Plastid Targeting of Rice $\alpha$-Amylase in Living Cells

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Introduction

Starch is the most remarkable and important storage material for energy and carbon source in plants. It consists of two simple glucan polymers, amylose and amylopectin, organized to form crystalline, insoluble granules with an internal lamellar structure. This granular structure is relevant when considering the mechanism of starch degradation, because many glucan-metabolizing enzymes appear to be unable to act on intact granules as a substrate. Most information on the structure of starch comes from studies of the starch-storing organs of crop species, but recent work has shown that leaf starch is similar to storage starches in many respects.

$\alpha$-Amylases (EC 3.2.1.1) in cereal are polymorphic enzymes. Ten distinct $\alpha$-amylase genes have been cloned and sequenced, and more than 20 native $\alpha$-amylase isoforms have been identified and characterized in rice (Table 1). As shown in Fig. 1, all the deduced amino acid sequences of rice $\alpha$-amylases contain signal sequences characteristic for ER translocation. The function of $\alpha$-amylase has been studied extensively in the endosperm of germinating cereal seed. It is generally accepted that $\alpha$-amylase is known to be the only enzyme that can digest intact starch granules and plays the major role for the mobilization of reserved starch in the endosperm of germinating seeds, although the contribution of each isoform in details is still obscure. In the germinating seeds, $\alpha$-amylases are secreted from the scutellar epithelium and the aleurone layer to the starchy endosperm, where these directly bind and degrade the starch granules in the dead cell amyloplasts (Fig. 2). Thus, $\alpha$-amylase is one of the best-characterized secretory enzymes in plant.

However, $\alpha$-amylase was also detected in none secretory tissues, such as shoot, leaf, and developing endosperm cells. What the function of $\alpha$-amylase in living cells is? This is our most interesting subject.

Characterization of Rice Plants with Suppressed Expression and Overexpression of $\alpha$-Amylase

Our interest is to determine the involvement of $\alpha$-amylase in starch degradation in rice living cell plastids. We attempted to generate a series of transgenic rice plants by Agrobacterium-mediated transformation with $\alpha$-amylase I-1 (AmyI-1) and II-4 (AmyII-4) cDNA under control of Cauliflower mosaic virus (CaMV) 35S promoter.

We obtained transgenic rice lines with suppressed expression of $\alpha$-amylase I-1. The 15-2 line contained 5 copies of the transgene and showed that the expression of $\alpha$-amylase I-1 was almost perfectly suppressed at both mRNA and protein levels (Fig. 3A). The seeds of the 15-2 line were subjected to determine the ability of seed germination and seedling growth. As shown in Fig. 3B and C, the germination and shoot elongation of this line markedly delayed in comparison with those of wild-type seeds. The germination times of 15-2 line and wild-type were calculated to be 3.3 and 2.1 days, respectively, when seeds with a minimum coleoptile length of 3 mm or a radicle length of 3 mm were defined as germinated seeds. The slow germination and seedling growth in the 15-2 line seem to be due to the reduction of carbon source supply by the amylolytic breakdown of reserve starch in the endosperm. Therefore, we examined the ability of seed germination under a sugar-supplemented condition. Indeed, the rate of germination and seedling growth of the 15-2 line with suppressed expression of $\alpha$-amylase I-1 was normalized under a sugar-supplemented condition. Thus, the phenotype of 15-2 line is resulted from the inactivation of $\alpha$-amylase I-1 expression.
We also produced and characterized rice plants overexpressing α-amylase I-1 and II-4. In both α-amylase I-1 and II-4 overexpression lines, A3-1 and D1-4, amylase activities were markedly increased in leaves of transgenic plants (Fig. 4A, B). When the starch accumulation in leaves harvested immediately after sunset was measured, the plants overexpressing α-amylases, particularly α-amylase I-1, exhibited low starch contents of the leaves, which was 33% of that in the wild-type plants (Fig. 4D). These results indicated that α-amylase I-1 is actually involved in the starch degradation in rice leaves.

We further examined the phenotype of rice grains overexpressing alpha-amylase. The α-amylase activities were markedly higher in ripening seeds of A3-1 and D1-4 than in the wild-type (Fig. 5). In particular, the level of α-amylase activity was much higher in the D1-4 line overexpressing α-amylase II-4 than in the A3-1 line during an early stage of ripening. Furthermore, the tissue growth of ripening seeds of transgenic rice was significantly delayed in comparison with the wild type. We then analyzed the phenotypic characteristics of mature seeds of the A3-1 and D1-4 lines. The dry weight of mature seeds of A3-1 and D1-4 was reduced 4% in A3-1 and 11% in D1-4 compared to the wild-type. The quality of seeds of A3-1 and D1-4 was evaluated by reflection (Fig. 6A) and transmission (Fig. 6B) views. Normal good grains showed clear and crystalline view. Dominant morphological phenotypes of abnormal seeds of A3-1 and D1-4 were white core and whole opaque white, respectively. Collectively, these results indicated that overexpression of α-amylase activity inhibited the accumulation of reserve starch and lowered the grain quality of rice.

Moreover, it was observed that the phenotypes of transgenic rice grains were similar to the white immature grains caused by high temperature stress during the period of ripening. Actually, the high temperature stress induced α-amylase activities in ripening seeds, so that we infer expression of α-amylase affect the accumulation of reserve starch and the grain quality of rice.

### Plastid Targeting Mechanism of Rice α-Amylase

In plant cells, the biosynthesis and degradation of starch occur in the plastids, those are chloroplasts in green leaves and amyloplasts in storage tissues. We had several lines of evidences to prove that the rice predominant α-amylase isoform I-1 is localized within the plastids in rice living leaf cells, though it is a well-known secretory glycoprotein. How does α-amylase target into the plastids in living cells? To clarify the targeting pathway of α-amylase into plastids, we analyzed the targeting of Amyl-1-GFP fusion proteins in onion epidermal cells. We examined the simultaneous expression of AmylI-1-GFP and plastid marker WxTP (Wx transit peptide)-DsRED in the onion cells. Large portion of AmylI-1-GFP was matched to the distribution of WxTP-DsRED, indicating that α-amylase I-1 can target to the plastids, and that the enzyme molecule contains a plastid targeting signal common to both rice and onion cells.

Sar1 and Arf1 GTPases have been shown to be necessary for the protein traffic between the ER and Golgi complex in plant cell. Expression of dominant mutants of AtArf1 and AtSar1 inhibits ER-to-Golgi traffic, therefore Golgi-resident proteins and secretory and vacuolar proteins are retained in ER network structure. We tested the effects of dominant mutants on expression and targeting of Golgi and plastid markers in onion epidermal cells, and confirmed that expression of dominant mutants of AtArf1 and AtSar1 arrest the traffic of ER-to-Golgi, but the translocation of WxTP-DsRED from cytosol to plastid is not prevented by these mutants. To determine the involvement of ER-to-Golgi traffic in the plastid targeting of α-amylase I-1, triple simultaneous expression of Amyl-1-GFP, WxTP-DsRED, and dominant mutants of AtSar1 and AtArf1, was performed. In the both mutants, punctate structures in addition to ER network were also green-fluorescent, which probably represented ER exit sites and never overlapped with plastids. These perturbation experiments using dominant mutants of AtArf1 and AtSar1 clearly indicated that the traffic of ER-to-Golgi is necessary for the targeting of α-amylase I-1 to the plastid.

Next question is “Is there a pathway from the Golgi to the plastids?” When simultaneous expression of the plastid WxTP-GFP and trans-Golgi ST-mRFP markers were carried out in the
onion epidermal cell overexpressing α-amylase I-1, many spots of the trans-Golgi marker becomes coincided with the fluorescence of the plastid maker. We obtained further evidence that there exists a communication between the Golgi bodies and the plastids by employing live imaging with ST-mRFP and WxTP-GFP. Based on all these information, we proposed a hypothetical targeting mechanism of α-amylase I-1 into plastid (Fig. 7). When an order of supplying alpha-amylase into the plastids were called, special Golgi bodies were formed, and moved to near around plastids, then contacted with the plastids. The merge of plastid and trans-Golgi markers as observed in live imaging suggests that fusion events between these two organelles might take place, but vesicular mechanism is not ruled out. One other puzzling problem is the topology of the targeted space. α-Amylase I-1 appears to find its final destination in the plastid stroma, where it degrades starch. However, membrane fusion between the Golgi or Golgi-derived vesicles and the outer envelope of plastids would result in the localization in the intermembrane space between the outer and inner envelopes. Another mechanism to enable stromal targeting remains to be elucidated.

Conclusion

- Analyses of transgenic rice plants with suppressed expression and/or overexpression of α-amylase I-1 revealed that α-amylase I-1 is involved in the starch breakdown in living cells.
- Electron microscopic studies, the expression and targeting of α-amylase I-1 fused with GFP in transgenic rice green cells, and cell fractionation studies showed that the glycoprotein α-amylase I-1 occurs in plastids.
- Studies of transient expression of Amyl-1-GFP in onion epidermal cells indicated that α-amylase I-1 is transported to the plastids via the secretory pathway.
- The starch binding region of α-amylase I-1 was concluded to be essential for its plastid targeting.
- There exists a communication between the Golgi apparatus and the plastid in higher plant cells.

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Beamer-Presentation shown at the meeting.

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