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Original Paper

Overexpression of an extraplastidic *β*-amylase which accumulates in the radish taproot influences the starch content of *Arabidopsis thaliana*

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Abstract Recent studies have revealed that *β*-amylases (EC 3.2.1.2) located in plastids participate in endogenous starch degradation. On the other hand, many plants possess a respectable amount of extraplastidic *β*-amylases. Because of their existence outside of the plastids, it has been believed that extraplastidic *β*-amylases do not contribute to starch degradation in plants. Here, we investigated the role of the major extraplastidic *β*-amylase of the radish (*Raphanus sativus*) (RsBAMY1) in starch metabolism. Amylase activity was elevated in the growing taproot of the radish. During the elevation of amylase activity, the starch content decreased, but the contents of soluble sugars such as maltose, glucose, fructose and sucrose increased. In the radish taproot, RsBAMY1 protein accumulated in the primary cambium and anomalous cambium of the storage parenchyma. The starch granules, however, were found just inside the cambium and adjacent to the anomalous cambium. When the *RsBAMY1* gene was overexpressed in *Arabidopsis*, the plants contained lower starch contents than the wild-type plant. These results suggest that the extraplastidic *β*-amylase may affect the starch metabolism in some plants.

Key words: Cambium, maltose, *Raphanus sativus*, storage organ.

β-Amylase (EC 3.2.1.2) is an amylolytic enzyme which hydrolyses *α*-1,4 glycosidic linkages at the non-reducing end to *β*-maltose. Recent molecular genetic studies have provided convincing evidence that plastidic *β*-amylases are involved in endogenous starch degradation in *Arabidopsis* leaves (Smith et al. 2005; Fettke et al. 2009; Zeeman et al. 2010). *β*-Maltose is a primary product of starch degradation in *Arabidopsis* leaves (Weise et al. 2005). The *Arabidopsis* genome has nine *β*-amylase genes. Among them, the four *β*-amylase proteins (BAMs 1–4) are localized in the plastids, and BAMs 1, 3, and 4 were shown to contribute to starch degradation (Kaplan and Guy 2005; Edner et al. 2007; Fulton et al. 2008; Valerio et al. 2011). The *bam3* or *bam4* mutant showed a strong starch excess phenotype (Kaplan and Guy 2005; Fulton et al. 2008). The *bam1* mutant showed no starch excess phenotype (Kaplan and Guy 2005; Fulton et al. 2008), but a multiple mutant which was produced by crossing the *bam1* mutant with the *bam3* or *bam4* mutant showed a higher starch content compared to *bam3* or *bam4* (Fulton et al. 2008). The isoforms BAM1 and BAM3 had amylolytic activities, but BAM4 did not (Li et al. 2009), suggesting that BAM4 may modulate the rate of starch degradation as a maltose sensor (Fulton et al. 2008). These results indicate that the plastidic *β*-amylases directly or indirectly contribute to starch degradation in plants.

On the other hand, it is known that a large amount of *β*-amylase activities, most of which are extraplastidic, are found in many vegetative organs (Beck and Ziegler 1989; Ziegler 1999), such as pea leaves (Ziegler and Beck 1986) and hedge bindweed rhizomes (Van Damme et al. 2001). Despite their abundance, the extraplastidic *β*-amylases have not been considered to degrade endogenous starch because they cannot approach starch granules in the plastid. Researchers have thus postulated that the abundant *β*-amylase may serve as a vegetative storage protein (Gana et al. 1998; Ziegler 1999; Van Damme et al. 2001).

In the case of *Arabidopsis*, more than 80% of the total amylolytic activity was localized outside of the plastid (Lin et al. 1988; Monroe and Preiss 1990; Monroe et al. 1991). A high proportion of the total amylolytic activity is contributed by one isoform, BAM5, which was shown

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; DAS, days after sowing; GFP, green fluorescent protein; RsBAMY1, *Raphanus sativus* *β*-amylase 1; *RsBAMY1*OE, *RsBAMY1* overexpressor; 5′UTR, 5′ untranslated region.

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to be present within phloem sieve elements (Wang et al. 1995). The *bam5* knock-out mutant showed a mild starch excess phenotype (Kaplan and Guy 2005), suggesting that BAM5 is related to starch metabolism in *Arabidopsis*. This report was the first suggestion that extraplastidic β-amylases may contribute to altering the starch metabolism. In order to confirm this possibility, it is necessary to clarify whether organs whose extraplastidic β-amylase activities are strong really manifest low-starch phenotypes.

Recently, we reported that radish (*Raphanus sativus*, Brassicaceae) taproots possess high amylase activity, almost all of which is β-amylase activity (Hara et al. 2009). After purification of the β-amylase, its cDNA designated as *RsBAMY1*, *Raphanus sativus β-amylase 1*, was cloned by using the partial amino acid sequences. The sequence of *RsBAMY1* revealed that the gene is very similar to *Arabidopsis BAM5*. In the radish plant, the RsBAMY1 protein was expressed mainly in the taproot (Hara et al. 2009), where more soluble sugars accumulate than starch (Hara et al. 2011). Given this, we speculated that RsBAMY1 may influence starch metabolism in the radish taproot during its development. Therefore, in this paper, we investigated the RsBAMY1 expression, its localization, and sugar contents during radish taproot development. We also obtained data on the starch contents of transgenic *Arabidopsis* overexpressing *RsBAMY1*. From these results, we propose that the extraplastidic β-amylase may contribute to decreasing starch contents in the radish and *Arabidopsis*.

Materials and methods

Plant materials

Radish (*Raphanus sativus* L., cv. 'Comet') seeds were purchased from Takii Seed (Kyoto, Japan), and sown in December. The cultivation was done twice, once in 2008 and once in 2009. Radish plants were grown in a plastic pot ($100\,cm^2 \times 10\,cm$) containing Peatban (Sakata Seed, Yokohama, Japan) in a naturally illuminated greenhouse at an uncontrolled temperature at Shizuoka University, Japan. The plants were watered every week with Hyponex solution (500 times dilution; Hyponex, Tokyo, Japan). Tissues from leaves, hypocotyls, roots, shoots, flowers and fruits were collected at different times. Hypocotyls were collected at various developmental stages [9, 22, 31, and 45 days after sowing (DAS)]. Etiolated seedlings were obtained by growing at 23°C in the dark. Seeds were sown on defatted cotton watered in plastic pots. The pots were then placed in aluminum containers and incubated for five days. For the centrifugation fractionation test, European red radishes purchased at a local market in Japan were used.

Arabidopsis thaliana (L.) Heynh (ecotype Columbia-0) plants were grown in 7-cm plastic pots filled with Peatban (Sakata Seed) in growth chambers with $100\,\mu mol\,m^{-2}\,s^{-1}$ light under long-day conditions (16 h light/8 h dark cycle) at 23°C. The density of planting was two plants per pot. The transformation of *Arabidopsis* plants was conducted via *Agrobacterium*-mediated transformation (Clough and Bent 1998). To measure the total leaf area, cotyledons and rosette leaves of 3-week-old plants were dissected and photographed. Their areas were measured with ImageJ software (http://rsb.info.nih.gov/ij/). For the determination of starch and soluble sugar contents, 3-week-old plants harvested at the end of the light period were used.

Crude enzyme extract

Radish tissues were homogenized with two volumes of deionized water by using a mortar and pestle on ice. The homogenate was centrifuged at $10{,}000\times g$ for 15 min at 4°C. The supernatant was a crude enzyme extract. *Arabidopsis* plants were homogenized with a cold buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 5 mM dithiothreitol (DTT). The homogenate was centrifuged at $18{,}000\times g$ for 15 min at 4°C. The supernatant was used. The extracts were maintained at −20°C until use. The amylase activity and antigenicity for an anti-RsBAMY1 antibody in the extracts did not change during 6 months of storage.

The protein amount was determined spectrophotometrically at 595 nm by using the Quick Start Bradford Protein Assay (Bio-Rad, Tokyo, Japan), with bovine γ-globulin as a standard protein. Assays were performed according to the manufacturer's instructions.

Amylase assay and immunoblot analysis

The amylase activity assay and RsBAMY1 immunoblot analysis were performed as described in a previous report (Hara et al. 2009). The procedures are briefly described. The amylase activity was determined by measuring reducing sugars which are released from a soluble starch. The enzyme solution (4 μl) was combined with a substrate solution (36 μl) consisting of 20 μl of 1% soluble starch and 16 μl of 100 mM sodium acetate buffer (pH 4.8). After the reaction mixture was incubated at 37°C for 5 min, 40 μl of the 3,5-dinitrosalicylic acid reagent containing 44 mM 3,5-dinitrosalicylic acid, 1 M sodium potassium tartrate, and 0.4 M sodium hydroxide were added to the mixture. The solution was heated at 100°C for 5 min. After cooling to room temperature, 360 μl of deionised water was added, and then the absorbance at 540 nm was measured. Calibration curves were produced by using maltose. One unit represented the generation of 1 μmol of reducing sugars per min.

For detecting RsBAMY1 by immunoblot analysis, protein samples, which were separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide gel), were transferred to a nitrocellulose membrane filter (Hybond-ECL; GE Healthcare, Tokyo, Japan) with a Mini Trans-Blot (Bio-Rad). The following procedure was done by the ECL Western Blotting Analysis System (GE Healthcare). The primary antibody (an anti-RsBAMY1 antibody; 1 : 5000 dilution) and the secondary antibody (a horseradish peroxidase-conjugated anti-rabbit immunoglobulin

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G; 1:5000 dilution) were used. The chemiluminescence signals were detected by a LAS-4000 Image Analyzer (Fujifilm, Tokyo, Japan).

Soluble sugars and starch analysis

Fresh tissues were treated twice with 10 volumes of 80% (v/v) ethanol at 80°C for 20 min. The extracts and insoluble materials were used for the soluble sugars analysis and the starch analysis, respectively. For the soluble sugars analysis, the ethanol extracts were evaporated and the residues dissolved in deionized water. The solution was passed through a Sep-Pak C18 cartridge (Millipore, MA, USA) that had been equilibrated with water to remove pigments and phenolics. The contents of glucose, fructose, and sucrose in the radish hypocotyls were determined using high performance liquid chromatography (HPLC; LC-2000; Jasco, Tokyo, Japan). Soluble sugars were separated at 30°C on an Asahipak NH_2P-50 4E column (4.6 mm×250 mm; Showa Denko, Tokyo, Japan), and the refractive index (RI) was detected using an RI detector (RI-2031; Jasco). The mobile phase was 75% acetonitrile in water. The flow rate was 1 ml min^{-1}. The contents of glucose, fructose, and sucrose in *Arabidopsis* leaves were measured in a coupled enzyme assay (Jones et al. 1977) with modifications. A soluble sugar extract (1 ml) was incubated in a mixture (0.5 ml) containing 100 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol, 9 mM ATP, 3 mM NADP, and 18 mM $MgCl_2$. The reaction was started by the successive addition of 2.4 U hexokinase and 1.2 U glucose-6-phosphate dehydrogenase, 7.5 U phosphoglucose isomerase, and 0.45 U invertase dissolved in 50 mM sodium citrate buffer (pH 4.6). After incubating at 37°C for 5 min, the absorbance at 340 nm was measured. The contents of the corresponding sugars were determined by the absorbance changes. The maltose content was determined as described previously (Shirokane et al. 2000). Extracted soluble sugars (100 μl) were incubated in a mixture containing 50 mM sodium phosphate buffer (pH 7.0), 1.2 mM NADP, 0.15 mM D-glucose 1,6-diphosphate, 20 mM KCl, 2 mM $MgSO_4$ and 0.25% (v/v) Triton X-100. The reaction was started by the addition of 5 U maltose 1-epimerase, 5 U maltose phosphorylase, 1.5 U β-phosphoglucomutase, and 5 U glucose-6-phosphate dehydrogenase. After incubating at 37°C for 5 min, the absorbance at 340 nm was measured. For the starch content determination, the ethanol insoluble residue was extracted by an equal volume of 0.4 M KOH at 80°C for 60 min. After the extract was neutralized, soluble starch was digested by 10 U α-amylase and 7 U amyloglucosidase. Glucose formation was determined by a glucose oxidase- and peroxidase-based enzyme assay (Papadopoulos and Hess 1960). Starch content was calculated based on the released glucose.

In the carbohydrate analyses, recoveries determined by the standard addition method were applied to calculate the contents of the corresponding sugars.

ADP-glucose pyrophosphorylase (AGPase) assay

The AGPase activity was determined as described in a previous report (Nakamura et al. 1989). The enzyme solution (50 μl) was mixed with a substrate solution (400 μl) containing 50 mM HEPES-NaOH (pH 7.5), 1.2 mM ADP-glucose, 5 mM pyrophosphate, 6 mM $MgCl_2$, and 3 mM DTT. The mixture was incubated at 30°C for 20 min, and then heated at 100°C for 1 min. After cooling to room temperature, 290 μl of 50 mM HEPES-NaOH (pH 7.5) and 100 μl of 6 mM $NADP^+$ were added, and then the absorbance at 340 nm was measured. The sample was mixed with an enzyme solution (10 μl) containing 50 mM HEPES-NaOH (pH 7.5), 0.4 U phosphoglucomutase, and 0.35 U G6P dehydrogenase. After incubating at 30°C for 10 min, the absorbance at 340 nm was measured. One unit represents 1 μmol NADPH generation per min.

Tissue printing, immunohistochemistry, and starch stain

A tissue printing technique was performed as described previously (Hara et al. 2000) with modifications. Transverse cut ends of hypocotyls were printed onto the nitrocellulose membrane filter (Hybond-ECL; GE Healthcare) for 15 s. The filters were blocked with 5% (w/v) skimmed milk protein in PBST which consisted of phosphate-buffered saline (PBS, 10 mM potassium phosphate buffer pH7.4 and 137 mM NaCl) and 0.05% (v/v) Tween-20. Then the filters were probed with the primary antibody (the anti-RsBAMY1 antibody; a 1:1000 dilution). After being washed, the membranes were incubated in the secondary antibody (anti-rabbit IgG conjugated with alkaline phosphatase; Promega, WI, USA) at a 1:5000 dilution in PBST. The color was developed by adding NBT/BCIP Stock Solution (Roche Diagnostics, Mannheim, Germany). A control membrane incubated with pre-immune serum instead of the anti-RsBAMY1 antibody did not show any signal (data not shown).

For immunocytochemistry, taproots were fixed with 2% (v/v) glutaraldehyde in PBS for 4 h at 4°C. Thin sections of the tissue prepared by hand were dehydrated and rehydrated in the ethanol series. The sections were blocked with 5% (w/v) skimmed milk protein in PBST, and then probed with the anti-RsBAMY1 antibody at a 1:1000 dilution. For the control, a pre-immune serum was used. The secondary antibody and detection procedures were the same as above. Sections were observed under a microscope (SZX7; Olympus, Tokyo, Japan) equipped with a digital photographic camera (C3040Z; Olympus).

Starch in the tissue sections was detected by incubating the fixed sections in a solution containing 10 mM I_2 and 14 mM KI for 20 min at room temperature. Observation was done by microscope. For a whole plant starch staining, the areal parts of *Arabidopsis*, which were decolorized in hot 80% (v/v) ethanol, were stained with iodine solution. After the plant was rinsed with water, photographs were taken.

Green fluorescent protein (GFP) assay

The *GFP-RsBAMY1* construct was produced by inserting the open reading frame (ORF) of *RsBAMY1* into the C-terminus

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of GFP in *35S-Ω-sGFP (S65T)* plasmid gifted by Dr. Y. Niwa. The *RsBAMY1* sequence was produced by PCR with 5′-TCT AGA GGA TCC ATG GCT GCA AAT TAC AACG-3′ and 5′-CCC TTG CTC ACC ATG GCA CCA TCA ACC TTCATG-3′ as primers. The *RsBAMY1-GFP* construct was prepared by inserting the ORF of *RsBAMY1* into the N-terminus of GFP in the *35S-Ω-sGFP*. In this case, the *RsBAMY1* sequence was amplified by PCR with 5′-ATG GAC GAG CTG TAC AAG ATG GCT GCA AAT TAC AAC-3′ and 5′-CCG CTT TAC TTG TAC ATC AAC CAT CAA CCTTCATG-3′ as primers. The constructs were produced by In-Fusion Technology (Takara-Clontech, Kyoto, Japan). For transient expression, onion (*Allium cepa*) epidermal cells were placed on Murashige and Skoog (MS) medium [MS salts, MS vitamins, and 3% (w/v) sucrose (pH 5.7)] solidified with 0.8% (w/v) agar. Then, 1.5 mg of gold particles was coated with the 1 μg plasmid by co-precipitation in 20 μl of 0.1 M spermidine and 50 μl of 2.5 M $CaCl_2$. Plasmid DNA was introduced into cells using a gene gun (PDS-1000/He; Bio-Rad) with the following parameters: vacuum 28 inch Hg; distance 9 cm; helium pressure 650 psi. After bombardment, tissues were maintained in the dark at 22°C for 18 h. Fluorescent cells were imaged by confocal laser-scanning microscopy (TCS SL; Leica, Tokyo, Japan).

Overexpression in Arabidopsis

The ORF of *RsBAMY1* was amplified by PCR and cloned into the vector pRI 101-AN DNA (Takara Bio, Shiga, Japan) under the control of the sequence of the CaMV 35S promoter. The *RsBAMY1* gene was inserted at the 3′ terminus of the 5′ untranslated region (5′UTR) of the *Arabidopsis* alcohol dehydrogenase gene to enhance the efficiency of translation (Sugio et al. 2008; Matsui et al. 2012). The KOD DNA Polymerase (Toyobo, Osaka, Japan) used for the PCR. Primers were 5′-CAC TGT TGA TAC ATA TGG CTG CAA ATT AC-3′ and 5′-TTC AGA ATT CGG ATC CTC AAC CAT CAA CCT-3′. The construct was produced by In-Fusion Technology (Takara-Clontech).

The transcript levels of *RsBAMY1* were analyzed by an RT-PCR system (AMV Reverse Transcriptase XL; Takara Bio). Total RNA was extracted from the various tissues with the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Five hundred nanogram of RNA was used. Reverse transcription was performed at 45°C for 30 min. PCR was done with the *RsBAMY1* specific primers (5′-GCT GCA AAT TAC AAC GAG AAG C-3′ and 5′-CAA CGG CAC AAT CTC ATG CC-3′). The PCR conditions were as follows: 1 cycle at 94°C for 1 min; 18 cycles at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 90 s. The *Arabidopsis ACTIN2 (ACT2)* gene was amplified as an internal control. The primers used were 5′-ACC TTG CTG GAC GTG ACC TTA CTG AT-3′ and 5′-GTT GTC TCG TGG ATT CCA GCA GCT T-3′. The PCR was performed as follows: 1 cycle at 94°C for 1 min; 22 cycles at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 30 s. The putative sizes of the RT-PCR products for *RsBAMY1* and *ACT2* were 1,389 bp and 298 bp, respectively. The amplified products were analyzed by 1% agarose gel

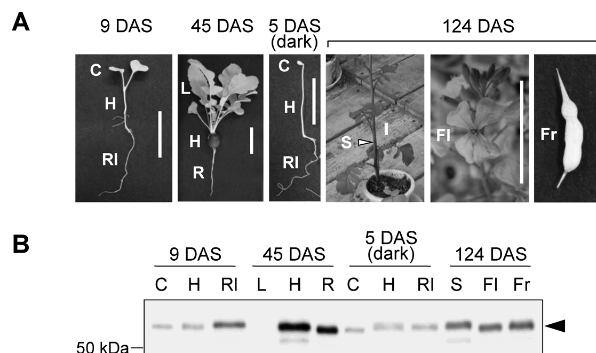

Figure 1. RsBAMY1 proteins detected in different organs of the radish. (A) Radish organs tested in this study. C, cotyledon; H, hypocotyl; Rl, rootlet; L, leaf: R, root; S, stem; Fl, flower; Fr, fruit (immature). Bars represent 5 cm. (B) Immunoblot to detect RsBAMY1 proteins (5 μg of total protein in each lane) in the different organs.

electrophoresis and the results were documented by a LAS-4000 Image Analyzer.

Statistical analysis

Data for p values were analyzed by Student's t test at a significance level of 0.05.

Results

Organ distribution of RsBAMY1

We previously reported that the RsBAMY1 protein is the major β-amylase expressed in the radish taproot (Hara et al. 2009). The RsBAMY1 protein accumulated in the taproot, but there was little accumulation in the areal part. The expression in the other organs, however, was not investigated. In the present study, we detected RsBAMY1 proteins in 12 organs of young and mature radish plants. The 12 organs are shown in Figure 1A. The immunoblot analysis showed that the hypocotyls and roots at 45 DAS contained a large amount of RsBAMY1 protein (Figure 1B). Since there were size variations in proteins which were reacted with the anti-RsBAMY1 antibody, radish may have RsBAMY1-related isoforms which were derived from its homologous genes and/or generated by post-translational modifications.

Changes in the RsBAMY1 expression and sugar contents during taproot growth

As shown in Figure 1B, the hypocotyl at 9 DAS contained a small amount of the RsBAMY1 protein, but the mature hypocotyl at 45 DAS contained a large amount of the protein, suggesting that the RsBAMY1 protein accumulation increased during taproot growth. The developmental stages of the radish taproot are indicated in Figure 2A. We numbered the growth stages while the radish plant grew. The fresh weight of the taproot drastically increased between stage 3 and stage 4 (Figure 2B). Figures 2C and 2D show the changes

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in the RsBAMY1 protein and total amylase activity ($U\,mg^{-1}$ protein) during the growth of the radish taproot. The immunoblot analysis showed that the amount of RsBAMY1 protein increased throughout the developmental stages, and a large amount of the protein was contained at stage 4 (Figure 2C). In this immunoblot, the same amount of crude protein was loaded in each lane. The time–course change of the amylase activity expressed as $U\,mg^{-1}$ protein was similar to that of the RsBAMY1 protein amount analyzed by the immunoblot (Figure 2D). On the other hand, if the amylase activity was expressed as $U\,g^{-1}$ fresh weight, the activity reached a peak at stage 3, and then decreased at stage 4 (Figure 2E). After the total protein content peaked at stages 2 and 3, the protein content decreased to a low level (Figure 2F).

The contents of starch and soluble sugars during the taproot development were determined (Figure 3). Although the starch contents were remarkably elevated at stage 2, the contents dropped to a low level at stage 3 (Figure 3A). ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) is known to regulate starch biosynthesis (Li et al. 2011). The activity reached the highest levels at stages 2 and 3, and then decreased quickly to the level of stage 1 (Figure 3B). The contents of maltose, which is a direct product of β-amylase, reached a peak at stage 3 (Figure 3C). We previously found that glucose, fructose, and sucrose are abundant soluble sugars in the taproots of radish cultivars (Hara et al. 2011), and therefore investigated the time courses of the glucose, fructose, and sucrose contents during taproot growth (Figures 3D–F). Elevation of the glucose accumulation continued during taproot development (Figure 3D). The change in the fructose content (Figure 3E) was similar to that in the glucose content (Figure 3D). The sucrose content gradually increased during taproot development (Figure 3F).

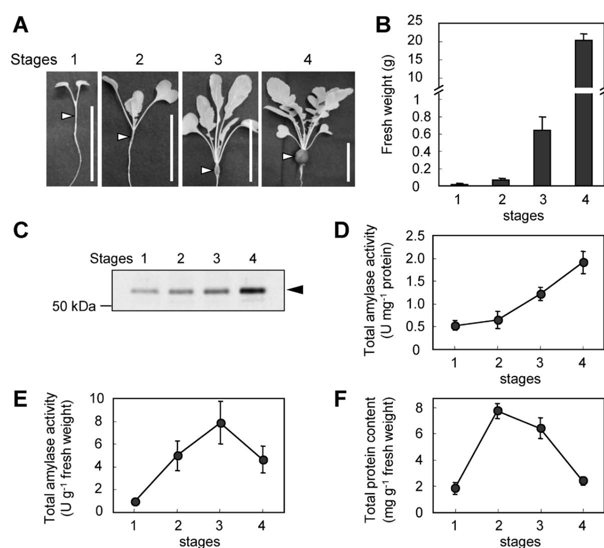

Figure 2. RsBAMY1 proteins and total amylase activities during development of radish taproots. (A) Pictures of radishes at different growth stages. The numbers attached to the pictures represent the corresponding stages. Arrowheads indicate the positions of hypocotyls. Mature taproots are derived from hypocotyls. Bars represent 10 cm. (B) Time-courses of fresh weight are shown. Values and bars represent means±SD (n=3). (C) Immunoblot to detect RsBAMY1 proteins (5 μg of total protein in each lane). Total amylase activities expressed as $U\,mg^{-1}$ protein (D) and as $U\,g^{-1}$ fresh weight (E), respectively. (F) Total protein contents. Values and bars represent means±SD (n=3).

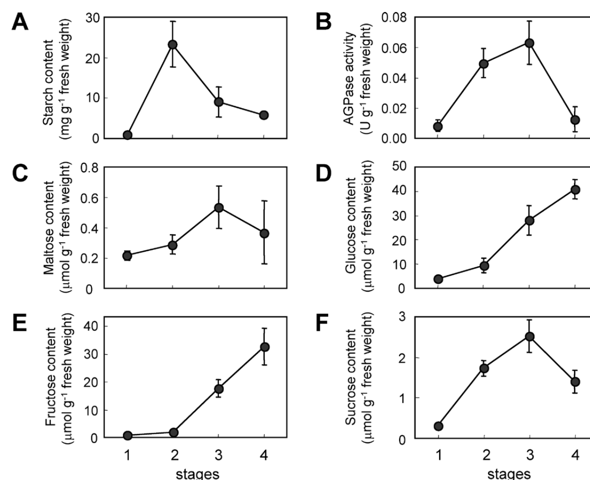

Figure 3. Sugar contents and ADP-glucose pyrophosphorylase (AGPase) activity during development of the radish taproot. Contents of starch (A), maltose (C), glucose (D), fructose (E), and sucrose (F) are shown. AGPase activity is represented in (B). Stage numbers (1 to 4) refer to the growth stages depicted in Figure 2A. Values and bars represent means±SD (n=3).

Localization of RsBAMY1 and starch granules in the taproot

To grasp the rough localization of the RsBAMY1 protein accumulation in the swollen taproot of the radish (stage 4), we first applied immuno-tissue printing using the anti-RsBAMY1 antibody to transverse sections of the taproot (Figure 4A). The staining was observed as a circular line around the inside of the taproot peeling and as dots scattered in the xylem (Figure 4A, right half). For a close-up analysis, immunohistochemistry was analyzed for transverse sections of the taproot (Figures 4B, C). Staining was located in the primary cambium (red arrowheads) and the anomalous cambiums (white arrowheads), which are dispersed in the storage parenchyma of the xylem. No specific signal was detected when pre-immune serum was used (Figure 4D). In addition, we investigated the localization of starch in the taproot sections using iodine solution. The staining occurred just inside the primary cambium and adjacent to the anomalous cambiums (Figures 4E, F). This means that the zone of the starch staining and that of the RsBAMY1 staining did not overlap each other, indicating that starch and RsBAMY1 are not co-localized to the same tissues.

BAM5 is the major extraplastidic isoform of β-amylase

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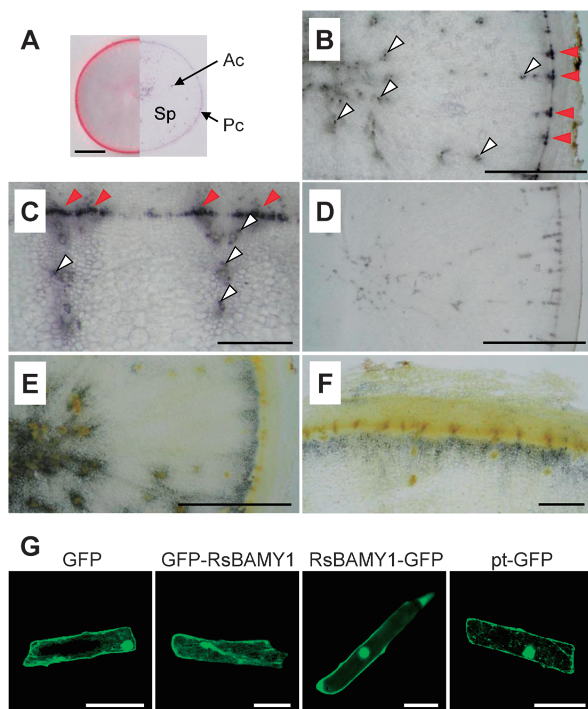

Figure 4. Localization of RsBAMY1 and starch in the radish taproot. (A) Tissue printing of RsBAMY1 in the radish taproot. Transverse sections prepared from a radish taproot at stage 4 were examined using anti-RsBAMY1 antibody. The left half and right half show a fresh slice and a tissue printing, respectively. Ac, anomalous cambium; Pc, primary cambium; Sp, storage parenchyma. A bar represents 1 cm. (B–D) Immunohistochemical observations of RsBAMY1 in the radish taproot. Transverse sections prepared from a radish taproot at stage 4 were examined using the anti-RsBAMY1 antibody (B, C) and pre-immune antiserum (D). Primary cambium (red arrowheads) and anomalous cambium (white arrowheads) are shown. Bars represent 5 mm (B, D) and 0.5 mm (C), respectively. (E, F) Tissue localization of starch in the radish taproot at stage 4. Sections were stained with iodine solution. Black dots indicate starch granules. Bars represent 5 mm (E) and 1 mm (F), respectively. (G) Subcellular localization of RsBAMY1 and green fluorescent protein (GFP) fusion proteins in onion epidermal cells. Epidermal cells were bombarded with either GFP alone, N-terminus GFP fusion (GFP-RsBAMY1), C-terminus GFP fusion (RsBAMY1-GFP), or plastid targeting signal (pt)-GFP fusion. Bars represent 200 μm.

in *Arabidopsis*. BAM5, whose sequence possesses no plastid transit peptide, was found to be localized in the cytoplasm or the vacuole (Monroe and Preiss 1990; Monroe et al. 1991). Because RsBAMY1 is a close ortholog of BAM5, it is postulated that RsBAMY1 is also an extraplastidic β-amylase. To confirm that, transient green fluorescence protein (GFP) assay and centrifugation fractionation were performed (Figure 4G; Supplemental data 1). In the onion epidermal cells, GFP alone was detected in the nucleus and cytoplasm (Figure 4G). This pattern is consistent with previous observations of the GFP localization in plant cells (Haseloff and Amos 1995; Chiu et al. 1996). Fluorescence of fused proteins, i.e. GFP-RsBAMY1 and RsBAMY1-GFP, was also detected in the nucleus and cytoplasm. The

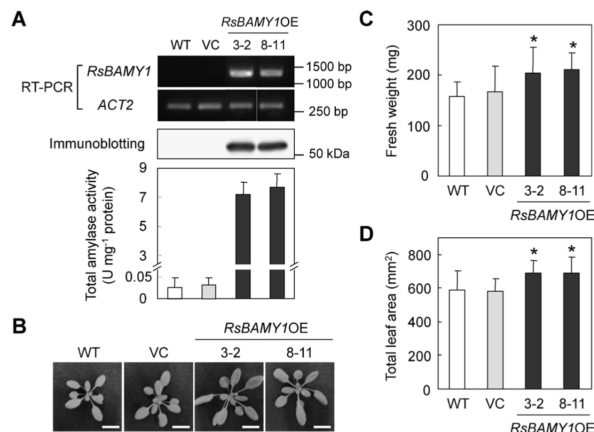

Figure 5. Growth of transgenic *Arabidopsis* plants overexpressing *RsBAMY1*. (A) *RsBAMY1* expression, RsBAMY1 accumulation, and total amylase activity in *Arabidopsis* plants. Wild type (WT), vector control (VC), and *RsBAMY1* overexpressors (*RsBAMY1*OEs, lines 3-2 and 8-11) were tested. *ACTIN2* (*ACT2*) was used as an internal control of the gene expressions. Values and bars represent means±SD (n=5) of amylase activity. (B) Three-week-old plants are exhibited. Bars represent 2 cm. (C, D) Fresh weight (C) and total area (D) of rosette leaves are shown. Values and bars represent means±SD (n=10 for C and n=12 for D). * Significant difference ($p<0.05$) in comparison to WT determined by Student's t-test.

localization of RsBAMY1 fused proteins was different from the localization of plastid targeting (pt)-GFP. Similar localizations were observed in the tests using radish mesophyll cells expressing the RsBAMY1 fused constructs (Supplemental data 1A). The centrifugation fractionation using the radish taproot indicated that RsBAMY1 was detected only in the soluble fraction (Supplemental data 1B, SF). These results suggest that RsBAMY1 is an extraplastidic β-amylase, as is BAM5.

Analyses of transgenic plants

Transgenic *Arabidopsis* expressing RsBAMY1 was produced to reveal the effects of RsBAMY1 on the starch content *in planta*. For strong expression of *RsBAMY1*, we used the sequence of cauliflower mosaic virus 35S promoter and the 5′UTR of the *Arabidopsis* alcohol dehydrogenase gene to enhance transcription and translation (Sugio et al. 2008; Matsui et al. 2012). An empty vector was transformed to generate a control line (a vector control, VC). Although we obtained eight independent transgenic lines, we conducted the following tests with the two lines that showed the first and second highest *RsBAMY1* expressions [*RsBAMY1* overexpressors (*RsBAMY1*OEs) 3-2 and 8-11]. Figure 5A indicates that the *RsBAMY1*OEs contained the *RsBAMY1* transcripts and the RsBAMY1 proteins, neither of which were detected in the wild-type (WT) and VC plants. Moreover, *RsBAMY1*OEs showed more than 200-fold increases in amylase activities compared to WT and VC. Centrifugation fractionation showed that the RsBAMY1 protein was mainly found in the soluble fraction of the

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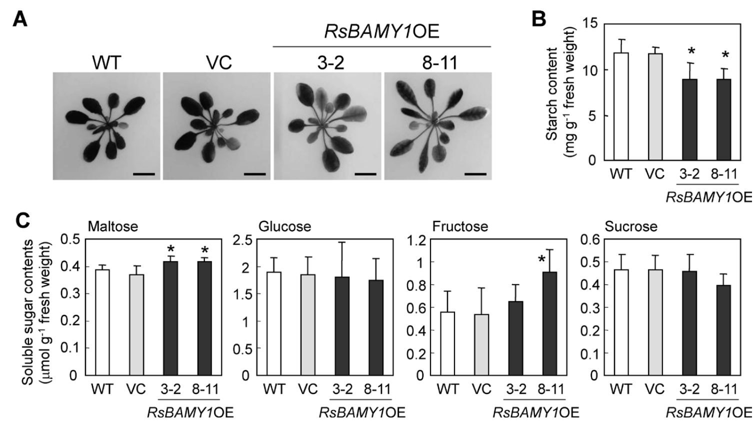

Figure 6. Starch and soluble sugars in transgenic *Arabidopsis* plants overexpressing *RsBAMY1*. Three-week-old plants harvested at the end of the light period were analyzed. (A) Starch staining. Plants were decolorized in hot 80% (v/v) ethanol and stained with iodine solution. Bars represent 1 cm. (B) Starch contents. (C) Soluble sugar contents. In B and C, values and bars represent means±SD (n=5). * Significant difference (p<0.05) in comparison to WT determined by Student's t-test.

*RsBAMY1*OE 8-11 (Supplemental data 2), suggesting that RsBAMY1 was extraplastidic in *Arabidopsis*. Visible observation indicated that the 2 *RsBAMY1*OEs had larger rosette leaves than WT and VC (Figure 5B). The fresh weights of the *RsBAMY1*OEs were 130% (line 3-2) and 135% (line 8–11) of those of WT, respectively (Figure 5C). The increase in the leaf fresh weights resulted from that in the total leaf areas (Figure 5D). Regarding the sugar contents in the transgenics, the *RsBAMY1*OEs showed significantly lower starch contents than WT and VC (Figures 6A, B). In addition, the *RsBAMY1*OEs contained more maltose than WT and VC (Figure 6C, the left-most graph). However, the contents of glucose, fructose, and sucrose were not different between the *RsBAMY1*OEs and the control plants (WT and VC) except that *RsBAMY1*OE 8–11 contained more fructose than WT and VC (Figure 6C).

Discussion

It has been thought that extraplastidic β-amylases cannot participate in the endogenous degradation of starch, because they are localized outside of the plastid. Because some β-amylases abundantly accumulate in the vegetative organs, such as sweet potato tubers (Nakamura et al. 1991), alfalfa taproots (Gana et al. 1998), and hedge bindweed rhizomes (Van Damme et al. 2001), it has been postulated that extraplastidic β-amylases may be a vegetative storage protein. However, a recent study indicated that a knockout mutant of *bam5*, the extraplastidic β-amylase most expressed in *Arabidopsis*, showed a slight starch excess phenotype (Kaplan and Guy 2005), suggesting that the extraplastidic β-amylase may also participate in metabolizing starch in *Arabidopsis*.

To confirm this idea, we decided to investigate the starch contents in the organs where the extraplastidic β-amylase activity is increasing. Here we used the radish taproot where the expression of RsBAMY1 (a radish ortholog of *Arabidopsis* BAM5) was increasing during growth. Although the RsBAMY1 sequence is highly similar to the BAM5 sequence, it has not been demonstrated that RsBAMY1 is really extraplastidic. Thus we conducted the following investigation. First, a sequence analysis showed that RsBAMY1, like BAM5, possesses no plastid transit peptide. Second, the subcellular localization test using GFP suggested that the fused proteins between RsBAMY1 and GFP were located in the extraplastidic spaces of the onion and radish cells (Figure 4G; Supplemental data 1A). Third, a centrifugation fractionation using the radish taproot indicated that most RsBAMY1 was detected in the soluble fraction, but little RsBAMY1 was found in the plastid-rich fraction (Supplemental data 1B). Taken together, these results suggested that RsBAMY1 is an extraplastidic β-amylase.

The amylase activity expressed as $U g^{-1}$ fresh weight reached a maximum at stage 3 (Figure 2E), where the starch content ($mg g^{-1}$ fresh weight) drastically decreased (Figure 3A). The starch synthesis was indicated to proceed strongly at stage 3, because the highest AGPase activity was marked at this stage (Figure 3B). The time–course curve of the amylase activity ($U g^{-1}$ fresh weight) was similar to that of the content of maltose ($\mu mol g^{-1}$ fresh weight) which is a direct product of β-amylase (Figures 2E, 3C). These results suggest that the extraplastidic RsBAMY1 may be involved in the starch metabolism of the radish taproot.

The BAM5 protein was first described as a protein which accumulated in the phloem sieve elements (Wang et al. 1995). RsBAMY1 was present in the cambial tissues as well (Figure 4). The radish taproot is a swollen organ whose inside is filled with the xylem parenchyma, which is surrounded by the primary cambium. Many secondary anomalous cambiums are dispersed in the

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xylem parenchyma of the mature taproot. RsBAMY1 was located in both types of cambium. Starch did not exist at the site where RsBAMY1 accumulated. These observations also support the idea that RsBAMY1 may be related to the starch metabolism.

In some *Arabidopsis* mutants, the phenotypes with excess starch are positively correlated with growth inhibition (Delatte et al. 2006; Lloyd et al. 2005). Inversely, it is considered that the enhancement of starch degradation may provide growth promotion in *Arabidopsis*. Since the transgenic lines overexpressing *RsBAMY1* showed lower starch contents and higher growth in the rosette leaves than WT (Figures 5, 6), it is possible that *RsBAMY1* degrades starch *in vivo*. Given all these findings, we conclude that the extraplastidic *β*-amylases, at least RsBAMY1 and BAM5, contribute to the starch metabolism in plants.

Overexpression of *RsBAMY1* also provided morphological alterations in *Arabidopsis*. The most noticeable change was found in the areal part of the plant. The rosette leaves of the *RsBAMY1*OEs were larger than those of WT and VC (Figure 5B). The *RsBAMY1*OEs showed higher fresh weights and larger leaf areas than WT and VC (Figures 5C, D). A comparison of the cell sizes between the *RsBAMY1*OEs and WT or VC indicated that the mesophyll cells of the *RsBAMY1*OEs were more greatly expanded than those of WT or VC (data not shown). The amylase activity in the radish taproot ($U\,g^{-1}$ fresh weight) remained at high levels during the growing stages of the taproot (Figure 2E, stages 3 and 4), when the taproot diameter was increasing as the xylem parenchyma was developing (Figure 2B). Since sufficient concentrations of endogenous soluble sugars are needed to maintain continuous cell division and expansion, RsBAMY1 may supply soluble sugars to the growing organs by digesting starch. A recent study on metabolite profiling in *Arabidopsis* demonstrated that the increase in biomass was well correlated with the decrease in several metabolites, especially starch (Sulpice et al. 2009). This suggested that RsBAMY1 may increase plant biomass by enhancing starch mobilization.

Although it was demonstrated that RsBAMY1 is a major *β*-amylase expressed in the radish taproot (Hara et al. 2009), it is likely that radish may possess various kinds of extraplastidic *β*-amylase isoforms similar to RsBAMY1, because the size variations of positive signals were found in the immunoblot analysis (Figure 1B). This suggests that not only RsBAMY1 but also other extraplastidic *β*-amylases may participate in the starch metabolism of radish.

It is still not known how the extraplastidic *β*-amylase RsBAMY1 regulates the starch content in *Arabidopsis* and possibly in the radish taproot. No RsBAMY1 was co-located with starch at the tissue level. On the other hand, the maltose production increased while the amylase activities were elevated during the development of the radish taproot. This suggests that RsBAMY1 may participate in degrading starch in cells where starch is synthesized. Since it is hypothesized that the subcellular compartmentation may partially loosen in the vascular cells which are actively dividing, RsBAMY1 may have an opportunity to access starch. Otherwise, it is also possible that RsBAMY1 may reduce the starch content without directly degrading starch. Recently it was reported that two minor extraplastidic *β*-amylases, BAM7 and BAM8, acted as transcription factors that regulated shoot growth and development through crosstalk with brassinosteroid signaling (Reinhold et al. 2011). RsBAMY1 may control the starch metabolism by regulating gene expression(s) as well as BAM7 and BAM8. Elucidating the mechanisms by which RsBAMY1 regulates the starch content will improve our understanding of starch metabolism in plants.

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