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Identification and Characterization of a Phloem-Specific β-Amylase[1]

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A monoclonal antibody, RS 5, was raised by injecting sieve elements isolated from tissue cultures of *Streptanthus tortuosus* (Brassicaceae) into BALB/c mice and screening resultant hybridoma supernatants for the labeling of phloem using immunofluorescence microscopy. The RS 5 monoclonal antibody identifies a 57-kD protein on immunoblots, which is present in phloem-forming tissue cultures of *S. tortuosus* but is absent in cultures that lack phloem. Purified 57-kD protein of *S. tortuosus* is demonstrated to be a phloem-specific β-amylase. Partial peptide sequences of the 57-kD protein of *S. tortuosus* are shown to be 96% identical with the corresponding portions of a deduced sequence reported for a major form of β-amylase in *Arabidopsis thaliana*. The RS 5 antibody cross-reacts with the major form of *A. thaliana* β-amylase on immunoblots, and the antibody also binds to the sieve elements of *A. thaliana* using immunofluorescence microscopy. The results suggest that the major form of *A. thaliana* β-amylase is a phloem-specific enzyme.

Phloem transports carbohydrates, amino acids, and other nutrients from "sources," the places where photoassimilates are formed, to "sinks," the places where the nutrients are stored or metabolized. The fact that many unknown proteins are present in the translocation streams of wheat (Fisher et al., 1992) and castor beans (Sakuth et al., 1993) suggests that the identification of phloem-specific gene products may help us to understand the underlying mechanism(s) of phloem function as well as to reveal the evidence of metabolic pathways not presently associated with the phloem.

Several phloem proteins and genes encoding phloem proteins have been identified by investigators using different approaches. One method based on the collection of phloem exudate has led to the identification and purification of a filamentous P-protein (Eschrich et al., 1971; Sabnis and Hart, 1976; Kollmann, 1980) and a phloem lectin (Sabnis and Hart, 1978; Bostwick and Thompson, 1993). Because phloem exudates are not readily available from many plants, such as *Arabidopsis thaliana*, and because phloem exudates do not contain many of the membrane proteins, organelles, or enzymes (Geigenberger et al., 1993) that are sequestered in the phloem pathway, this approach has some limitations.

Another method uses biochemical and molecular techniques to identify proteins known or believed to function in phloem transport. Examples include the identification of a Suc-binding protein using a Suc analog as a probe (Grimes et al., 1992) and a Suc transporter pS21 (SoSUT1) identified by a functional assay based on complementation of yeast mutants (Riesmeier et al., 1992). Similar complementation of yeast also resulted in the identification of a potato Suc transporter StSU1 (Riesmeier et al., 1993). The expression of StSUT1 has been demonstrated in the minor veins using in situ hybridization (Riesmeier et al., 1993), and the analysis of antisense mutants indicated that this potato Suc transporter plays a vital role in phloem loading (Riesmeier et al., 1994).

Other enzymes, such as a stachyose synthase (Holthaus and Schmitz, 1991), a galactinol synthase (Beebe and Turgeon, 1992), and a phloem-specific Suc synthase (Nolte and Koch, 1993), have been localized in the phloem by immunohistochemistry using antibodies prepared against purified proteins. Several promoters have also been found to direct gene expression predominantly in the phloem by histochemical staining using GUS activity after the plants were transfected with the translational fusion genes. Examples include the promoters of a maize Suc synthase-1 gene (*Sh*) (Yang and Christou, 1990), an *A. thaliana* H^+-ATPase gene (DeWitt et al., 1991), an *A. thaliana* Suc synthase gene (Martin et al., 1993), and a rice Suc synthase-1 gene (Shi et al., 1994).

Although these methods are powerful, they are largely based on existing paradigms for phloem function. We have explored an alternative approach based on the production of monoclonal antibodies against phloem cells, with the goal of identifying previously unknown phloem gene products. We induced phloem differentiation in the callus cultures of *Streptanthus tortuosus* (Brassicaceae) and isolated sieve elements from the cultures that were used to immunize BALB/c mice. We then screened the mouse hybridoma supernatants for the production of phloem-specific monoclonal antibodies (Tóth et al., 1994). In this paper, we report that a monoclonal antibody, RS 5, recognizes a

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Abbreviations: CHA, cyclohexaamylose; dd, double distilled; FITC, fluorescein isothiocyanate; TBST, Tris-buffered saline; 10 mM Tris, 500 mM NaCl, and 0.05% [v/v] Tween 20, pH 7.5, with Tween 20.

sieve element-specific form of β-amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2) in vegetative organs of *S. tortuosus* (Brassicaceae) and *A. thaliana*.

MATERIALS AND METHODS

Plant Materials

Streptanthus tortuosus (Brassicaceae) plants were grown under natural light in the greenhouse at the University of Iowa (Iowa City). Plants of Columbia ecotype of *Arabidopsis thaliana* were grown in the growth chamber at 22°C under alternating periods of 8 h of light (cool-white fluorescent) and 16 h of dark.

Plant Tissue Culture and Isolation of Sieve Elements

The conditions for tissue cultures and the protocols leading to the isolation of sieve elements were described earlier (Tóth et al., 1994).

Immunization and Antibody Production

The method of monoclonal antibody production was reported earlier (Tóth et al., 1994). Polyclonal antibodies were raised according to the method of Monroe and Preiss (1990).

Immunohistochemistry

Stem sections cut with a razor blade were incubated in blocking buffer (2.5% nonfat dry milk in PBS) for 45 min, washed in blocking buffer, and then incubated in RS 5 monoclonal antibody diluted in blocking buffer (1:10–1:1000) for 45 min, washed in blocking buffer, and incubated in goat anti-mouse secondary antibody conjugated to FITC (Sigma) or rhodamine (Pierce) (diluted 1:200 in blocking buffer) for 45 min. After the sections were washed, they were examined with a Zeiss Photomicroscope III equipped with a mercury light source and filters (Omega Optical, Inc., Brattleboro, VT) for separate excitation of different fluorochromes. The fluorescent images were recorded with a Hamamatsu (Park Ridge, IL) silicon-intensifier target tube camera and digitized with a PixelPipeline board (Perceptics Corp., Knoxville, TN) and a Macintosh IIfx computer. The images were processed with the NIH Image program (written by Wayne Rasband and available via Internet by anonymous file transfer protocol from zippy.nimh.nih.gov). For composite images of separate fluorochromes, the NIH Image files were converted into separate color channels and formed into composite images using Photoshop 2.5 (Adobe Systems Inc., Mountain View, CA) and the Macintosh IIfx computer. The computer images were printed on a Tektronix (Willsonville, OR) Phaser IISDx printer or a Polaroid (Cambridge, MA) Digital Palette CI 5000S film recorder.

Extraction of Total Proteins

Ten grams of cultures of *S. tortuosus* were frozen in liquid nitrogen, ground in 2.5 mL of extraction buffer containing 50 mM Tris-HCl (pH 8.5), 1% β-mercaptoethanol, and 1 mM PMSF, and centrifuged at 12,000*g* at 4°C for 30 min. The supernatant was collected and concentrated to a final protein concentration of 1 mg/mL using Amicon Centriprep-10 tubes (Amicon, Inc., Beverly, MA).

Gel Electrophoresis and Immunoblot Assay

A 4% stacking gel and a 12% separating gel were used for SDS-PAGE according to the method of Laemmli (1970). SDS-PAGE-separated proteins were transferred onto a nitrocellulose membrane using either a TE42 Transphor wet transfer unit or a TE70 SemiPhor semidry transfer unit (Hoefer Scientific Instruments, San Francisco, CA). For immunoblotting using monoclonal antibodies, blots with transferred proteins were incubated in blocking buffer (2.5% nonfat dry milk in TBST) for 2 h, rinsed with dd H_2O and incubated overnight in either RS 5 monoclonal antibody (1:50 diluted in 1% BSA/TBST) or mouse preimmune serum (1:1000 in 1% BSA/TBST). The blots were washed in blocking buffer for 30 min and incubated in goat anti-mouse secondary antibody conjugated to alkaline phosphatase (diluted 1:4000 in blocking buffer) for 1 h. After the blots were washed for 30 min in blocking buffer, they were rinsed in dd H_2O and stained in bromochloroindolyl phosphate/nitroblue tetrazolium developer. Immunoblotting using polyclonal antibodies was carried out essentially as described above except that 5% nonfat dry milk/TBST was used as the blocking buffer, the blots were incubated in the primary antibody (diluted 1:2000 in 5% nonfat dry milk/TBST) for 1 h, and a goat anti-rabbit alkaline phosphatase-conjugated secondary antibody was used.

Purification of p57

The purification was monitored by immunoblotting using RS 5 antibody. Three hundred sixty grams of 21-d-old phloem (+) cultures were frozen in liquid nitrogen and ground in 90 mL of extraction buffer containing 50 mM Tris-HCl (pH 8.5), 1% β-mercaptoethanol, and 1 mM PMSF, followed by a 12,000*g* centrifugation at 4°C. The total protein extract was subjected to $(NH_4)_2SO_4$ precipitation and the 35 to 45% (w/v) fraction was collected. The sample was dialyzed against dd H_2O and further separated in a Rotofor IEF preparative cell (Bio-Rad) according to pI (1% Bio-lyte, pH 3–10, 12 W, 4 h). Rotofor-separated fractions containing p57 were pooled. The p57 protein was further purified to homogeneity using a CHA column (Vretblad, 1974; Monroe and Preiss, 1990) after it was determined to be a β-amylase.

Peptide Sequencing of p57

After Rotofor separation the partially purified p57 was subjected to SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The immunoreactive 57-kD band was excised from the membrane and used for N-terminal sequencing. The excised 57-kD protein was also partially digested with trypsin according to the method of Stone and Williams (1993) and used for internal sequencing. The N-terminal sequencing and internal sequencing were carried out at the University of Iowa Protein Facility by Edman

degradation on an Applied Biosystems 475A protein sequenator.

Enzyme Assays

Amylopectin was made as a 10-mg/mL solution in dd H_2O. The p57 or β-amylase was diluted in 80 mM succinate buffer (pH 5.6); 200 μL of amylopectin were mixed with 200 μL of enzyme-containing succinate buffer and incubated at 25°C for 15 min. Then 400 μL of the color reagent (1% dinitrosalicyclic acid, 30% sodium potassium tartrate in 0.4 M NaOH) was added to stop the reaction, and the mixture was heated for 5 min in a 100°C water bath. The A_{540} was measured with a spectrophotometer. A dilution series of maltose was used to obtain a standard curve. One unit of β-amylase is that liberating 1 μmol of maltose per minute at 25°C.

β-Limited dextrin was made by adding 40 units of sweet potato β-amylase to a 100-mL boiled starch solution (2%, w/v). The solution was incubated at room temperature overnight and heated to 100°C for 5 min to inactivate the added β-amylase.

To determine the optimum pH of the amylase activity of p57, 0.025 unit of p57 was diluted in 400 μL of buffers at different pHs, each containing 4 mg of amylopectin. Three sets of buffers were used to cover different pH ranges: 50 mM succinate buffer (pH 3.8–6.0), 50 mM Mes buffer (pH 5.3–6.2), and 50 mM sodium phosphate buffer (pH 5.4–8.0). The reaction was carried out at 25°C for 15 min. The enzyme activity was determined as above.

Enzyme activity neutralization assays of p57 were carried out according to the method of Monroe and Preiss (1990).

RESULTS

A Phloem-Specific Monoclonal Antibody, RS 5, Was Generated by Immunizing Mice with Isolated Sieve Elements

We cultured explants of *S. tortuosus* to form callus tissues that differentiate phloem sieve elements and companion cells. Sieve elements were isolated from the callus tissues (Tóth et al., 1994) and injected into BALB/c mice to produce monoclonal antibodies (Köhler and Milstein, 1975). A phloem-specific monoclonal antibody, RS 5, was selected by screening the hybridoma supernatants using hand-cut stem sections of *S. tortuosus* and indirect immunofluorescence miscroscopy (Fig. 1).

RS 5 Recognizes a 57-kD Phloem-Specific Protein in Tissue Cultures of *S. tortuosus*

Immunoblotting was used to identify the protein that is recognized by RS 5. A 57-kD protein was specifically labeled on immunoblots of proteins extracted from cultures that support phloem differentiation [phloem (+)] (Fig. 2, lane 4) but not on the blots of proteins from cultures that lack phloem [phloem (−)] (Fig. 2, lane 3). A 57-kD protein can also be recognized (arrow, Fig. 2) in the Coomassie blue-stained gels of proteins from phloem (+) cultures (Fig. 2, lane 2) but not in the gels of proteins extracted from phloem (−) cultures (Fig. 2, lane 1).

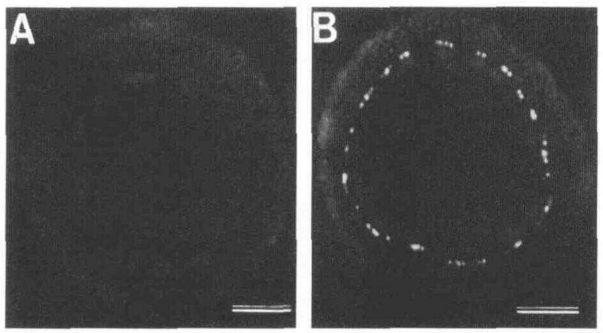

Figure 1. RS 5 monoclonal antibody specifically labels phloem cells in stem sections of *S. tortuosus*. A, *S. tortuosus* stem sections incubated in BALB/c mouse preimmune serum were not labeled. Bar = 0.3 mm. B, A low-magnification image shows that RS 5 labels phloem tissue in a *S. tortuosus* stem cross-section. Bar = 0.3 mm.

The Protein Identified by RS 5 Exists in the Phloem of *A. thaliana*

The RS 5 monoclonal antibody also specifically labels the phloem of *A. thaliana* stem as determined by indirect immunofluorescence microscopy (Fig. 3B). The phloem of *A. thaliana* stem is not labeled in control sections incubated in preimmune serum (Fig. 3A). The phloem-specific labeling is also shown at higher magnification (Fig. 3, C and D). The fluorescence recorded in the outer cortical parenchyma cells of some samples (Fig. 3, A and B) is the result of autofluorescence of chloroplasts in this tissue that was not filtered out because of the broad spectrum of the filters used in the experiment. Although the red autofluorescence of the Chl is clearly different from the green, specific fluorescence of the FITC-conjugated antibodies in the phloem, the monochrome video camera records both equally.

The Protein Labeled by RS 5 Is Sieve Element Specific and Is Not a Cell Wall Protein

Stem sections of *S. tortuosus* were labeled with RS 5 and a goat anti-mouse secondary antibody conjugated to FITC. After the section was imaged using epifluorescence microscopy, a second separate image of the same section was also captured using substage (white) illumination. The two images were assigned two separate, pseudo colors (pink and blue, respectively), and a composite image was generated in the computer (Fig. 4A). The composite image shows that RS 5 labels an antigen that is present in the collapsed sieve element protoplast, which appears pink, but is not present in the cell walls (blue).

A dual-labeling procedure was used to demonstrate the cellular localization of the antigen identified by RS 5. Longitudinal free-hand sections of *A. thaliana* stems were incubated with the RS 5 monoclonal antibody, followed by a rhodamine-conjugated secondary goat antibody. The labeled sections were mounted on slides in a solution (0.1%) of aniline blue to stain the callose (Eschrich and Currier, 1964) of sieve element sieve plates. The dual-labeled sec-

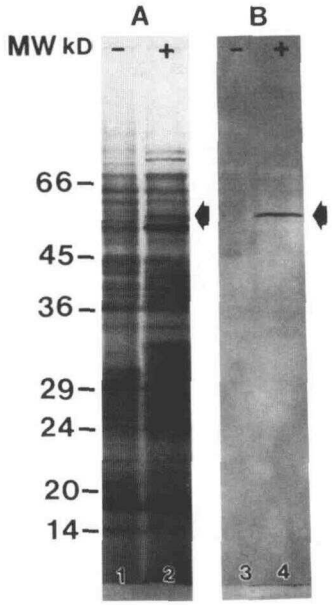

Figure 2. Proteins were extracted from 14-d-old *S. tortuosus* tissue cultures that either do (+) (lanes 2 and 4) or do not (−) (lanes 1 and 3) support phloem differentiation, separated on SDS-PAGE (A) and probed using the RS 5 antibody (B). Each lane contained 25 μg of proteins.

tions were examined in the microscope, and a composite image was formed in the computer by assigning red to RS 5 labeling and blue to the UV-excited fluorescence of callose. The resulting composite image shows that RS 5 labeling occurs only in the sieve elements, the cells that are identified by the presence of sieve plates (Fig. 4B). No labeling of adjacent companion cells or parenchyma cells was observed. The few particles labeled outside the sieve elements are interpreted as having been dragged out of the cells during cutting. Although four sieve plates were stained by aniline blue in Figure 4B, only sieve elements actually sectioned by the razor blade are labeled by the RS 5 antibody, suggesting that the RS 5 antibody can only gain access to the antigen when the sieve elements are sectioned.

p57 Is a Sieve Element-Specific β-Amylase

We used the RS 5 antibody and immunoblotting to follow the purification of the p57 protein. We screened proteins from phloem (+) cultures at different stages of callus and found that relatively old cultures (about 21 d) contain high levels of p57 (Q. Wang, J. Monroe, and R.D. Sjölund, unpublished data). An extraction buffer containing a reducing agent was used to extract total water-soluble proteins from the 21-d-old-phloem (+) tissue cultures (Fig. 5, lane 1). The extracted proteins were precipitated by 35 to 45% (w/v) $(NH_4)_2SO_4$ (Fig. 5, lane 2) and were separated in a Rotofor cell (Bio-Rad) that separates proteins based on their pI values (Fig. 5, lane 3).

The partially purified p57 was further separated using SDS-PAGE (Fig. 5, lane 3, arrow) and transferred onto a polyvinylidene fluoride membrane. The immunoreactive p57 band was excised from the membrane and sequenced. A 20-amino acid sequence was obtained from the N terminus (Fig. 6) that matches exactly a reported amino acid sequence deduced from a β-amylase cDNA of *A. thaliana* (Monroe et al., 1991). In addition, we made a partial digestion of the p57 sample and obtained several internal amino acid sequences, which also closely match the reported *A. thaliana* β-amylase sequence (Fig. 6).

To test whether p57 is a β-amylase, for our first step we made a CHA affinity column, which is specific for β-amylase but not for α-amylase (Vretblad, 1974; Monroe and Preiss, 1990). The protein sample after Rotofor purification (Fig. 5, lane 3) was passed through the affinity column. Proteins that were washed through the column prior to elution (Fig. 7, lane 1) showed no detectable β-amylase activity and were not labeled by RS 5 on the corresponding immunoblots (Fig. 7, lane 3). The proteins that were eluted from the column with a CHA solution (Fig. 7, lane 2) had strong β-amylase enzyme activity and contained a 57-kD protein that was recognized by RS 5 on immunoblots (Fig. 7, lane 4).

CHA column-purified p57 had the highest β-amylase enzyme activity with an amylopectin solution among nine substrates tested (Table I). The p57 was unable to digest β-limited dextrin or the unboiled starch, properties typical of a β-amylase (Lizotte et al., 1990; Monroe and Preiss, 1990). The optimal pH of p57 activity was 5.6. The whole purification process was repeated and monitored by an enzyme activity assay instead of immunoblots, and a pu-

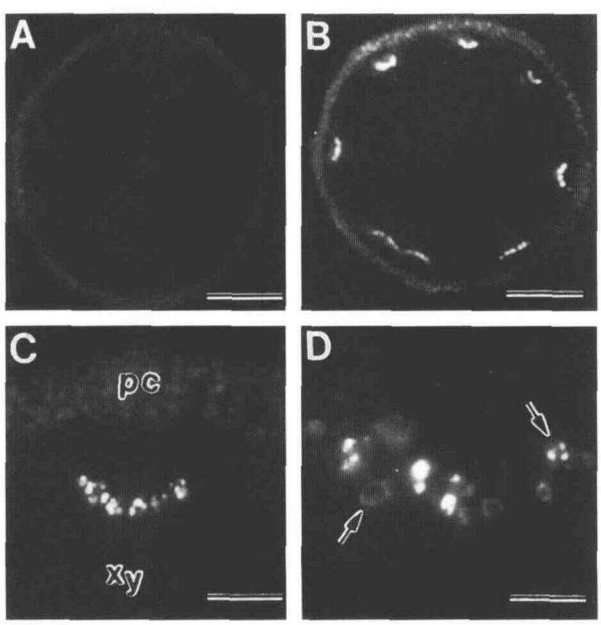

Figure 3. RS 5 recognizes phloem specifically in sections of *A. thaliana* stems. A, Sections incubated in mouse preimmune serum fail to show labeling above the background level. Bar = 0.3 mm. B, Low-magnification image shows that RS 5 labels the phloem tissue. Bar = 0.3 mm. C, Image at higher magnification showing a vascular bundle. The phloem tissue is identified by RS 5. Xylem vessels (xy) and parenchyma cells (pc) have a low level of autofluorescence. Bar = 75 μm. D, Image at higher magnification shows that the RS 5 binding has a "particulate" pattern (arrow). Bar = 10 μm.

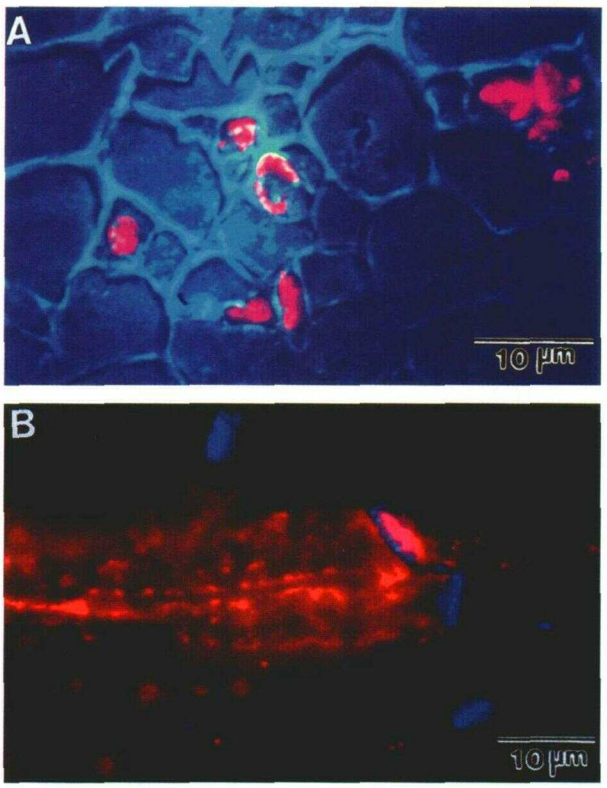

Figure 4. Dual labeling demonstrating that RS 5 does not label a cell wall protein but an antigen that is sieve element specific. A, Composite image shows that RS 5 labels a protein (in pink) in the collapsed protoplast of phloem cells of *S. tortuosus* stems. The cell walls are imaged in blue and are not labeled by RS 5. B, Dual-labeled image of an *A. thaliana* stem in longitudinal section showing that RS 5 specifically recognizes sieve elements with a "particulate" pattern (in red). Aniline blue-stained sieve plates are shown in blue.

N-terminal peptide sequence

YNEKLLLNYVPVYVMLPLGV

Internal peptide sequence

WVRDVGNSDPDIYYTNR
DN

AGHPEWDLPE

DGYRPIAR

Figure 6. The peptide sequences of p57 show identity to the published sequence of β-amylase of *A. thaliana* with the exception of the two amino acids underlined. The differing amino acids from the β-amylase of *A. thaliana* are given in bold.

rification scheme was obtained (Table II). The p57 was purified to homogeneity after 50-fold purification, indicating that p57 is a relatively abundant protein in the *S. tortuosus* phloem (+) cultures that are rich in sieve elements.

The Major Form of the β-Amylase in *A. thaliana* Is a Phloem Enzyme

Rabbit polyclonal antibodies were raised against p57. The polyclonal antibodies were judged to be specific against p57 on immunoblots (Q. Wang, J. Monroe, and R.D. Sjölund, unpublished data). Figure 8 shows a neutralization assay of the β-amylase activity of p57 using the polyclonal antibodies against both p57 and the *A. thaliana* β-amylase (Monroe and Preiss, 1990). The β-amylase activity of p57 was inhibited by both of the polyclonal antibodies used in the assay.

The partial amino acid sequences of p57 and the results of the neutralization assay suggest that p57 and the reported β-amylase of *A. thaliana* are closely related proteins. It is possible that the purified β-amylase of *A. thaliana* actually is the phloem-specific form of β-amylase that is

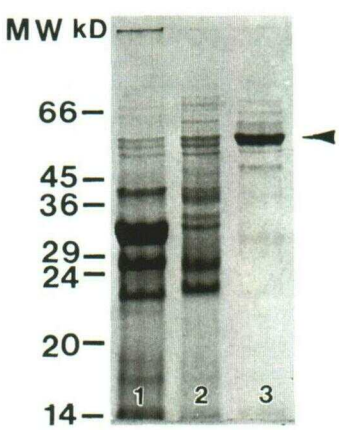

Figure 5. A Coomassie blue-stained SDS gel showing the partial purification of p57 (arrow). Total proteins were extracted from 21-d-old phloem (+) cultures (lane 1), precipitated by 35 to 45% $(NH_4)_2SO_4$ (lane 2), and further separated by a Rotofor cell (lane 3).

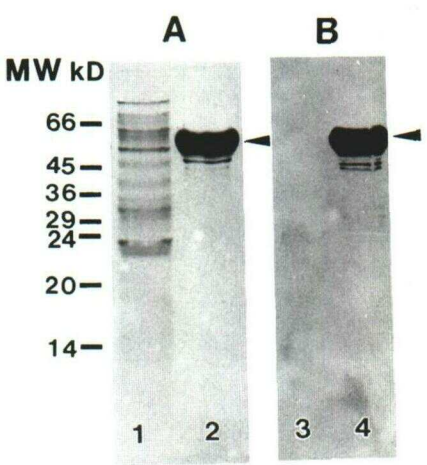

Figure 7. The p57 was further purified using an affinity column. A, The protein retained in the column (lane 2) and the pass-through proteins (lane 1) are shown in a Coomassie blue-stained SDS gel. B, The RS 5 antibody labels the column-retained protein (lane 4) but not the pass-through proteins (lane 3) on the immunoblot.

Table I. *Substrate specificity of p57*

All substrates were dissolved in dd H_2O at a concentration of 10 mg/mL. Assay time was 15 min at 25°C except for unboiled starch, β-limited dextrin and pullulan, which were incubated for 2 h at 25°C. The relative activity was calculated using boiled starch as a control, which was defined as 100.

Substrate	Relative Activity
Starch (unboiled, Sigma S-4251)	0
Starch (boiled, Sigma S-4251)	100
β-limit Dextrin[a]	0
Glycogen type III (rabbit, Sigma G-8876)	86
Glycogen type VIII (limpet, Sigma G-1633)	59
Pullulan (Sigma P-4516)	0
Amylose (corn, Sigma A-7403)	76
Amylose (potato, Sigma A-0512)	62
Amylopectin (potato, Sigma A-8515)	107

[a] Preparation of β-limited dextrin is described in "Materials and Methods."

recognized by the monoclonal antibody RS 5 in the sieve elements of *A. thaliana* using indirect immunofluorescence microscopy (Figs. 3 and 4B). This prediction was tested by further experiments using immunoblots. Figure 9 shows that the polyclonal antibody raised against p57 strongly cross-reacts with the purified β-amylase of *A. thaliana* (Monroe and Preiss, 1990) (Fig. 9, lane 4). The polyclonal antibody raised against the purified β-amylase of *A. thaliana* (Monroe and Preiss, 1990) binds strongly to the purified p57 (Fig. 9, lane 5). More importantly, both purified p57 and the purified β-amylase of *A. thaliana* were recognized by RS 5 on immunoblots (Fig. 9, lanes 1 and 2), suggesting that the phloem-specific epitope is preserved in both proteins. Because limited amounts of the *A. thaliana* β-amylase were available, the amount used was only one-tenth of the amount of the p57 loaded in the gel and the band in lane 2 is weaker than the band in lane 1. The difference between lanes 3 and 4 or between lanes 5 and 6 are not as obvious, because the polyclonal antibodies have much stronger affinity than the RS 5 monoclonal antibody does. The lower bands may represent the degraded proteins of the purified β-amylase, which were not seen on the Coomassie blue-stained gel but were labeled on immunoblots because of the high affinity of the polyclonal antibodies.

Table II. *Purification steps of p57 from S. tortuosus cultures that differentiate sieve elements*

Three hundred sixty grams of 21-d-old callus culture containing sieve elements were used, and the values are the means of three purifications.

Fraction	Total Protein	Total Activity	Specific Activity	Yield	Fold of Purification
	mg	*Units*	*Units/mg*	*%*	
Crude extraction	130	2733	21	100	1
35—45% $(NH_4)_2SO_4$ precipitation	29.1	1625	56	59	2.6
Rotofor (pH 3–10)	0.425	213	501	7.8	23.8
Affinity column (CHA)	0.210	210	1000	7.7	50

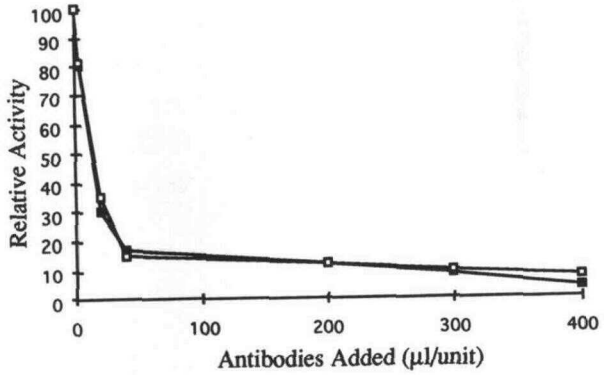

Figure 8. The β-amylase activity of p57 was neutralized both by the polyclonal antibody against p57 (■) and by the polyclonal antibody against the β-amylase of *A. thaliana* (□).

DISCUSSION

Previous localization studies of β-amylase have yielded conflicting results. A vacuolar site for the enzyme was suggested as the result of cell fractionation studies (Beck and Ziegler, 1989). Other studies based on polyclonal antibodies raised against β-amylase (Okamoto and Akazawa, 1979; Hagenimana et al., 1992) have suggested an association with starch grains. Polyclonal antibodies raised against an inhibitor of starch phosphorylase, which was later shown to be β-amylase (Pan et al., 1988), labeled cell walls and starch granules in paraffin sections of sweet potato (Chang and Su, 1986).

The demonstration here that RS 5, a monoclonal antibody selected for its specificity to the phloem, identifies a protein that shares a sequence identity with an *A. thaliana* β-amylase was an unexpected result. Because the *A. thaliana* β-amylase is recognized by RS 5 on immunoblots, this major form of *A. thaliana* β-amylase could actually be the

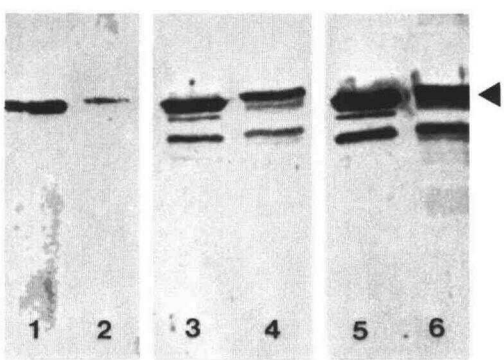

Figure 9. The RS 5 antibody recognizes the *A. thaliana* β-amylase on immunoblots. The purified p57 (lanes 1, 3, and 5, 1 µg/lane) and the purified β-amylase of *A. thaliana* (lanes 2, 4, and 6, 0.1 µg/lane) were probed by RS 5 (lanes 1 and 2), by the polyclonal antibody against p57 (lanes 3 and 4), or by the polyclonal antibody against the β-amylase of *A. thaliana* (lanes 5 and 6). The bands corresponding to p57 are indicated by an arrow.

phloem-specific enzyme as identified by RS 5 using immunofluorescence microscopy, although it was, at first, difficult to reconcile our strict phloem localization with earlier localization studies (Okamoto and Akazawa, 1979; Chang and Su, 1986; Beck and Ziegler, 1989; Hagenimana et al., 1992). One possible explanation for our results is that we used a monoclonal antibody rather than a polyclonal serum raised against β-amylase. In a careful comparison of sweet potato starch phosphorylase and sweet potato β-amylase, several monoclonal antibodies raised against either enzyme were shown to cross-react with both proteins in an ELISA assay (Chern et al., 1990), suggesting that β-amylase shared common epitopes with starch phosphorylase. It may follow, therefore, that polyclonal antibodies raised against β-amylase will also cross-react with starch phosphorylase and label both enzymes in histochemical or fluorescent localization studies. If, however, there are one or more epitopes in β-amylase that are not shared with starch phosphorylase, then it should be possible to raise a monoclonal antibody against a β-amylase-specific epitope that would not cross-react with, or label, starch phosphorylase on blots or in tissue sections. We believe that RS 5 is such a β-amylase-specific antibody. By imposing a very stringent screen for phloem specificity during our production of the monoclonal antibody, we may have screened not only for phloem specificity but also for the specific recognition of an unshared β-amylase epitope.

The presence of a β-amylase in sieve elements provides new information about the location of this enzyme. The role of β-amylase in sieve elements, however, is yet to be determined, partly because the role of β-amylase in any tissue is not yet known. Investigations of the biochemical properties of plant β-amylase have failed to identify the in vivo substrate of the enzyme, but pea epicotyl β-amylase is able to hydrolyze small maltodextrins containing four or more Glc residues (Lizotte et al., 1990). Maltodextrin hydrolysis might have a role in sieve elements to prevent the buildup of highly polymerized polysaccharides. If large starch grains of the kind typically formed in amyloplasts of storage cells were formed in sieve elements from loaded sugars, they would probably impede transport through sieve pores. Evidence of a requirement for preventing starch synthesis can be found in wound phloem development where parenchyma cells with large amyloplasts redifferentiate into sieve elements after the wounding of vascular bundles. The starch in the original parenchyma cell amyloplasts is degraded early in the development of wound phloem (Behnke and Schulz, 1983), and the parenchyma cell amyloplasts are converted into the unique form of sieve element plastids that do not store normal starch. Similar differences between parenchyma and phloem plastids have been reported (Sjölund, 1992) for the *S. tortuosus* callus tissues used in the study of RS 5.

If β-amylase has a role in the prevention of starch buildup during the translocation of sugars in phloem sieve elements, then increasing the rate of sugar loading might be expected to increase the level of β-amylase in sieve elements. It is noteworthy, therefore, that the level of β-amylase present in *A. thaliana* (Caspar et al., 1989; Mita et al., 1995) and sweet potato (Nakamura et al., 1991) has been shown to increase greatly following the incubation of cut leaves in Suc solutions.

The localization of β-amylase in phloem has built a bridge between the field of β-amylase research and the study of phloem. A re-examination of the properties and potential substrates of β-amylase from the perspective of sieve element function may advance our understanding about the physiological role of this enzyme. On the other hand, our knowledge of phloem gene expression may be greatly enhanced by the valuable information that is already available about the regulation of the β-amylase gene (Nakamura et al., 1991; Ishiguro and Nakamura, 1994; Mita et al., 1995).

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