Title

A major β-amylase expressed in radish taproots

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Abstract

Radish (*Raphanus sativus* L.) taproots display high amylolytic activity. Amylase zymograms using gels containing soluble starch and β-limit dextrin have indicated that β-amylase is a major amylolytic enzyme. We purified β-amylase with anion-exchange chromatography followed by glycogen precipitation. From the deduced amino acid sequences, a cDNA clone designated *Raphanus sativus* β-amylase 1 (RsBAMY1) was isolated. RsBAMY1 closely resembled *Arabidopsis thaliana* major β-amylase (At4g15210). Immunoblot analyses performed using an anti-RsBAMY1 antibody that was raised against a peptide sequence found in RsBAMY1 showed that the RsBAMY1 protein accumulated in the taproot. In addition, the transcriptional level and the protein accumulation of RsBAMY1 were enhanced during the taproot growth. These results suggest that RsBAMY1 is a major starch-digestive enzyme in the radish taproot.

Keywords: β-Amylase; Radish; *Raphanus sativus* L.; Taproot

1. Introduction

The radish (*Raphanus sativus* L.) is a Brassicaceae root vegetable. Although the radish is grown and consumed throughout the world, Far East Asian countries, such as Japan, Korea, and China, are the major consuming regions. In Japan, 1.65 million tons of radishes were harvested in 2006 (Preliminary Statistical Report on Agriculture, Forestry and Fisheries of Japan, 2007), and people in Japan consume 20 kg of radish per year (Talalay & Fahey, 2001). Radish taproots are eaten raw, pickled, and boiled. Radish sprouts are also consumed as herbs in Japanese dishes. Recently, researchers have become interested in the radish because extracts and compounds made from radishes have shown many health benefits. Glucoraphasatin, a major glucosinolate in the radish, quenched hydrogen peroxide and was efficiently oxidized to glucoraphenin, which is a minor glucosinolate in the radish (Barillari, Cervellati, Paolini, Tatibouët, Rollin, & Iori, 2005). Antioxidative effects of radish sprouts have been reported in rats (Ippoushi, Takeuchi, Ito, Horie, & Azuma, 2007). Glucosinolates are substrates of myrosinases, which convert the glucosinolates to the corresponding isothiocyanates when plant tissues are mechanically disrupted. Glucoraphasatin- and glucoraphenin-derived isothiocyanates induced apoptosis in cancer cells (Papi et al., 2008). A glucoraphasatin-derived isothiocyanate is a potent inducer of

detoxification enzymes in the HepG2 cell line (Hanlon, Webber, & Barnes, 2007). Radish myrosinases were purified (Shikita, Fahey, Golden, Holtzclaw, & Talalay, 1999), and their cDNAs were isolated (Hara, Fujii, Sasada, & Kuboi, 2000). These results suggest that the radish produces health benefits through the antioxidative and anticarcinogenic activities of glucosinolates and isothiocyanates.

Besides, it is probably that investigating the traditional use of the radish would result in the finding of additional functions. In Japan, people use a grated raw radish, known as daikon oroshi, which is a common garnish in Japanese dishes. They prefer the pungency of isothiocyanates generated by daikon oroshi because the pungency promotes their appetite. In addition, they add daikon oroshi to boiled rice, rice cakes, and noodles, because it is believed to help in the digestion of starch-containing foods. Indeed, amylolytic activity has been recorded in the radish taproot. The existence of potent diastase activity in the radish was first described in the early 19th century (Aoki, 2007). In general, plant starch-hydrolyzing enzymes include α-amylase, β-amylase, isoamylase, and limit dextrinase (Smith, Zeeman, & Smith, 2005). α-Amylase is an endoamylase which hydrolyzes internal α-1,4 linkages of linear or branched glucans. β-Amylase is an exoamylase which releases β-maltose from the non-reducing ends of α-1,4-linked glucans. β-Amylase cannot pass α-1,6 linkages, however, so it does not digest a β-limit dextrin. Isoamylase and limit dextrinase hydrolyze the α-1,6 linkages of amylopectin. A β-amylase was purified from an Egyptian radish (Rashad, Jwanny, El-Sayed, Mahmoud, & Abdallah, 1995), suggesting that the β-amylase is related to at least a portion of the amylolytic activity in radish taproots. In order to study the starch-hydrolyzing activity in the radish, it is necessary to show that the starch-hydrolyzing activity in radish taproots depends on β-amylase, and to obtain information about the primary structure of the β-amylase. In this paper, we report a cDNA for a major β-amylase expressed in the radish taproot. We also discuss the role of β-amylase in the radish as a functional vegetable.

2. Materials and methods

2.1. Plant materials

Radishes were grown in a greenhouse located at Shizuoka University, Japan. We cultivated radishes 3 times from 2006 to 2007 for this study. Radish seeds (*Raphanus sativus* L. cv. Comet, Takii, Kyoto, Japan) were sown in a plastic planter

containing vermiculite. The plants were watered with the Hyponex solution (500 times dilution) (Hyponex, Tokyo, Japan) every week, and harvested on the 8th day after sowing (DAS) (stage I), the 34th DAS (stage II), and the 44th DAS (stage III). The harvest periods were slightly different among cultivations, because the growth depended on the climate. The plants were used to extract crude enzyme, starch, and total RNA. For purification of β-amylase, European red radish was purchased at a local market in Japan.

2.2. Crude enzyme extract

Radish taproots (5 g fresh weight) were ground by a steel musher on ice until they became a paste. The paste was centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was a crude enzyme extract from the taproots. Leaves (1 g fresh weight) were homogenized in 2 ml of deionized water using mortar and pestle. The supernatant after centrifugation (10,000*g*, 15 min, 4°C) was a leaf crude enzyme extract. As an extraction from leaves, roots were homogenized and centrifuged to obtain a root crude enzyme extract. The extracts were kept at −20°C until use. The amylolytic activity and antigenicity for an anti-RsBAMY1 antibody in the extracts did not change during storage at −20°C for 6 months.

2.3. Determination of amylase activity

Glucan hydrolyzing activity was measured by the 3,5-dinitrosalicylic acid (DNSA) method described previously (Lizotte, Henson, & Duke, 1990) with slight modifications. An 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. The mixture was incubated at 37°C for 5 min. Immediately, 40 μl of the DNSA reagent containing 44 mM DNSA, 1 M sodium potassium tartrate, and 0.4 M sodium hydroxide was added to the reaction mixture. The solution was heated at 100°C for 5 min. After cooling to room temperature, 360 μl of deionized water was added. Then the absorbance was read at 540 nm. For the blank reaction, the DNSA reagent was combined with the substrate solution before the enzyme solution was added. Calibration curves were produced by reacting different concentrations of maltose with the DNSA reagent.

2.4. Protein quantification

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

2.5. Amylase zymography

Native polyacrylamide gel electrophoresis (native PAGE) and an amylase activity stain were done by the method of the previous report (Lin, Spilatro, & Preiss, 1988) with modifications. Proteins in the samples were separated by the native PAGE (7% polyacrylamide gel) containing 0.1% soluble starch. After electrophoresis, the gel was soaked in 100 mM sodium acetate buffer pH 4.8 containing 0.2% soluble starch at 37°C for 30 min. The starch in the gel was stained by immersing the gel in 1% acetic acid containing 10 mM I_2 and 14 mM KI for 3 min at room temperature. The presence of amylolytic activity was represented as a lytic band. For a β-limit dextrin-hydrolyzing activity, β-limit dextrin was substituted for soluble starch.

2.6. Sodium dodecyl sulfate-PAGE (SDS-PAGE)

Protein samples were fully denatured by boiling with 2-mercaptoethanol and SDS, and separated in a 12.5% polyacrylamide gel with the Mini-Protean III electrophoresis system (Bio-Rad). Gels were stained with colloidal Coomassie Blue (Bio-Safe, Bio-Rad).

2.7. β-Amylase purification

Crude enzyme extract (50 ml) was obtained from radish taproots (70 g fresh weight) by the method described above. The extract was dialyzed for 24 h against deionized water with SnakeSkin Pleated Dialysis Tubing (10,000 MWCO, PIERCE, IL, USA). The dialyzed sample was loaded onto the TOYOPEARL DEAE-650M (TOSOH, Tokyo, Japan) column (15x200 mm), equilibrated with deionized water. After the column was washed with three volumes of 10 mM Tris-HCl buffer pH 7.5 containing 1 mM dithiothreitol, bound protein was eluted by a 130-ml linear gradient of NaCl (0-500 mM) in 10 mM Tris-HCl buffer pH 7.5 containing 1 mM dithiothreitol. Fractions of 5 ml each were collected. The fractions showing amylase activity were combined. Affinity precipitation was done on ice according to the previous method

(Lizotte et. al., 1990) with slight modifications. Ethanol was added to the active fraction until the final concentration of ethanol reached 40%. After centrifugation at 10,000*g* for 20 min at 4°C, the supernatant (30 ml) was used for the affinity precipitation. The following reagents were added to the supernatant in order, with gentle stirring: 1.5 ml of 0.2 M sodium phosphate buffer pH 7.9, 2.1 ml of 2% oyster glycogen solution, and 3 ml ethanol. The suspension was agitated for 5 min, and then centrifuged at 2,000*g* for 6 min at 4°C. The pellet was washed twice with 10 mM sodium phosphate buffer pH 7.9 containing 40% ethanol. The washed pellet was suspended in 1 ml of 100 mM sodium acetate buffer pH 4.8 and kept at 37°C for 1 h to digest glycogen completely. After centrifugation (10,000*g*, 5 min, 4°C), the supernatant was subjected to the NAP-5 column (a Sephadex G-25 disposable column, GE Healthcare, Tokyo, Japan), which was equilibrated with 100 mM sodium acetate buffer pH 4.8. The purified β-amylase was stable at −20°C for 3 months.

2.8. Protein mass spectrometry

A purified protein, which was treated with 10 mM dithiothreitol and 55 mM iodoacetamide, was digested by trypsin. The fragments were analyzed by nanoflow liquid chromatography coupled to electrospray ionization quadrupole time-of-flight tandem mass spectrometry (nanoLC-ESI-Q-TOF/MS/MS). A database search was performed with the MASCOT MS/MS Ion Search against the NCBInr database in the taxonomy of *Arabidopsis thaliana*.

2.9. Immunoblot analysis

Protein samples were resolved by 12% SDS-PAGE as described above. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane filter (Hybond-ECL, GE Healthcare) with a Mini Trans-Blot (Bio-Rad). A blocked filter was incubated with a primary antibody, i.e. a rabbit polyclonal anti-RsBAMY1 antibody raised against a synthetic peptide (FKEAAAKAGHPEWDLPEDAGE), which is found in the sequence of RsBAMY1. Horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) was a secondary antibody. Positive signals were detected by the chemiluminescence technique with the ECL Western Blotting Detection System (GE Healthcare). The signals were detected by an LAS-4000 Image Analyzer (FUJIFILM, Tokyo, Japan).

2.10. Reverse transcription-polymerase chain reaction (RT-PCR)

The levels of RsBAMY1 transcripts in radish organs were analyzed by RT-PCR. Total RNA was extracted from the leaf, taproot, and root of the radish with the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). One microgram of RNA was subjected to a semiquantitative RT-PCR system (Goidin, Mamessier, Staquet. Schmitt, & Berthier-Vergnes, 2001) using the QuantumRNA 18S Internal Standards Kit (Ambion, TX, USA). In this system, both the target RNA and the 18S rRNA were amplified together. The 18S rRNA expression was used as an internal standard. Reverse transcription was performed at 45°C for 30 min, and PCR proceeded through 27 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The sequence of the sense primer was 5'-GTGAGGAAAATGCACGCTGA-3' which covers from 1381 bp to 1400 bp in the RsBAMY1 cDNA. The sequence of the antisense primer was 5'-TATCACCGTTCACTTCACAC-3' (from 1706 bp to 1725 bp in the RsBAMY1 cDNA). The ratio of 18S primer to competimer was 2:8. The PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. The deduced lengths of PCR products were 345 bp (RsBAMY1) and 315 bp (18S rRNA).

2.11. Starch analysis

Measuring of the starch content was done as described previously (Kötting, Pusch, Tiessen, Geigenberger, Steup, & Ritte, 2005) with slight modifications. Fresh-cut cubes (approximately 100 mm^3 each) prepared from radish taproots (1 g fresh weight) were extracted twice by refluxing each with 10 ml of 80% (v/v) ethanol for 20 min. In the case of hypocotyls, a fresh hypocotyl (1 g fresh weight) was cut into pieces (approximately 5 mm in length) and extracted as described above. Insoluble material was washed in 10 ml deionized water. One milliliter of 0.4 M potassium hydroxide was added to the hydrated insoluble material, and then incubated at 95°C for 1 h. The sample, which was neutralized with 2 M acetic acid, was centrifuged at 10,000g for 5 min at 4°C. The supernatant (1 ml) was digested by α-amylase (10 U) and amyloglucosidase (7 U) at 37°C for 18 h. Glucose formation was determined by using the hexokinase and glucose-6-phosphate dehydrogenase system described in the previous report (Hara, Oki, Hoshino, & Kuboi, 2003).

3. Results

3.1. Purification of radish taproot amylase

First, we detected amylase activity in the radish taproot (Fig. 1). Proteins from leaves and taproots were analyzed by zymography to detect the amylolytic activity. Proteins were separated by the native PAGE (Fig. 1A), and the gels were stained with iodine (Figs. 1B, C). When soluble starch was added to the polyacrylamide gel, a clear amylolytic band was detected in the taproot sample (Fig. 1B, lane TR, arrowhead), but no band was found in the leaf sample (Fig. 1B, lane L). On the other hand, native PAGE analysis using a polyacrylamide gel containing β-limit dextrin indicated that both samples (taproot and leaf) did not show any lytic bands (Fig. 1C). Similar results were shown when the amylolytic activity was determined by the formation of reducing ends (Figs. 1D, E). These results indicate that the major amylolytic enzyme in the radish taproot is a β-amylase, but not an α-amylase, an isoamylase, or a limit dextrinase. Thus we purified the β-amylase from the radish taproot. A crude extract of taproots was dialyzed and then subjected to anion exchange chromatography (TOYOPEARL DEAE-650M) with a linear gradient of NaCl (0-500 mM). Active fractions of amylase, which formed a major peak in the chromatography, were mixed with a glycogen solution to perform an affinity precipitation. A single protein band appeared in the SDS-PAGE analysis for the glycogen-bound fraction (Fig. 2A, lane 4, arrowhead). It is likely that the band consisted of a homogenous protein, because the glycogen-bound fraction showed a single band also in the native PAGE analysis (Fig. 2B, lane 1). An amylolytic band was observed at the same position where the protein was located (Fig. 2B, lane 2, arrowhead). Since no lytic band was found in the native gel containing β-limit dextrin (data not shown), it can be concluded that the purified protein is a β-amylase. Through the purification from the crude extract to the affinity precipitation step, purification magnifications were 133, and the yield was 32%. The mobility of the protein in the SDS-PAGE indicated that the molecular mass was 56.7 kDa. The optimum pH for the soluble starch-digesting activity was 5.0. The enzyme showed half of the maximum activity at pH 4.0 and 7.0. Although the optimum temperature of the activity was 50°C, 80% of the maximum activity at 50°C remained when the enzyme was reacted at 70°C. Soluble starch was the best substrate among several glucans tested. If the activity for soluble starch was standardized to 100%, the activities for rabbit glycogen, oyster glycogen, and potato amylose were 83%, 40%, and 27%, respectively. No activity was detected

in pullulan, α-cyclodextrin, or β-limit dextrin. The addition of ethylenediaminetetraacetic acid or cation (Zn^{2+}, Mg^{2+}, Ca^{2+}, or K^{+}) did not affect the enzyme activity.

3.2. cDNA cloning of radish amylase

To obtain data regarding the primary structure of the purified protein, trypsin-digested fragments of the protein were subjected to nanoLC-ESI-Q-TOF/MS/MS analysis combined with the MASCOT search program. Because the radish and *Arabidopsis thaliana* are members of Brassicaceae, it is expected that they may have similar genetic backgrounds. Thus, we selected *A. thaliana* as the taxonomy in the MASCOT search. Three sets of peptides, i.e. DGYRPIAR, MYGFTYLR, and YGHEIVPLK, which are portions of an Arabidopsis β-amylase (BAM5, At4g15210), matched the purified radish protein. Screening of the EST library which is produced from radish mRNA (Hara, Sugano, & Kuboi, 2003) revealed that there is one EST clone which has the three peptide sequences. Sequencing and a BLAST search suggested that the EST clone had a complete open reading frame encoding a protein which is closely related to plant β-amylases. Thus, we designated the clone *Raphanus sativus* β-amylase 1 (*RsBAMY1*, accession number: AB441862). *RsBAMY1* is 1737 bp in length and contains a 5' noncoding region (60 bp) and a 3' noncoding region (183 bp). The open reading frame of *RsBAMY1* encoded a protein whose calculated molecular weight is 56,394 with 498 amino acids. The deduced molecular weight corresponds to the molecular mass of the purified protein (56.7 kDa) described above. RsBAMY1 has two conserved catalytic residues (Glu189 and Glu383 in RsBAMY1) as determined by a structural analysis of a soybean β-amylase-maltose complex (Hirata, Adachi, Sekine, Kang, Utsumi, & Mikami, 2004). A phylogenetic tree based on amino acid sequences indicated that RsBAMY1 belongs to a plant β-amylase family (Fig. 3). RsBAMY1 is most similar to At4g15210, which was hit in the MASCOT search analysis described above.

3.3. Immunological detection of RsBAMY1

We raised a polyclonal anti-RsBAMY1 antibody to detect RsBAMY1 protein in the radish. To search an appropriate sequence for producing anti-peptide antibody, we checked the three-dimensional structure of a soybean β-amylase (NCBI

Structure Summary, MMDB ID: 55684). A junctional region between the fifth helix and a random segment adjacent to the C-terminus of the fifth helix in the soybean β-amylase was a candidate, because the region was a peripheral part of the tertiary structure of the β-amylase. We used the sequence FKEAAAKAGHPEWDLPEDAGE, which is located in the junctional region of RsBAMY1, as determined by comparing it to the sequence of the soybean β-amylase. Immunoblot analyses demonstrated that the anti-RsBAMY1 antibody could selectively detect the purified radish β-amylase (Figs. 4A, B). This shows that the purified protein is the *RsBAMY1* gene product. When a radish plant was divided into leaf, taproot, and root (Fig. 4D), RsBAMY1 protein accumulated in the taproot, but little RsBAMY1 was detected in the leaf and root (Fig. 4C). Although PCR products corresponding to 18S rRNA were found at similar levels in the three organs, PCR products derived from the *RsBAMY1* transcripts were detected only in the taproot sample (Fig. 4E). These results suggest that the taproot-specific accumulation of RsBAMY1 protein is regulated by the transcript accumulation of the *RsBAMY1* gene.

3.4. RsBAMY1 expression and starch accumulation in radish taproot

The levels of RsBAMY1 expression and starch accumulation were analyzed at the different stages of hypocotyl (taproot) growth in the radish. A hypocotyl grows to a swollen taproot in the radish cultivar used in this study (Fig. 5A). The hypocotyl in the early seedlings (stage I) had low β-amylase activity, a low RsBAMY1 protein level, and little transcripts accumulation (Figs. 5B, C, E, F). The hypocotyl at stage I contained little starch (Fig. 5D). β-amylase activity, RsBAMY1 amount, and *RsBAMY1* transcripts accumulation were enhanced at the growing stage of the taproot (stage II), but these values decreased at the mature stage of the taproot (stage III). The starch contents did not change between stage II and stage III.

4. Discussion

In this study, we report for the first time the cDNA for a major taproot-specific β-amylase in the radish. To obtain the cDNA, we purified and characterized β-amylase from radish taproots. The characteristics of the radish β-amylase purified in the present study, such as molecular mass, optimum pH, substrate specificity, and insensitivity to cations, are similar to those of plant β-amylases described previously (Ziegler, 1999; Lizotte et al., 1990; Monroe & Preiss, 1990;

Wang, Monroe, & Sjölund, 1995). However, an Egyptian radish β-amylase was found to be remarkably activated by cations (Rashad et al., 1995), suggesting that Rashad et al. might have purified a different type of β-amylase. They purified the radish β-amylase in four steps including three column chromatography procedures after a crude extract preparation. Our purification system, however, consisted of three steps including one column chromatography procedure. This means that the efficiency of the purification method of radish β-amylase was improved.

Recent studies on the health-promoting properties of the radish have highlighted the functions of glucosinolates and isothiocyanates, because these compounds show antioxidative and anticarcinogenic activities (Barillari et al., 2005; Papi et al., 2008; Hanlon et al., 2007). Thus, it is obvious that the glucosinolates and isothiocyanates are major health-promoting factors in the radish. In this study, we show an additional function of the radish, i.e., the potent amylolytic activity of the β-amylase RsBAMY1. It is currently widely believed that the activity of the digestive system declines due to complicated stresses. RsBAMY1 in the radish may be a natural digestive to that helps in the digestion of starch-containing foods. Daily consumption of radishes may support the function of the digestive organs. Because the highest activity of RsBAMY1 was observed during the growing stages of radish taproots (Fig. 5), young taproots are useful when a radish containing high amylase activity is needed.

It is known that β-amylase is related to the sweetening of vegetables by degrading endogenous starch during cooking. Indeed, the radish becomes sweet when it is cooked. Moreover, when grated raw radish (daikon oroshi) is prepared, it gradually becomes sweet. This phenomenon seems to reflect the action of β-amylase. Consumers have a tendency to prefer sweet radishes, and the *RsBAMY1* gene may be a useful marker in the selection and breeding of sweeter radishes.

In conclusion, we summarize the present results as follows. 1) The potent amylolytic activity in the radish taproot is due to the β-amylase RsBAMY1. 2) A simple purification process for RsBAMY1 was established. 3) Young taproots contain high amylolytic activity. 4) The RsBAMY1 gene may be a useful marker in the production of sweeter radishes. These results are expected to provide useful information regarding the development of products to which the properties of radish β-amylase can be applied.

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Figure Legends

Fig. 1. Amylolytic activity in radish taproots. A-C, native PAGE. Crude extract (5 μg each) from taproots (TR) or leaves (L) was subjected to native PAGE. A, image of a polyacrylamide gel stained with colloidal Coomassie Blue. B, zymogram of amylolytic activity in a soluble starch-containing gel. C, zymogram of amylolytic activity in a β-limit dextrin-containing gel. D and E, amylolytic activity

determined by the DNSA method described in Materials and methods. Substrates are soluble starch (D) and β-limit dextrin (E). Crude extracts are prepared from taproots (TR) and leaves (L). Values and bars represent means ± S.D. of 4 plants.

Fig. 2. Purification of β-amylase from radish taproots. A, SDS-PAGE analyses of active fractions in purification steps. Lane 1, crude extract (100 μg protein); lane 2, dialysis (10 μg protein); lane 3, TOYOPEARL DEAE-650M (10 μg protein); lane 4, glycogen affinity precipitation (5 μg protein). B, native PAGE analysis (lane 1, 5 μg) and zymography in a soluble starch-containing gel (lane 2, 0.1 μg) of the purified protein. Arrowheads indicate the position of the purified protein.

Fig. 3. Phylogenetic tree based on amino acid sequences showing relationships in β-amylases. Accession numbers or locus codes for the clones are as follows. Gm, *Glycine max* BAA09462; Ms, *Medicago sativa* O22585; Hv, *Hordeum vulgare* AAX37357; Os, *Oryza sativa* BAC83773; Zm, *Zea mays* AAD15902; At2g32290, *Arabidopsis thaliana* BAM6; Ib, *Ipomoea batatas* P10537; Cs, *Calystegia sepium* AAG44882; Rs, *Raphanus sativus* RsBAMY1 (open circle, present study); At4g15210, *Arabidopsis thaliana* BAM5; Cr, *Chlamydomonas reinhardtii* XP_001691372; Ma, *Musa acuminata* AAZ94622; Bc, *Bacillus cereus* BAA75890; Bm, *Bacillus megaterium* CAB61483; Cb, *Clostridium botulinum* ACA57155. The unrooted phylogenetic tree was produced by ClustalW (the DDBJ homepage) with a Phylodendron application (http://iubio.bio.indiana.edu/treeapp/treeprint-sample2.html). Bar indicates the distance of a 10% difference in amino acid sequences.

Fig. 4. RsBAMY1 accumulation in different radish organs. A, SDS-PAGE analyses of crude protein extract (lane C, 100 μg protein) and purified β-amylase (lane P, 5 μg) from radish taproots. The gel was stained with colloidal Coomassie Blue. Arrowhead indicates the position of the purified β-amylase. B and C. immunoblot analyses with an anti-RsBAMY1 antibody. B, taproot crude extract (lane C, 3 μg protein) and purified β-amylase (lane P, 20 ng). C, crude protein extracts from taproots (lane TR), leaves (lane L), and roots (lane R) are analyzed. Three micrograms of protein was loaded. D, the radish used in this experiment. Leaves (L), taproot (TR), and roots (R) are shown. E, RT-PCR analyses. Total RNA was extracted from the taproots (TR), leaves (L), and roots (R). Details of

experiments are described in Materials and methods. Closed and open arrowheads indicate the positions of RT-PCR products from *RsBAMY1* transcripts and 18S rRNA, respectively.

Fig. 5. Changes in RsBAMY1 expression and starch content during taproot growth. A, Growth of radish taproot. Brackets indicate hypocotyl and taproot used in this experiment. Bars represent 2 cm. Roman numerals represent growth stages. B, amylase activities in the different growth stages. C, specific activity of amylase. D, starch content. Values and bars represent means ± S.D. of 4 plants. E, immunoblot analyses with an anti-RsBAMY1 antibody. Crude protein extract from taproots (1 μg each) was analyzed. F, RT-PCR analyses. Taproots at different growth stages were used for total RNA extraction. Details of experiments are described in Materials and methods. Closed and open arrowheads indicate the positions of RT-PCR products from *RsBAMY1* transcripts and 18S rRNA, respectively.

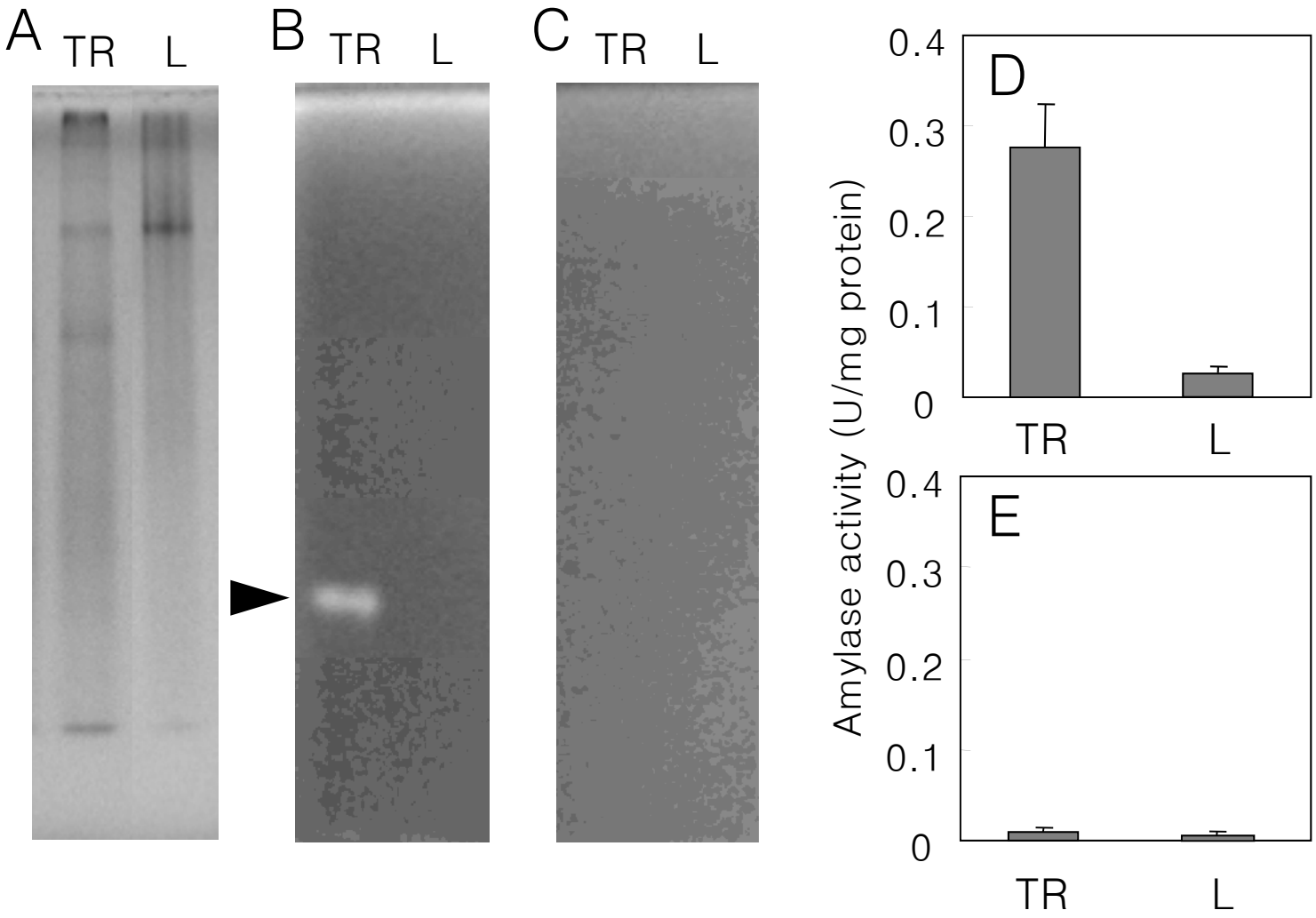
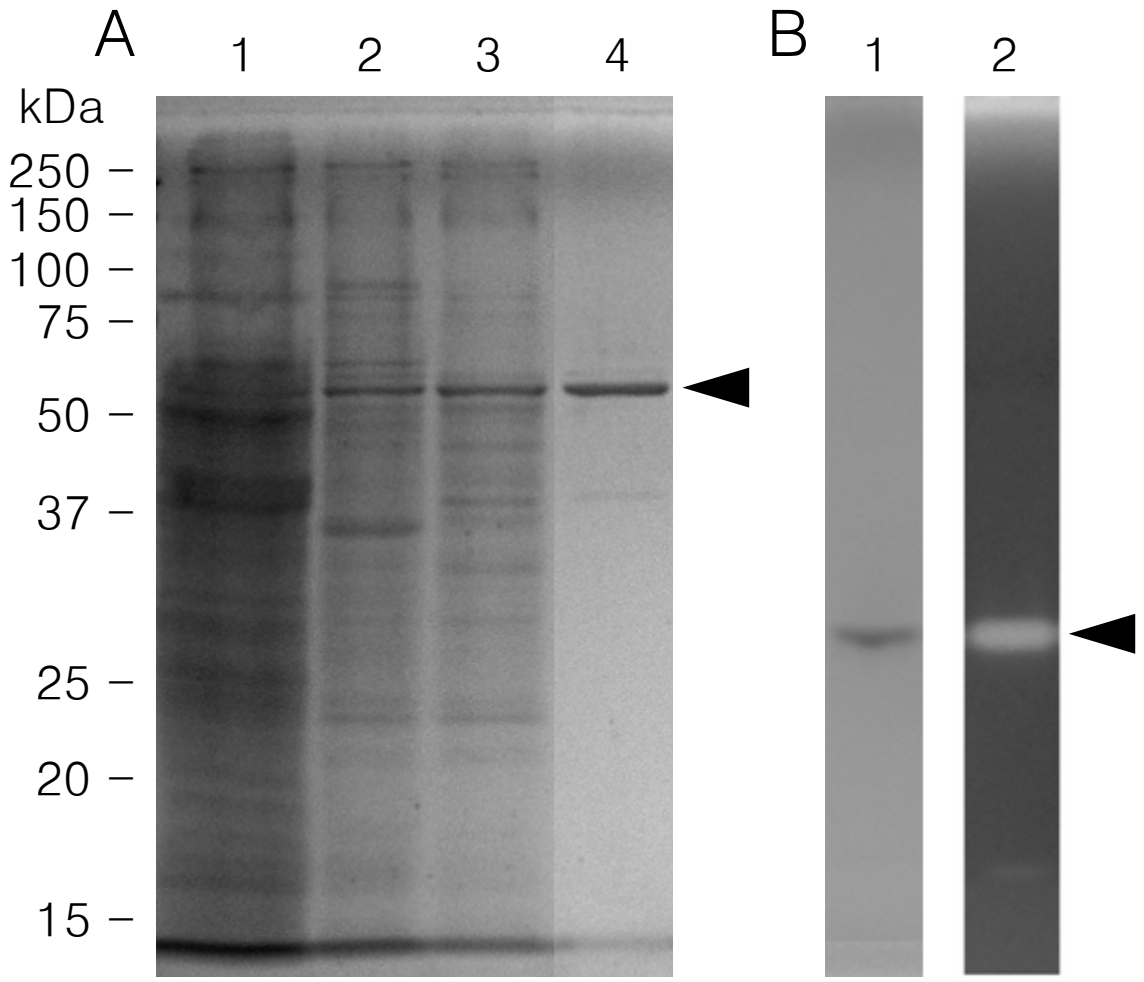


Fig. 1 Hara et al.



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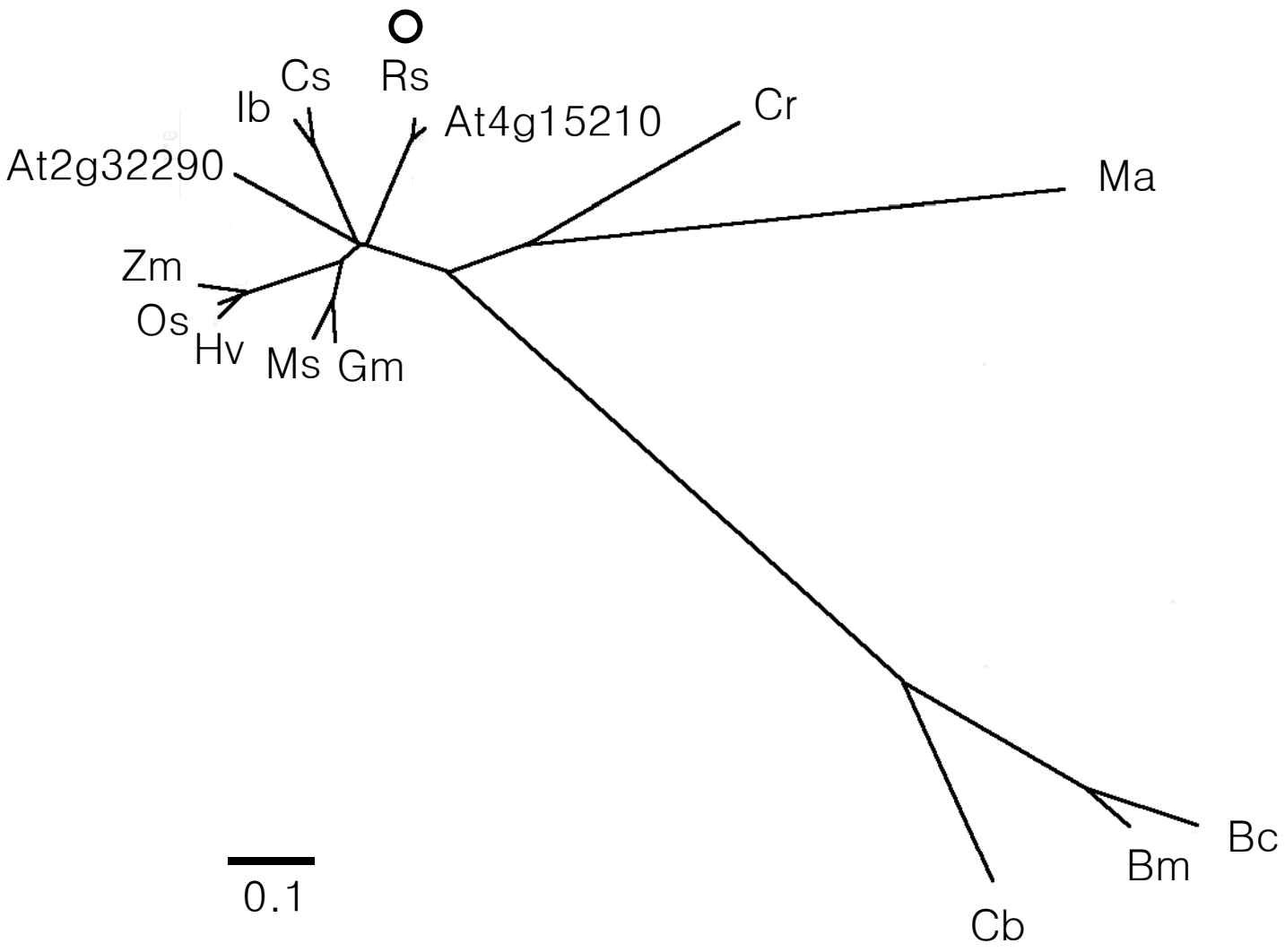
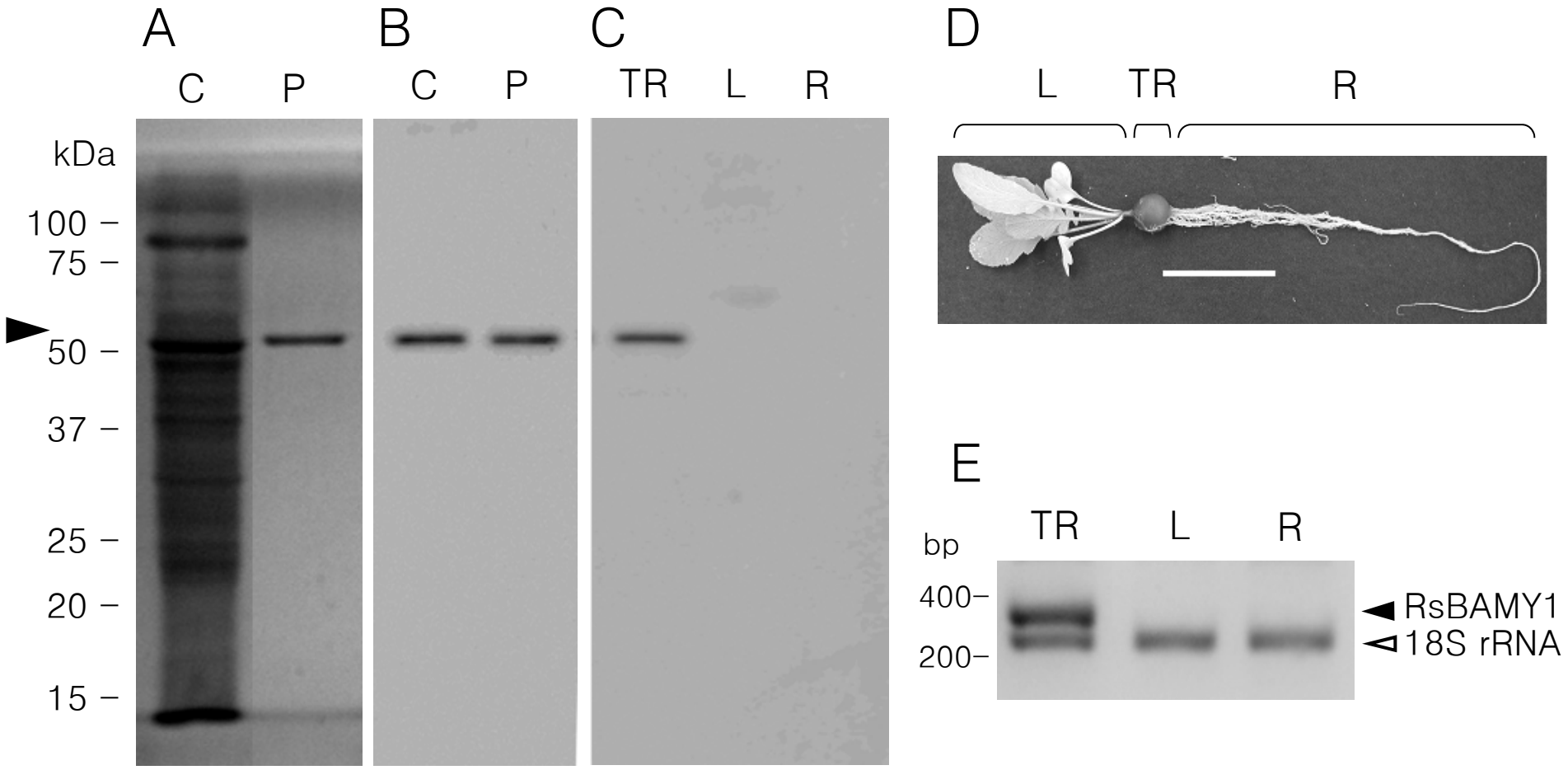


Fig. 3 Hara et al.



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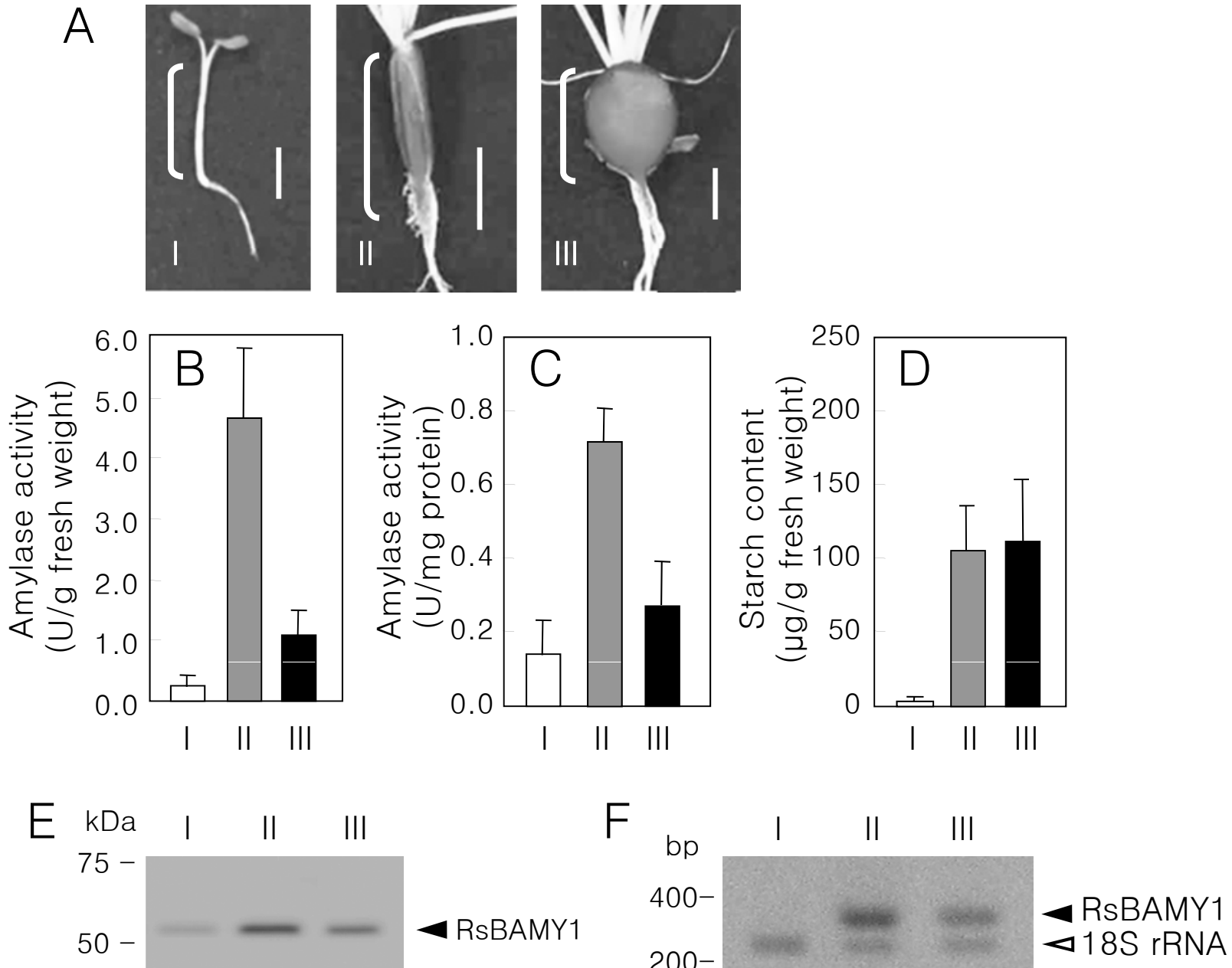

Fig. 5 Hara et al.