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.

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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 and the reactions or subcellular locations of several of them were assayed.

Wisconsin 211 was obtained from the Wisconsin Arabidopsis Knock-out (Torrey Mesa Research Institute, San Diego, CA), and the mutant SAIL_642 G08 were isolated from seeds of the Syngenta SAIL collection (University (Columbus, OH). The mutant lines SAIL_613 D12 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 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 specific for Arabidopsis AtAMY3, RT-PCR was performed according to the genbank sequence (GenBank™ accession number A0003406). RT-PCR was performed with RNA isolated from rice leaves (Oryza sativa cv. TMG67) 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 glyceraldehyde-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 phosphoglucone isomerase and phosphoglucone aldolase 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 N₂, 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) and sugars (in the soluble fraction) were conducted as described by Critchley et al. (14).

Results

Cloning of the Arabidopsis a-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 a-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 CERES119981 and CERES3059 and GenBank™ accession number AY050398, respectively).

We isolated 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) for 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 a-amylases, but the N-terminal domain (~50 kDa) shares no similarity to previously described a-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
The rice corresponding rice cDNA. Genomic sequence analysis indicated that apple (MdAMY10, 9). We investigated whether similar domain similar to that of AtAMY3 has been reported from a 12-h day/12-h night cycle (Fig. 3). Several different time points from leaves of plants grown under a 12-h day/12-h night cycle (Fig. 3) showed 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. 3A). 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. 3B) 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. 4A, 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 amylpectin 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. 4B). A second band with greater mobility (a putative isomer of β-amylase, as it is unable to hydrolyze β-limit dextrin) was present only in the homogenate indicating that it is an extraplastidal 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 (AtAMY3-2, included amino acids 497–887). After separation by SDS-PAGE both recombinant proteins could be detected by immunoblotting with anti-AtAMY3 antibody (Fig. 5A) and could be renatured into an active state (Fig. 5B). Soluble starch was digested with these recombinant proteins, and the end products were analyzed by thin-layer chromatography (Fig. 5C). 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 plastidal 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 (Atamy3-3, -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...
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. 6A), and renaturation of enzyme activities in amylepectin-containing gels revealed that the 93-kDa endoamylase activity was also abolished (e.g. Fig. 6B). Electrophoresis of crude extracts under non-denaturing conditions in polyacrylamide gels containing amylopectin revealed that the /H9251-amylase band A2 was missing (e.g. Fig. 6C). This is the /H9251-amylase 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. 7A). 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. 7B). 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 /H9251-amylase genes, AtAMY1 and AtAMY2 (Fig. 8A). In both cases, the T-DNA insertion site was confirmed by sequence
analysis and loss of expression verified using RT-PCR (Fig. 8B). 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. 8C). 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 at the 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
AtAMY1 and genes; exons are depicted as (SALK_094382) and of RT-PCR analysis of Arabidopsis type grown under the same conditions (Fig. 9 was degraded in the dark in the triple mutant similarly to wild

9778 AtAMY3, the only known chloroplast-targeted hypothesis we have investigated the nature and role of transitory starch in chloroplasts (1, 4, 18, 27, 28). To test this endosperm of germinating cereal seeds (2) and it is often sug-

an important role in the degradation of storage starch in the

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 investigat-

diastal endoamylases participating in starch breakdown or that 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 (hand designated 1A; 11, 27). The cellular location of the protein and the nature of the gene encoding this endoamylase were not char-

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 sec4. However, we also demonstrate that disruption of AtAMY3 through insertionional mutagenesis does not affect starch degradation or cause
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 sequence similarity to the N-terminal domain of GWD1 (Fig. 2). Possible functions for the N-terminal sequences are that they

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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