

Carbohydrate metabolism in rice during callus induction and shoot regeneration induced by osmotic stress

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Abstract. We are interested in the cellular physiological events taking place during shoot regeneration in rice (*Oryza sativa* L. cv. Tainan 5) callus induced by osmotic stress. At first, the sucrose and starch metabolisms in rice callus were studied because carbohydrates are the main energy source in plant tissue culture. The results showed that fresh weight, water content, cellular water, and osmotic potentials all decreased significantly in highly regenerable callus which was induced on MS basal medium supplemented with 10 μ M 2,4-D and 0.6 M mannitol (TN5-M₆). Besides, the starch and soluble sugar contents in TN5-M₆ callus were higher than in un-regenerable callus, induced on the same medium without mannitol. Then, a sudden increase of glucose content was found in TN5-M₆ the first day after the callus was transferred to regeneration medium. Simultaneously, the activities of sucrolytic enzymes, sucrose synthase, and acid invertase were higher, and they may have responded to the increase of glucose content. It is suggested that the sudden increase of glucose content may play an important role in shoot regeneration.

Keywords: Carbohydrate metabolism; *Oryza sativa*; Osmotic stress; Regeneration related factors; Shoot regeneration.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; α -Amy, α -amylase; AGPase, ADP-glucose pyrophosphorylase; Bound-IT, cell wall-bound form invertase; HEPES, N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]; MS, Murashige and Skoog; RSus, rice sucrose synthase; Sol-IT, soluble form invertase; SPase, starch phosphorylase.

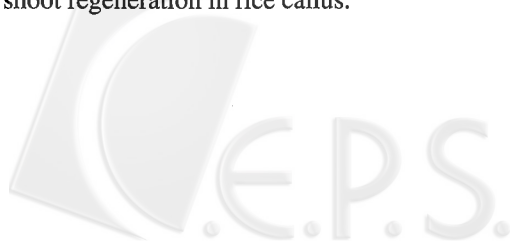
Introduction

Plant cells possess totipotency, i.e., whole plants can be regenerated from single cells by modulating culture conditions (Reinert, 1959). The mechanisms of totipotency, however, are little understood so far, and are mainly discussed in relation to the concentration and ratio of phytohormones (Toonen and De Vries, 1996). It has been reported that osmotic stress affects callus growth, colony formation, shoot regeneration, somatic embryogenesis, and the metabolism of specific compounds (Maretzki et al., 1972; Klenovska, 1973). In previous studies, we discovered that shoot regeneration frequency was dramatically different among rice callus induced from different varieties (Lai and Liu, 1982). Additionally, the shoot regeneration ability of un-regenerable callus could be promoted by osmotic stress treatment (Lai and Liu, 1986; 1988; Liu and Lai, 1991). This provides an alternative concept that the growth and differentiation of cells could be modulated by the cellular physiological water status. We are thus interested in what cellular physiological events occurred during this process.

Carbohydrate supplied to a medium not only acts as a source of carbon and energy, but also as an osmotic agent during organogenesis (Thorpe and Murashige, 1970; Verma and Dougall, 1977). However, very little is known about carbohydrate metabolism in cultured cells. Our preliminary histological study showed that starch granules increased in highly regenerable rice callus. After being transferred to regeneration medium, the callus was able to regenerate shoots in several days, and those starch granules disappeared (Liu and Lee, 1996). The correlation between starch metabolism and shoot formation was reported in tobacco (Thorpe and Murashige, 1968; Thorpe and Meier, 1974; Thorpe et al., 1986), sugarcane (Ho and Vasil, 1983), and *Begonia* (Mangat et al., 1990). However, there is no further information about carbohydrate metabolism in rice callus. Moreover, no link between osmotic stress, carbohydrate metabolism, and shoot regeneration has been explored.

In this study, callus growth and cellular water status under osmotic stress were measured. Then, the contents of carbohydrates and the activities of enzymes related to sucrose and starch metabolism during callus induction and shoot regeneration were further examined, to clarify the relationship between osmotic stress, carbohydrate metabolism, and shoot regeneration in rice callus.

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Materials and Methods

Callus Induction and Shoot Regeneration

Rice (*Oryza sativa* L. cv. Tainan 5) was used in this experiment. Primary callus was induced on 10 to 12-day-old immature embryos on MSD₁₀ (TN5-M₀, MS basal medium plus 10 μ M 2,4-D) or MSD₁₀M₆ medium (TN5-M₆, MS basal medium plus 10 μ M 2,4-D and 0.6 M mannitol) (Murashige and Skoog, 1962), according to our earlier experiments (Lai and Liu, 1982; 1986; 1988). Mannitol was used as the osmotic agent. After two weeks, callus was transferred to MSK₂₀N₁₀ regeneration medium (MS basal medium plus 20 μ M kinetin and 10 μ M NAA) for shoot regeneration. All cultures were incubated at 25°C and kept under continuous fluorescent light with an intensity of approximately 70 μ mol/m²/s. The results in this study were obtained from three independent experiments. Shoot regeneration was recorded after being transferred to regeneration medium for four weeks. The shoot regeneration frequency was calculated as (callus number with shoot / total callus number) \times 100%.

Measurements of Callus Growth and Water Content

Callus induced on MSD₁₀ and MSD₁₀M₆ medium was collected each week for four weeks, except that the first week was replaced by the 10-day-old callus. After being transferred to regeneration medium, calluses were collected each day or every two days for 13 days. These collected calluses were fixed and stored in a -70°C freezer until analysis or were weighed directly for their fresh weight. The fresh weights were averaged from 10 calluses per experiment. Dry weights were obtained from these weighed calluses that were dried in a ventilating oven at 80°C for 24 h. Water content was determined from (Fresh weight—Dry weight / Fresh weight) \times 100% (Lai and Liu, 1988).

Water Status Measurements with a Psychrometer

Water potential (ψ_w) and osmotic potential (ψ_s) were measured using a Wescor Dew Point Microvoltmeter HR-33T and a Wescor thermocouple hygrometer sample chamber C-52. Our preliminary experiments showed that leaving samples in the sealed chamber for 30 min equilibration before measurement was sufficient with rice callus. The method used to measure ψ_w and ψ_s in callus was as described earlier (Brown and Thorpe, 1980). The pressure potential (ψ_p) was calculated from ψ_w and subtracting ψ_s .

Measurement of Carbohydrate Contents

Starch and the content of soluble sugars, sucrose and glucose, were measured in this study. The collected calluses, dried by lyophilization, were homogenized and extracted twice with 80% (v/v) hot ethanol. The supernatant and the pellet were used for soluble sugar and starch measurement, respectively, following the partially modified methods of Ou-Lee and Setter (1985).

The glucose oxidase-peroxidase coupled reaction method was used. For glucose content, PGO reagent (50 mM HEPES, 3 mg/ml p-hydroxybenzoic acid, 0.1 mg/ml 4-aminoantipyrine, 0.5 units peroxidase, and 1.5 units glucose oxidase, pH 7.0) was added to the ethanol-extracted supernatant and kept at room temperature for 15 min. The absorption value of 490 nm was obtained by Microplate autoreader (EL311, Bio-TEK). Glucose was used as the standard. For sucrose content, ethanol-extracted sample was hydrolyzed by invertase (I4504, Sigma) before PGO reagent was added. These procedures are credible enough for analysis (Ou-Lee and Setter, 1985; Cheng, 1994). The absorption value included both sucrose and glucose, so the glucose content should be subtracted first from this determination to obtain sucrose content. For starch content, the pellet was re-suspended with H₂O and boiled for 20 min. The amyloglucosidase buffer (90 mM sodium acetate, 0.1% NaN₃, and 25 units amyloglucosidase, pH 4.6) was added and incubated at 37°C for 40 h. The supernatant was collected after centrifugation and quantified as mentioned above for glucose content measurement.

Extraction and Assays of Carbohydrate Metabolism Related Enzymes

The collected callus was homogenized and extracted with 10 mM Tris-HCl buffer (pH 7.0) containing 5 mM β -mercaptoethanol, 0.1 mM EDTA, and 1% polyvinyl pyrrolidone. After centrifugation, the supernatant was dialyzed with Tris-HCl buffer by a microdialysis system (1200MD, BRL) at 4°C overnight, and used for all related enzymes assays except Bound-IT. In the present study, both Sol-IT and RSus activities were assayed by the Somogyi-Nelson method, described by Liou (1990). Besides this, three starch metabolism-related enzymes,

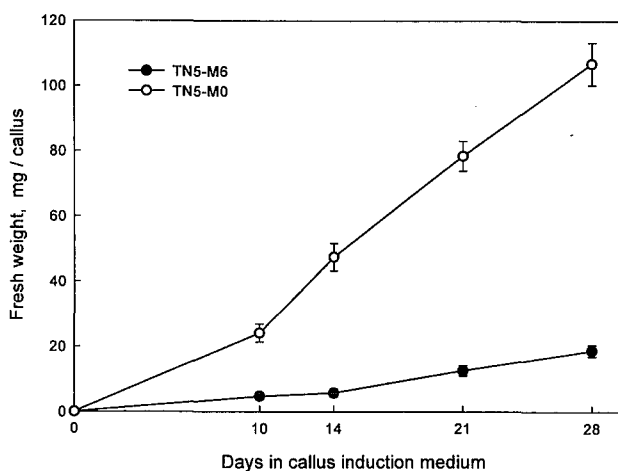


Figure 1. Changes of fresh weight in rice callus induced from MSD₁₀ medium without (TN5-M₀) or with 0.6 M mannitol (TN5-M₆) treatment. Vertical bars represent standard errors (n = 3). Only those standard bars larger than the symbol are shown.

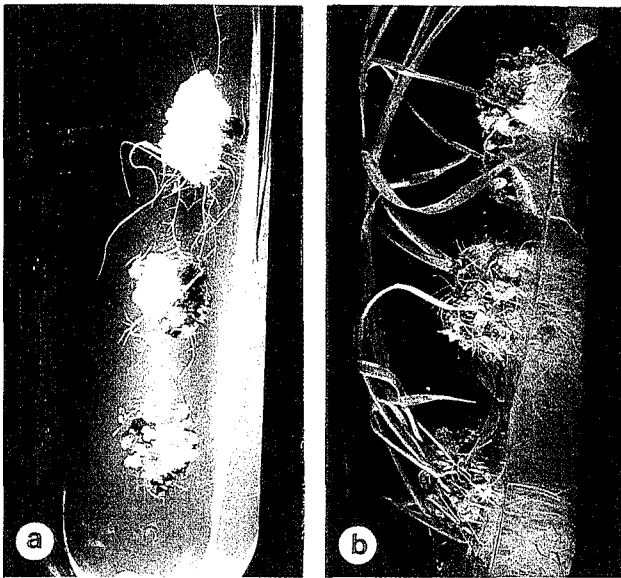


Figure 2. Shoot regeneration of TN5 rice callus induced from MSD₁₀ medium without (a) or with 0.6M mannitol (b) treatment. The fourteenth-day callus was transferred to MSK₂₀N₁₀ regeneration medium for 28 days.

AGPase, SPase, and α -Amy, were determined by the methods of Chang (1995). The pellet was washed and its cell wall bound proteins eluted with 10 mM Tris-HCl buffer containing 1 M NaCl. The supernatant was collected after centrifugation and used to determine Bound-IT activity by the Somogyi-Nelson method (Liou, 1990). The protein content was determined by the Coomassie blue dye-binding method described by Bradford (1976) using bovine serum albumin as the standard.

Results

Callus Growth and Shoot Regeneration

The fresh weight of the callus induced from MSD₁₀ medium increased greatly following culture inoculation; however, it increased less when the callus was induced from MSD₁₀M₆ medium (Figure 1). After being transferred to regeneration medium, no shoots were regenerated in TN5-M₀, but the regeneration frequency increased to approximately 75% in TN5-M₆. In general, green spots emerged between the third and sixth day, and shoots could be seen between the tenth and thirteenth day (Figure 2).

Shoot Regeneration and Water Relations

To clarify the correlation between callus growth, shoot regeneration, and cellular water status, the cellular water content values ψ_w , and ψ_s were measured. First, the ψ_w of MSD₁₀ medium was about -0.6 MPa. However, it decreased to approximately -2.5 MPa of MSD₁₀M₆ medium. The callus induced from MSD₁₀M₆ medium possessed lower water content (Figure 3a) and greater (more negative) ψ_w , and

ψ_s (Figure 4a-b) than that from MSD₁₀ medium during callus induction. These results suggested that callus growth was inhibited by high osmotic stress. On the other hand, water content increased; ψ_w , and ψ_s of TN5-M₆ callus quickly became less negative after being transferred to regeneration medium; and there was no significant difference with TN5-M₀ from the seventh to the ninth day on regeneration medium (Figure 3b; Figure 4d-e). The ψ_p values, however, were higher, both during callus induction and shoot regeneration, in TN5-M₆ than in TN5-M₀ callus (Figure 4c, f).

Carbohydrate Contents during Callus Induction and Shoot Regeneration

Changes of sucrose, glucose, and starch contents during callus induction and shoot regeneration were determined. The results showed that sucrose, glucose, and starch contents were all higher at the initial stage of culture and maintained higher contents longer in TN5-M₆ than in TN5-M₀ callus (Figure 5a-c). After being transferred to regeneration medium, glucose content increased prominently during the first day in TN5-M₆ callus. Although glucose levels decreased quickly after three days, higher levels were maintained in TN5-M₆ than in TN5-M₀ callus on regeneration medium (Figure 5e). The phenomenon of higher glucose content in TN5-M₆ callus was very consistent in several repeat experiments. On the other hand, both sucrose and starch contents were not significantly different between TN5-M₆ and TN5-M₀ callus during shoot regeneration (Figure 5d, f).

Activities of Enzymes for Sucrose and Starch Metabolism

In this experiment, three sucrolytic enzymes—RSus, Sol-IT, and Bound-IT—as well as three starch metabolism-related enzymes—AGPase, SPase, and α -Amy—were analyzed. We could hardly detect the soluble form of alkaline invertase in our whole study (data not shown).

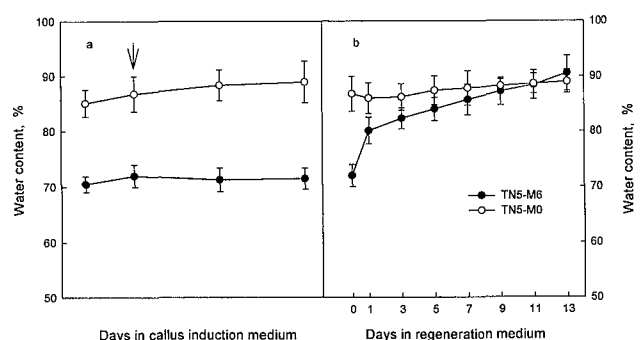


Figure 3. Changes of water content in rice callus at callus induction (a) and shoot regeneration stages (b). The arrow indicates the timing of callus transferred to MSK₂₀N₁₀ regeneration medium. The symbols in this figure are the same as those in Figure 1. Vertical bars represent standard errors (n = 3).

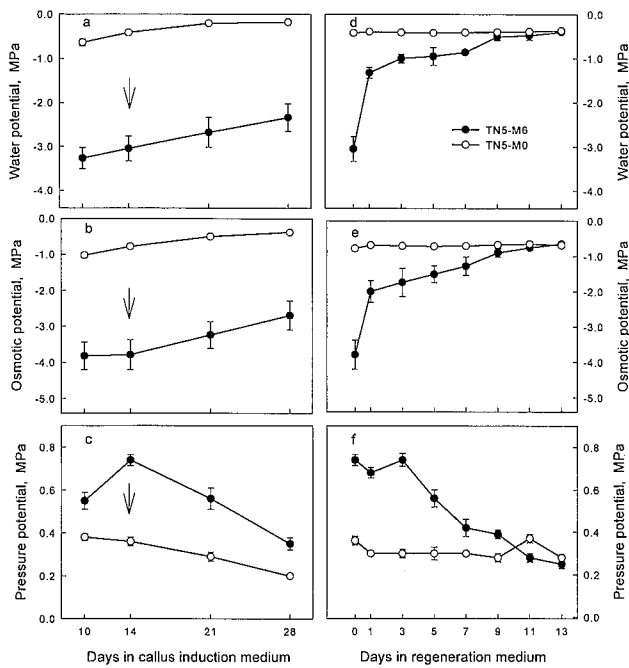


Figure 4. Changes of cellular water, osmotic, and pressure potentials in rice callus at callus induction (a, b, c) and shoot regeneration stages (d, e, f). The arrows in figure a, b, c, indicate the timing of callus transferred to $MSK_{20}N_{10}$ regeneration medium. The symbols in this figure are the same as those in Figure 1. Vertical bars represent standard errors ($n = 3$). Only those standard bars larger than the symbol are shown.

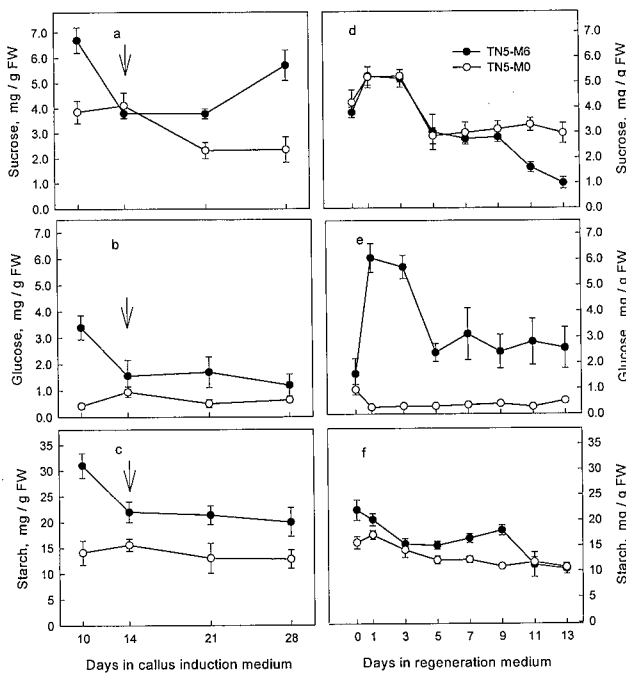


Figure 5. Changes of sucrose, glucose, and starch contents in rice callus at callus induction (a, b, c) and shoot regeneration stages (d, e, f). The arrows in figure a, b, c, indicate the timing of callus transferred to $MSK_{20}N_{10}$ regeneration medium. The symbols in this figure are the same as those in Figure 1. Vertical bars represent standard errors ($n = 3$). Only those standard bars larger than the symbol are shown.

During callus induction, higher Bound-IT and lower α -Amy activities in TN5-M₆ were observed than in TN5-M₀ callus (Figure 6b; Figure 7c). However, the Sol-IT, RSus, AGPase, and SPase activities in TN5-M₆ callus were all similar to those activities in TN5-M₀ callus (Figure 6; Figure 7). On the other hand, higher activities of sucrolytic or starch metabolism-related enzymes, except for α -Amy in TN5-M₆ callus, were observed after transfer to regeneration medium (Figure 6; Figure 7). The α -Amy activity of TN5-M₆ didn't show higher activity until the seventh day in the medium (Figure 7f). These results suggest that highly regenerable rice callus possesses a more efficient carbohydrate metabolism. Whether this has any meaning for callus growth and shoot regeneration needs further study.

Discussion

The shoot regeneration frequency of rice callus could be promoted significantly by highly osmotic stress treatment (Figure 2; Jain et al., 1996; Lai and Liu, 1986; 1988) as has been reported for other species (Binzel et al., 1996; Brown et al., 1989; Etienne et al., 1993; Lou and Kako, 1994; Roberts, 1991). Besides, we found that the osmotic stress-induced callus TN5-M₆ always maintained a lower water content ψ_w and ψ_s (Figure 3; Figure 4). We found that Ai-Nan-Tsao 39, a highly regenerable variety without osmotic stress, also had a lower water content ψ_w and ψ_s

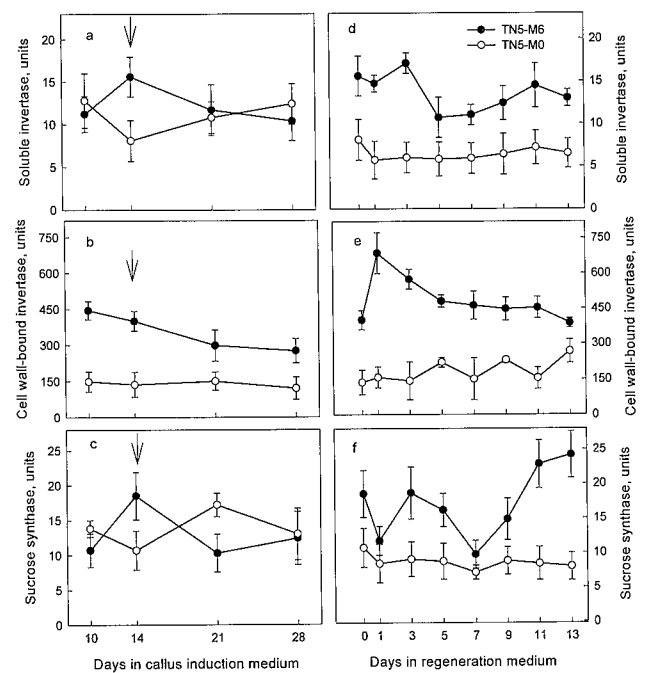


Figure 6. Changes of specific activity of sucrolytic enzymes in rice callus at callus induction (a, b, c) and shoot regeneration stages (d, e, f). One unit of activity represents 1 μ g reducing sugars produced per mg protein, per min. The arrows in figure a, b, c, indicate the timing of callus transferred to $MSK_{20}N_{10}$ regeneration medium. The symbols in this figure are the same as those in Figure 1. Vertical bars represent standard errors ($n=3$).

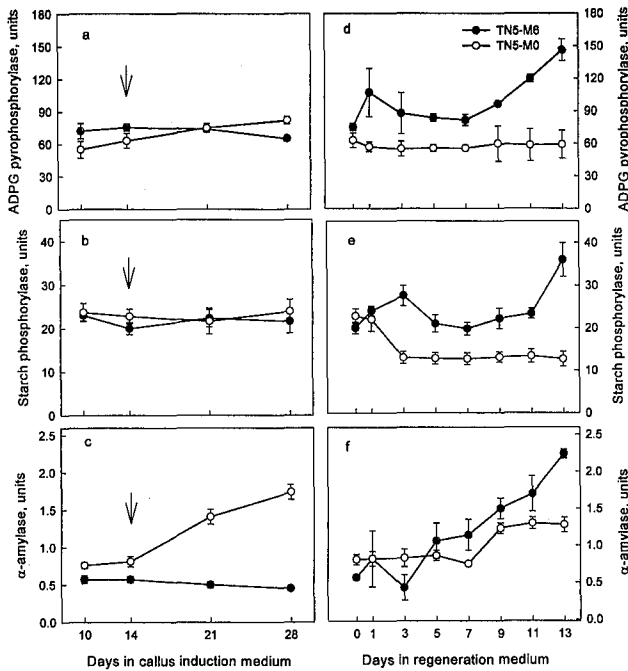


Figure 7. Changes of specific activity of starch metabolism-related enzymes in rice callus at callus induction (a, b, c) and shoot regeneration stages (d, e, f). One unit of AGPase and SPase activity represents 1 μM Pi produced per mg protein, per min. One unit of α -Amy activity represents 1 OD produced per mg protein, per min. The arrows in figure a, b, c, indicate the timing of callus transferred to $\text{MSK}_{20}\text{N}_{10}$ regeneration medium. The symbols in this figure are the same as those in Figure 1. Vertical bars represent standard errors ($n = 3$). Only those standard bars larger than the symbol are shown.

than the non-regenerable callus TN5- M_0 (Huang and Liu, unpublished data), thus suggesting that shoot regeneration is closely related to cellular water status. This osmotic requirement for embryogenesis or organogenesis has also been found in carrot (Wetherell, 1984) and tobacco (Brown et al., 1979; Brown and Thorpe, 1980). Furthermore, the timing of the osmotic requirement in highly regenerable callus precedes the changes of several physiological reactions and callus morphology. It is speculated that osmotic effect might be correlated to physiological change and shoot regeneration. Brown and Thorpe (1980) have postulated that osmotic adjustment is probably involved in the initiation of organogenesis in tobacco callus. However, the mechanisms of shoot regeneration induced by osmotic stress are still little understood although the increased efficiency of isolated mitochondria for energy production has been observed (Brown and Thorpe, 1982).

Higher soluble sugar and starch contents were accumulated in TN5- M_6 during callus induction. They decreased at the early regeneration stage, and a sudden increase in glucose content was found at the same time (Figure 5). The phenomenon of starch accumulation and disappearance were also observed in tobacco (Thorpe and Murashige, 1968), sugarcane (Ho and Vasil, 1983), carrot

(Wurtele et al., 1988), and *Begonia* (Mangat et al., 1990). The accumulated starch is probably an energy reserve for the high energy process of organogenesis and provides for osmotic agents in the form of free soluble sugars (Thorpe et al., 1986). In our experiments, we found a higher correlation between regeneration ability and glucose content at the initiation stage of shoot regeneration in rice callus (Figure 5e). In our other rice regeneration system, induced by a high concentration of sucrose, we found a similar tendency (Huang and Liu, unpublished data). We, therefore, postulate that the glucose content at the early regeneration stage may be an indicator for shoot regeneration in rice callus. To our knowledge, ours is the first study to mention this relationship. We also conclude here that the starch content at the callus induction stage and the glucose content at the initial stage of shoot regeneration were both important "regeneration-related factors" in rice callus (Huang and Liu, 1998).

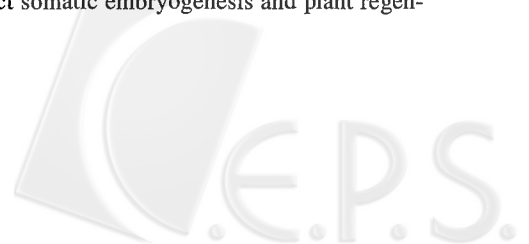
During callus induction in TN5- M_6 , the higher soluble sugar content might be due to the increase of sucrose uptake from the medium resulting from Bound-IT activity (Figure 6b). However, the higher starch content was mainly caused by lower degradation through α -Amy (Figure 7c). Increased IT activity promoted by osmotic or water stress has been reported in pea (Castrillo, 1992), sweet potato (Wang et al., 1999), and *Craterostigma plantagineum* (Schwall et al., 1995). In addition, higher soluble sugars probably inhibit α -Amy expression and cause starch accumulation. It has been demonstrated that the expression of α -Amy is enhanced by sugar deficit and reduced by sugar supply in rice suspension cells (Yu et al., 1991; 1992). Moreover, the mechanism of starch accumulation in rice callus is different than in tobacco and carrot systems. The accumulated starch in these two culture systems is caused by increasing biosynthesis (Thorpe and Meier, 1974; Wurtele et al., 1988).

According to the enzyme analysis, it is suggested that the higher glucose content during the first day on regeneration medium in TN5- M_6 callus results from reserved sucrose and starch degradation and uptake from culture medium. Bound-IT is responsible for sucrose uptake from the medium either at the callus induction or shoot regeneration stages. It is closely related to cellular carbohydrate content and the subsequent shoot regeneration. Further research is necessary to clarify the roles of Bound-IT and the following metabolism of glucose in rice during callus induction and shoot regeneration.

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滲透壓誘導水稻癒合組織植株再生過程中碳水化合物之代謝

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本研究主要探討滲透壓誘導水稻癒合組織植株再生過程中，細胞生長與碳水化合物之代謝情形。結果發現，若把水稻未成熟胚培養在含 10 μ M 2,4-D 及 0.6 M 甘露糖醇 (mannitol) MS 培養基中誘導癒合組織，其鮮重、水分含量、及細胞內水勢能與滲透勢能明顯低於培養在不含甘露糖醇培養基中，若將此癒合組織移入分化培養基後，植株再生率大幅提高至 75% 左右。此外，此高分化能力的細胞在癒合組織誘導階段，可溶性糖及澱粉含量顯著增加，主要是因為蔗糖吸收增加及澱粉分解減少所致。另外，植株再生階段初期，細胞內葡萄糖含量急速增加，可能是來自於培養基吸收及貯存於細胞內澱粉與蔗糖分解而來，此高葡萄糖現象，亦可作為水稻癒合組織植株再生與否的指標。

關鍵詞：碳水化合物代謝；滲透壓逆境；水稻；再生相關因子；植株再生。

