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α-Amylase Is Not Required for Breakdown of Transitory Starch in *Arabidopsis* Leaves*

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The *Arabidopsis thaliana* genome encodes three α-amylase-like proteins (AtAMY1, AtAMY2, and AtAMY3). Only AtAMY3 has a predicted N-terminal transit peptide for plastidial localization. AtAMY3 is an unusually large α-amylase (93.5 kDa) with the C-terminal half showing similarity to other known α-amylases. When expressed in *Escherichia coli*, both the whole AtAMY3 protein and the C-terminal half alone show α-amylase activity. We show that AtAMY3 is localized in chloroplasts. The starch-excess mutant of *Arabidopsis sex4*, previously shown to have reduced plastidial α-amylase activity, is deficient in AtAMY3 protein. Unexpectedly, T-DNA knock-out mutants of *AtAMY3* have the same diurnal pattern of transitory starch metabolism as the wild type. These results show that AtAMY3 is not required for transitory starch breakdown and that the starch-excess phenotype of the *sex4* mutant is not caused simply by deficiency of AtAMY3 protein. Knock-out mutants in the predicted non-plastidial α-amylases AtAMY1 and AtAMY2 were also isolated, and these displayed normal starch breakdown in the dark as expected for extraplastidial amylases. Furthermore, all three AtAMY double knock-out mutant combinations and the triple knock-out degraded their leaf starch normally. We conclude that α-amylase is not necessary for transitory starch breakdown in *Arabidopsis* leaves.

α-Amylase (EC 3.2.1.1) is an endoamylolytic enzyme that hydrolyzes the α-1,4-glucosidic linkages of starch. It is found in most reserve tissues during periods of starch mobilization (1, 2). In germinating cereal seeds, α-amylase secreted from the aleurone cells initiates the degradation of starch granules in the non-living endosperm. It liberates soluble glucans that can be further degraded by other hydrolytic enzymes such as debranching enzymes and β-amylases. In most plant tissues, however, starch degradation occurs within living cells. Leaf starch, for example, is synthesized inside chloroplasts during photosynthesis and degraded during the subsequent dark period. The importance of endoamylases in the pathway of starch degradation in plant tissues such as leaves is not known (3–5).

The capacity of endoamylase to initiate the degradation of intact starch granules *in vitro* suggests that it could be an important component of the starch-hydrolyzing pathway. However, there is no direct evidence for the involvement of α-amylase in starch degradation in plastids. Several cell fractionation studies have indicated that there is α-amylase activity inside chloroplasts (6–8), but only recently have genes encoding α-amylases with putative plastidial targeting signals been discovered (9). Most published DNA sequences encoding plant α-amylases predict proteins that have a signal peptide for extracellular localization and a molecular mass of ~45–50 kDa (7). Presumably, extrachloroplastic amylases are not involved in the metabolism of transitory starch in chloroplasts.

Electrophoresis of crude extracts under non-denaturing conditions has shown that *Arabidopsis* leaves contain several enzymes with amylolytic activity, including α-amylases, β-amylases, and debranching enzymes (8, 10–12). One α-amylase activity (designated A2), was found to be plastidial, and this activity was significantly reduced in amount in the starch-excess mutant *sex4* (8). Because other amylolytic enzyme activities were still present in the *sex4* mutant, it was suggested that the deficiency in chloroplastic α-amylase was the cause of the high starch phenotype, indicating an important role for the enzyme in the degradation of transitory starch.

Other factors important for starch degradation in leaves have been identified recently. Forward and reverse genetic approaches in *Arabidopsis* and potato have shown that β-amylase (13) and disproportionating enzyme (DPE1, 14) are directly involved in and necessary for normal rates of starch degradation, as are a maltose transporter in the chloroplast envelope (MEX1, 15) and a transglucosidase that metabolizes maltose (16–18). Normal rates of degradation also require a glucan, water dikinase (GWD1 or R1, 19–21), which phospho-

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rylates glucosyl residues of amylopectin. Potato and *Arabidopsis* plants with reduced activities of this enzyme have very high starch contents consistent with reduced rates of starch degradation, but the reasons for this are not understood. None of these enzymes is known to attack the starch granule *in vivo*, and the reactions or subcellular locations of several of them preclude such a role. A chloroplastic β-amylase important in starch degradation in potato leaves has recently been shown to attack starch granules isolated from potato tubers (13). In this study we tested the hypothesis that α-amylase plays a role in starch degradation in leaves. We report the functional characterization of the only predicted plastidial α-amylase in *Arabidopsis* (AtAMY3) and describe the effects of mutation of this gene and of the other two genes encoding α-amylases (AtAMY1 and AtAMY2) on starch metabolism.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—*Arabidopsis thaliana L.* plants (ecotypes Columbia and Wasserilewskija) were grown in soil in one of two growth regimes: (*a*) 23 °C, 70% relative humidity, with continuous illumination at 100 μmol quanta M^{-2} s^{-1} and (*b*) 20 °C, 75% relative humidity, with a 12-h photoperiod at 150 μmol quanta M^{-2} s^{-1}. Plants at growth stage 3.90 (31) were used for all experiments unless otherwise stated. *Arabidopsis*-expressed sequence tag (EST)[1] clones and SALK_005044, SALK_094382, and SALK_008656 seeds were obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH). The mutant lines SAIL_613 D12 and SAIL_642 G08 were isolated from seeds of the Syngenta SAIL collection (Torrey Mesa Research Institute, San Diego, CA), and the mutant Wisconsin 211 was obtained from the Wisconsin Arabidopsis Knock-out Facility (University of Wisconsin, Madison, WI). The *sex4-2* mutant was isolated from ethyl methanesulfonate mutagenized M2 *Arabidopsis* seeds obtained from Lehle Seeds (Round Rock, TX) and the *sex4–1* mutant was obtained from an x-ray generated population (8).

General Molecular Analysis—Standard cloning, DNA blot, and RNA blot techniques were as described by Sambrook *et al.* (32). The actin2 gene (At3g18780) was used as a constitutively expressed control in RT-PCR experiments. DNA sequencing was performed with double-stranded plasmids using an ABI 377 DNA sequencer (PE Biosystems, Foster City, CA). The DNA and protein sequences were analyzed with the GCG program (Genetics Computer Group, Madison, WI).

Isolation of cDNA Clones—To isolate the cDNA clone of *Arabidopsis AtAMY3*, an *Arabidopsis* cDNA library (Stratagene) was screened with a probe isolated from EST cDNA clone 135M15T7. To isolate cDNA clones of rice *OsAMY3*, primers were designed according to the genomic sequence (GenBank™ accession number AO003408). RT-PCR was performed with RNA isolated from rice leaves (*Oryza sativa* cv. TNG67) and *Pfu* DNA polymerase (Stratagene) as recommended by the suppliers. The PCR products from two independent reactions were cloned and sequenced.

Isolation of Chloroplasts—Chloroplasts were isolated either via protoplasts as described in Fitzpatrick and Keegstra (33) for immunoblotting or mechanically as described in Zeeman *et al.* (8) for renaturation gels. The chloroplast marker enzyme glyceradehyde-3-phosphate dehydrogenase was assayed as described in Zeeman *et al.* (8). Chloroplasts isolated via the former method were purified on a Percoll gradient and treated with protease. Activity gel assays for phosphoglucose isomerase and phosphoglucomutase were conducted as described by Yu *et al.* (34).

Measurement of Carbohydrates—Leaves were stained for starch using an iodine solution after first decolorizing them in 80% (v/v) ethanol. For quantitative measurements, samples comprising all the leaves of individual plants were harvested, immediately frozen in liquid N_2, and stored at −80 °C. Samples were extracted using perchloric acid as described in Critchley *et al.* (14). Quantitative assays of starch (in the insoluble fraction) and sugars (in the soluble fraction) were conducted as described by Hargreaves and ap Rees (35) and Kunst *et al.* (36), respectively. Malto-oligosaccharides (in the soluble fraction) were separated using high performance anion exchange chromatography on a Carbopac PA-100 column (Dionex) and measured using a pulsed amperometric detector as described in Critchley *et al.* (14).

End Product Analysis of AtAMY3 Recombinant Proteins—To express AtAMY3 protein (included amino acids 57–887) and AtAMY3-ΔN protein (included amino acids 497–887), these regions were amplified by PCR and subcloned into BamHI site of an *Escherichia coli* expression vector pET30A (Novagen, Madison, WI). The constructs were confirmed by DNA sequencing and were transformed into *E. coli* strain BL21/DE3. The proteins expressed in inclusion body fraction after the isopropyl 1-thio-β-D-galactopyranoside induction were isolated and separated by SDS-PAGE in 10% (w/v) polyacrylamide gels as recommended by Novagen. Renaturation of the gel was performed as described above, and bands were excized, minced, and incubated with 2% (w/v) soluble starch. Separation of end products and standards by thin-layer chromatography on a silica gel 60 plate (Merck) developed with *n*-butanol:ethanol:water (5:3:2), and detection of oligosaccharides by diphenylamine and aniline was performed according to methods described by White and Kennedy (37).

Non-denaturing and SDS-renaturing Gel Analysis—For non-denaturing (native) gels, the method described in Zeeman *et al.* (8) was used. For the renaturation of enzyme activity from SDS-denatured proteins, two different methods were used.

Proteins from crude extracts of leaves were separated in 7.5% SDS-polyacrylamide gels containing 0.1% potato amylopectin or 0.1% β-limit dextrin. Gels were washed three times in 100 ml of incubation medium containing 100 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and then incubated for 16 h in the same medium. Amylase activities were detected with 0.67% (w/v) I_2 and 3.33% (w/v) KI.

Recombinant proteins expressed in *E. coli* (see above) were separated in 10% SDS-polyacrylamide gels containing 0.2% soluble starch. To renature proteins, gels were washed at room temperature three times with 100 ml of 40 mM Tris-HCl, pH 7.5, for 3 h and twice with 6 M urea, 1 mM dithiothreitol, 50 mM Tris-HCl, pH 8.0, for 1 h. Gels were then washed with several changes of 1 mM dithiothreitol, 50 mM Tris-HCl, pH 8.0, for 18 h at 4 °C. The renatured gels were incubated in 0.1 M Tris-HCl, pH 7.0, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol overnight at 37 °C, and amylase activities were detected with 0.67% (w/v) I_2 and 3.33% (w/v) KI.

Immunoblot Analysis—Standard immunology techniques were used as described by Harlow and Lane (38). Proteins from leaf extracts were separated by SDS-PAGE in 7.5% or 10% (w/v) polyacrylamide gels and electroblotted to nitrocellulose membranes. Rabbit antisera were prepared against the *E. coli* expressed *Arabidopsis* AtAMY3 antigen and used as probes for the immunoblot analyses. The *Arabidopsis* AtAMY3 antigen was isolated from an *E. coli* strain carrying a plasmid with an EcoRI-HindIII 0.8-kb fragment of EST clone 135M15T7 into pET30C (Novagen). The primary antibody was detected with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

RESULTS

Cloning of the Arabidopsis α-Amylase cDNA AtAMY3—BLAST searches of the *Arabidopsis* sequence data bank identify three genomic sequences (GenBank™ accession numbers AL161562, AC009978, and AC010675) that have sequence similarity to known α-amylase genes. These sequences have been designated *AtAMY1*, *AtAMY2*, and *AtAMY3*, respectively (9). Full-length cDNA clones corresponding to these genomic sequences were also identified (Ceres clones CERES119931 and CERES3059 and GenBank™ accession number AY050398, respectively).

We obtained the full-length cDNA for *AtAMY3* by screening an *Arabidopsis* cDNA library using the EST 135M15T7 as a probe. As reported by Stanley *et al.* (9), comparison of the genomic sequence and the full-length cDNA sequence shows that it contains 12 introns (Fig. 1) with typical GT-AG dinucleotide splicing junctions in each case. The *AtAMY3* gene (At1g69830), encodes a protein of 887 amino acids (Fig. 1), predicted to contain a chloroplast transit peptide of 56 amino acids (ChloroP, 22). The molecular mass of the predicted protein is 99.7 kDa, or 93.5 kDa following the cleavage of its transit peptide. The C-terminal domain (amino acids 497–887) shows ~46% identity to rice extracellular α-amylases, but the N-terminal domain (~50 kDa) shares no similarity to previously described α-amylase proteins.

Two domains of the novel N-terminal region (amino acids 31–160 and 235–375) have appreciable sequence similarity to domains of the N-terminal region of GWD1 of *Arabidopsis* and

[1] The abbreviations used are: EST, expressed sequence tag; RT, reverse transcription.

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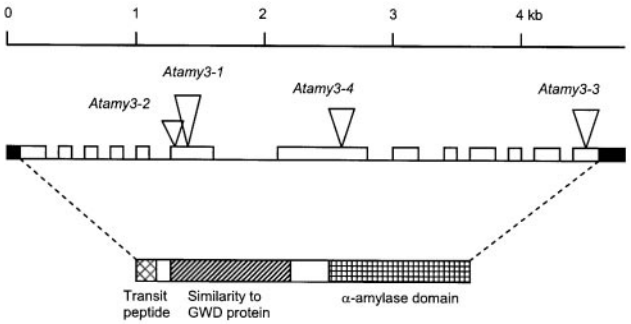

FIG. 1. **Structure of the *AtAMY3* gene (*above*) and protein (*below*).** Exons are depicted as *open boxes* and the 5′- and 3′-untranslated regions as *black boxes*. *Triangles* mark the sites of the T-DNA insertions in four independent knock-out lines, *Atamy3-1* (SAIL_642 G08), *Atamy3-2* (SAIL_613 D12), *Atamy3-3* (SALK_005044), and *Atamy3-4* (Wisconsin H_211). The domains of the AtAMY3 protein with homology to other α-amylases or to GWD1 are marked.

Solanum tuberosum (Fig. 2*A*). As with AtAMY3, the C-terminal region of GWD1 is believed to be responsible for its catalytic activity, and the function of the N-terminal region is not known (21). Further analysis revealed that the two domains of similarity between AtAMY3 and GWD1 also represent repeated motifs of ~90 amino acids within each of the proteins (Fig. 2, *A* and *B*). A second GWD1-like protein of *Arabidopsis* (GWD2, At4g24450) also contains a single copy of this conserved sequence in its N-terminal region (Fig. 2*B*).

The proteins encoded by *AtAMY1* (At4g25000) and *AtAMY2* (At1g76130) do not contain predicted chloroplast transit peptides or N-terminal regions similar to that of AtAMY3. Analysis using SignalP (23) indicates the presence of a 24-amino-acid signal peptide that would target AtAMY1 to the secretory pathway (9).

Presence of Sequences Similar to AtAMY3 in Other Plant Species—A gene encoding an α-amylase with an N-terminal domain similar to that of AtAMY3 has been reported from apple (MdAMY10, 9). We investigated whether similar α-amylase genes are present in other plants. A rice genomic sequence (*OsAMY3*, GenBank™ accession number AP003408) is similar to the *AtAMY3* gene. Using RT-PCR, we obtained the corresponding rice cDNA. Genomic sequence analysis indicated that the rice *OsAMY3* gene has an identical intron/exon structure to *AtAMY3* and encodes a protein with 64% amino acid identity to AtAMY3 (data not shown). By BLAST-searching DNA data bases for sequences significantly similar to the part of the *AtAMY3* cDNA encoding the unique N-terminal domain, we also identified ESTs from *Lycopersicon pennellii* (GenBank™ accession number AW399728), *Lycopersicon hirsutum* (GenBank™ accession number AW616948), *Medicago truncatula* (GenBank™ accession numbers AW689974, AW691339, BE322876), *Glycine max* (GenBank™ accession number BE801868), and *Hordeum vulgare* (GenBank™ accession number BG299837). The sequence similarity between these ESTs and *AtAMY3* was not restricted to the GWD-like regions. Thus, it appears that *AtAMY3*-like genes belonging to the α-amylase family 3 proposed by Stanley *et al.* (9) are present and expressed in both dicots and monocots.

Expression, Localization, and Activity of AtAMY3—Previous microarray studies have shown that AtAMY3 mRNA shows strong diurnal fluctuations that are under circadian control. Amounts increase in the latter part of the light period and decrease early in the dark period (24, 25). We confirmed this pattern of expression using RNA gel blots with RNA isolated at several different time points from leaves of plants grown under a 12-h day/12-h night cycle (Fig. 3*A*). Protein isolated at the same time points was analyzed with immunoblots using an anti-AtAMY3 antibody raised against a recombinant AtAMY3 protein expressed in *E. coli* (see "Experimental Procedures"). In contrast to the mRNA level, we found that neither the amount of AtAMY3 protein (Fig. 3*B*) nor the amylolytic activity of AtAMY3 present in the leaf extracts (assayed by native gels, data not shown) showed strong diurnal fluctuations.

Two experiments were carried out to confirm the chloroplastic localization of the AtAMY3 protein. First, we performed immunoblot analyses on *Arabidopsis* chloroplasts that had been purified on a Percoll gradient and treated with protease. These chloroplasts were essentially free of cytosolic contamination (using activity gel assays for the cytosolic isoforms of phosphoglucose isomerase and phosphoglucomutase activity, data not shown). Immunoblotting of chloroplast extracts and total leaf homogenates with anti-AtAMY3 antibody revealed that both contained a reactive protein of 93 kDa (Fig. 4*A*, *first* and *fourth lanes*), which corresponds well to the predicted size of the mature protein. The amount of chloroplast protein analyzed on the blot was one-seventh the amount of protein of the total leaf homogenate, but the amount of AtAMY3 protein in these two fractions on the blot was comparable. This indicates strongly that the protein is chloroplastic. Second, we performed renaturation experiments in SDS-gels containing amylopectin using leaf homogenates and chloroplasts isolated from the homogenates. The gels were loaded such that each lane contained equal amounts of the chloroplast marker enzyme glyceraldehyde-3-phosphate dehydrogenase. After electrophoresis and removal of SDS, an α-amylase activity with a molecular mass of 93 kDa could be renatured. This activity was present in a leaf homogenate and in chloroplasts prepared from the homogenate (Fig. 4*B*). A second band with greater mobility (a putative isoform of β-amylase, as it is unable to hydrolyze β-limit dextrin) was present only in the homogenate indicating that it is an extraplastidial enzyme. These two experiments thus confirm that AtAMY3 is located in the chloroplast.

To investigate the activity of AtAMY3, we isolated *E. coli*-expressed AtAMY3 protein (included amino acids 57–887) and the C-terminal domain of the protein (AtAMY3-ΔN, included amino acids 497–887). After separation by SDS-PAGE both recombinant proteins could be detected by immunoblotting with anti-AtAMY3 antibody (Fig. 5*A*) and could be renatured into an active state (Fig. 5*B*). Soluble starch was digested with these recombinant proteins, and the end products were analyzed by thin-layer chromatography (Fig. 5*C*). Both the full-length and the C-terminal domain of AtAMY3 liberated short malto-oligosaccharides together with trace amounts of glucose. The end products were similar to those released by commercially available Bacillus α-amylase. These results show first that the AtAMY3 protein has an endoamylolytic activity and second that the N-terminal domain of the protein is not required for this activity.

Leaves of the *Arabidopsis* mutant *sex4,* which have increased levels of starch, have previously been shown to contain reduced levels of a plastidial α-amylase activity (8). Immunoblot and renaturation gel analysis confirmed that AtAMY3 protein and activity levels were reduced in this mutant (Fig. 4, *A* and *B*).

Transitory Starch Is Degraded Normally in Null Mutants of All Three AtAMY Genes—If AtAMY3 is an important enzyme for transitory starch degradation, null mutants lacking AtAMY3 would be expected to have reduced rates of starch degradation at night and should thus contain elevated levels of leaf starch. To discover whether this is the case, we isolated four independent *AtAMY3* mutants with T-DNA insertions in the gene (*Atamy3-1, -3-2, -3-3*, and *-3-4*). Sequence analysis of each line revealed the T-DNA insertion sites in the *AtAMY3* gene (Fig. 1), and expression analysis using either RNA gel

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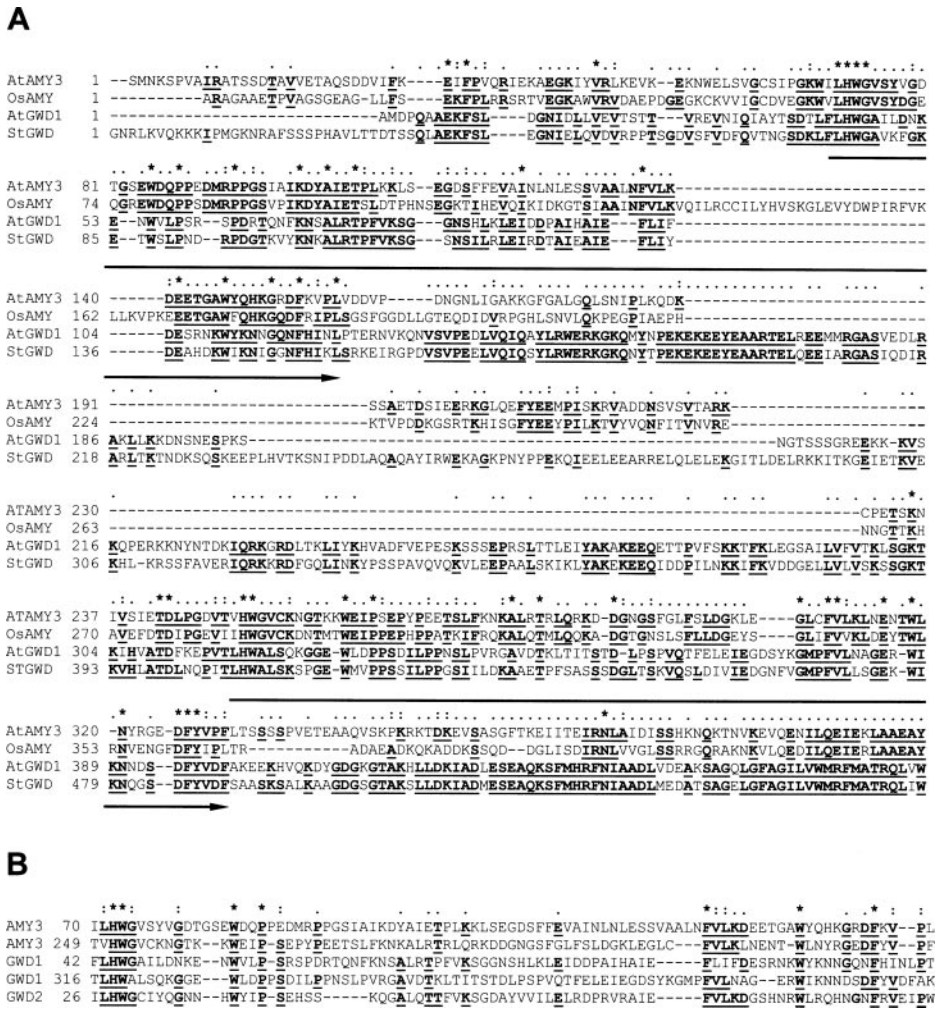

FIG. 2. **Sequence comparisons of the N-terminal domains of plastidial α-amylases from *Arabidopsis* and rice, and of glucan, water dikinases from *Arabidopsis* and potato.** *A*, the predicted chloroplast transit peptides were removed, and the N termini of *Arabidopsis* (AtAMY3, At1g69830) and rice (OsAMY3, GenBank™ accession number AP003408) plastidial α-amylases were aligned with those of GWD1 sequences from *Arabidopsis* (AtGWD1, SEX1 protein, At1g10760) and potato (StGWD, R1 protein, GenBank™ accession number AY027522) using ClustalX (39). Amino acids conserved between either the two AMY3 or GWD proteins are indicated in *bold* and *underlined*. *Asterisks* above the alignment indicate residues conserved in all four sequences, *colons* three, and *dots* two. *Arrows* indicate repeated regions that are aligned in *B*. *B*, repeated N-terminal regions of *Arabidopsis* proteins AtAMY3 and AtGWD1 and a single sequence found in GWD-like protein AtGWD2 (At4g24450) were aligned using ClustalX. Conserved amino acids are indicated in *bold* and *underlined*. An *asterisk* indicates all five residues conserved, a *colon* four, and a *dot* three.

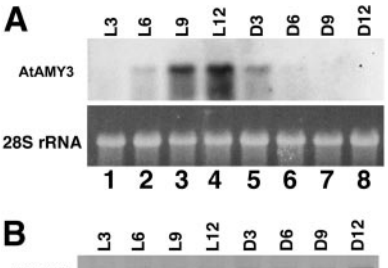

FIG. 3. **RNA gel blot and immunoblot analysis of *AtAMY3* expression in wild type plants grown in a 12-h/12-h light/dark cycle.** *A*, RNA gel blot of total leaf RNA (20 μg) isolated from the wild type plants was probed with radiolabeled *Arabidopsis AtAMY3* probe. The leaves were harvested at the time point indicated (*e.g. L3* represents leaves harvested from plants that had been illuminated for 3 h). *B*, immunoblot of leaf protein (20 μg) probed with the AtAMY3 antibody. *D*, dark.

blots or RT-PCR revealed that the *AtAMY3* transcript was missing in all four lines (data not shown). The protein recognized by the AtAMY3 antiserum was missing in all of the lines (*e.g.* Fig. 6*A*), and renaturation of enzyme activities in amylopectin-containing gels revealed that the 93-kDa endoamylase activity was also abolished (*e.g.* Fig. 6*B*). Electrophoresis of crude extracts under non-denaturing conditions in polyacrylamide gels containing amylopectin revealed that the α-amylase band A2 was missing (*e.g.* Fig. 6*C*). This is the α-amylase band that is reduced in amount in the *sex4* mutant (8).

Leaves of the four mutant lines and their respective wild types were harvested at the end of the day and at the end of the night. Qualitative staining for the presence of starch (iodine staining) and quantitative measurements (by enzymatic digestion of starch to glucose) revealed no significant differences in leaf starch content in any of the mutants compared with the respective wild types (data not shown). In a more detailed experiment using the mutant line *Atamy3-1*, the starch content in leaves was measured throughout the diurnal cycle and compared with that of the corresponding wild type grown under the same conditions. The pattern of accumulation of starch during the light period and degradation during the dark period was very similar in the two lines (Fig. 7*A*). We measured maltooligosaccharides at two time points during the day (8 h and 12 h) and at two points during the night (16 h and 20 h) using high performance anion exchange chromatography-pulsed amperometric detector. Amounts of oligosaccharides from maltose to maltohexaose were not significantly different in wild type and mutant plants (data not shown). In a second experiment we measured the sucrose and free hexose contents of leaves. Minor differences between the wild type and mutant were detected at some time points. However, the overall sugar contents of the two lines were similar (Fig. 7*B*). These results show that other starch-degrading enzymes can accomplish transitory starch degradation in the absence of AtAMY3 and that sugar metabolism, which is sustained by the products of starch breakdown at night, is not perturbed in any major way.

We identified T-DNA insertion mutations in the remaining two α-amylase genes, *AtAMY1* and *AtAMY2* (Fig. 8*A*). In both cases, the T-DNA insertion site was confirmed by sequence

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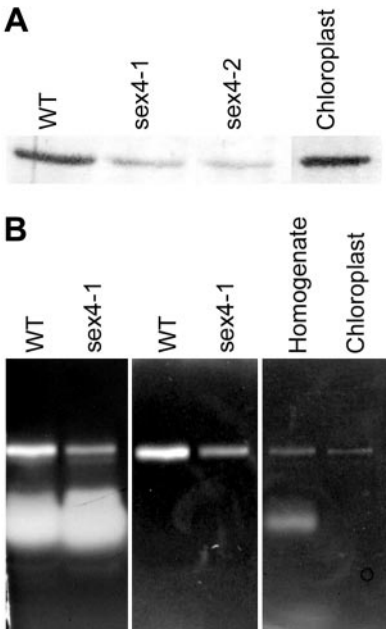

FIG. 4. **Plastidial localization of the AtAMY3 protein and its expression in the *sex4* mutant.** *A*, a blot of soluble proteins isolated from leaves from the wild type (*WT*) (20 μg), *sex4* mutants (20 μg), and purified chloroplasts (3 μg) was probed with the AtAMY3 antibody. *B*, renaturation of amylolytic activity after SDS-PAGE in gels containing either amylopectin (*first* and *third panels*) or β-limit dextrin (*second panel*). Soluble proteins were extracted from leaves from the wild type or *sex4* mutants. Chloroplasts were prepared as described under "Experimental Procedures." Homogenate and chloroplast lanes from wild type leaves (*third panel*) were loaded such that each lane contained equal amounts of the chloroplast marker enzyme glyceraldehyde-3-phosphate dehydrogenase.

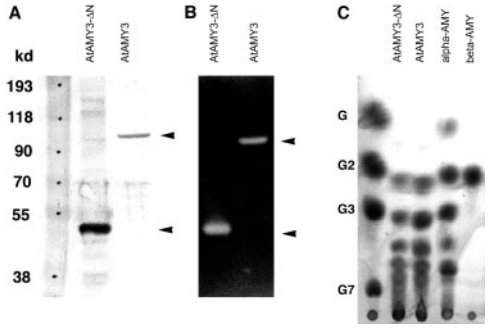

FIG. 5. **End products analysis of soluble starch digested with AtAMY3 proteins.** *A*, immunoblot of *E. coli* expressed proteins of AtAMY3 and the C-terminal domain of this protein (AtAMY3-ΔN). Insoluble proteins were separated by 10% SDS-PAGE and probed with AtAMY3 antibody. *Arrows* indicate AtAMY3 and AtAMY3-ΔN proteins. *B*, AtAMY3 and AtAMY3-ΔN proteins were separated by 10% SDS-PAGE containing soluble starch and renatured for the amylolytic activity assay. *Arrows* indicate the AtAMY3 and AtAMY3-ΔN amylolytic activity. *C*, soluble starch digested with AtAMY3, AtAMY3-ΔN, Bacillus α-amylase (alpha-AMY), or wheat β-amylase (β-AMY). The end products and oligosaccharide standards were separated by thin-layer chromatography and detected with diphenylamine and aniline. *G*, glucose.

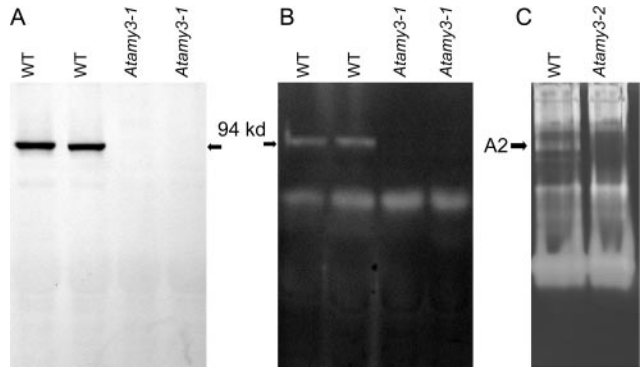

FIG. 6. **Loss of AtAMY3 in T-DNA insertion mutants.** *A*, immunoblot blot of soluble proteins isolated from leaves of wild type (*WT*) and *Atamy3-1* plants. Proteins were separated in a 7.5% SDS-PAGE, blotted onto nitrocellulose, and probed with AtAMY1 antibody. *B*, renaturation of amylolytic activity in gels containing amylopectin after SDS-PAGE of soluble proteins isolated from leaves of wild type and *Atamy3-1*. After removal of SDS, gels were incubated at room temperature for 16 h and stained with an iodine solution. *C*, non-denaturing PAGE of starch-hydrolyzing enzymes in crude extracts of wild type and *Atamy3-2* leaves using gels containing 0.1% (w/v) amylopectin. After electrophoresis, gels were incubated at room temperature for 16 h and stained with an iodine solution.

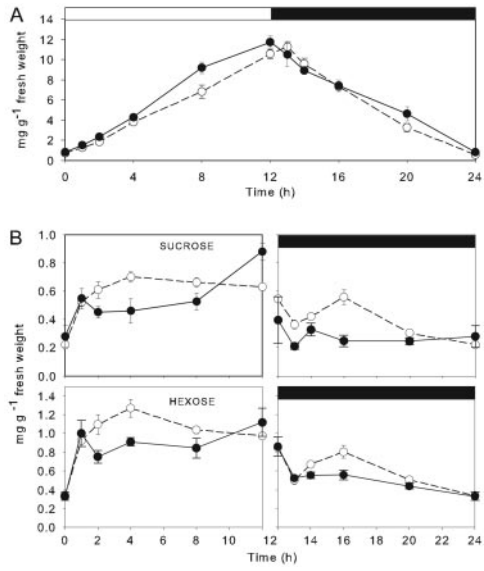

FIG. 7. **Starch and sugar contents of leaves of wild type and *Atamy3-1* knock-out plants.** Samples comprising all the leaves of individual plants (0.2–0.4g fresh weight) of wild type (*closed symbols*) and knock-out mutant *Atamy3–1* (*open symbols*) were harvested into liquid N_2 and extracted in perchloric acid. Starch in the insoluble fraction was determined enzymatically after hydrolysis to glucose. Sugars in the neutralized, soluble fraction of the extract were assayed enzymatically. Values are means ± S.E. of six measurements, each made on a separate plant. Results for starch (*A*) are from a different batch of plants from those for sugars (*B*). Plants for analysis for starch were harvested at time points from the start of the light period onwards (*closed box*, dark period), whereas those for sugars were harvested from the start of the dark period onwards. The sugar contents are displayed from the start of the light period for comparison with the starch results (thus 0 and 24 h time points are the same data). *A*, starch content. *B*, content of sucrose (*upper panels*) and hexoses (sum of glucose and fructose contents, *lower panels*).

analysis and loss of expression verified using RT-PCR (Fig. 8*B*). Starch accumulation in the leaves of these mutants was assessed by iodine staining at the end of the day and at the end of the night (Fig. 8*C*). In both mutants, starch was present at the end of the day and absent at the end of the night indicating that starch metabolism was unaffected in these lines. No changes in the growth rate or morphology of these plants relative to the wild types were observed.

To determine whether there is redundancy of function within the AtAMY family, we created all double mutant combinations and the triple T-DNA insertion mutant (*Atamy1*:*Atamy2*:*Atamy3-2*). Iodine staining of leaves at the end of the day and end of the night indicated that starch accumulation and breakdown was normal in all double mutants (data not shown). For the triple mutant, starch content was assayed throughout the diurnal cycle. Starch accumulated normally in the light and

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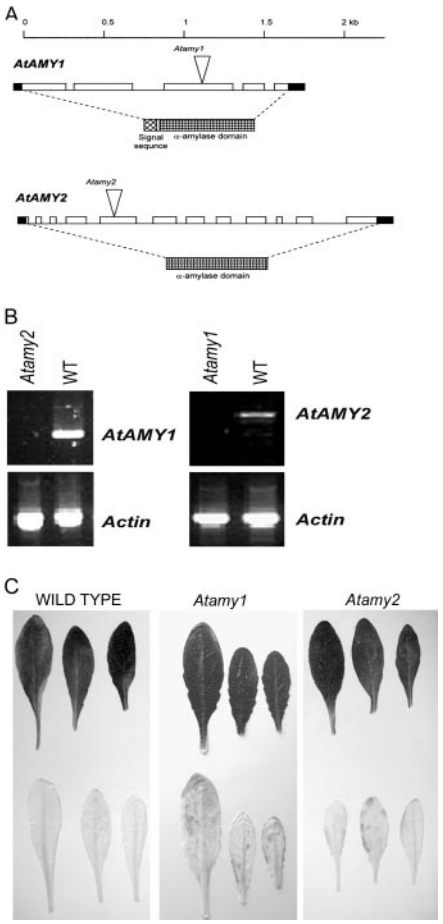

FIG. 8. **Structure and T-DNA insertions in the *Arabidopsis AtAMY1* and *AtAMY2* loci.** *A*, structure of the *AtAMY1* and *AtAMY2* genes; exons are depicted as *open boxes* and the 5′- and 3′-untranslated regions as *black boxes*. *Triangles* mark the site of T-DNA insertions. *B*, RT-PCR analysis of *AtAMY1* expression in the wild type and *Atamy1* (SALK_094382) and of *AtAMY2* expression in the wild type and *Atamy2* (SALK_008656). PCR products were separated in agarose gels and stained with ethidium bromide. *C*, iodine-stained leaves harvested at the end of the day (*top*) or end of the night (*bottom*) from the wild type and knock-out mutants *Atamy1* and *Atamy2*.

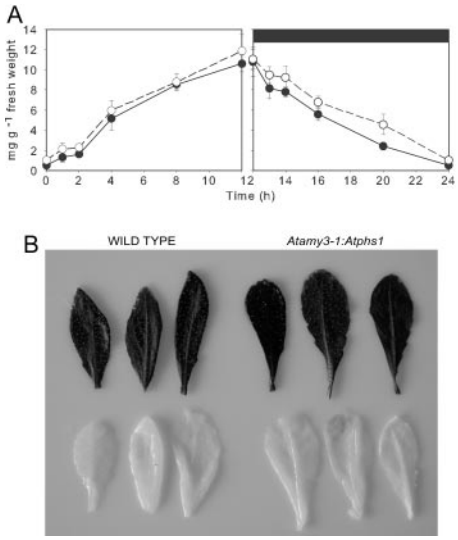

FIG. 9. **Starch breakdown in multiple-knock-out mutants.** *A*, samples comprising all the leaves of individual plants (0.2–0.4g fresh weight) of wild type (*closed circles*) and *Atamy1:Atamy2:Atamy3-2* triple knock-out mutant (*open circles*) were harvested into liquid N_2 and extracted in perchloric acid. Starch in the insoluble fraction was determined enzymatically after hydrolysis to glucose. Values are means ± S.E. of three or four measurements, each made on a separate plant. *B*, iodine-stained leaves harvested at the end of the day (*top*) or end of the night (*bottom*) from the wild type and *Atamy3-1:Atphs1* double knock-out mutant.

was degraded in the dark in the triple mutant similarly to wild type grown under the same conditions (Fig. 9*A*).

We have shown that plastidial starch phosphorylase (AtPHS1) is not required for starch breakdown in *Arabidopsis* (26). To discover whether the lack of a starch degradation phenotype in *Atphs1* and *Atamy3* mutants is the result of mutual redundancy, we tested for epistasis by creating a *Atphs1:Atamy3-1* double mutant. Iodine staining revealed that starch accumulated normally in the light and was broken down normally in the dark in this double mutant (Fig. 9*B*).

DISCUSSION

The Role of α-Amylase—It is accepted that α-amylase plays an important role in the degradation of storage starch in the endosperm of germinating cereal seeds (2) and it is often suggested that α-amylase plays a similar role in the degradation of transitory starch in chloroplasts (1, 4, 18, 27, 28). To test this hypothesis we have investigated the nature and role of AtAMY3, the only known chloroplast-targeted α-amylase in *Arabidopsis*. Our results confirm that AtAMY3 has α-amylase activity, is localized in chloroplasts, and is the A2 amylase reduced in the starch-accumulating mutant line *sex4*. However, we also demonstrate that disruption of *AtAMY3* through insertional mutagenesis does not affect starch degradation or cause starch to accumulate. Thus, we conclude that AtAMY3 is not essential for starch breakdown in chloroplasts under the growth conditions we use.

It is possible that AtAMY3 does participate in starch breakdown in wild type leaves but that other enzymes can compensate in its absence. We tested whether AtAMY1 or AtAMY2 might be able to compensate for lack of AtAMY3, even though neither of these enzymes is predicted to be targeted to the chloroplast. Neither of these enzymes is required for normal rates of starch degradation, and starch degradation is normal even when all three AtAMY genes are disrupted, indicating that starch degradation does not require any α-amylase. Our results indicate either that there are other, undiscovered plastidial endoamylases participating in starch breakdown or that enzymes other than endoamylases may initiate the degradation of intact starch granules in chloroplasts. Starch phosphorylase can attack leaf starch granules *in vitro* (29), but we have shown that it is not required for starch breakdown in *Arabidopsis* leaves under our growth conditions (26). We investigated if AtPHS1 and AtAMY3 are functionally redundant by creating a double mutant lacking both of these enzymes. However, this plant had normal leaf starch degradation. Therefore, the enzyme(s) responsible for attacking intact starch granules in *Arabidopsis* leaves remains unknown. β-amylase is a candidate (13) but analysis of β-amylase function in *Arabidopsis* is complicated by the presence of at least nine genes (25).

AtAMY3 Gene Expression and Structure—The mRNA level of *AtAMY3* increases in the light period and decreases in the dark period. This pattern of expression, like those of several genes believed to be involved in starch metabolism (25), is under circadian control (24, 30). These data could be interpreted to mean that gene expression is timed to coincide with the onset of starch mobilization. Interestingly, an endoamylase with diurnally fluctuating activity that coincides with the expression pattern observed for *AtAMY3* was found in *Arabidopsis* leaves using non-denaturing amylopectin-containing gels (band designated 1A; 11, 27). The cellular location of the protein and the nature of the gene encoding this endoamylase were not char-

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acterized. However, in contrast to the pattern of transcription, we found that the amount of AtAMY3 protein and enzyme activity did not fluctuate throughout the diurnal cycle. This indicates that the diurnally fluctuating endoamylase activity observed by Kakefuda and Preiss (11) and the AtAMY3 protein probably represent two different enzymes.

AtAMY3 is unusual among α-amylases not only in having a plastid-targeting signal but also because it is 93 kDa, whereas other α-amylases are typically about half this mass. The C-terminal half of the protein contains the α-amylase domain (Fig. 1) and is sufficient to give the protein α-amylase activity (Fig. 5). The function of the N-terminal region is unknown, but other plant species contain genes encoding α-amylases that are conserved in this region. This suggests that this type of plastidial α-amylase may be a feature of higher plants in general. Interestingly, the N-terminal region of AtAMY3 shows sequence similarity to the N-terminal domain of GWD1 (Fig. 2). Possible functions for the N-terminal sequences are that they bind to starch or to other proteins or that they catalyze other unknown reactions, but these hypotheses need to be tested.

The Arabidopsis Mutant sex4—The *Arabidopsis* mutant *sex4* accumulates increased levels of starch in the leaves and is deficient in plastidial α-amylase activity. No reductions in any other starch-metabolizing enzymes were detected in *sex4*, leading to the suggestion that the plastidial α-amylase is required for normal degradation of transitory starch (8). We have shown that *sex4* is deficient in the AtAMY3 protein and plastidial α-amylase activity, confirming and extending the original observations. Genetic mapping indicated that the *SEX4* locus lies on chromosome three (8), whereas *AtAMY3* is on chromosome one, so *SEX4* clearly does not encode AtAMY3. Thus, the protein encoded at the *SEX4* locus regulates the synthesis or stability of the *AtAMY3* protein, but the mechanism underlying this effect remains to be established. However, we can conclude that deficiency in AtAMY3 is not the primary cause of the starch-excess phenotype of the *sex4* mutant because knock-out mutants of AtAMY3 when grown in the same conditions as those used in the characterization of *sex4* accumulate and degrade starch normally.

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