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Distribution and Cellular Localization of Aspartate Aminotransferase Isoenzymes in Rice

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Aspartate aminotransferase (EC 2.6.1.1, AAT) occurs as two forms in rice bran (from *Oryza sativa* cv. Koganemasari), AAT-1 and AAT-2. Immunotitrations with antisera against the AAT isoenzymes indicated an immunological distinction between AAT-1 and AAT-2. Sequential titration of total AAT activity showed that AAT-1 and AAT-2 comparised 82% and 18% of the total activity in the crude extract of rice bran, respectively. AAT-1 and AAT-2 comprised 45% and 55% of the total AAT activity in the crude extract of top tissues of the rice plant. AAT-1 was mainly in the cytosolic fraction of the rice plant cells. On the other hand, AAT-2 was the major isoenzyme of AAT in mitochondria and peroxisomes. Minute activity of AAT-1 was found in the membrane fractions of the mitochondria and peroxisomes and was solubilized with Triton X-100.

Plants contain two or three isoenzymes of aspartate aminotransferase (EC 2.6.1.1, AAT). The localization in intracellular organelles and distribution in plant tissues of these AAT isoenzymes have been studied by many workers, because AAT is important in plants, including transferring of fixed carbon from mesophyll cells to bundle sheath cells.[1] In *Eleusine coracana*[2] and *Panicum maximum* Jacq. var. *trichoglume*,[3] which are C_4 plants and showed three activity bands of AAT, AAT-1, which migrates at the slowest rate in native-PAGE, is primarily located in the cytosol of mesophyll cells, and AAT-3, which migrates at the fastest rate, is in the mitochondria of bundle sheath cells. In the root nodules of alfalfa[4] and lupin,[5] which are C_3 plants and contained two AAT isoenzymes, AAT-1 and AAT-2 have been reported to be the major form of AAT in root and the predominant form in nodules, respectively. AAT-1 in carrot (suspension cultures), which is a C_3 plant and contained three forms of AAT, has been suggested to be a cytosolic enzyme.[6]

Rice plants also contained two AAT isoenzymes: although stems of rice plant showed another activity band between the AAT isoenzymes in the native-PAGE, the enzyme of the activity band have not been identified as AAT. One isoenzyme, which migrates more slowly than the other isoenzyme in the native PAGE, have been suggested to be a mitochondrial isoenzyme, and the other isoenzyme to be a cytosolic one.[7] However, immunological studies on localization of rice AAT isoenzymes have not been done. We have purified two AAT isoenzymes, AAT-1 and AAT-2, to homogeneity from rice bran and prepared rabbit antisera against these AAT isoenzymes. The amino acid compositional relatedness of AAT-1 and AAT-2 among cytosolic and mitochondrial AAT isoenzymes of pig and chicken and preliminary protein sequence analysis suggested that AAT-1 and AAT-2 are cytosolic and mitochondrial isoenzymes, respectively.[20]

Thus, the objectives of these studies were to: (a) evaluate the antigenic relatedness of AAT-1 and AAT-2, (b) identify through immunological techniques the distribution of AAT-1 and AAT-2 in the rice bran and top tissues, and (c) locate these isoenzymes intracellularly.

Materials and Methods

Plant materials. Rice (*Oryza sativa* [L] cv. Koganemasari) seeds were surface-sterilized with 70% ethanol and then 10% hypochlorous acid, and then grown at 25°C for 2 weeks in a sterilized glass bottle containing 0.1% (v/v) Hyponex shielded from light to prevent formation of chloroplasts.

Reagents. Cytochrome *c* (Type III), hydroxypyruvate lithium salt, Percoll, and fatty acid-free bovine serum albumin were purchased from Sigma. Zysorbin was obtained from Zymed Laboratories Inc. Rabbit antisera against AAT-1 and AAT-2 were prepared with the homogeneous preparations of AAT-1 and AAT-2 as described previously.[8] All other materials were obtained from commercial sources and were of reagent grade or better.

Preparation of crude extract. All procedures were done at 0–5°C, unless otherwise described. Rice bran (5 g, dry weight), which had been thoroughly defatted by extraction with diethyl ether, was mixed with 20 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM 2-oxoglutarate, 0.01 mM pyridoxal 5'-phosphate, and 0.01% 2-mercaptoethanol (Buffer S). The mixture was incubated at 37°C for 30 min with agitation, and then an extract was pressed with a piece of double-layered gauze. The extract was centrifuged at 10,000 × *g* for 10 min to obtain a supernatant, which was used as a crude exract of rice bran. Top tissues (5 g, wet weight) of rice plants cut into small pieces were suspended in 10 ml of Buffer S. The suspension was homogenized for 5 min with a Polytron homogenizer operating at 60% of full power. The homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant obtained was used as a crude extract of rice plants. Crude extracts from organelles were prepared as described below.

Preparation of mitochondrial and peroxisomal fractions. Fresh tops (5 g, wet weight) of rice seedlings grown in the dark were washed twice with 50 ml of distilled water and then cut into about 3-mm pieces with a razor. The plant material was suspended in 30 ml of 0.1 M HEPES–KOH buffer (pH 7.5) containing 0.4 M mannitol, 1 mM EDTA, 0.1% bovine serum albumin, and 0.6% polyvinylpyrrolidone. The suspension was homogenized for 30 min with a Polytron homogenizer for 30 s operating at 50% of full power. The homogenate was filtered through a piece of double-layered gauze. Then the filtrate obtained was centrifuged at 1000 × *g* for 10 min. The supernatant obtained was centrifuged again at 10,000 × *g* for 10 min. The precipitate was suspended in 1 ml of Buffer A (20 mM HEPES–KOH buffer, pH 7.5, containing 0.3 M mannitol and 0.1% fatty acid-free bovine serum albumin). The suspension was centrifuged at

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Abbreviations: AAT, aspartate aminotransferase, EC 2.6.1.1.

10,000 × *g* for 10 min to obtain a precipitate. The suspension and centrifugation steps were repeated 5 times to wash the precipitate thoroughly. The precipitate (crude organelles) was finally suspended in 1 ml of Buffer A with a Teflon homogenizer and then centrifuged on a Percoll gradient.

The crude organelle suspension (0.5 ml) was put onto a discontinuous gradient consisting of 1-, 2-, and 1-ml Percoll solutions of 10, 28, and 45% (v/v), respectively; all Percoll solutions contained 20 mM MOPS–KOH buffer (pH 7.2), 0.25 M sucrose, and 0.2% (w/v) fatty acid-free bovine serum albumin. The tubes were centrifuged at 30,000 × *g* for 30 min with an SW 55 Ti rotor (Beckman) and fractionated from the top. The fractions (300 μl each) were homogenized at 40% efficiency for 30 s with a Heat System Ultrasonic W-220 sonic oscillator. The AAT activity and protein concentration of the homogenates were measured after an addition of 0.01 mM (final concentration) pyridoxal 5′-phosphate followed by incubation at 30°C for 30 min.

Preparation of membrane fraction of the organelles. The mitochondrion and peroxisome fractions were washed 3 times with 3 ml of Buffer A by repeating the centrifugation and suspension. The organelle suspensions (0.5 ml, each) finally obtained were homogenized with the sonic oscillator as described above. The homogenates were centrifuged on a Percoll gradient and fractionated as described above.

Solubilization of AAT from the organelle membranes. The membrane fractions of mitochondria and peroxisomes were washed twice with 20 mM MOPS–KOH buffer (pH 7.2) by repeating the centrifugation (10,000 × *g*, 10 min) and suspension. The washed membrane fractions (200 μl, each) suspended in 20 mM MOPS–KOH buffer (pH 7.2) were mixed with an equal volume of 20 mM MOPS–KOH buffer (pH 7.2) containing 0–1.0% (v/v) Triton X-100. The mixtures were incubated at 30°C for 5 min, and then centrifuged at 25,000 × *g* for 5 min. The protein concentration and AAT activity of the supernatant solutions were measured after the addition of 0.01 mM (final concentration) pyridoxal 5′-phosphate followed by incubation at 30°C for 30 min.

Enzyme and protein assay. AAT activity was measured by a coupled malate dehydrogenase method as described previously.[9] The activities of catalase (EC 1.11.1.6) and cytochrome *c* oxidase (EC 1.9.3.1) were measured by the method described by Kamiryo *et al.*[10] Fumarase (EC 4.2.1.2) and hydroxypyruvate reductase (EC 1.1.1.29) were assayed using fumarate[11] and hyroxypyruvate[12] as substrates, respectively. One unit of these enzymes was defined as the amount that catalyzed the formation of 1 μmol of the product from the substrate per min.

Protein concentrations were measured by the method of Lowry *et al.*[13] using bovine serum albumin as the standard. A modified method[14] was also used for protein assay of the samples containing Triton X-100.

Ouchterlony double diffusion test. The immunodiffusion test was done by th method of Ouchterlony.[15] After precipitin bands were formed, the agar plate was washed thoroughly with 0.9% NaCl and then stained with 0.5% Amide Black solution dissolved in 7% acetic acid. The agar plate was destained with 7% acetic acid.

Immunotitration of AAT activity. The crude extracts (50 μl, each) from rice bran and the top tissues, and mitochondrial and peroxisome and the mixtures of anti-AAT-1 antiserum (50 μl) and 0–100 μl of anti-AAT-2 antiserum. The volume of all the reaction mixtures was adjusted to 200 μl with 0.85% NaCl. The anti-AAT-1 and -AAT-2 sera obtained from rabbits had been diluted 2.5- and 5-fold, respectively with 0.85% NaCl to make the working antiserum solutions. The control reaction mixture contained 50 μl of the crude extract, 50 μl of 2.5-fold diluted preimmune serum, and 100 μl of 5-fold diluted preimmune serum. After an incubation at 37°C for 60 min, 20 μl of Zysorbin (18 mg/ml) was added, and then the mixtures were incubated at 37°C for another 10 min. The mixtures were centrifuged at 25,000 × *g* for 5 min to remove antigen-antibody precipitates. AAT activity in the supernatants obtained was measured.

Immunotitration of purified AAT-1 and AAT-2 isoenzymes was done with constant quantities of activity of the purified enzymes and varying quantities of anti-AAT-1 and anti-AAT-2 antisera. To 20 μl (about 0.03 unit, each) of AAT-1 and AAT-2 mixture was added 0–50 μl of the antisera. The volume of the mixture was adjusted to 90 μl with 0.85% NaCl. After an incubation at 37°C for 1 h, 10 μl of Zysorbin (18 mg/ml) was added. The reaction mixture was incubated for another 10 min, and then centrifuged at 25,000 × *g* for 5 min. The AAT activity of the supernatant was assayed.

Results

Antigenic relatedness of AAT-1 and AAT-2

The Ouchterlony double diffusion test showed that antibodies to AAT-1 recognize AAT-1 but not AAT-2 and antibodies to AAT-2 recognize AAT-2 but not AAT-1 (Fig. 1). Even more conclusive evidence for the lack of similarity between the two isoenzymes of AAT was demonstrated by the immunotitration of purified AAT-1 and AAT-2 activities with anti-AAT-1 and anti-AAT-2 antisera (Fig. 2). Antiserum against AAT-1 immunoprecipitated 100% of the AAT-1 activity, while none of AAT-2 activity was immunoprecipitated by anti-AAT-1 antiserum. Addition of 50 μl of anti-AAT-2 antiserum removed 100% of the purified AAT-2 activity. Little of an equal amount of AAT-1 activity was removed by the same serum treatment. Addition of 50 μl of preimmune serum removed neither AAT-1 activity nor AAT-2 activity. Thus, the two antisera could effectively discriminate between the AATs in rice bran.

Then, the antisera were used to evaluate the relative contribution of AAT-1 and AAT-2 to the total AAT activity found in the crude extracts from the rice bran (Fig. 3) and top tissues of rice plant (Fig. 4). Because the crude extracts were prepared without prevention of disruption of organelles, the crude extracts should contain organelle AAT as well as cytosolic AAT. Addition of anti-AAT-1 antiserum to the brain extracts resulted in a 82% loss in activity, while

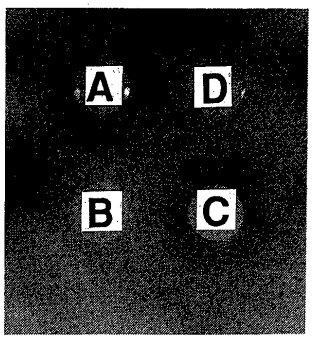

Fig. 1. Ouchterlony Double Diffusion Test of Antisera against Rice Aspartate Aminotransferase Isoenzymes.

The wells, A and C, contained AAT-1 (15 μg) and AAT-2 (13 μg), respectively. The wells, B and D, contained the undiluted antisera against AAT-1 and AAT-2, respectively.

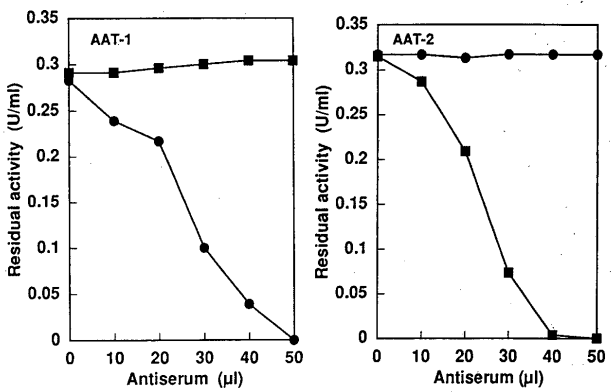

Fig. 2. Immunotitration of Purified AAT Isoenzymes from Rice Bran Using Antisera against the AAT Isoenzymes.

Anti-AAT-1 (●) and anti-AAT-2 (■) antisera were added to the solutions containing the purified AAT-1 (left column) and AAT-2 (right) isoenzymes. After the immune complexes were precipitated in the presence of Zysorbin, AAT activity remaining in the supernatant was measured.

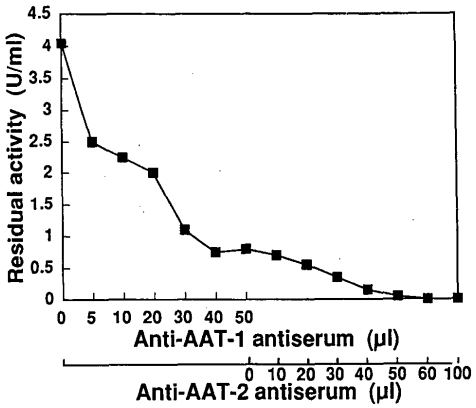

Fig. 3. Immunotitration of AAT Activity in the Crude Extract of Rice Bran.

The crude extracts (50 μl, each) were mixed with the antisera and incubated at 37°C for 1 h. After the immunoprecipitates were removed, AAT activity in the supernatant was measured.

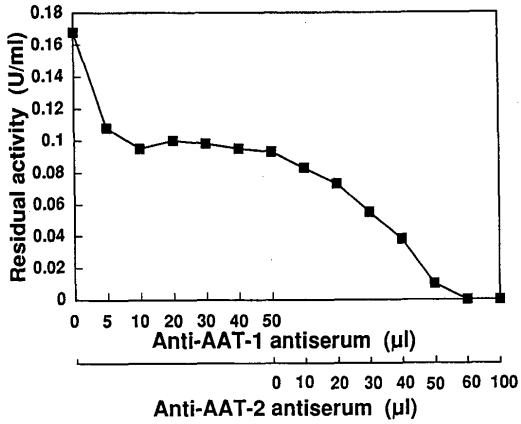

Fig. 4. Immunotitration of AAT Activity in the Crude Extract of Top Tissues of Rice Plant.

The crude extracts (50 μl, each) were mixed with the antisera.

addition of anti-AAT-2 antiserum resulted in a 18% loss in activity. These titrations account for 100% of the AAT activity in the crude extract of rice bran. Titration of the crude extracts from the top tissues with anti-AAT-1 and anti-AAT-2 antisera resulted in a 45% and 55% loss in activity, respectively. These titrations account for 100% of the AAT activity in the crude extract. The result also showed that AATs in rice bran have the same antigenicity as those in rice plant and that anti-AAT-1 and anti-AAT-2 antisera can be used to immunoprecipitate cellular AATs in rice plants.

Localization of AAT isoenzymes in rice organelles

Cell fractionation experiments were done to investigate the subcellular localization of AAT-1 and AAT-2 in rice organelles. We have established the conditions of Percoll density gradient centrifugation that can separate mitochondrial and peroxisomal fractions of rice tissue (Fig. 5). The mitochondrial fraction, which showed high activities of cytochrome *c* oxidase and fumarase, was sedimented at the boundary of 10% and 28% Percoll. The peroxisomal fraction, which showed high activities of catalase and hydroxypyruvate reductase, was sedimented at the boundary of 28% and 45% Percoll. AAT activity was found in both mitochondrial and peroxisomal fractions, suggesting that either AAT-1 or AAT-2 is an organelle-specific isoenzyme.

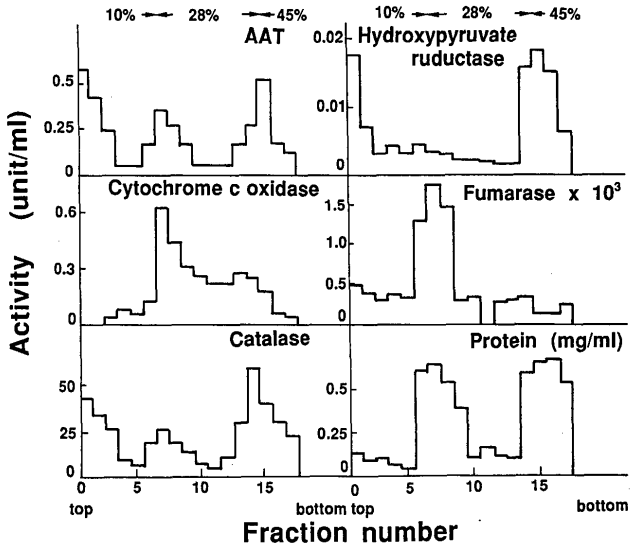

Fig. 5. Preparation of Mitochondrial and Peroxisomal Fractions of Rice Plant Top Tissues by Percoll-density Gradient Centrifugation.

The gradient was centrifuged at 30,000 rpm for 30 min at 4°C with a Beckman 55 Ti rotor. About 300-μl fractions were collected from the top of the gradient.

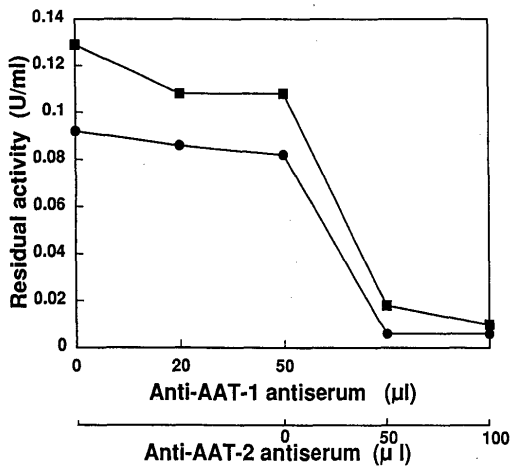

Fig. 6. Immunotitration of AAT Activity in the Mitochondrial and Peroxisomal Fractions of Rice Tissues.

■, mitochondrial fraction; ●, peroxisomal fraction.

Crude extracts from the mitochondrial and peroxisomal fractions were immunotitrated with the anti-AAT-1 and anti-AAT-2 antiserum to evaluate the relative contribution of AAT-1 and AAT-2 to the total AAT activity (Fig. 6). Addition of anti-AAT-1 serum to the mitochondrial extracts resulted in a 15% loss in AAT activity while addition of anti-AAT-2 serum resulted in a 77% loss in AAT activity. Those titrations account for 92% of the AAT activity in the mitochondrial extract. Titration of the crude extract from the peroxisomal fraction with anti-AAT-1 and anti-AAT-2 antisera resulted in a 11% and 82% loss in activity, respectively. These results showed that AAT-2 is a mjaor form of AAT in mitochondria and peroxisomes of rice tissues.

Membrane associated form of AAT

AAT-1 was a minor form of AAT in mitochondria and peroxisomes of rice tissues. The membrane fractions were prepared from these organelles to examine the possibility that AAT-1 may associate with or bind to membranes of these organelles. The membrane fractions were precipitated on the bottom of centrifugal tubes by the Percoll density

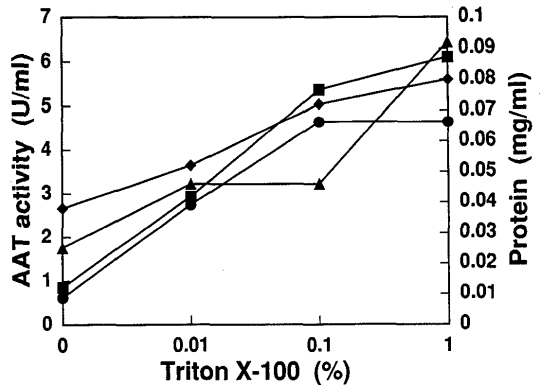

Fig. 7. Solubilization of AAT Activity and Protein from Membrane Fractions of Rice Mitochondria and Peroxisomes.

AAT activity released from the membranes of mitochondria (■) and peroxisomes (●) was measured. Protein released from membranes of mitochondria (▲) and peroxisomes (◆) was measured by the modified Lowry procedure.

gradient centrifugation. The membrane fractions were treated with various concentrations of Triton X-100 (Fig. 7). AAT activity and proteins were solubilized almost in parallel with the concentration of the detergent. The solubilized AAT was identified as AAT-1 by native PAGE and immunotitration.

Discussion

Our data demonstrated that AAT-1 and AAT-2 isoenzymes are immunologically distinct. AAT-2 was the major isoenzyme of AAT in the organelles. The results coincided well with the enzymatic properties of AAT-2 that suggest AAT-2 is a mitochondrial isoenzyme.[20] Since peroxisomes also contained AAT-2, it may be preferable to designate AAT-2 as an organelle isoenzyme. Although part of AAT-1 bound to membranes of the organelles, AAT-1 was mainly localized in the cytosolic fraction. Thus, AAT-1 may be designated as a cytosolic isoenzyme.

Rice AAT-1, a putative cytosolic isoenzyme, had a higher p*I* value and slower migration rate in the native PAGE than those of rice AAT-2, a putative organelle isoenzyme. In other plants, one AAT isoenzyme, which is a slow-migrating isoenzyme in the native PAGE and corresponds to the rice AAT-1, has been shown to be a cytosolic isoenzyme, and the other, which is a fast-migrating isoenzyme and corresponds to the rice AAT-2, to be a mitochondrial or plastid isoenzyme.[1,3,16] On the contrary, all cytosolic AATs of animals and a yeast so far studied migrate faster than mitochondrial ones, showing that the mitochondrial isoenzymes are more basic (having higher p*I*) than cytosolic ones. Hartmann *et al.*[17] have pointed out that p*I* of most of animal mitochondrial isoprotein is higher than that of its cytosolic counterpart. The difference in p*I* relationship of isoenzymes between animals and plants may correlate with the difference in function of animal and plant mitochondria. Further studies on other plant enzymes that have mitochondrial and cytosolic isoenzymes are required to generalize the difference in the p*I* relationship of isoenzymes between animals and plants.

AAT-2 in the mitochondria and peroxisomes could not be differentiated from each other by the immunotitration and the native PAGE. However, it is possible that the AAT-2 in these organelles are not identical in primary structures, and that the AAT-2 consists of two or more isoenzymes of AAT. Serine : pyruvate aminotransferase (EC 2.6.1.51, SPT) is also found in mitochondria and peroxisomes of rat liver cells. The primary structures of mitochondrial and peroxisomal SPTs are almost identical. The only difference detected between them is that the N-terminal amino acid of peroxisomal SPT is blocked while that of mitochondrial one is methionine. The different organelle distribution arises from transcription from different initiation sites: the mitochondrial SPT is synthesized as a protein having mitochondrial targeting N-terminal extension peptide.[18] Further studies, including cloning and sequencing analysis of cDNA encoding AAT isoenzymes of rice plants are required to identify AAT-2 in the rice organelles. Our antibody against AAT-2 should be useful in these studies.

A part of AAT-1 activity was on the organelle membranes. Treatment with detergent was necessary to remove AAT-1 activity from the membranes, suggesting that AAT-1 is buried in the membranes. These results account for the previous results that the mitochondrial fraction showed AAT-1 and AAT-2 activities.[7] It has been shown that mitochondrial AAT of animals binds to inner mitochondrial membranes, and inner mitochondrial membrane contains a binding protein that binds to mitochondrial AAT.[19] Since AAT-1 of rice is a cytosolic enzyme, the binding site of AAT-1 may be outer side of the organelles. However, our methods are not sufficient to conclusively demonstrate the membrane localization of AAT-1. Access to AAT-1 and AAT-2 antibodies for *in situ* staining should allow for more precise and accurate localization of the AAT isoenzymes of rice.

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