PCR and restriction digests. The resulting *AMY1-GFP* construct and unmodified pCAMBIA1302 were then transformed independently into *Agrobacterium tumefaciens* strain gr3101P as previously described (Tzfira *et al*. 1997). The presence of the plasmids in *A. tumefaciens* was confirmed by PCR and restriction analysis. Transgenic *Arabidopsis* Col-0 and *amy1-2* plants were then generated using *Agrobacterium*-mediated floral spray transformation as previously described (Chung, Chen & Pan 2000). PCR was used to identify homozygous T2 lines for further analysis.

Protoplast generation

Protoplasts were prepared from leaves of 6- to 8-week-old plants, according to the method of Katagiri (2002). Protoplasting enzymes (macerase, cellulysin) were obtained from EMB Biosciences (San Diego, CA, USA). Protoplasts were washed in 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM MES, pH 5.6, resuspended in native polyacrylamide gel electrophoresis (PAGE) loading buffer (Dinges *et al*. 2001) and then electrophoresed on native, starch-containing gels as described next.

Native, starch-containing PAGE

To analyse plants for starch-degrading activity, native PAGE was conducted using a method modified from Dinges *et al*. (2001). Resolving gels contained 7% acrylamide (30:0.8 acrylamide-bisacrylamide used throughout), 375 mM Tris, pH 8.8 and 0.3% starch, while stacking gels contained 4% acrylamide and 63 mM Tris, pH 6.8. To prepare the samples, leaf tissue was combined with 1 μL of loading buffer (4.5% glycerol, 17.5 mM Tris, 134 mM glycine, pH 8.8, 2 mg mL^{-1} bromphenol blue) per milligram leaf tissue and homogenized in 1.5 mL tubes. Equal leaf weights were electrophoresed at constant current of 18–20 mA at 4 °C in an electrode buffer of 25 mM Tris, 192 mM glycine, pH 8.8. After the dye front ran off of the gels, the gels were run for an additional 40 min. Gels were incubated in an assay buffer (50 mM sodium acetate, 5 mM $CaCl_2$, pH 5.2) at 37 °C for 6 h to overnight. After incubation, the gels were rinsed with deionized water and then stained with iodine (5.7 mM I_2, 43.4 mM KI, 0.2 M HCl). Following staining, the gels were either photographed using a digital camera or scanned with a flatbed scanner, resulting in subtle differences in gel appearance and colouration.

© 2007 James Madison University

Protein gel blotting

Protein gel blots were performed to determine if the transgenic plants contained GFP. Leaf discs were obtained with a #5 cork borer, frozen in liquid nitrogen and then ground to a powder. To each leaf disc, 100 μL of sodium dodecyl sulphate (SDS) loading buffer (Laemmli 1970) supplemented with 6 M urea was added and the powder was extracted. The extracts were boiled for 5 min and then centrifuged for 5 min in a microcentrifuge at 14 000 *g*. Proteins were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes in 10 mM cyclohexylaminopropane sulfonic acid (CAPS) (pH 11) and 10% methanol. Membranes were blocked overnight at 4 °C in TBST (0.5 M NaCl, 20 mM Tris, pH 7.5, 0.05% Tween-20) supplemented with 5% (w/v) fat-free dry milk powder and 1% (v/v) goat serum (Sigma, St. Louis, MO, USA). Membranes were then incubated in 1:1000 monoclonal anti-GFP (Becton Dickinson Biosciences, Palo Alto, CA, USA) in the same blocking buffer for 1 h at room temperature. The membranes were washed with TBST three times for 5 min each and then incubated in 1:3000 goat anti-mouse horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, CA, USA) in TBST supplemented with 5% (w/v) fat-free dry milk powder for 30 min at room temperature. After washing in TBST as before, the membranes were incubated in ECL Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to manufacturer's instructions, exposed to X-ray film and then developed.

Leaf infiltrations

For GFP localization experiments, leaves were pressure-infiltrated by syringe with water or water containing 500 μg mL^{-1} proteinase K in the presence and absence of 1% Triton X-100 (w/v). After 30 min at 24 °C, the leaf discs were obtained with a #5 cork borer, frozen, and then processed for protein gel blotting as described earlier. For the Triton X-100-treated samples, leaves were heated to 70 °C for 5 min prior to extraction. To determine the effect of *Pseudomonas syringae* pv. tomato infection on A1 levels, *P. syringae* pv. tomato DC3000 pVSP61 (*avrRpm1*) were grown on *Pseudomonas* Agar F (Fisher Scientific, Pittsburgh, PA, USA) supplemented with 50 μg mL^{-1} kanamycin and 100 μg mL^{-1} rifampicin at 28 °C. Cells were resuspended in 1 mM $MgCl_2$ and pressure-infiltrated by syringe at a density of 10^4 or 10^8 cfu mL^{-1} into leaves of 3- to 4-week-old wild-type plants. Leaves that were infiltrated with the low dose were extracted after 4 d for native gel assays. For systemic analyses, two leaves per plant were infiltrated with the high dose of cells and after 4 d, the other leaves on the plants were extracted for native gel assays.

Quantitative real-time PCR (Q-PCR) methods

RNA was extracted from leaf tissue with RNAwiz reagent or Tri reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions and treated with DNA-free (Ambion), using the manufacturer's protocol for rigorous treatment, to remove residual genomic DNA. Total RNA (1.5 μg) was transcribed into cDNA with the iScript cDNA synthesis system (Bio-Rad). The DyNAmo SybrGreen qPCR kit (New England Biolabs, Ipswich, MA, USA) was used with an Opticon 2 DNA engine (Bio-Rad) for quantitative PCR. Triplicate reactions with two primer sets were set up for cDNA samples and for the corresponding 'no RT' controls. The cDNA reactions were diluted 1:1 and 5 μL was used in each qPCR reaction. Primers for the AMY1 gene were as follows: At-AMY1-F, 5′-CTTTGGCTTCCTCCT CCTTCTCAA-3′ and At-AMY1-R, 5′-CTTTCCTCTCAG CTGTTCTGTGGT-3′. A second primer set amplified a product from the *A. thaliana L23a* gene (GenBank Accession Number AF034694), a ribosomal protein gene that served as the calibrator sample. Primers for the *L23a* gene were as follows: AtL23a-F, 5′-AGTACCCACTCACCA CTGAATCTG-3′ and AtL23a-R, 5′-TCTGGTGTAAGC CTCATGTAAGCC-3. Cycling conditions were 95 °C, 10 s; 60 °C, 20 s; 72 °C, 20 s for 40 cycles. Relative levels of gene expression were calculated using the comparative C_T method ($\Delta\Delta C_T$ method, Applied Biosystems 1997).

RESULTS

Phylogenetic analysis indicates plants contain three functionally distinct types of α-amylases

Arabidopsis contains three genes with strong similarity to previously characterized α-amylase genes, named *AMY1*, *AMY2* and *AMY3* (Lloyd *et al*. 2005). *AMY1* (At4g25000), the focus of this study, encodes a protein of 423 amino acids with a predicted mass of 47 kDa. It has a typical signal sequence, as predicted by PSORT (Nakai & Kanehisa 1992; http://psort.nibb.ac.jp/) and TargetP (Emanuelsson *et al*. 2000; http://www.cbs.dtu.dk/services/TargetP/), indicating that it likely enters the secretory pathway. *AMY2* (At1g76130) also encodes a 47 kDa protein but it contains no predicted targeting signals. *AMY3* (At1g69830) is considerably larger, encoding a protein of about 100 kDa with a predicted chloroplast transit peptide. None of these proteins are more than 48% identical to each other and are thus not likely to have arisen from recent gene duplications. The proteins are also not likely to be functionally redundant, given their putative cellular localizations.

In order to compare these three *Arabidopsis* α-amylases with those from other plants, we aligned their amino acid sequences using ClustalW (http://www.ebi.ac.uk/clustalw) and then constructed an unrooted phylogenetic tree (data not shown). As observed by Stanley *et al*. (2002), the sequences clustered into three clades that correlate perfectly with their predicted molecular masses and the presence or absence of specific targeting sequences. Clade I includes *AMY1* and contains sequences that range from 421 to 445 amino acids, each with a putative signal sequence. Clade II includes *AMY2* and consists of sequences that range in size from 407 to 414 amino acids with no predicted targeting signals, while Clade III includes *AMY3* and contains sequences that are all much larger than the others,

© 2007 James Madison University

ranging from 887 to 906 amino acids. Each member of clade III contains a putative chloroplast transit peptide and aligns with the smaller α-amylases in their C-terminal halves. It is worth noting that the three sequenced plant genomes of *Arabidopsis*, rice and black cottonwood all contain at least one member in each of the three clades, suggesting that each clade is important to plant function. Stanley *et al.* (2002) also noted that the *Pinus taeda* expressed sequence tag (EST) collection included sequences from each clade.

AMY1 encodes amylase A1 activity

Two T-DNA insertion mutants in *AMY1* were obtained from the SALK collection and named *amy1-1* (SALK_094382; described in Yu *et al.* 2005) and *amy1-2* (SALK_014462). PCR analysis confirmed that the insertions were in the *AMY1* gene (data not shown). Segregation analysis revealed that the first mutant, *amy1-1*, had T-DNA integration at only a single locus, but *amy1-2* contained insertions at two independent loci. To eliminate the second insertion, we crossed an *amy1-2* plant with a wild-type plant and isolated a line with the insert in *AMY1* that yielded seeds with a 3:1 ratio of kanamycin-resistant to sensitive progeny, indicating that it contained T-DNA insertions at only a single locus. Sequencing across the *AMY1*/T-DNA insertion border confirmed that the inserts in *amy1-1* and *amy1-2* were in the third exon and the third intron, respectively (Fig. 1a). For all subsequent experiments, we used homozygous *amy1-1* and *amy1-2* mutant plants.

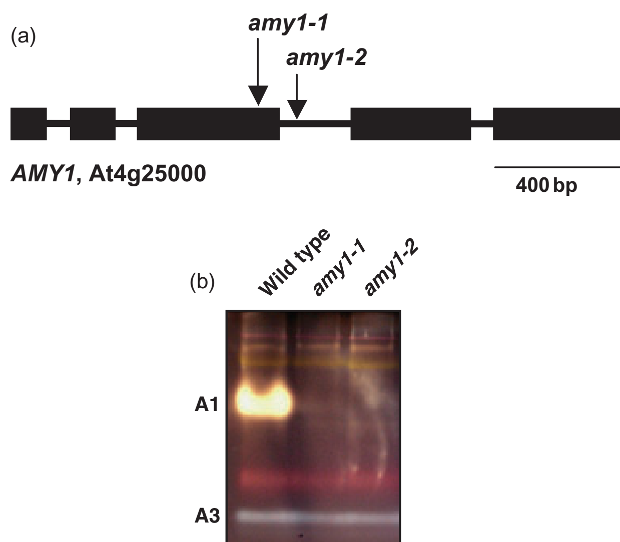

Figure 1. Location of T-DNA insertions in *AMY1* and their effect on amylase activity detected using native, starch-containing polyacrylamide gel electrophoresis (PAGE). (a) Map of the *AMY1* intron/exon structure illustrating the location of two independent T-DNA insertions designated *amy1-1* (SALK_094382) and *amy1-2* (SALK_014462). (b) Native, starch-containing PAGE of equal leaf weights from 4-week-old wild-type (Col-0) and T-DNA insertion mutants floated on 50 mM abscisic acid (ABA) for 24 h. Amylases A1 and A3 are as identified by Lin *et al.* (1988).

Leaf extracts from mutant and wild-type plants were subjected to native, starch-containing PAGE and the mutants were found to lack a yellow to white-coloured band previously identified by Lin *et al.* (1988) as 'amylase A1', while other starch-degrading activities remained largely unchanged (Fig. 1b). In order to visualize the difference between *amy1* mutant and wild-type extracts more clearly, all of the protein extracts used in the gel illustrated in Fig. 1a were obtained from leaves pre-treated by floating on 50 μM ABA for 24 h, which induces A1 activity (see further discussion). Both *amy1-1* and *amy1-2* mutant leaves always lacked A1, with or without exogenous ABA treatment, indicating that the mutants were not simply unresponsive to ABA. In starch zymograms, the colour of bands generated is indicative of the type of enzyme that acted on the starch (Kakefuda & Duke 1984) and in this case, the colour of the A1 band is consistent with it being an α-amylase. The red band below A1 and several other starch hydrolases including a phloem-localized grey β-amylase (Monroe & Preiss 1990; Wang, Monroe & Sjolund 1995) were present in both mutants and wild-type leaves, as were several other unidentified minor bands.

AMY1 localizes to the apoplast

The presence of a putative signal peptide at the N-terminus of AMY1 suggests that it enters the secretion pathway. Vacuum infiltration followed by centrifugation has been used to extract apoplastic α-amylases from pea stems (Beers & Duke 1988) or tobacco leaves (Heitz *et al.* 1991). However, due to the small size of *Arabidopsis* leaves and stems, we had only marginal success using this approach to isolate apoplastic fluids from *Arabidopsis*. Instead, we attempted to localize the A1 protein using two approaches: analysing washed protoplasts for the presence of A1 activity and localizing GFP in transgenic plants expressing an AMY1-GFP fusion protein.

If A1 activity is localized in the apoplast, digestion of the cell wall during preparation of protoplasts should release A1 activity into washing solutions. The analysis of broken protoplasts on native, starch-containing PAGE should result in the absence of A1 activity. Crude leaf extracts from wild-type leaves contained A1 and A3 activity, while washed protoplasts prepared from leaves of the same age contained amylase A3 but lacked A1 almost entirely, indicating that it may be extracellular or loosely associated with the cell wall and/or plasma membrane (Fig. 2a). Additional starch-degrading activities observed in the protoplast lane were due to contaminating starch hydrolases present in the enzyme preparations used to make the protoplasts, as seen by comparison to lane 3 (Fig. 2a).

To localize AMY1 using GFP, the *AMY1* coding sequence with its native signal sequence was inserted into the vector pCAMBIA1302 between the 35S promoter and *GFP*, creating *AMY1-GFP* (Fig. 2b). *AMY1-GFP* was then introduced into wild-type *Arabidopsis* plants using *Agrobacterium*-mediated floral transformation. Control plants were transformed with unmodified pCAMBIA1302

© 2007 James Madison University

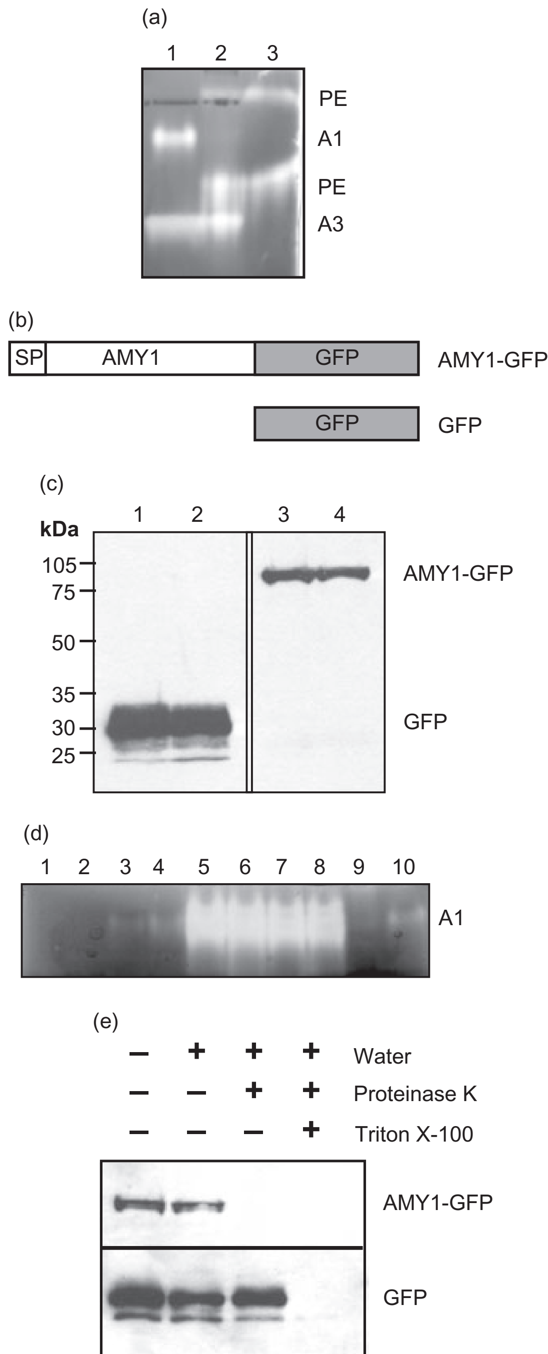

Figure 2. Analysis of wild-type *Arabidopsis* leaf protoplasts and transgenic plants expressing AMY1-GFP indicate that A1 is secreted. (a) Leaf protoplasts were prepared from equal leaf weights of wild-type plants and subjected to native, starch-containing polyacrylamide gel electrophoresis (PAGE). Lane 1 contains a crude extract prepared from wild-type (Col-0) leaves. Lane 2 contains wild-type leaf protoplasts, while lane 3 contains a 1:50 dilution of the protoplasting enzymes (macerase and cellulysin) used during protoplast preparation. Amylases A1 and A3 are as identified by Lin *et al*. (1988), while PE indicates protoplasting enzymes. (b) Schematic of the gene constructs used to transform wild-type and *amy1-2 Arabidopsis* plants. SP is the native AMY1 signal peptide. (c) Protein gel blot of extracts prepared from equal leaf weights of transgenic plants harbouring AMY1-GFP and GFP alone. After sodium dodecyl sulphate (SDS)-PAGE and transfer to nitrocellulose, the membrane was probed with an anti-GFP antibody. Lanes 1 and 2 contain leaf extracts from independent GFP transgenic lines, while lanes 3 and 4 contain leaf extracts from independent AMY1-GFP transgenic lines, all in wild-type background. Protein masses in kDa are indicated on the left. (d) Native gel of AMY1-GFP and GFP transgenic lines. Lanes 1 and 2 are leaf extracts from independent GFP transgenics in the *amy1-2* background while lanes 3 and 4 are from independent GFP transgenics in wild-type background. Lanes 5 and 6 are leaf extracts from independent AMY-GFP plants in the *amy1-2* background, while lanes 7 and 8 are from independent AMY1-GFP plants in wild-type background. Lane 9 is a leaf extract prepared from *amy1-2* leaves, and lane 10 is a leaf extract from wild-type leaves. (e) Leaves from AMY1-GFP plants and GFP plants in wild-type background were infiltrated with water, proteinase K and Triton X-100 in varying combinations. Leaf extracts were prepared and equal leaf weights were subjected to SDS-PAGE followed by protein gel blotting with the anti-GFP antibody.

containing *GFP* (Fig. 2b). AMY1-GFP migrated at the predicted molecular mass in SDS-PAGE immunoblots (Fig. 2c) and exhibited A1 activity in native, starch-containing PAGE (Fig. 2d). However, the A1 activity we detected in AMY1-GFP transgenic lines migrated as a triplet of protein bands in about the same position as A1, suggesting alternative folding or proteolytic processing had occurred (Fig. 2d). When *AMY1-GFP* was inserted into the *amy1-2* background, A1 activity was restored, confirming that A1 activity is encoded by the *AMY1* gene (Fig. 2d). Unfortunately, fluorescence microscopy failed to show a strong GFP signal above the autofluoresence normally observed in wild-type plants, in either the GFP or the AMY1-GFP plants. The lack of fluorescence was most likely due to the type of GFP (mGFP5) employed in this study (Roberts *et al*. 1998). We therefore used an alternative strategy to determine if AMY1-GFP was in the apoplast. We reasoned that cell membranes should protect the cytosolic, control GFP from degradation by infiltrated proteinase K, while AMY1-GFP would be sensitive to degradation only if it was located in the apoplast. Proteinase K ($500\,\mu g\,mL^{-1}$) pressure-infiltrated by syringe into leaves of both types of transgenic plants resulted in the degradation of the GFP of the AMY1-GFP fusion protein, but had no effect on the cytosolic GFP after 30 min (Fig. 2e). To expose intracellular forms of GFP to degradation by proteinase K, the plasma membrane was solubilized by first infiltrating with 1% Triton X-100 and proteinase K and then heating the leaf discs to 70 °C for 5 min. After this treatment, the GFP in both AMY1-GFP and GFP was degraded (Fig. 2e). The results of the protoplasting experiments and the leaf infiltration experiments both indicate that AMY1 is located in the apoplast of leaves.

A1 is developmentally regulated and induced by biotic and abiotic stress

Young and mature leaves of wild-type *Arabidopsis* plants contained amylase A1 at low levels as determined by native

© 2007 James Madison University

starch-containing PAGE, but the level was highly elevated with age and in senescing leaves (Fig. 3a). Levels of the other starch hydrolases visible in this native gel system generally declined in older leaves. Amylase A3, a cytosolic β-amylase expressed in phloem tissue (Wang *et al*. 1995), increased from 4 to 8 weeks and then declined in the 10-week-old plants. Q-PCR analysis revealed that the *AMY1* mRNA level increased over 240-fold between 4- and 8-week-old plants, suggesting that *AMY1* expression is regulated at the level of transcription (Fig. 3b).

Leaf senescence is an organized developmental programme influenced by plant age as well as environmental conditions such as nutrient supply, shading or temperature (Park *et al*. 1998; Quirino, Normanly & Amasino 1999; Buchanan-Wollaston *et al*. 2003; Lim, Woo & Nam 2003). Phytohormones such as ABA, ethylene or methyl jasmonate can also induce senescence (Park *et al*. 1998). Certain stresses and hormones such as ABA are also able to induce genes that are directly responsive to those treatments (Weaver *et al*. 1998). Because many senescence-associated genes (SAGs) are regulated by ABA (Park *et al*. 1998; Weaver *et al*. 1998), we tested the effect of ABA on the levels of A1 activity and *AMY1* mRNA levels. When detached leaves were floated on 50 μM ABA for 48 h, the level of A1 activity increased slightly after the first 6 h of treatment and dramatically after 2 d, while the levels of other starch-degrading activities remained constant (Fig. 3c). In control leaves treated with the same level of ethanol used to dissolve the ABA, A1 activity did not change over the 2 d period. The ABA effect could be observed with as little as 1 μM ABA, but with 10 and 100 μM ABA the induction was much stronger (Fig. 3d). Q-PCR revealed that 48 h after spraying with 100 μM ABA, the level of the *AMY1* mRNA increased almost 300-fold in 4-week-old plants, while in 6-week and 8-week-old plants the levels of *AMY1* mRNA increased 1200-fold and 3400-fold, respectively (Fig. 3e).

ABA accumulates in plants in response to a wide range of biotic and abiotic stresses, and in turn it regulates a large number of genes (Hoth *et al*. 2002). We examined two such stresses that we reasoned would induce *AMY1* and lead to cell death or membrane leakage such that the secreted amylase might gain access to starch and influence cell survival, infection by a bacterial pathogen and heat stress. Leaves of 3-week-old wild-type plants were pressure-infiltrated with 10^4 or 10^8 cfu mL^{-1} of *P. syringae* pv. tomato DC3000 expressing *avrRpm1*. This bacterial strain induces localized and systemic disease resistance in *Arabidopsis* Col-0 plants containing the RPM1 resistance protein. Leaves were inoculated with *P. syringae* pv. tomato (*avrRpm1*) at a low inoculum (10^4 cfu mL^{-1}) to monitor any slow changes in amylase activity that might occur over time. In contrast, leaves were inoculated with a high concentration (10^8 cfu mL^{-1}) of the bacteria to induce a rapid, localized cell death response (referred to as the HR) and systemic acquired resistance. In leaves infected with the low dose of bacteria, A1 was elevated 4 d after inoculation as compared with mock-inoculated leaves (Fig. 3f). Leaves infiltrated with the high dose of bacteria exhibited HR within 48 h. After 4 d, A1 activity was induced in the uninfected leaves of the same plant (Fig. 3g), indicating that pathogen-induced disease resistance led to systemic A1 induction. While inductions of A1 activity were moderate, they were consistently observed in repeated experiments.

Heat stress also led to elevated A1 activity. After 16 h in darkness at 37 °C, amylase A1 was induced as compared with plants held in darkness at 24 °C (Fig. 3h). In order to determine if the A1 enzyme itself was heat stable, we prepared a crude leaf extract in water (1:1, w/v) to mimic the conditions in a dead cell milieu, where no buffer, no additional ions, reductant or protease inhibitors would be found. When held at 37 °C for up to 60 min, we could detect no major differences in the type or quantity of starch hydrolases in a native gel (Fig. 3i). At 45 °C, the red band was lost by 30 min, while A1 and A3 activities were unchanged after 60 min. At 65 °C, only amylase A1 activity was resistant to heat denaturation.

Because the expression of the *AMY1* gene was induced both by senescence and by infection with avirulent *P. syringae* pv. tomato (*avrRpm1*), we decided to investigate the level of expression in the *cpr5-2* mutant of *Arabidopsis*, which constitutively expresses systemic acquired resistance (Bowling *et al*. 1997) and is hypersenescent (Yoshida *et al*. 2002). Q-PCR revealed that the expression of the *AMY1* gene was up-regulated 40-fold in 5-week-old *cpr5-2* plants relative to wild-type plants of the same age (Fig. 4).

Despite numerous attempts, we have not been able to observe consistent phenotypic differences between wild-type and AMY1-deficient mutants in terms of their starch levels throughout senescence, in response to stress or ABA treatment, or in the growth of avirulent *P. syringae* pv. tomato (*avrRpm1*) in infected leaves (data not shown).

DISCUSSION

Arabidopsis contains an α-amylase gene, *AtAMY1*, that contains a putative signal sequence, suggesting it may be secreted to the apoplast. The function of such secreted α-amylases remains unclear, because living cells synthesize and degrade plastidic starch using pathways that do not involve α-amylases (Lloyd *et al*. 2005; Yu *et al*. 2005), let alone ones that are secreted. Two T-DNA mutants with insertions in this gene lacked a prominent starch hydrolase band on native, starch-containing polyacrylamide gels (Fig. 1b). This band was previously identified as 'amylase A1' but was not fully characterized (Lin *et al*. 1988). Here we link A1 activity to the *AtAMY1* gene and demonstrate that *AMY1* is induced during senescence (Fig. 3b), by the hormone ABA (Fig. 3c) by heat stress (Fig. 3h), and after infection by avirulent *P. syringae* pv. tomato (*avrRpm1*) (Fig. 3f,g). Heitz *et al*. (1991) hypothesized that the tobacco apoplastic α-amylase induced during TMV infection might act on plastidic starch after HR with the resulting sugars being available to neighbouring cells. Our working hypothesis is that AMY1 breaks down starch after cell death resulting from any biotic or abiotic stress, possibly

© 2007 James Madison University

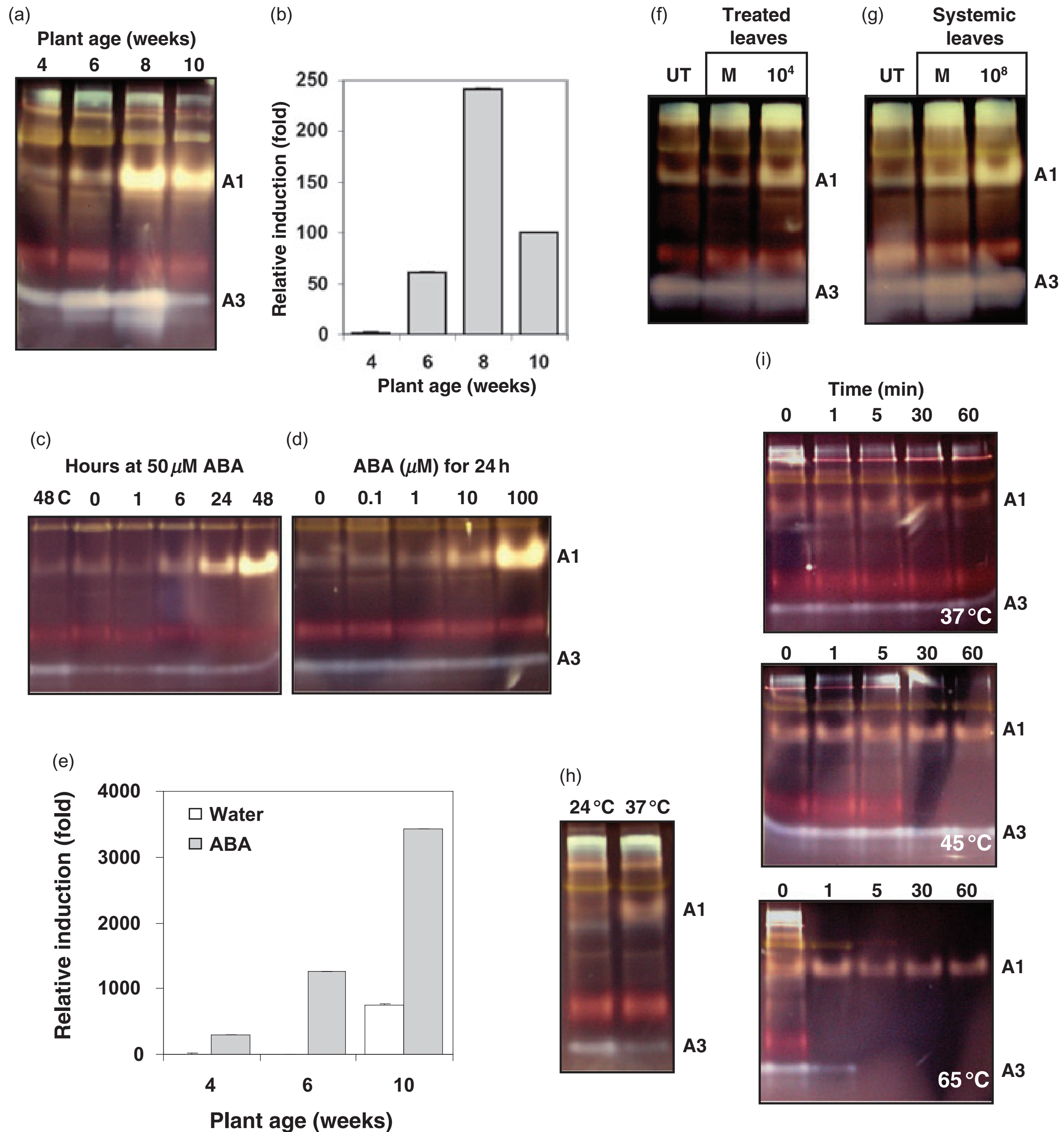

Figure 3. Amylase A1 is heat stable and its expression is developmentally regulated and affected by abscisic acid (ABA), *Pseudomonas syringae* and heat. (a) Native, starch-containing polyacrylamide gel electrophoresis (PAGE) of equal leaf weights prepared from wild-type (Col) plants of increasing ages. Amylases A1 and A3 are as identified by Lin *et al*. (1988). (b) Relative *AMY1* gene expression as measured using real-time quantitative PCR (Q-PCR). Total RNA was isolated from wild-type (Col) plants of varying ages, reverse-transcribed and used as template in PCR. Relative induction was calculated using the comparative C_T method, calibrating to the 4-week-old sample. The *L23a* ribosomal protein gene was used for standardization. Data are the average of triplicate reactions and are from one typical experiment. Error bars are the SD of the $\Delta\Delta C_T$ value and are sometimes too small to visualize on the scale of the graph. (c) Detached leaves were floated on control solution for 48 h (48 C) or on 50 μM ABA for various lengths of time and then equal leaf weights were subjected to native, starch-containing PAGE. (d) Detached leaves were floated on increasing concentrations of ABA for 24 h and then equal leaf weights were subjected to native, starch-containing PAGE. (e) Relative *AMY1* gene expression 48 h after plants were sprayed with 100 μM ABA as measured using Q-PCR as described for (b). (f) Leaves were infiltrated with 1 mM $MgCl_2$ (M, mock) or 10^4 cfu mL^{-1} *P. syringae* pv. tomato expressing *avrRpm1* in 1 mM $MgCl_2$ (10^4) 4 d prior to leaf extraction and native, starch-containing PAGE. UT, untreated plants. (g) Same as (f) but two leaves per plant were infiltrated with 10^8 cfu mL^{-1} of the same bacteria and other leaves on the same plants were used for the analysis 4 d later. (h) Whole plants were incubated at 24 or 37 °C for 16 h, after which leaf extracts were prepared and equal leaf weights were subjected to native, starch-containing PAGE. (i) Crude leaf extracts from wild-type plants were prepared in water (1:1, w/v), held at varying temperatures for up to 60 min and then equal leaf volumes were subjected to native, starch-containing PAGE.

© 2007 James Madison University

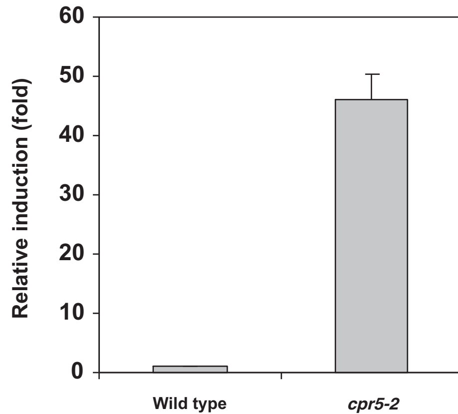

Figure 4. Expression of the *AMY1* gene in *cpr5-2*, a hypersenescent disease resistance mutant. Total RNA was isolated from 5-week-old wild-type (Col) and mutant plants, reverse-transcribed and used as template in quantitative, real-time PCR. Relative *AMY1* gene expression was calculated using the comparative C_T method, calibrating to the wild-type sample and using the *L23a* ribosomal protein gene for normalization. Data are the average of triplicate reactions, and are from one typical experiment. Error bars indicate the SD of the $\Delta\Delta C_T$ value.

eliminating starch as a nutrient source for a pathogen or providing glucose for utilization by neighbouring cells. It is noteworthy that the expression of *STP4*, which encodes a monosaccharide H^+ symporter, is up-regulated in response to wounding, treatment with chitin or with *P. syringae* pv. tabaci elicitors, or exposure to fungal pathogens (Truernit *et al*. 1996).

A phylogenetic analysis of plant α-amylases indicates that there are three functionally distinct clades of these genes. Because rice, black cottonwood and *Arabidopsis* all contain members of each clade, they are probably all important to plant function in some way. *AMY1* encodes an α-amylase that is more closely related to the secreted cereal α-amylases that function in dead tissues than it is to the two other α-amylase genes in *Arabidopsis*. Using washed protoplasts and proteinase K infiltration, we showed that the *AMY1* gene product is secreted. Although protoplast preparation is a stressful process, due to the robust stability of A1 activity, we believe it is unlikely that the lack of A1 activity in washed protoplasts was simply due to degradation. Our experiments clearly support the localization of AMY1 in the apoplast, however, in rice several α-amylases belonging to the same class as AMY1 are targeted to both the secretory pathway and to plastids (Chen *et al*. 2004; Asatsuma *et al*. 2005). In both the protoplasting and leaf infiltration experiments, our experimental design would allow for the identification of AMY1 that was localized in plastid, assuming no significant protein degradation occurred, which is not likely given the stability of AMY1. To further investigate this possible dual targeting, new AMY1-GFP transgenics are being created with a brighter form of GFP.

Senescing leaves accumulate AMY1 (Fig. 3a), suggesting a role for the enzyme in senescence-associated starch degradation. However, using iodine staining, we observed that starch disappeared late in leaf senescence in both wild-type and *amy1* mutants, indicating that *AMY1* is not the only enzyme involved in starch degradation in unstressed, senescing leaves (data not shown). Senescing leaves accumulate a wide range of defence molecules, even in the absence of pathogen (Quirino *et al*. 1999) and may be more likely to be damaged by biotic or abiotic stress during which AMY1 could play a role in starch mobilization.

The response of the *AMY1* gene to ABA suggests a general role for A1 activity during stress responses. ABA causes a moderately slow but robust induction of *AMY1* (Fig. 3c), and because ABA accumulates in response to many stresses, this alone could account for the induction of *AMY1* we observed after different stresses. The lack of *AMY1* induction in the ABA signalling mutant *abi1-1* during senescence and after heat stress supports this conclusion (Hoth *et al*. 2002). The observation that *AMY1* expression is induced both during senescence and after infiltration with *P. syringae* pv. tomato (*avrRpm1*) is not unusual. Many genes have been identified that are induced both during senescence and during defence responses (Butt *et al*. 1998; Schenk *et al*. 2005). A subset of defence response genes is induced during senescence in the absence of pathogen (Quirino *et al*. 1999).

Our data suggest a role for AMY1 in stress tolerance in *Arabidopsis*. A1 activity is extremely heat stable, a feature not uncommon for ABA-induced proteins (Ried & Walker-Simmons 1990). Of the leaf starch hydrolases observable in native, starch-containing polyacrylamide gels, A1 is the only enzyme that survives more than a few minutes at 65 °C in a water extract (Fig. 3i). The heat stability of A1 may allow it to function in cells that die from biotic or abiotic stress. This may serve as an additional defence feature in *Arabidopsis*, which may be particularly susceptible to heat-induced cell death due to its basal rosette leaves being close to the soil (Nilsen & Orcutt 1996).

A role for AMY1 in response to abiotic and biotic stress is also supported by an analysis of the promoter region of the gene using the PLACE database (Higo *et al*. 1999). As illustrated in Fig. 5, the *AMY1* promoter includes three ABA-responsive-element-like sequences (ABRE-like elements), one ASF-1 binding site, one CCAAT box which is found in the promoter of heat shock protein genes (Rieping & Schoffl 1992), one Elicitor Responsive Element, and two GT-1 motifs which are associated with pathogen- and salt-induced gene expression (Park *et al*. 2004). Also present are one ABA-responsive core of a low temperature responsive element, one MYB recognition site, five MYC recognition sites, a Pyrimidine box, a TCA-1 motif which is associated with salicyclic acid-inducible gene expression (Goldsbrough, Albrecht & Stratford 1993) and four W-boxes, which are bound by WRKY transcription factors, associated with both pathogen defence and senescence (Eulgem *et al*. 2000).

We focused on *AMY1* gene expression during senescence and during induction by abiotic and biotic stresses; however, the *AMY1* gene is also the major α-amylase gene

© 2007 James Madison University

```
-1000  GTCATATTCAAGTACTACAAAACTCCACTAGTGGTTGATGAGACAGAGAA
        Y  V  I  F  K  Y  Y  K  T  P  L  V  V  D  E  T  E
-950   GCCCAAAACGGTGTCGGATCATTCCATCAACATGGTCAAGCTTTCATCTA
         K  P  K  T  V  S  D  H  S  I  N  M  V  K  L  S
-900   CTCCGGCTTCTGGTGATCTTACGGTTCAGCCGCAGACTAATCCTGACGTG
       S  T  P  A  S  G  D  L  T  V  Q  P  Q  T  N  P  D
-850   AGTCATCCTATTAAAACTCATGGTGGTGACTTAGAGGACCAGATGGACAA
        V  S  H  P  I  K  T  H  G  G  D  L  E  D  Q  M  D
-800   GAAAATGCCAAACTAAGTCAAGATTTAGTTTTCAATTATGAATTAGTGAT
         K  M  P  N  *
-750   TGACTTTAATGTTGAACTGTTGAGTCTGGTTTCCTAATTTGTCTTTTATT
-700   TTCTCATCATCTTCTTTCGAAATATCTCGAAATTGTACTATCCTTCAAAA
-650   ATTTATGTGCGTCGTTTAGAATTTTGTAAAATAGTTTTTGATGCCTCTGT
-600   TCTCAGTAATACTTAAAAGTTTATTAGTCCGGCTAATTAAAAAGGAAATG
-550   AAAATTCAAAGTAAGTTAGATAAACATGATCATTCACAGGTCAGATGTTT
-500   TAAAAAAAAATCATTATGGTGTACATCACATGTAGACAATACTTCAGAAT
-450   TCATCTGGACTACCAGAATTGAGTTACCTAGTACTTCTCAATTCTATTTT
-400   ACCCTAACGTCTAATAAATAACAAGTACTCTAGCCTCTTCGTTTTATGAT
-350   TCCTCTAGGAAAAGTTAATGTTACGGCCCAATCACTTTTTTTAACAGCCC
-300   AAACAACATATATTAGCTCCAAATATCATTTTTTCCCCTAGAATATTCTC
-250   AACCTATTGTCCACTCAAAACGTGACAAATGGAGGTCTAAAGGGAGACCA
-200   TACTTGACTCATTTTAGAGCTAGGATCAGACAGAGTAGATTTTTTGCCAT
-150   AACTCCTTGTAAATGTATTCACATTTCATTCCCAAGAAAAATAGACTGAT
-100   GAAGAAATATATCAGATATGACAAGGCCGTGTCGTTTAGGTTACGTAACT
-50    CTACAAGGTTTAGGGTCTCAATATAAACACACAAAGCAGATAGAAGAAGC
+1     AAACCATTCACAATCAGACAATGACATCTCTCCATACGTTACTCTTCTCT
                            M  T  S  L  H  T  L  L  F  S
+51    TCTCTTCTTTTCTTCATCGTCTTTCCAACCTTCACGTTTTCCTCCACCTT
        S  L  L  F  F  I  V  F  P  T  F  T  F  S  S  T
```

Figure 5. Nucleotide sequence of the 1000 bp upstream region of *AMY1* (At4g25000). The transcription start site (+1) and start codon (ATG) for *AMY1* are shaded near the bottom. A putative TATA box is also shaded. The C-terminal sequence of At4g25010 is illustrated at the top of the figure. Underlined and in bold are all of the putative promoter elements found on either strand that are involved in senescence, biotic or abiotic stress, or abscisic acid (ABA)-induced gene expression as identified using the PLACE database (http://www.dna.affrc.go.jp/PLACE/). These elements include three ABA-responsive-element-like (ABRE-like) sequences (ACGTG at –855, –231 and +83), one ASF-1 binding site (TGACG at –857), one CCAAT box (CCAAT at –323), one Elicitor Responsive Element (TTGACC at –917), two GT-1 motifs (GAAAAA at –115 and –271, one ABA-responsive core of low temperature responsive element (CCGAC at –938), one MYB recognition site (WAACCA at –724), five MYC recognition sites (CANNTG at –811, –509, –474, –449 and –224), a Pyrimidine box (TTTTTTCC at –272), a TCA-1 motif (TCATCTTCTT at –694) and four W-boxes (TTGAC at –916, –784, –751 and –197).

expressed during seed development (Kim *et al*. 2005). The *AMY1* gene is expressed in developing siliques 7 to 9 d after pollination. *AMY1* expression is also induced by applying exogenous GA_4 to the siliques during seed development (Kim *et al*. 2005). The role of gibberellins in *AMY1* expression during the rest of development remains to be investigated.

The *Arabidopsis* secreted α-amylase might act on some unknown carbohydrate normally found in cell walls or generated by microorganisms, so we are presently attempting to characterize its substrate specificity. However, our data on the expression of *AMY1* are consistent with a role for the enzyme in starch degradation in dead cells. Such a role would require that the enzyme be resistant to proteolysis and this was found to be the case for the secreted pea and tobacco α-amylases (Beers & Duke 1990; Heitz *et al*. 1991). However, despite numerous attempts, we have not been able to detect a deficiency in starch degradation in the mutants either during senescence, after severe abiotic stress or after infection with *P. syringae* pv. tomato (*avrRpm1*). It is likely that when cells die, other starch hydrolases remain active for some time (Fig. 3i), so it is possible that we simply have not yet identified the conditions in which *AMY1* becomes essential to the process of starch degradation.

ACKNOWLEDGMENTS

This work was supported by NSF Grant IBN 0238053 to Jonathan D. Monroe. The authors would like to acknowledge the expert technical assistance of Blake Jones, who assisted with the sequencing of the T-DNA insertion mutants, and B. Grant Marshall and S. Nick Schroeder, who assisted with the Q-PCR analysis. We would also like to thank the ABRC for seed stocks of the *cpr5-2* mutant and the T-DNA mutants SALK_094382 and SALK_014462.

REFERENCES

Applied Biosystems (1997) *ABI PRISM 7700 Sequence Detection System User Bulletin Number 2: Relative Quantitation of Gene Expression*. Applied Biosystems, Foster City, CA, USA.

Asatsuma S., Sawada C., Itoh K., Okito M., Kitajima A. & Mitsui T. (2005) Involvement of α-amylase I-1 in starch degradation in rice chloroplasts. *Plant Cell Physiology* **46,** 858–869.

Baunsgaard L., Lutken H., Mikkelsen R., Glaring M.A., Pham T.T. & Blennow A. (2005) A novel isoform of glucan, water dikinase phosphorylates pre-phosphorylated α-glucans and is involved in starch degradation in *Arabidopsis*. *Plant Journal* **41,** 595–605.

Beck E. & Ziegler P. (1989) Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **40,** 95–117.

Beers E.P. & Duke S.H. (1988) Localization of α-amylase in the apoplast of pea (*Pisum sativum* L.) stems. *Plant Physiology* **87,** 799–802.

Beers E.P. & Duke S.H. (1990) Characterization of α-amylase from shoots and cotyledons of pea (*Pisum sativum* L.) seedlings. *Plant Physiology* **92,** 1154–1163.

Bowling S.A., Clarke J.D., Liu Y., Klessig D.F. & Dong X. (1997) The *cpr5* mutant of *Arabidopsis* expresses both NPR-1 dependent and NPR-1 independent resistance. *Plant Cell* **9,** 1573–1584.

Buchanan-Wollaston V., Earl S., Harrison E., Mathas E., Navabpour S., Page T. & Pink D. (2003) The molecular analysis of leaf senescence – a genomics approach. *Plant Biotechnology Journal* **1,** 3–22.

Butt A., Mousley C., Morris K., Beynon J., Can C., Holub E., Greenberg J. & Buchanan-Wollaston V. (1998) Differential expression of a senescence-enhanced metallothionein gene in *Arabidopsis* in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*. *Plant Journal* **16,** 209–221.

Caspar T., Huber S.C. & Somerville C. (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity. *Plant Physiology* **79,** 11–17.

Chen M-H., Huang L-F., Li H-M., Chen Y-R. & Yu S-M. (2004) Signal peptide-dependent targeting of a rice α-amylase and cargo proteins to plastids and extracellular compartments of plant cells. *Plant Physiology* **135,** 1367–1377.

Chung M.H., Chen M.K. & Pan S.M. (2000) Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. *Transgenic Research* **9,** 471–476.

© 2007 James Madison University

Commuri P.D. & Duke S.H. (1997) Apoplastic α-amylase in pea is enhanced by heat stress. *Plant and Cell Physiology* **38,** 625–630.

Delatte T., Umhang M., Trevisan M., Eicke S., Thorneycroft D., Smith S.M. & Zeeman S.C. (2006) Evidence for distinct mechanisms of starch granule breakdown in plants. *Journal of Biological Chemistry* **281,** 12050–12059.

Dinges J.R., Colleoni C., Myers A.M. & James M.G. (2001) Molecular structure of three mutations at the maize *sugary1* locus and their allele-specific phenotypic effects. *Plant Physiology* **125,** 1406–1418.

Emanuelsson O., Nielsen H., Brunak S. & von Heijne G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300,** 1005–1016.

Eulgem T., Rushton P.J., Robatzek S. & Somssich I.E. (2000) The WRKY superfamily of plant transcription factors. *Trends in Plant Science* **5,** 199–206.

Goldsbrough A.P., Albrecht H. & Stratford R. (1993) Salicylic acid-inducible binding of a tobacco nuclear protein to a 10 bp sequence which is highly conserved amongst stress-inducible genes. *Plant Journal* **3,** 563–571.

Heitz T., Geoffroy P., Fritig B. & Legrand M. (1991) Two apoplastic α-amylases are induced in tobacco by virus infection. *Plant Physiology* **97,** 651–656.

Higo K., Ugawa Y., Iwamoto M. & Korenage T. (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research* **27,** 297–300.

Hoth S., Morgante M., Sanches J.P., Hanafey M.K., Tingey S.V. & Chua N.H. (2002) Genome-wide gene expression profiling in *Arabidopsis thaliana* reveals new targets of abscisic acid and largely impaired gene regulation in the *abi1-1* mutant. *Journal of Cell Science* **115,** 4891–4900.

Kakefuda G. & Duke S.H. (1984) Electrophoretic transfer as a technique for the detection and identification of plant amylolytic enzymes in polyacrylamide gels. *Plant Physiology* **75,** 278–280.

Kakefuda G., Duke H. & Hostak M.S. (1986) Chloroplast and extrachloroplastic starch-degrading enzymes in *Pisum sativum* L. *Planta* **168,** 175–182.

Katagiri F. (2002) How to study gene function. In *Arabidopsis: A Laboratory Manual* (eds D. Weigel & J. Glazebrook), pp. 300–304. Cold Spring Harbor Laboratory Press, Woodbury, NY, USA.

Kim Y.C., Nakajima M., Nakayama A. & Yamaguchi I. (2005) Contributions of gibberellins to the formation of *Arabidopsis* seed coat through starch degradation. *Plant and Cell Physiology* **46,** 1317–1325.

Kötting O., Pusch K., Tiessen A., Geigenberger P., Steup M. & Ritte G. (2005) Identification of a novel enzyme required for starch metabolism in *Arabidopsis* leaves. The phosphoglucan, water dikinase. *Plant Physiology* **137,** 242–252.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lim P.O., Woo H.R. & Nam H.G. (2003) Molecular genetics of leaf senescence in *Arabidopsis*. *Trends in Plant Science* **8,** 272–278.

Lin T.P., Spilatro S.R. & Preiss J. (1988) Subcellular localization and characterization of amylases in *Arabidopsis* leaf. *Plant Physiology* **86,** 251–259.

Lloyd J.R., Kossmann J. & Ritte G. (2005) Leaf starch degradation comes out of the shadows. *Trends in Plant Science* **10,** 130–137.

Lorbeth R., Ritte G., Willmitzer L. & Kossmann J. (1998) Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. *Nature Biotechnology* **16,** 473–477.

Monroe J.D. & Preiss J. (1990) Purification of a β-amylase that accumulates in *Arabidopsis thaliana* mutants defective in starch metabolism. *Plant Physiology* **94,** 1033–1039.

Nakai K. & Kanehisa M. (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* **14,** 897–911.

Nilsen E.T. & Orcutt D.M. (1996) *The Physiology of Plants Under Stress: Abiotic Factors*, p. 689. John Wiley and Sons, New York, NY, USA.

Nittylä T., Messerli G., Trevisan M., Chen J., Smith A.M. & Zeeman S.C. (2004) A previously unknown maltose transporter essential for starch degradation in leaves. *Science* **303,** 87–89.

Okita T.W., Greenburg E., Kuhn D.N. & Preiss J. (1979) Subcellular localization of the starch degradative and biosynthetic enzymes of spinach leaves. *Plant Physiology* **64,** 187–192.

Park J.H., Oh S.A., Kim Y.H., Woo H.R. & Nam H.G. (1998) Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in *Arabidopsis*. *Plant Molecular Biology* **37,** 445–454.

Park H.C., Kim M.L., Kang Y.H., *et al*. (2004) Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiology* **135,** 2150–2161.

Quirino B.F., Normanly J. & Amasino R.M. (1999) Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen-independent induction of defense-related genes. *Plant Molecular Biology* **40,** 267–278.

Ried J.L. & Walker-Simmons M.K. (1990) Synthesis of abscisic acid-responsive, heat-stable proteins in embryonic axes of dormant wheat grain. *Plant Physiology* **93,** 662–667.

Rieping M. & Schoffl F. (1992) Synergistic effect of upstream sequences, CCAAT box elements, and HSE sequences for enhanced expression of chimaeric heat shock genes in transgenic tobacco. *Molecular and General Genetics* **231,** 226–232.

Ritte G., Lloyd J.R., Eckermann N., Rottmann A., Kossmann J. & Steup M. (2002) The starch-related R1 protein is an α-glucan, water dikinase. *Proceedings of the National Academy of Sciences of the USA* **99,** 7166–7171.

Roberts C.S., Rajagopal S., Smith L.A., et al. (1998) A comprehensive set of modular vectors for advanced manipulations and efficient transformation of plants by both *Agrobacterium* and direct DNA uptake methods. *CAMBIA Vector Release Manual, Version 3.05*.

Saeed M. & Duke S.H. (1988) Chloroplastic regulation of apoplastic α-amylase activity in pea seedlings. *Plant Physiology* **93,** 131–140.

Saeed M. & Duke S.H. (1990) Amylases in pea tissue with reduced chloroplast density and/or function. *Plant Physiology* **94,** 1813–1819.

Scheidig A., Fröhlich A., Schulze S., Lloyd J.R. & Kossman J. (2002) Downregulation of a chloroplast-targeted β-amylase leads to a starch-excess phenotype in leaves. *Plant Journal* **30,** 581–591.

Schenk P.M., Kazan K., Rusu A.G., Manners J.M. & Macclean D.J. (2005) The *SEN1* gene of *Arabidopsis* is regulated by signals that link plant defence responses and senescence. *Plant Physiology and Biochemistry* **43,** 997–1005.

Smith A.M., Zeeman S.C. & Smith S.M. (2005) Starch degradation. *Annual Review of Plant Biology* **56,** 73–98.

Stanley D., Fitzgerald A.M., Farnden K.J.F. & MacRae E.A. (2002) Characterisation of putative α-amylases from apple (*Malus domestica*) and *Arabidopsis thaliana*. *Biologia Bratislava* **11,** 137–148.

Stitt M., Bulpin P.V. & ap Rees T. (1978) Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. *Biochimica et Biophysica Acta* **544,** 200–214.

Truernit E., Schmid J., Epple P., Illig J. & Sauer N. (1996) The sink-specific and stress-regulated *Arabidopsis STP4* gene:

© 2007 James Madison University

enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. *Plant Cell* **8,** 2169–2182.

Tzfira T., Jensen C.S., Wang W., Zucker A., Vinocur B., Altman A. & Vainstein A. (1997) Transgenic *Populus tremula*: a step-by-step protocol for its *Agrobacterium*-mediated transformation. *Plant Molecular Biology Reporter* **15,** 219–235.

Wang Q., Monroe J. & Sjolund R.D. (1995) Identification and characterization of a phloem specific β-amylase. *Plant Physiology* **109,** 743–750.

Weaver L.M., Gan S., Quirino B. & Amasino R.M. (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Molecular Biology* **37,** 455–469.

Weintraub M. & Ragetti H.W.J. (1964) An electron microscope study of tobacco mosaic virus lesions in *Nicotiana glutinosa* L. *Journal of Cell Biology* **23,** 499–509.

Weise S.E., Weber A. & Sharkey T.D. (2004) Maltose is the major form of carbon exported from the chloroplast at night. *Planta* **218,** 474–482.

Yoshida S., Ito M., Nishida I. & Watanabe A. (2002) Identification of a novel gene *HYS1/CPR5* that has a repressive role in the induction of leaf senescence and pathogen-defence responses in *Arabidopsis thaliana*. *Plant Journal* **29,** 427–437.

Yu T.S., Kofler H., Hausler R.E., *et al*. (2001) The Arabidopsis sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *Plant Cell* **13,** 1907–1918.

Yu T.S., Zeeman S.C., Thorneycroft D., *et al*. (2005) α-Amylase is not required for breakdown of transitory starch in Arabidopsis leaves. *Journal of Biological Chemistry* **280,** 9773–9779.

Received 18 August 2006; received in revised form 24 October 2006; accepted for publication 13 November 2006

© 2007 James Madison University