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Expression of α-amylases, carbohydrate metabolism, and autophagy in cultured rice cells is coordinately regulated by sugar nutrient

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Summary

A rice suspension cell culture system has been established to study how sugar depletion regulates α-amylase expression, carbohydrate metabolism, and other physiological and cellular changes. It is shown here that a group of 44 kDa α-amylases are constitutively expressed whether or not the cells are starved of sucrose. However, expression of a new group of α-amylases of 46 kDa is dramatically induced when cells are starved of sucrose. Cellular sugar and starch were rapidly consumed and metabolic activity was decreased in the starved cells. Extensive autophagy also occurred in the starved cells, which caused an increase in vacuolar volume and degradation of cytoplasmic constituents including amyloplasts. Immunocytochemical studies revealed that α-amylases are localized in starch granules within amyloplasts, in cell walls, and in some of the vacuoles. The presence of putative signal sequences in the N-termini of nine rice α-amylases suggests hitherto unidentified pathways for import of α-amylases into amyloplasts. The studies show that differential α-amylase expression, carbohydrate metabolism, metabolic activity, and vacuolar autophagy are coordinately regulated by the sugar level in the medium. As the starved suspension cells exhibit some sugar-regulated characteristics of α-amylase expression in germinating rice embryos as well as physiological changes similar to those in senescing cells, this system represents an ideal tool for studying cellular, biochemical, and molecular biological aspects of α-amylase gene regulation, carbohydrate metabolism, senescence, and protein targeting in plants.

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Introduction

Two types of starch, the most important storage carbohydrate in plants, can be distinguished: transitory (assimilatory) starch which is located in chloroplasts, and reserve starch which is deposited in amyloplasts. Research into starch metabolism has focused mostly on the nature and regulation of biochemical reactions involved (Beck and Ziegler, 1989; Steup, 1988). The cellular and physiological aspects of starch metabolism have been less well investigated. Although there is physiological evidence that starch degradation is regulated (Beck and Ziegler, 1989; Steup, 1988), the pathway of starch breakdown and the regulatory mechanisms are largely unknown.

Gene regulation and biosynthesis of starch-degrading enzymes in seeds, in particular the hormonal induction of α-amylase gene expression and the secretion of the enzymes in germinating cereal grains, has been extensively investigated (Fincher, 1989). In this system, α-amylases are secreted by aleurone cells into the starchy endosperm where they degrade the starch grains. Because at this stage the cells of the starchy endosperm are already dead and have lost their ultrastructural integrity, no studies have been made of the subcellular aspects of starch degradation. Starch is also stored in leaves, stems, and roots. The levels of starch fluctuate, indicating control mechanisms that may operate via the regulation of gene expression, the accumulation of starch-degrading enzymes, or the subcellular distribution of existing enzymes.

We previously reported that expression of the α-amylase gene family in rice suspension cells is suppressed by sugar present in the culture medium and induced by its absence (Yu *et al.*, 1991, 1992). Both transcriptional and post-transcriptional control mechanisms are found to be important in the metabolic regulation of α-amylase gene expression in the suspension cells (Sheu *et al.*, 1994). Sugars have also been found to suppress the expression of α-amylase genes in germinating embryos of rice seeds (Karrer and Rodriguez, 1992). We are interested in understanding the control mechanism of α-amylase expression and starch degradation in cereals. As a first step toward this end, we investigated the physiological and cellular aspects of amylase expression and starch metabolism in cultured rice cells because the sugar level can be controlled easily by manipulating the culture medium. In this report, we demonstrate that sucrose starvation leads to an increase in specific α-amylases, a decrease in starch, and

an increase in vacuolar autophagy. By using immunocytochemistry and peptide sequence analysis, we suggest that α-amylases are targeted to amyloplasts, probably through a novel translocation pathway.

Results

Expression of new species of α-amylases depends on levels of sucrose in medium

α-Amylases that had accumulated in cells grown in sucrose-containing and sucrose-free medium were analyzed by SDS–PAGE and Western blot analysis. When cells were grown in sucrose-containing medium, only the 44 kDa α-amylases were found in the cellular protein extracts (Figure 1, lane 1). However, after cells had been transferred to sucrose-free medium for 1 day, in addition to the 44 kDa α-amylases, accumulation of a new group of α-amylases of 46 kDa was also observed (Figure 1, lanes 2). If the starved cells were shifted back to sucrose-containing medium, accumulation of the 46 kDa α-amylases decreased significantly after 1 day (Figure 1, lane 3) and only the 44 kDa α-amylases were present in the cellular protein extracts after 2 days (Figure 1, lane 4). These results show that expression of a new group of α-amylases appeared after cells had been starved of sucrose, and that accumulation of these α-amylases disappeared after cells had been replenished with sucrose.

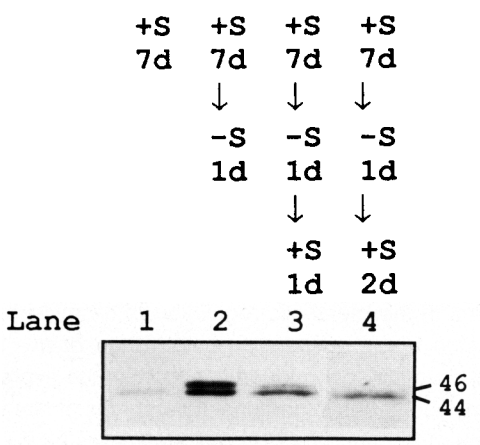

Figure 1. Effect of sucrose on the accumulation of cellular α-amylases.
Cells were grown in sucrose-containing medium for 7 days, then washed and resuspended in sucrose-free medium for 1 day, and shifted back to sucrose-containing medium for another 2 days. Cells were collected at various times and proteins were extracted. Ninety micrograms of total proteins were loaded in each lane and α-amylases were detected by Western blot analysis using anti-rice α-amylase antibodies. 'd' indicates days in sucrose-containing (+S) or sucrose-free (–S) medium. The molecular masses of α-amylases are presented in kDa.

Starved cells rapidly consume cellular sugars and starch

To determine the status of carbohydrate in rice cells cultured under the two regimes, we analyzed their sugar and starch contents. Decreases of 10% and 20% in starch and sugar levels, respectively, were found 1 day after cells had been transferred to fresh sucrose-containing medium, but the levels increased gradually thereafter (Figure 2). In contrast, in cells grown in sucrose-free medium, the levels of both starch and sugar decreased rapidly, especially during the first 3 days (Figure 2). Only about 30% and 10% of the original levels of starch and sugars, respectively, remained in cells 3–4 days after starvation. These results indicate that the cells rapidly consumed most of their reserve starch and sugars during sucrose starvation.

Threshold of starvation period for survival

To determine whether cells could recover from starvation, cells were starved of sucrose for various times and then transferred to medium containing sucrose. Growth curves (Figure 3a) show that control cells that had not been starved grew fastest. Starvation of cells for 1, 2 and 3 days resulted in a slight lag in growth and a somewhat decreased growth rate after the cells had been shifted back to the medium containing sucrose. Starvation of cells for 4 days significantly prolonged the lag period of growth, which was resumed 3 days later. Cells starved for 5 and 6 days could not be rescued.

The capacity of cells to reduce triphenyl tetrazolium chloride (TTC) has been used to measure cellular

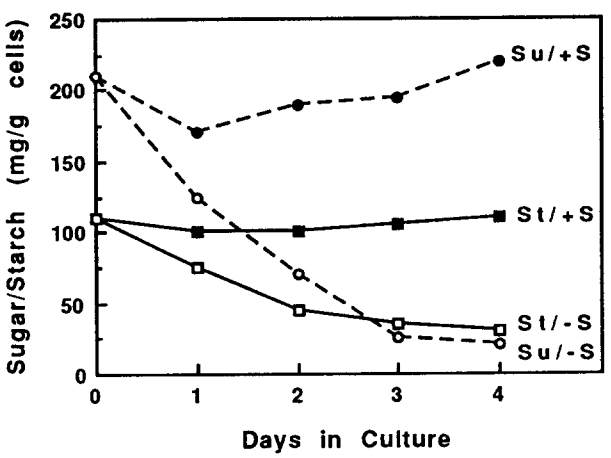

Figure 2. Effect of sucrose starvation on the cellular starch and sugar levels.
Cells were grown in sucrose-containing medium for 7 days, then transferred to sucrose-containing or sucrose-free medium, and incubated for various times. Cells were collected at various times and starch and soluble sugar contents were determined as described in Experimental procedures. The experiment was done in triplicate. Su (dash line) and St (solid line) indicate sugar and starch levels, respectively. +S and –S indicate presence or absence of sucrose in medium, respectively.

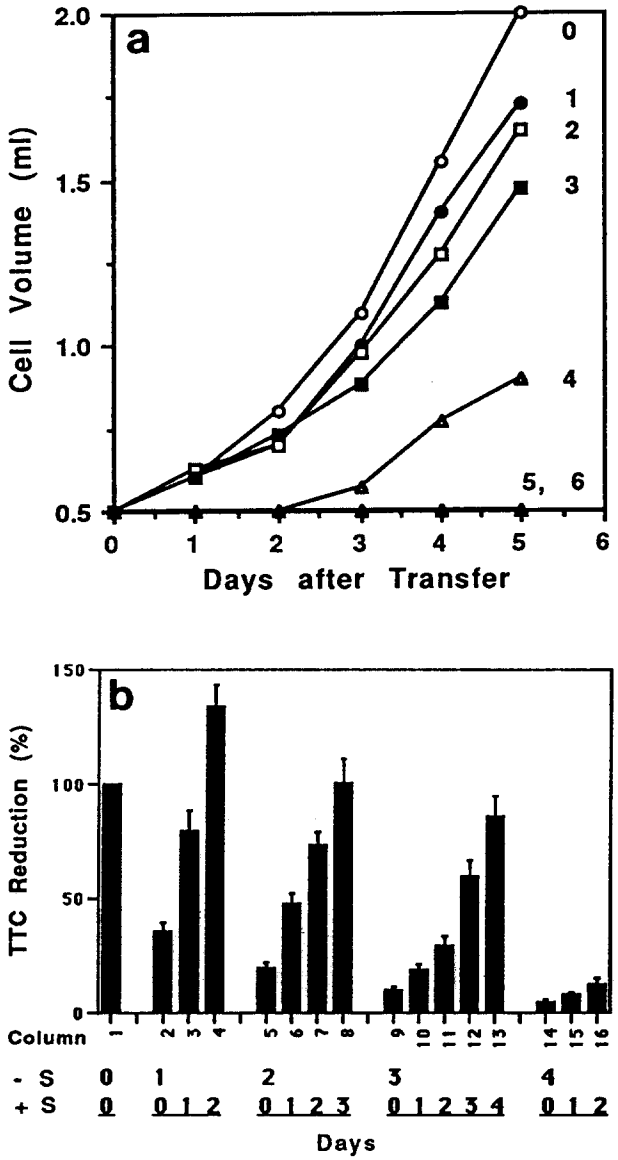

Figure 3. Effect of starvation period on the survival and metabolic activity of cells.

Cells grown in sucrose-containing medium for 7 days were transferred to sucrose-free medium and incubated for various times. A 0.5 ml volume of cells was then transferred to 25 ml sucrose-containing medium and growth curves and capacity for TTC reduction were determined.

(a) Growth curves of cells were determined by measuring the volume of cells on each day for a 5 day growth period. Cells collected on each day were placed in a 5 ml graduated tube and medium was added to a final volume of 2 ml. The volume of cells was determined by subtracting the volume of medium required to fill up the 2 ml cell suspension. The experiment was done in triplicate. Number associated with each curve indicates days of incubation in sucrose-free medium prior to transfer to sucrose-containing medium.

(b) Histograms showing the reduction of TTC (as absorbance at 530 nm in 100 mg fresh weight of cells) by rice suspension cells. The experiment was done in triplicate and data are presented as mean values plus standard errors of the mean. TTC reduction in cells which have not been starved is expressed as 100%. –S and +S indicate that cells were first incubated in sucrose-free medium for 0–4 days and then in sucrose-containing medium for various days (underlined).

metabolic activity (Benson *et al.*, 1992; Steponkus and Lanphear, 1967). The assay depends upon the reduction of tetrazolium by cellular dehydrogenases; the reduced dye product is measured spectroscopically. Cells starved for 1–4 days (Figure 3b, columns 2, 5, 9 and 14) had lower TTC reduction capacities compared with control non-starved cells (Figure 3b, column 1). The decrease in TTC reduction capacity correlated with increased length of starvation period. After cells had been shifted back to sucrose-containing medium, the capacity for TTC reduction resumed most rapidly in cells starved for 1 day (Figure 3b, columns 3 and 4), slightly more slowly in cells starved for 2 days (Figure 3b, columns 6–8) and 3 days (Figure 3b, columns 10–13) and most slowly in cells starved for 4 days (Figure 3b, columns 15 and 16). This result is consistent with the curve for cell growth (Figure 3a) showing that cells can survive sucrose starvation for up to 4 days.

Ultrastructural changes in sucrose-starved cells

To determine how sucrose starvation affects the subcellular organization of cells, we examined their ultrastructure. Cells provided with sucrose contained many amyloplasts/starch grains and other organelles including mitochondria, Golgi apparatus, endoplasmic reticulum (ER), and small vacuoles (Figure 4a and b). In cells starved of sucrose for 2 days, the vacuolar volume became extremely large and in most cells the cytoplasm and organelles were confined to a narrow area adjacent to the cell walls (Figure 4c). Most amyloplasts/starch grains and other organelles disappeared from the starved cells (Figure 4d). Each section of a normal cell examined with the electron microscope (EM) usually contained at least two to four amyloplasts/starch grains. However, most starved cells did not contain amyloplasts/starch grains. This result is consistent with the loss of starch from the starved cells as shown in Figure 2.

Since the amyloplasts containing starch granules disappeared and vacuolar volume increased during starvation, we investigated when and how vacuolar volume increased and whether amyloplasts were degraded within vacuoles during starvation. Sections of cells starved for various times were prepared and examined. Increase in vacuolar volume began 6–12 h after starvation and starch granules appeared in the vacuoles at approximately the same time. Starch granules were usually closely packed within amyloplasts in the cytoplasm (Figures 4a, b and 5c). However, they became separated after being present in vacuoles and the amyloplast membranes were difficult to identify (some representative micrographs are shown in Figure 5). These starch granules were sometimes slightly separated (Figure 5a), or quite far apart (Figure 5b), or dis-

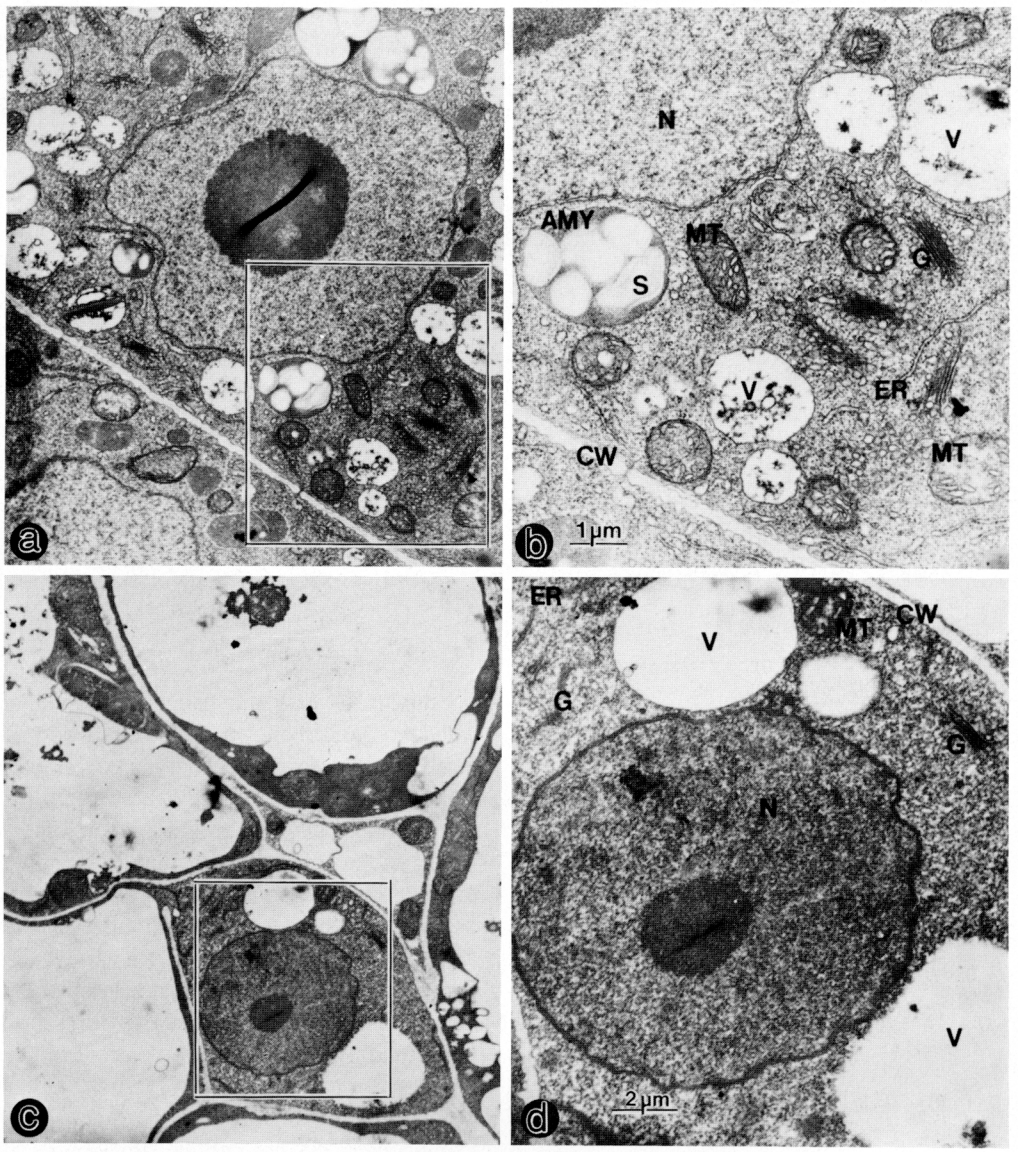

Figure 4. Electron micrographs of rice cells grown in sucrose-containing or sucrose-free medium.
Cells grown in sucrose-containing medium for 7 days were transferred to sucrose-containing or sucrose-free medium and incubated for 2 days. Cells were collected, fixed, sectioned and examined as described in Experimental procedures.
(a) Presence of amyloplasts and other organelles in cells provided with sucrose.
(b) Higher magnification of the boxed area in (a) reveals the morphology of organelles.
(c) Sucrose-starved cells containing large vacuoles and localized cytoplasm.
(d) Higher magnification of one cell in boxed area in (c) reveals cytoplasm and organelles.
AMY, amyloplast; CW, cell wall; ER, endoplasmic reticulum; G, golgi body; MT, mitochondria; N, nucleus; S, starch granule; V, vacuole.

persed (Figure 5c and d) within vacuoles. Fusion of vacuoles and discharge of vacuolar contents from starch granule-containing vacuoles into vacuoles with clearer vacuolar sap were commonly observed (Figure 5b and d, arrowheads). These observations suggest that expansion of vacuolar volume might be carried out by fusion of vacuoles and degradation of amyloplasts occurred within vacuoles.

α-Amylases are detected in amyloplasts, vacuoles, and cell walls of the starved cells

The subcellular localization of α-amylases was determined by immunocytochemistry using polyclonal antibodies against the purified rice α-amylases which recognized proteins of 44 and 46 kDa (Figure 6a, lane 3). Although the antibodies also reacted with an 85 kDa cellu-

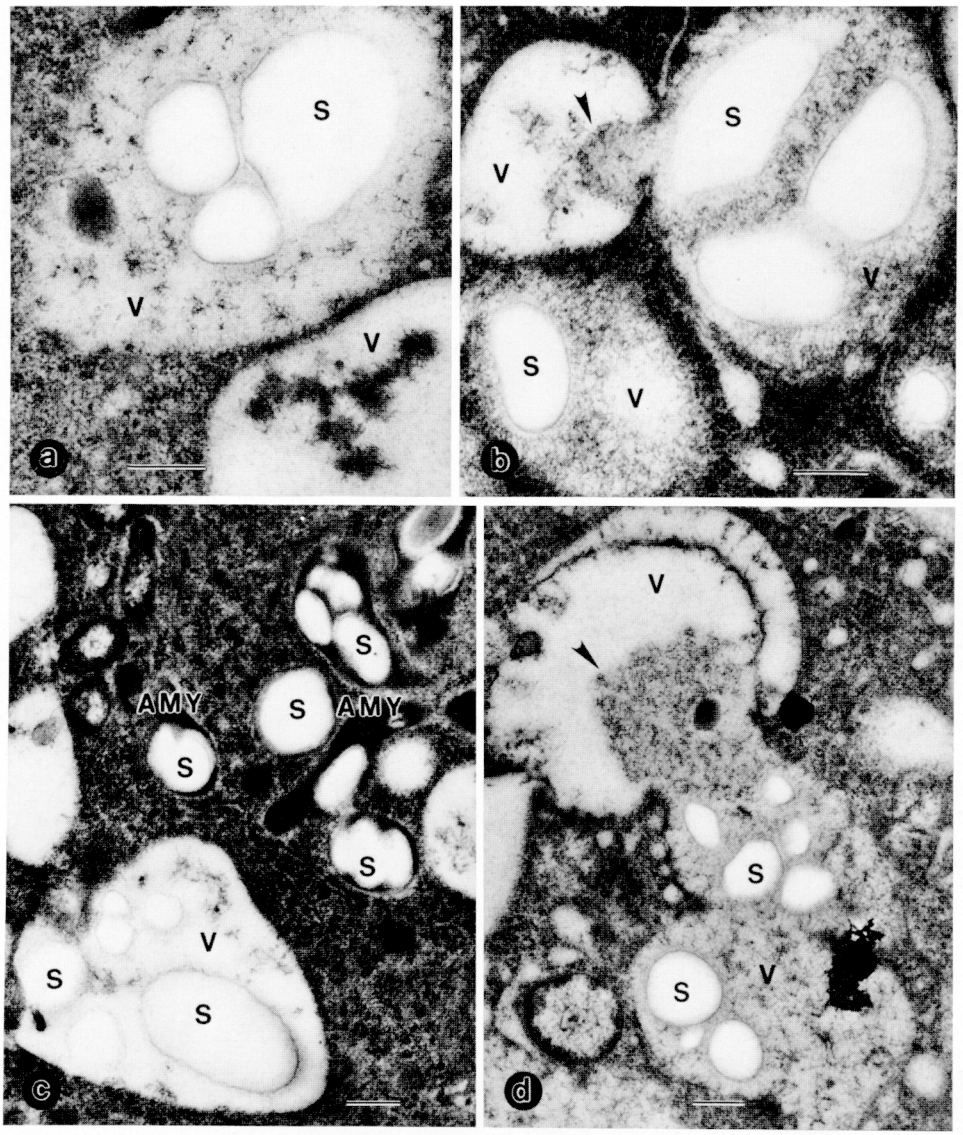

Figure 5. Electron micrographs of rice cells grown in sucrose-free medium for 12 h.
Cells grown in sucrose-containing medium for 7 days were transferred to sucrose-free medium and incubated for 12 h. Cells were collected and fixed as described in Experimental procedures. Sections were examined with an electron microscope.
(a)–(d) Starch granules within vacuoles. (a) Contact sites of three starch granules within the upper vacuole are slightly apart. (b) Three starch granules within the right vacuole are completely separated. (c) Starch granules are dispersed within the lower vacuole. Several intact amyloplasts containing starch granules are in the neighboring area. (d) Starch granules are dispersed within the lower vacuole. Note that the vacuoles containing starch granules in (b) and (d) have denser vacuolar sap, which is being discharged into another emptier vacuole (arrowheads).
AMY, amyloplast; S, starch granule; V, vacuole. Bar = 0.5 μm.

lar protein (Figure 6c, lane 1), we found that the pre-immune serum also reacted with this protein (Figure 6c, lane 3). Therefore, the α-amylase antibodies were used for immunolocalization of α-amylases and the pre-immune serum was used as a control.

The preservation of ultrastructure was not good in cells embedded in acrylic LR White resin (Figures 5 and 7) compared with the epoxy resin (Figure 4), but the hydrophilic properties of acrylic resins yielded superior labeling results. Label was found over starch granules within the intact amyloplasts of cells even before cells were starved of sucrose, mainly at the periphery and in the cavities of starch granules (Figure 7a), and over the cell walls of these cells (Figure 7a). In cells starved of sucrose for more than 12 h, label was detected within some vacuoles either inside engulfed amyloplasts (Figure 7b) or associated with protein aggregates (Figure 7c). Labeling of amyloplasts, cell walls and some vacuolar contents was very specific; gold particles were hardly ever observed in the cytoplasm and other organelles. No specific immuno-

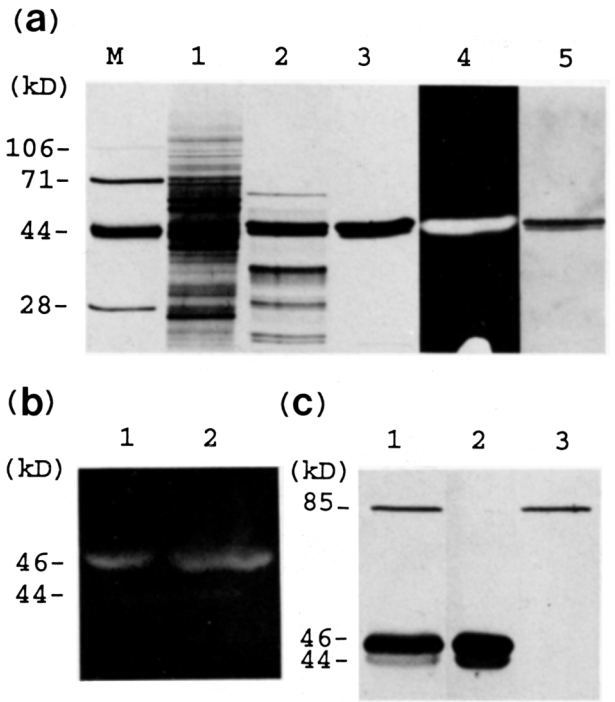

Figure 6. SDS–PAGE analysis of the rice α-amylases.
Cells grown in sucrose-containing medium for 7 days were transferred to sucrose-free medium and incubated for 2 days. Proteins were extracted from cells or harvested from media. α-Amylases were purified as described in Experimental procedures. Proteins or α-amylases were fractionated on SDS–PAGE and subjected to the following analyses.
(a) Analysis of α-amylases by staining, activity or immunoblotting assays. Lane 1, 3 μg total cellular proteins, silver stained. Lane 2, 2 μg total medium proteins, silver stained. Lane 3, 1 μg purified α-amylases, silver stained. Lane 4, zymogram of 2 μg purified α-amylases performed by a published method (Lacks and Springhorn, 1980). Lane 5, Western blot analysis of 0.2 μg purified α-amylases using anti-rice α-amylase antibodies. M, protein molecular weight marker.
(b) Zymogram of α-amylases after fractionation by gradient SDS–PAGE. Total medium proteins, 10 μg loaded in lane 1, 30 μg in lane 2.
(c) Western blot analysis of antibody specificity. Lanes 1 and 2, cellular and medium proteins, respectively, reacting with the anti-rice α-amylase antibodies. Lane 3, cellular proteins reacting with the rabbit pre-immune serum.

gold labeling was detected in parallel experiments using pre-immune serum as the primary antibody (Figure 7d). It is not known why the 85 kDa protein was not labeled in these cells by the pre-immune serum. Similar results of α-amylase localization were obtained with a monoclonal antibody that recognizes the 44 and the 46 kDa α-amylases (data not shown).

Analysis of the amino (N-) and carboxy (C-) terminal amino acid sequences of the rice α-amylase isozymes

α-Amylases of mammals, plants and bacteria are generally secretory proteins. The function of the N-terminal signal sequences of some α-amylase isozymes from barley (Düring *et al.*, 1990) and rice (Kumagai *et al.*, 1990) has been demonstrated. We therefore first compared the N-terminal sequences of nine rice α-amylase isozymes and the predicted signal peptide cleavage sites (von Heijne, 1986). Signal peptides typically have three structurally distinct regions: a positively charged N-terminal region, a central hydrophobic region, and a more polar C-terminal region (von Heijne, 1985). Results show that the predicted amino acid sequences of all the rice α-amylase isozymes contain typical signal sequences at their N-termini (Figure 8a). Analysis of the C-terminal amino acid sequences revealed that *αAmy3*, *αAmy6*, *αAmy8*, *RAmy3A*, and *RAmy3C* have 10 or 11 amino acid extensions at their C-termini and that these extensions have 50–90% identity with each other (Figure 8b). *RAmy2A* also has a 15 amino acid extension at the C-terminus, but it has no similarity with the sequences of the other five C-terminal extensions. The C-terminal 66 amino acid sequence of *RAmy1B* has almost no similarity with sequences in the corresponding regions of the other eight α-amylase isozymes and was not included in the sequence comparison. A common characteristic of these C-terminal extensions is that they are all rich in hydrophobic and positively charged amino acids. In addition, the C-terminal extension of *RAmy2A* is also rich in the hydroxylated amino acids serine and threonine.

Discussion

Starvation induces expression of a new group of α-amylases

Since the rice α-amylase isozymes are encoded by a family of at least 10 genes (Huang *et al.*, 1990), each of the two groups of α-amylases reported here probably consists of several species of α-amylase isozyme. Although accumulation of α-amylases significantly increased after cells were starved of sucrose, expression of the two groups of α-amylases was differentially regulated (Figure 1). The 44 kDa α-amylases were constitutively expressed regardless of the presence or absence of sucrose in the medium, but the 46 kDa α-amylases were detected only after cells had been starved of sucrose. Provision of sucrose to the starved cells abolished accumulation of the 46 kDa α-amylases. It is not known whether the 44 and 46 kDa α-amylases are encoded by different α-amylase genes or originate from post-translational modifications of a single or different gene product(s). Identification of genes encoding the 44 and 46 kDa α-amylases and study of the mechanisms by which expression of these α-amylases is differentially regulated should lead to a better understanding of the function of these amylases in cultured cells. We have, therefore, recently generated monoclonal antibodies which recognize different

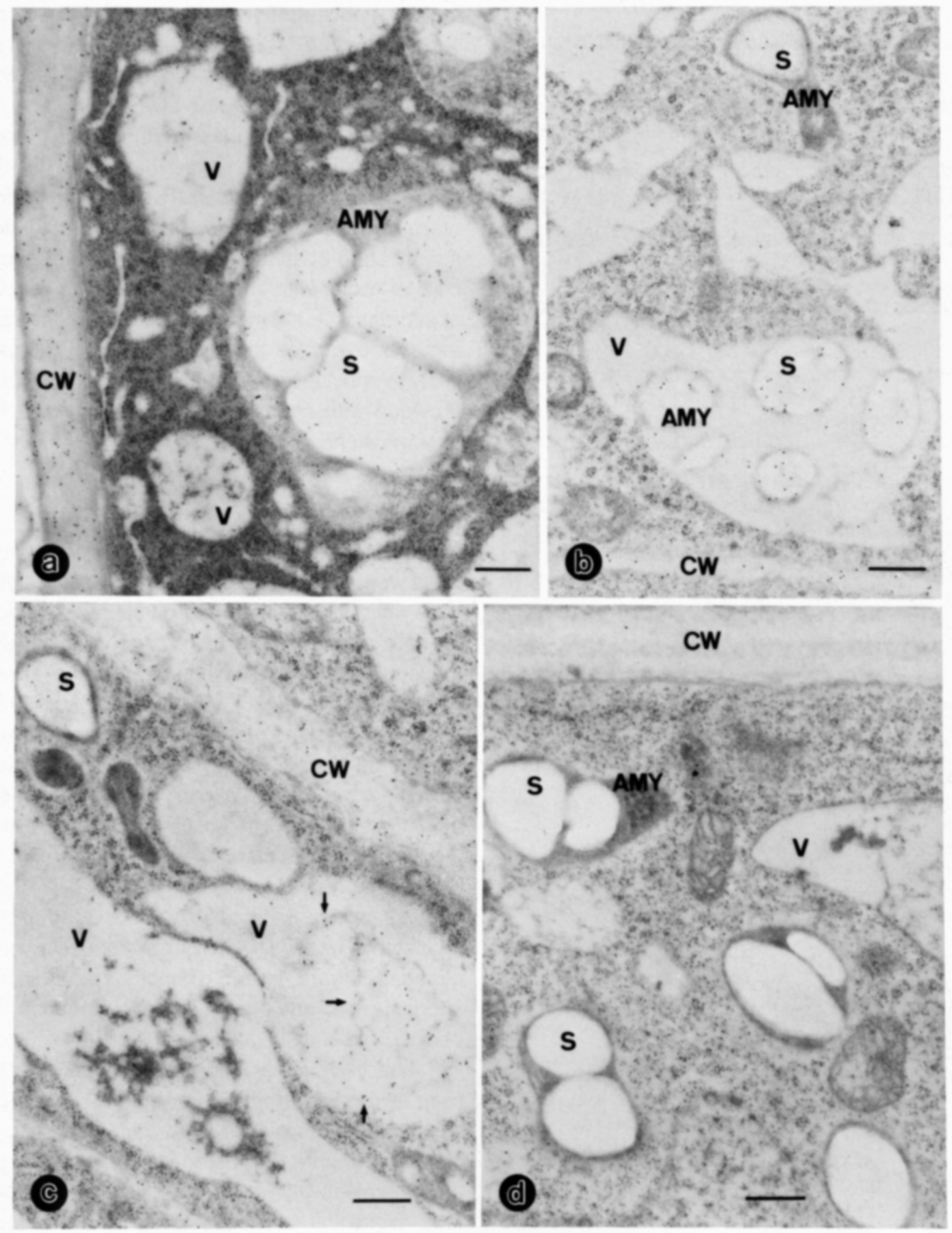

Figure 7. Immunocytochemical localization of α-amylases in cultured rice cells.
Cells were grown in sucrose-containing medium for 7 days and transferred to sucrose-free medium for various times. Cells were collected, fixed, sectioned, treated with the anti-rice α-amylase antibodies, and examined as described in Experimental procedures.
(a) Labeling of the amyloplast/starch granules and cell wall in cells 0 h after sucrose starvation.
(b) Amyloplasts free in cytoplasm or enclosed within a vacuole in cells 12 h after sucrose starvation. The amyloplasts are labeled.
(c) Gold particles associated with protein aggregates (indicated by arrows) within vacuoles 12 h after sucrose starvation. The amyloplast and cell wall are also labeled.
(d) Section from control cells 0 h after sucrose starvation and treated with the pre-immune serum.
AMY, amyloplast; CW, cell wall; S, starch granule; V, vacuole. Bar = 0.5 μm.

members of the 44 and 46 kDa α-amylases and purification and amino acid sequencing of proteins belonging to each of these two groups are presently underway.

Starvation induces degradation of cellular starch

A reverse correlation between α-amylase level and starch content in the starved cells suggests the possibility that

(a)

Genes N-terminal amino acid sequences

```
                 ++oo  o  ooo    ooo o     ooo
αAmy10   XXXSKRFL.SLS.LLI...LLLGF.SSSLAAG*QVLFQ/GF
           | || |||  ||     |||  || |||| ||||| ||
         oo ++oo  o  ooo    ooo o     ooo
RAmy1B   MVANKHFL.SLS.VLI...VLLGL.SSNLAAG*QVLFQ/GF
         || ||||| ||| |||    |||||  |||| ||  ||||| ||
          o  ++oo  o  ooo    ooo o     o o
αAmy7    MV.NKHFL.SLS.VLI...VLLGL.SSNLTAG*QVLFQ/GF
         |         ||   ||     || |  || |     ||||| ||
          o++oo o   oo   oo   o oo o    +oo o
αAmy6    MAKRIASMSSLL..LI..A.LLCL.SSHLAQA*QVLFQ/GF
         | |                  |  ||| ||  |||| ||||| ||
           +++o    o  ooo    oo o oo    oo o
αAmy8    MGKHHVTLCCVV.F....AV.LCLASS.LAQA*QVLFQ/GF
         | ||  |  |   |      | |||  | |||| ||||| ||
          o++   o  oo oo      ooo o    +oo o
RAmy3C   MAKHSTIMSCLL.FF....VLLCLG.SHLAQA*QVLFQ/GF
         |   |    |||         | ||         || ||||| ||
          +       o ooooo     o o  o        o
αAmy3    MKNTS..SLCLLLLV....V.LCSLTCNSGQA*QVLFQ/GF
         |        ||  |||                    | ||| ||
            ++ ooo  oooooooooo    oo+   o
RAmy3A   M.GKHMAALCGFLLVALLWLTPDVAH...A*QTQILFQ/GF
         |        |   ||  ||  |     |           |||| ||
            o  ++o oooooooo o     o
RAmy2A   M..ATGRRLSMILLLLLLGL....A....SG*DKILFQ/GF
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(b)

Genes C-terminal amino acid sequences

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                      ooooo ++     o o
RAmy2A   HGNGYAVWEKSAAAAADHRTSSSASL
         ||||||||||

αAmy10   HGNGYAVWEKI
         || ||| ||||

αAmy7    HGDGYAIWEKI
         ||  | ||||
                      o+ooo   +++
αAmy6    HGNNYCIWEKSGLRVPA..GRHHY
         ||| || ||| ||||||  || ||
                      o+ooo   +++
αAmy8    HGNDYCVWEKEGLRVPA..GRKHY
         ||| |||||| ||||||  || |
                      o+ooo   +++
RAmy3C   HGNNYCVWEKSGLRVPAl.GRRH
         ||| ||||||||||||   |||
                      o+oo o  ++
RAmy3A   HGNDYCVWEKSGLRVPEPEGRR
         || || ||||  ||||   ||
                      o+ooo   ++o
αAmy3    HGKDYSVWEKGSLRVPA..GRHL
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Figure 8. Amino acid sequence comparison of rice α-amylase genes. Amino acid sequences were predicted from nucleotide sequences collected from GenBank or EMBL (*αAmy3* = *RAmy3D*; *αAmy6* = *RAmy3B*; *αAmy7* = *RAmy1A*; *αAmy8* = *RAmy3E*). Gaps (dots) are introduced to maximize sequence identity. (a) Alignment of the N-terminal amino acid sequences. (b) Alignment of the C-terminal amino acid sequences. Asterisks (*) indicate the putative signal peptide cleavage sites predicted from a statistical method (von Heijne, 1986). Slashes (/) indicate the intron splice site. Vertical bars (|) indicate identical amino acid sequences. Amino acids with hydrophobic (O) or positively charged (+) chain are indicated. X indicates undetermined amino acid sequence.

starch was digested by α-amylases and used as an alternative carbon source. However, α-amylases were present at low concentration when cells were provided with sucrose (Figure 1). EM study also shows that α-amylases were already present in the starch granules within amyloplasts before sucrose starvation took place (Figure 7a). An intriguing question is why the level of starch in cells provided with sucrose was not significantly affected (Figure 2) since α-amylases were also present in amyloplasts of these cells. One possible explanation is that a steady state of synthesis and turnover of starch exists in cells provided with sucrose. As soon as cells are starved of sucrose, the balance is affected and the rate of starch turnover overrides the rate of starch synthesis. Alternatively, α-amylases present in the amyloplasts within cells provided with sucrose do not degrade starch until cells become starved.

Starvation induces vacuolar autophagy

Cells were able to maintain metabolic activities for up to 4 days without a supply of carbon source. The doubling time of rice suspension cells grown in sucrose-containing medium is 2 days. The results, that the growth rates of cells starved for 1–3 days were only slightly affected (Figure 3a) and the metabolic activities in these cells were resumed three to fourfold within 2 days (Figure 3b) after cells had been shifted back to the sucrose-containing medium, all suggest that cells were still alive at least within 3 days of sucrose starvation. The decreased growth rate of cells starved for 4 days suggests that a large proportion of these cells probably died. Cells starved of sucrose for 2 days have already undergone extensive morphological changes (Figure 4c and d). Increase of vacuolar volume suggests that autophagic-like invaginations of the tonoplast might have occurred. Fusion of vacuoles and increase in vacuolar volume were easily detected after cells were starved for longer than 12 h and presence of starch granules within the vacuoles was commonly observed (Figure 5). The amyloplasts/starch grains might be non-selectively engulfed by vacuolar phagocytosis, or they might first be sequestered by the autophagosome and subsequently fuse with vacuoles (lysosomes) as in animal systems (Seglen *et al.*, 1990) during sucrose starvation.

The appearance of starch granules without an intact amyloplast membrane inside vacuoles (Figure 5) suggests that amyloplasts, and probably other organelles, were degraded within vacuoles during starvation. Interestingly, association of α-amylases with these naked starch granules could still be observed (Figure 7b). In yeasts, proteolysis in the vacuoles is essential for protein turnover and nturient deficiency has been shown to induce extensive autophagic degradation of cytosolic components in vacuoles (Takeshige *et al.*, 1992). Plant vacuoles are rich

in hydrolases and vacuolar autophagy has been observed in plants undergoing senescence (Matile, 1975). The appearance of chloroplasts inside vacuoles in senescing wheat leaves has been suggested to be a mechanism for the degradation of chloroplasts during senescence (Wittenbach *et al.*, 1982). The extensive vacuolar autophagy that occurred in cells starved of sucrose may account for the physiological turnover and recycling of cytoplasmic and organellar constituents as energy substrate. In this regard starved cells are similar to senescing cells in plants.

α-Amylases may be translocated via unidentified pathways

Our previous study shows that α-amylases are secreted extracellularly (Yu *et al.*, 1991) and here we show that α-amylases are localized in cell walls as well as in starch granules within amyloplasts. Questions thus arise regarding the pathways of α-amylase translocation in the cultured cells. Amyloplasts have been considered as non-photosynthetic chloroplasts because both organelles originate from proplastids and appear to be interconvertible (Gillott *et al.*, 1991). Translocation of proteins to chloroplasts and extracellular compartments via different pathways is generally accepted (Verner and Schatz, 1988). Import into chloroplasts of a nuclear-encoded protein requires an N-terminal 'transit' sequence as a targeting signal (Schmidt and Mishkind, 1986). The transit sequence of a waxy protein localized in maize amyloplasts has been shown to mediate transport of a reporter protein *in vitro* into chloroplasts (Klösgen *et al.*, 1989). The deduced N-terminal amino acid sequences of nine rice α-amylases do not contain canonical chloroplast transit peptides. Instead, they all contain typical signal sequences characteristic for translocation of protein across the ER membrane (Figure 8a). The pathway of α-amylase secretion in barley aleurone cells has been shown to involve rough ER and Golgi apparatus (Gubler *et al.*, 1986; Zingen-Sell *et al.*, 1990). In addition, we have transferred and expressed the *Escherichia coli* β-glucuronidase (GUS) gene linked to the promoter and signal sequence of one of the rice α-amylase genes, *αAmy8*, in rice (Chan *et al.*, 1993). GUS was glycosylated and secreted into the culture medium from suspension-cultured transgenic rice cells (Chan *et al.*, 1994), suggesting that the predicted signal sequence of *αAmy8* directs translocation of GUS through the secretory pathway. Therefore, we propose that an alternative pathway for import of α-amylases into amyloplasts may exist. By this pathway, the α-amylases are translocated to amyloplasts via ER and other sorting machinery. This hypothesis is currently being tested.

α-Amylases were also detected in some vacuoles in association with protein aggregates (Figure 7c). In plant cells the default pathway for polypeptides that have entered the ER is secretion of the exoplasmic space, and targeting to the vacuole requires additional information (Chrispeels, 1991). Proteins that have both vacuolar and extracellular isoforms often have a C-terminal extension on one isoform that functions as a vacuolar targeting signal (Chrispeels and Raikhel, 1992). The C-terminal extensions of those vacuolar isoforms are rich in hydrophobic amino acids. The C-terminal extension of a vacuolar form cucumber chitinase has been shown to be sufficient for directing a normally secretory form of chitinase into the vacuole (Neuhaus *et al.*, 1991). Comparison of the deduced amino acid sequences indicates that some of the rice α-amylases have C-terminal extensions which are rich in hydrophobic and positively charged amino acids (Figure 8b). Whether or not the C-terminal extensions of some α-amylases serve as extra signals for further targeting to vacuoles or amyloplasts is currently also being tested.

Rice suspension cell culture as a model system

Under certain environmental conditions, e.g. in the dark, annual resting seasons, leaf senescence, or seed germination, photosynthesis is turned off or operates to a lower degree, and plants have to rely on internal carbon sources such as reserved starch to maintain metabolic activities. The cell physiology and regulatory mechanisms of starch metabolism remain largely unknown in higher plants owing to a lack of good experimental model systems. The multicellular nature of higher plants and the dynamic translocation of sugars between source and sink or different carbon storage pools all make it difficult to study how the regulation of starch mobilization is controlled *in vivo*. We have recently found that expression of two α-amylase genes, *αAmy3* and *αAmy8*, in germinating rice seeds is regulated by sugar level in the embryos and is related to the degradation of starch in embryos (Yu *et al.*, manuscript submitted). The expression of α-amylases and degradation of starch in cultured cells can be easily manipulated by altering the sugar level in the medium, which would be useful for studying the regulatory mechanisms of α-amylase gene expression and starch metabolism under defined conditions. Our additional finding that cells undergo autophagy under sucrose starvation could serve as a model system for studying the regulatory mechanisms of vacuolar autophagy and the biochemical responses of cells under nutrient starvation and during senescence. Localization of α-amylases in different compartments of the cultured cells also facilitates studies of sorting and targeting mechanisms for α-amylase translocation.

Experimental procedures

Plant material

Suspension cell culture of rice (*Oryza sativa* cv. TN5) was performed as described previously (Yu *et al.*, 1991). Cells were collected by filtration through a 400 mesh nylon sieve, blot-dried on paper towels, and weighed. The medium was collected by filtration through a piece of Whatman no. 1 filter paper. The collected cells or media were quick-frozen in liquid N_2 and stored at −70°C until use.

Measurement of soluble sugar and starch levels in cultured cells

Sugar and starch levels were determined by a modified anthrone reagent method (Yoshida *et al.*, 1976). Soluble sugars were extracted by grinding 200 mg of frozen cells in a 1.5 ml Eppendorf tube with a motor-driven micropestle for 30 sec, then placing the mixture into a 15 ml centrifuge tube and adding 7.5 ml of 80% ethanol. The sample was kept in a water bath at 80–85°C for 30 min. Cell debris was pelleted by centrifugation in a clinical centrifuge for 10 min, and the supernatant was collected. This extraction was repeated one more time. The combined alcohol extract was evaporated on a water bath at 80–85°C until most of the alcohol was removed and distilled water was added to make up a total volume of 100 ml. The sample was filtered through a piece of Whatman filter paper and used for sugar determination. Starch was extracted by first drying the cell residue left in the centrifuge tube during soluble sugar extraction in an oven at 80°C. Then 2 ml of distilled water were added to the dried residue, and the sample was placed in a bath of boiling water for 15 min and stirred occasionally. When the sample was cool, 2 ml of 9.2 M $HClO_4$ were added with constant stirring. The solution was stirred occasionally for 15 min, centrifuged for 10 min, and the supernatant was collected. Two milliliters of 4.6 M $HClO_4$ were added to the cell residue again for a repeated extraction. The suspension was stirred for 15 min, centrifuged for 10 min, and the supernatant was combined with that collected previously. The solution was then made up to 100 ml with distilled water, filtered through a piece of Whatman filter paper, and used for starch determination. A volume of 2 ml of the sugar or starch extract was put in a 15 ml tube and kept in an ice bath. A volume of 5 ml of the anthrone reagent (2 g of anthrone in 1 l of concentrated H_2SO_4 and prepared freshly before use) was added to the sample. After gentle mixing, the test tube was put into a bath of boiling water for exactly 7.5 min and then immediately cooled in ice. When the sample was cool, the absorbance at 630 nm was measured and the value of sugars was calculated according to the glucose standards.

Tetrazolium reduction

Metabolic activity of cells was estimated by reduction of TTC (Steponkus and Lanphear, 1967). One hundred milligrams (fresh weight) of cells were placed in a test tube, 5 ml of 0.8% (w/v) TTC in 0.05 M Na_2HPO_4–KH_2PO_4 buffer (pH 7.4) containing 0.1% (v/v) Tween 20 were added, and the sample was infiltrated under vacuum for 10 min. The sample was incubated at 30°C in darkness for 12 h. TTC solution was drained and cells were rinsed twice with distilled water. Cells were extracted with 10 ml of 95% (v/v) ethanol in a bath of boiling water for 20 min. Cells were removed by brief spin in a clinical centrifuge, the extract was cooled, and made up to a 10 ml volume with 95% ethanol. Absorbance at 530 nm was determined.

Purification of α-amylases and preparation of antibodies

Rice cells were suspension-cultured in sucrose-containing medium for 7 days and transferred to sucrose-free medium for 2 days. Cells were removed by filtration and the culture medium was centrifuged at 16 000 ***g*** for 10 min. The supernatant was heated at 70–72°C for 16 min, cooled on ice, and centrifuged at 16 000 ***g*** for 10 min. Ethanol was added to the supernatant to a final concentration of 40% and the sample was kept on ice for 30 min, then centrifuged at 16 000 ***g*** for 10 min. Two milliliters of rabbit liver glycogen (Sigma) solution (2% w/v) were added to the supernatant, which was then stirred at 4°C for 10 min. The sample was centrifuged at 16 000 ***g*** for 10 min. The pellet was dissolved in 1 ml of 10 mM Tris, pH 6.8, containing 10 mM $CaCl_2$. These steps produced partially purified α-amylases from the medium. Analysis with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Yu *et al.*, 1991) and silver staining (Merril *et al.*, 1981) revealed that the protein preparation contained two putative α-amylases with molecular masses of 44 and 46 kDa (Figure 6a, lane 3) when compared with the unpurified proteins present in cells (Figure 6a, lane 1) or in the medium (Figure 6a, lane 2). The 44 and 46 kDa proteins were shown to possess amylolytic activities (Figure 6a, lane 4, and Figure 6b) and react with the anti-barley α-amylase polyclonal antibodies (a gift from Dr Tuan-Hua David Ho, Washington Unviersity, St. Louis) (data not shown). Therefore, the 44 and 46 kDa proteins were confirmed as α-amylases. The polyacrylamide gel strips containing the 44 and 46 kDa α-amylases stained with Coomassie blue were excised, fragmented, and injected into rabbits to raise α-amylase polyclonal antibodies according to methods described by Harlow and Lane (1988). Specificity of the antibodies was checked by Western blot analysis and the antibodies were shown to react with the 44 and 46 kDa purified α-amylases (Figure 6a, lane 5) and α-amylases present in cell extracts (Figure 6c, lane 1) or in the medium (Figure 6c, lane 2).

Electron microscopy

Cells were freshly collected from the culture medium and prefixed in 2% (v/v) glutaraldehyde in 50 mM cacodylate buffer at pH 7.2 for 4 h at 4°C then washed three times for 10 min each wash in the same buffer. Cells were postfixed in 1% (w/v) OsO_4 in the same buffer for 1 h at room temperature and washed twice in the same buffer for 30 min each wash. Cells were then stained with 2.5% uranyl acetate at room temperature for 2 h, dehydrated in a 50–100% series of ethanol bath, and infiltrated with Spurr's resin. The resin was changed several times over a 2 day period and polymerized at 70°C for 12 h in a vacuum. Thin sections were cut with a diamond knife on an Ultracut E microtome (Reichert-Jung, Vienna, Austria), mounted on formvar-coated nickel grids (Polysciences, Warrington, PA), stained with alkaline lead citrate, and examined on a Zeiss 902 transmission electron microscope at 80 kV.

Immunocytochemistry

Cells were collected from the culture medium and immediately immersed in a freshly prepared fixation solution containing 1% (v/v) glutaraldehyde and 4% formaldehyde in 100 mM sodium phosphate buffer, pH 7.4, and incubated at 4°C for 4 h. Cells were

rinsed three times with the same buffer and postfixed in 1% (w/v) OsO_4 in the same buffer at room temperature for 1 h. Cells were rinsed in the same buffer twice for 30 min each, dehydrated in a graded ethanol series (30, 50, 70 and 90%) for 15 min each, in 100% ethanol twice for 15 min each, and finally embedded in L.R. White resin (SPI Supplies). The L.R. White resin was polymerized at 50°C for 24 h in a chamber filled with nitrogen. Thin sections were cut as described in the previous section.

Localization of α-amylases in cells was performed by an indirect immunogold labeling procedure (Lin and Langenberg, 1983). Thin sections on grids were incubated in 3% normal goat serum in PBS (pH 7.4) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4-$7H_2O$, 1.4 mM KH_2PO_4) for 10 min, labeled for 1 h with a 1:200 dilution of the primary antibodies (rabbit anti-α-amylase antiserum) in PBS (pH 7.4). The sections were then treated for 30 min with colloidal gold (10 nm diameter)-conjugated goat anti-rabbit IgG secondary antibodies. After immunolabeling, the sections were washed with distilled water and stained with 6% (w/v) uranyl acetate for 20 min, followed by alkaline lead citrate for 10 min, and finally examined with an electron microscope. For control treatment, sections were incubated with pre-immune serum under the same conditions.

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