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Osmotic stress stimulates shoot organogenesis in callus of rice (*Oryza sativa* L.) via auxin signaling and carbohydrate metabolism regulation

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Abstract This study aimed to clarify the possible mechanism of endogenous phytohormone signaling and carbohydrate metabolism during shoot organogenesis induced by osmotic stress in rice (*Oryza sativa* L. cv. Tainung 71) callus. Non-regenerable calli derived from Tainung 71 immature embryos were inoculated on Murashige and Skoog medium containing 10 μM 2,4-D. They turned to highly regenerable calli (HRC) (regeneration frequency more than 75 %) with lower calli fresh weight and water content when 0.6 M sorbitol was supplemented into the medium. The regeneration frequency was prominently decreased to 25 % while an auxin transport inhibitor, 2,3,5-triiodobenzoic acid (TIBA), was added into the sorbitol-treated medium. It suggested that endogenous auxin signal may be involved in the induction of HRC under osmotic stress treatment. As well, HRC showed high levels of glucose, sucrose, and starch and high expression of *cell wall-bound invertase 1*, *sucrose transporter 1* (*OsSUT1*), *OsSUT2*, *PIN-formed 1*, and *late embryogenesis abundant 1* (*OsLEA1*) genes. Their expressions are all dramatic inhibited except *OsLEA1* under TIBA treatment. It suggests a key role of auxin may be linked to the effect of shoot regeneration under osmotic stress treatment. Therefore, we present a putative hypothesis for regenerable calli induction by osmotic stress treatment in rice. Osmotic stress may regulate endogenous levels of auxin interacting with abscisic acid, then affect carbohydrate metabolism to trigger callus initiation and further shoot regeneration in rice.

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Abbreviations

ABA	Abscisic acid
AnA	Anthranilic acid and ABA combined
2,4-D	2,4-dichlorophenoxyacetic acid
CIM	Callus induction medium
CIN	Cell wall-bound invertase
HRC	Highly regenerable calli
IAA	Indole-3-acetic acid
LEA	Late embryogenesis abundant
MS	Murashige and Skoog
NAA	1-Naphthalene acetic acid
NRC	Non-regenerable calli
ORR1	*Oryza sativa response* regulator 1
PGRs	Plant growth regulators
PIN	PIN-formed protein
RM	Regeneration medium
SUT	Sucrose transporter
TIBA	2,3,5-Triiodobenzoic acid

Introduction

Cyto-differentiation is a complex morphological transition process in plant tissue culture. Plantlets regenerated through the embryogenic or organogenic pathway is well established in hundreds of plant species. However, the mechanism of totipotency is still less understood. Many factors affect shoot regeneration in plant tissue culture: such as genotype (Huang et al. 2002; Glowacha et al. 2010; Park et al. 2011), exogenous and endogenous hormones (Jiménez 2005; Barreto et al. 2010; Sun and Hong 2010;

Huang et al. 2012), carbon sources (Huang and Liu 1998; 2002; Iraqi et al. 2005; Huang et al. 2006; Silva 2010; Feng et al. 2010), and osmotic requirements (Geng et al. 2008; Huang and Liu 2002; Pan et al. 2010; Huang et al. 2012). Despite many shoot regeneration and transformation protocols developed in rice culture, the regeneration frequency is low and varies highly among cultivars (Al-Khayri et al. 1996; Huang et al. 2002; Hoque and Mansfied 2004; Khaleda and Al-Forkan 2006; Zhao et al. 2011). The regeneration ability of non-regenerable rice callus could be promoted by treatment with an osmotic agent such as sorbitol or mannitol (Huang and Liu 2002; Huang et al. 2002; Geng et al. 2008; Feng et al. 2011). Osmotic stress affects plant cells growth and physiological metabolism. Some kinds of compatible solutes are accumulated under osmotic stress treatment for example abscisic acid (ABA), free amino acids, and soluble sugars (Wang et al. 1999; Huang and Liu 2002; Jiménez 2005). However, the mechanism of osmotic stress inducing shoot regeneration has not been well investigated.

During tissue culture, exogenous carbohydrates are the main energy sources in the medium. Numerous studies have focused on the effects of different kinds and concentrations of supplemented carbohydrates for cell differentiation (Iraqi et al. 2005; Feng et al. 2010; Geng et al. 2008; Silva 2010). There are only scarce studies discussed the signaling and metabolic pathway of carbohydrates during cell culture (Schmitz and Lorz 1990; Huang and Liu 1998, 2002; Huang et al. 2006). Sucrose is generally used as the main exogenous carbohydrate source as well as osmotic agent in plant tissue culture; sucrose uptaken from the medium in explants is hydrolyzed into glucose and fructose for subsequent metabolism. Thus, cell wall-bound invertase (CIN) and sucrose transporter (SUT) were considered the main routes for sucrose uptake and transportation. CIN is involved in early seedling development, inflorescence differentiation, and grain filling in plants (Roitsch 1999; Hirose et al. 2002; Cho et al. 2005; Ji et al. 2005; Wang et al. 2008, 2010). SUT was found to have similar functions as CIN; it was also related to seed development and plant growth (Kaur et al. 2000; Scofield et al. 2007; Chen et al. 2010; Siao et al. 2011; Siahpoosh et al. 2012). However, the effects of these sucrose metabolism-related genes during cell culture under osmotic stress treatment in rice are still unknown. In our previous studies, the cellular carbohydrate contents were increased and metabolism-related enzyme activities were modulated by osmotic stress. They were highly related to shoot organogenesis but the underlying molecular mechanism was still unclear (Huang and Liu 2002; Huang et al. 2006).

Plant growth regulators (PGRs) have an important role in cell development. Many studies have shown the effects of PGRs on tissue culture (Jiménez 2005; Yin et al. 2008; Zhang et al. 2008; Barreto et al. 2010; Feng et al. 2010; Huang et al. 2012). Auxin and cytokinin are considered key factors to shoot differentiation in callus culture (Skoog et al. 1965; Pernisová et al. 2009; Su et al. 2009; Cheng et al. 2010; Vanneste and Friml 2009; Zhao et al. 2010). Besides, though ABA is considered an inhibitor of plant growth, while acting with other PGRs, it has a positive effect on plantlet development (Rai et al. 2011; Huang et al. 2012). In our previous studies, endogenous auxin, zeatin and ABA were at high levels in highly regenerable rice callus (Liu and Lee 1996; Huang et al. 2012). Auxin might be the main factor controlling cell differentiation (Bassuner et al. 2007; Petrásek and Friml 2009; Rademacher et al. 2012). Our previous studies indicated that endogenous auxin levels in rice calli may play critical roles during shoot regeneration (Huang et al. 2012). However, how endogenous auxin changes affect regenerable calli induction and shoot regeneration is still unknown.

Again, many studies have been indicated the expression levels of plant hormone-responsive genes could represent the endogenous levels of hormones (Mason et al. 2005; Xu et al. 2006; Huang et al. 2010; Shih et al. 2010). The auxin efflux carrier gene family, PIN-formed (PINs), is the key factor for auxin polar transport (Petrásek et al. 2006; Wang et al. 2009). *PIN* gene expression may represent auxin accumulation level (Xu et al. 2006; Huang et al. 2010). *OsPIN1* is detected in rice calli (Xu et al. 2006) and is related to organogenesis (Huang et al. 2010; Wang et al. 2009). Similarly, B-type response regulator (B-RR) proteins are positive signal regulators for cytokinin signaling (Müller and Sheen 2007) and the expression level of B-RR gene can be representive of endogenous cytokinin level (Mason et al. 2005). The B-RR *Oryza sativa response regulator (ORR1)* affects cytokinin signaling in rice (Ito and Kurata 2006). Late embryogenesis abundant (LEA) proteins are an ABA-dependent protein family. The proteins can be detected in embryo and tissue with water stress (NDong et al. 2002; Grelet et al. 2005; Shih et al. 2010). Because *OsLEA1* can be detected in rice callus and is an ABA-induced gene (Shih et al. 2010), the gene expression could present as the endogenous ABA level.

Many studies discussed the possible role of phytohormones, sugar sensing, and osmotic stress during shoot organogenesis, respectively (Huang and Liu 2002; Huang et al. 2002; Hartig and Beck 2006; Pernisová et al. 2009). However, no reports have elucidated the underlying mechanism among these factors. In this study, we present a working hypothesis to clarify the possible mechanism of endogenous phytohormone signaling and carbohydrate metabolism during shoot organogenesis induced by osmotic stress in rice callus. The callus growth and shoot organogenesis frequency were measured under osmotic stress treatment. The auxin transport inhibitor, 2,3,5-

triiodobenzoic acid (TIBA) was added into the sorbitol-containing medium to clarify the possible role of endogenous auxin on shoot regeneration (Liu and Lee 1996). The cellular carbohydrate contents were determined and the gene expression profiles of sucrose-uptake enzymes and plant hormone-responsive genes were further analyzed.

Materials and methods

Plant material, callus induction, and shoot regeneration

The most popular aromatic rice cultivar (*Oryza sativa* L. cv. Tainung 71; TNG71) in Taiwan was used in this study. Primary calli derived from 12 to 14 day-old immature seeds were inoculated on three different callus induction media (CIM): control, MSD_{10} (MS basal medium (Murashige and Skoog 1962) containing 3 % sucrose, 10 μM 2,4-D); osmotic stress treatment, $MSD_{10}S_6$ (MSD_{10} medium supplemented with 0.6 M sorbitol) (Huang et al. 2012); and $MSD_{10}S_6T_5$ ($MSD_{10}S_6$ medium with 5 μM TIBA). TIBA is a common inhibitor for indole -3-acetic acid (IAA) transportation (Liu and Lee 1996). After 2 weeks, calli were transferred to shoot regeneration medium (RM) composed of MS basal medium plus 3 % sucrose, 20 μM kinetin and 10 μM 1-naphthalene acetic acid (NAA). In our previous studies showed that regeneration frequency will gradual decrease following the cultural period in CIM. Thus, the 14-day-old callus derived from CIM is suitable for biochemical analysis and shoot regeneration (Huang and Liu 2002). Both culture stages were maintained at 27–28 °C and 200 μM photons m^{-2} s^{-1} with a 12 h light/12 h dark photoperiod. Because calli <7 days old are too small and difficult to collect, they were harvested and weighed as fresh weight only at the 10th and 14th days in CIM. The collected calli were dried in a ventilating oven at 80 °C for 48 h to constant weight. Water content (%) determination and shoot organogenesis frequency (%) evaluation were performed according to our previous studies (Huang et al. 2012; Lee and Huang 2013). The results were obtained from at least three independent experiments.

Extraction and determination of sucrose, glucose, and starch

Samples were harvested and weighed after inoculation at the 4th, 7th, 10th and 14th days on CIM and the 1st, 3rd, 5th and 7th days on RM. The dried samples were extracted twice with 80 % ethanol. The supernatant and pellet were used for soluble sugars (sucrose and glucose) and starch measurement, respectively (Huang and Liu 2002). The Glucose Assay Kit (GAGO-20, Sigma, USA) was used for glucose content determination. All the preparation and determination procedures are done according to Lee and Huang (2013). Each sample was tested at least 3 times.

RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from collected samples by the TRIzol reagent method (Invitrogen, USA) and treated with TURBO DNA-free DNase (Ambion, TX, USA) to remove residual genomic DNA (Lee and Huang 2013). First-stranded cDNA was synthesized from 1 μg total RNA with use of an oligo-dT primer (ImPro-II Reverse Transcription System, Promega, USA). An aliquot of the first-stranded cDNA mixture corresponding to 10 ng total RNA was used as a template. qRT-PCR involved the IQ^2 Fast qPCR System (Bio-Genesis) on the ECO real-time PCR machine (Illumina, USA). PCR amplification was 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, then, 95 °C for 15 s and 55 °C for 15 s for melting curve identification. To increase the specificity of gene amplification, primer sets were designed with use of Vector NTI (v9.0) with the 3′ UTR sequence for each gene. Relative mRNA expression of target genes was normalized to that of an internal control, *OsUBI* (D12629), and calculated as $2^{-\triangle\triangle Cq}$ values in comparison to unstressed MSD_{10} calli (Livak and Schmittgen 2001; Yin et al. 2009). The NormFinder program was used (http://moma.dk/normfinder-software) to normalize the expression levels of all target genes. All the gene-specific primers information is described at previous study (Lee and Huang 2013). All the amplified sequences are single product and the sequences are corrected after commercial DNA sequencing service (data not shown). All analyses involved three replicates of amplification with three independent batches of total RNA samples.

Statistical analysis

Results are shown as mean ± SE from at least three independent experiments. Data were analyzed by Fisher's least significant difference (LSD) test with SPSS v17.0 for Windows (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Osmotic stress affects callus growth, water content, and organogenesis frequency

To understand the effects of osmotic stress and auxin transport inhibitor TIBA on TNG71 rice callus induction and growth, we examined the fresh weight and water content during callus induction with different media. The

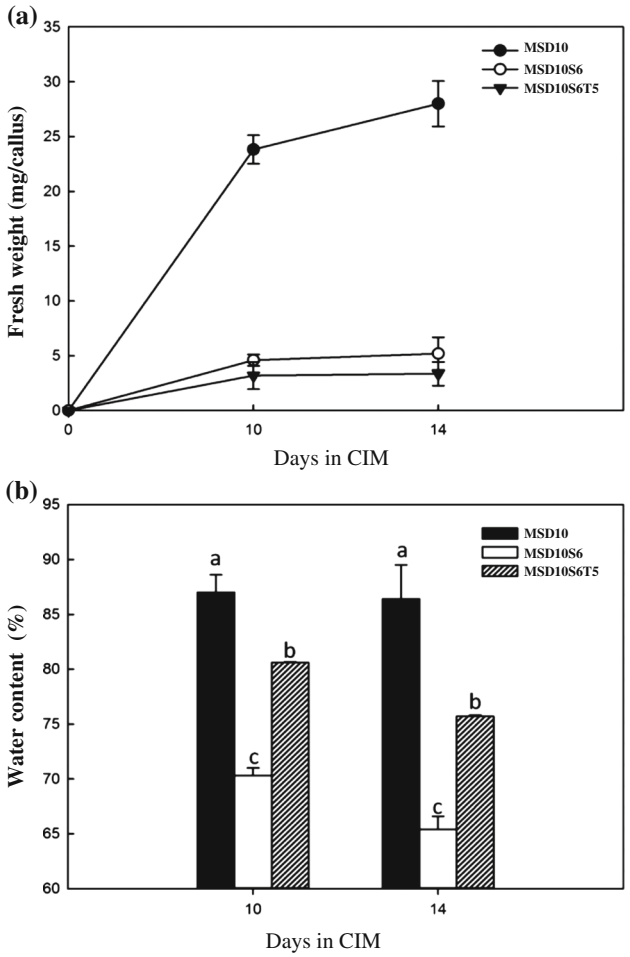

Fig. 1 Callus fresh weight (**a**) and water content (**b**) of rice Tainung 71 calli from 12 to 14-day-old immature seeds inoculated on callus induction medium (CIM). MS containing 10 μM 2,4-D alone (MSD_{10}; control), MSD_{10} supplemented with 0.6 M sorbitol ($MSD_{10}S_6$), or 0.6 M sorbitol and 5 μM TIBA ($MSD_{10}S_6T_5$). Data are mean ± SE (n = 3). *Bars* with different *lowercase letters* indicate significant difference at 5 % level

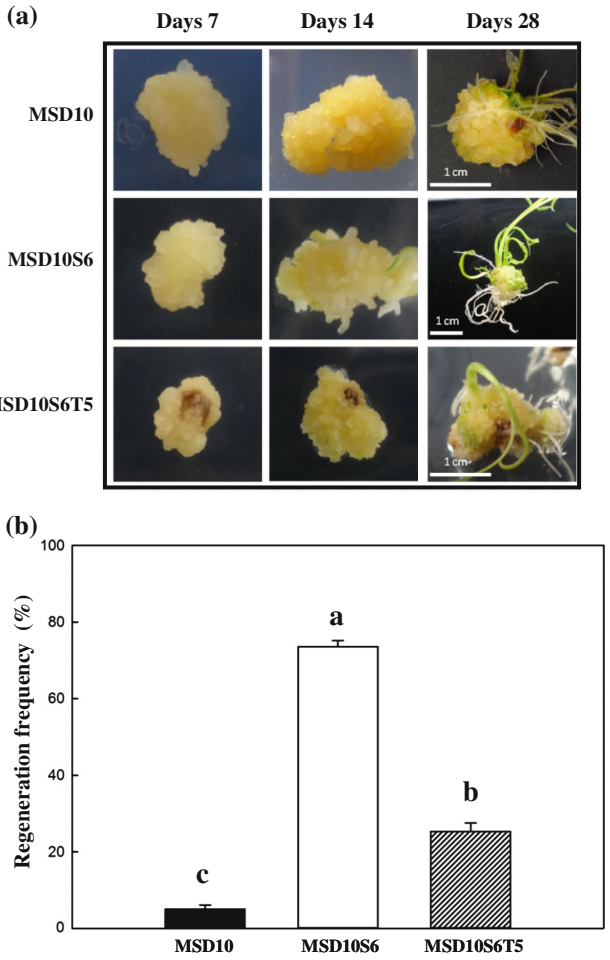

Fig. 2 Shoot organogenesis of 14-day-old callus induced from MSD_{10}, $MSD_{10}S_6$, and $MSD_{10}S_6T_5$ medium transferred to regeneration medium (RM) for 4 weeks. **a** Morphology, **b** shoot organogenesis frequency (%). Plantlets taller than 1 cm were recorded. Data are mean ± SE (n = 3). *Bars* with different *lower case letters* indicate a significant difference by LSD test at 5 % level

callus started to initiate from immature seeds on MSD_{10} medium at the 4th days and continued to enlarge after the cultural period; however, callus from $MSD_{10}S_6$ and $MSD_{10}S_6T_5$ medium did not appear until at the 10th days. The mean fresh weight of each callus at the 14th day was approximately 28.3 ± 2.4 mg. However, the callus fresh weight with $MSD_{10}S_6$ and $MSD_{10}S_6T_5$ medium was less increased, with fresh weight at the 14th day being 5.0 ± 0.8 and 4.2 ± 0.6 mg, respectively (Fig. 1a). The calli initiation and formation were severely disrupted when the immature embryos were inoculated on $MSD_{10}T_5$ medium (data not shown). We are thus omitted this treatment in the following experiment. The water content of MSD_{10} callus was >85 % and showed no significant fluctuation during callus induction (Fig. 1b). In contrast, water content decreased to 70 and 65 % at the 10th and 14th days, respectively, in callus from $MSD_{10}S_6$ medium. Moreover, the water content was slightly enhanced to 80 and 77 % with TIBA supplemented into $MSD_{10}S_6$ medium at the 10th and 14th days (Fig. 1b).

$MSD_{10}S_6$-derived calli showed green spots and shoot primordia emerging at the 10th–14th days, with multiple shoots were seen at the 28th days after transfer to RM (Fig. 2a), and the organogenesis frequency was approximately 75 % (Fig. 2b). $MSD_{10}S_6T_5$-derived calli showed no shoot primordia emerging at the 14th days and the organogenesis frequency was only about 25 %. However, when MSD_{10} calli were transferred to RM, the callus was quickly amplified and showed many regenerated adventitious roots. The regeneration frequency was <3 % (Fig. 2). Besides, the regenerated shoot numbers per explant is approximate 2.3 shoots in $MSD_{10}S_6$ but is only 1.4 shoots in $MSD_{10}S_6T_5$. Therefore, the osmotic-induced shoot

regeneration ability was highly related to callus growth and cellular water status (Huang and Liu 2002; Huang et al. 2002). The relationship between shoot regeneration and cellular water status was also showed in the regeneration system induced by exogenous of ABA and IAA precursor, anthranilate, and combined treatment (Huang et al. 2012). The inhibition of callus growth was intensified by extra TIBA treatment but not water content. Therefore, the callus derived from sorbitol-containing medium might have some compatible solute accumulation, including carbohydrates, and would be affected by IAA signals. The difference in shoot regeneration ability in rice callus may be mediated by carbohydrate metabolism efficiency and levels of phytohormones.

Relation of carbohydrate content and shoot organogenesis ability

To clarify the relationship between shoot organogenesis and carbohydrate metabolism, we examined glucose, sucrose and starch contents at callus induction and early shoot regeneration. Glucose, sucrose and starch contents were low and did not significantly fluctuate at callus induction or early shoot regeneration stage in MSD_{10} calli; however, glucose, sucrose and starch contents were significantly increased in sorbitol-treated calli ($MSD_{10}S_6$) during callus induction (Fig. 3a, b, c). The accumulated carbohydrates were gradually consumed and were maintained at higher levels in $MSD_{10}S_6$- than MSD_{10}-derived calli after transfer to RM at the 7th days (Fig. 3d, e, f). When TIBA was supplemented into the medium ($MSD_{10}S_6T_5$), the starch content was markedly decreased during the whole evaluation period. As well, the levels of glucose and sucrose were gradually decreased and similar to the contents with MSD_{10} at the late callus stage (Fig. 3a, b, c). Glucose, sucrose, and starch contents of $MSD_{10}S_6T_5$-derived calli slowly increased but were still lower than in $MSD_{10}S_6$-derived calli after transfer to RM, except for glucose content at the 3rd days and later (Fig. 3d, e, f). The correlation between carbohydrate metabolism and regeneration ability induced by osmotic stress had been mentioned (Huang and Liu 2002; Huang et al. 2006). We also found that higher soluble sugars content under osmotic stress treatment prominently is caused by the increase activity of cell wall-bound invertase and the uptake of sucrose from the medium. However, higher starch content was mainly caused by lower degradation through α-amylase (Huang and Liu 2002). It suggested that osmotic stress might have an effect on sucrose uptake and hydrolysis from the medium related to callus growth and cell differentiation. The gene expressions of sucrose metabolism related enzymes were further measured below.

Gene expression of cell wall-bound invertase (OsCIN1) and sucrose transporters (OsSUT) in rice calli

We determined the mRNA expression of *OsCIN1* and *OsSUTs* during callus induction and early shoot regeneration to identify the possible roles of sucrose metabolism on cell differentiation induced by osmotic stress. The expression of *OsSUT3, OsSUT4,* and *OsSUT5* was low and did not differ among all treatments (data not shown). Therefore, we compared only the expression profiles of *OsSUT1* and *OsSUT2*. The expression of *OsCIN1* and *OsSUT2* in MSD_{10} calli were low and gradually decreased; however, that of *OsSUT1* was low and slightly increased in CIM (Fig. 4a, b). In contrast, the expression of *OsCIN1* and *OsSUT1* was markedly enhanced with sorbitol supplemented into CIM, especially on the 14th days. However, the level of *OsSUT2* tended to decrease in CIM (Fig. 4d). The expression of *OsCIN1*, *OsSUT1* and *OsSUT2* was repressed and similar to the levels with MSD_{10} when TIBA was included in the $MSD_{10}S_6$ medium (Fig. 4a, b, c).

After transfer to RM, the expression profiles of *OsCIN1*, *OsSUT1* and *OsSUT2* in MSD_{10} calli were still low and did not significantly fluctuate. However, levels of these 3 genes were greatly enhanced in $MSD_{10}S_6$-derived calli in RM. *OsCIN1* and *OsSUT2* were upregulated during shoot regeneration; *OsSUT1* expression was slowly down-regulated after transfer to RM, but the expression was still much higher than in MSD_{10}-derived calli (Fig. 4d–f). The expressions of *OsSUT1* and *OsSUT2* were severely inhibited by TIBA treatment in CIM and RM, even though the inhibitor was not included in the RM (Fig. 4b, c, e, f). However, the *OsCIN1* expression was only slightly inhibited (Fig. 4a, b). Changes in these genes expression levels suggested that osmotic stress may upregulate *OsCIN1* and *OsSUT1* expressions to increase sucrose uptake from the medium and result in the accumulation of cellular sucrose, glucose, and starch.

Sorbitol affected cytokinin, auxin, and ABA signaling to promote shoot organogenesis

In previous studies, we found high levels of endogenous IAA and ABA but low levels of zeatin/zeatin riboside in highly regenerable rice calli induced by osmotic stress would quickly decrease after transfer to RM (Huang et al. 2012). To clarify the relationship between plant hormone signals and shoot organogenesis under osmotic stress in rice calli, we determined the expression patterns of the auxin efflux carrier *OsPIN1*, B-type response regulator of cytokinin signaling *ORR1*, and ABA-induced late embryogenesis abundant *OsLEA1*. At callus induction, *OsPIN1* had the highest expression at the 4th days in MSD_{10} medium then gradually decreased, but the opposite was observed in $MSD_{10}S_6$-derived

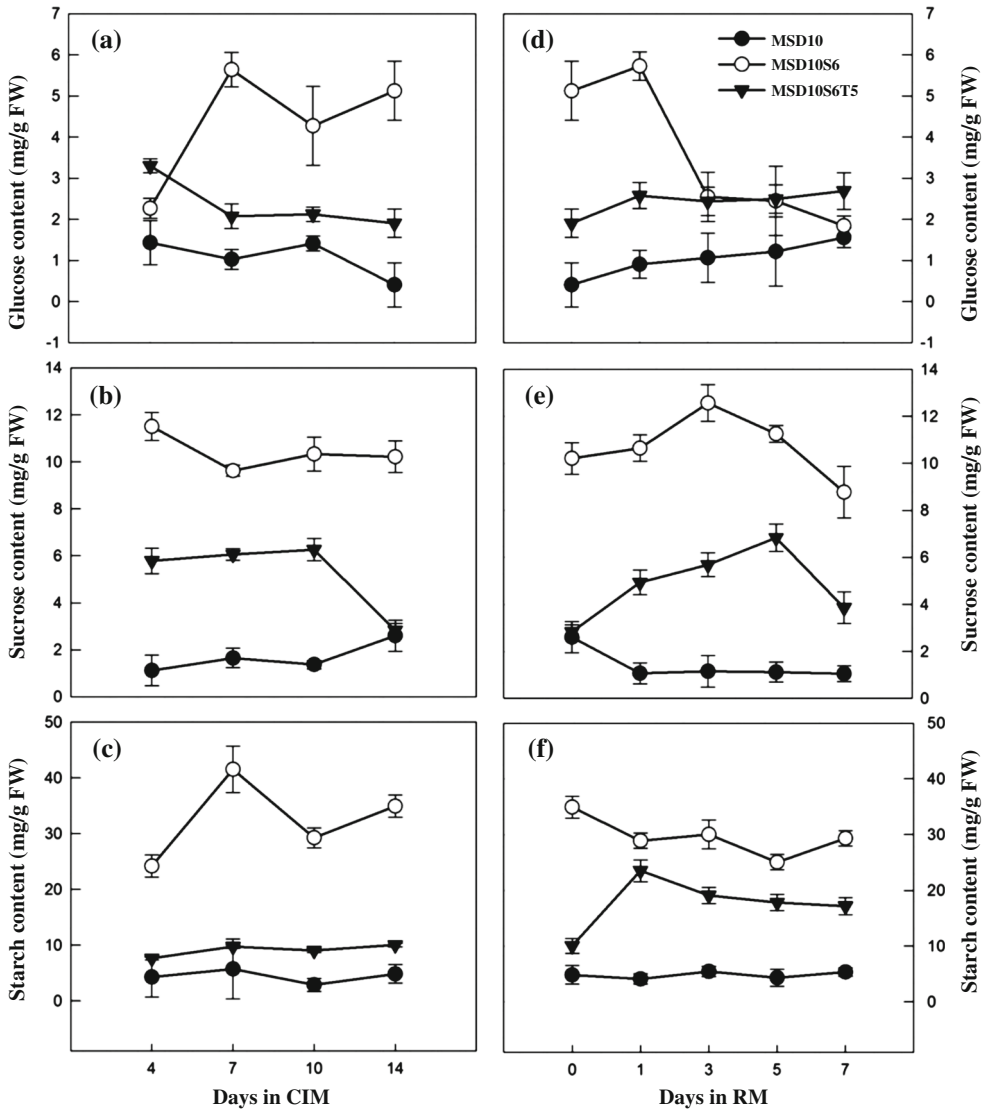

Fig. 3 Carbohydrate content during callus induction stage in TNG71 rice. Glucose, sucrose and starch content in calli inoculated in CIM (**a–c**) and after transfer to RM at 7 days (**d–f**). Data are mean ± SE (n = 3)

calli (Fig. 5a). The expression of *OsPIN1* could be enhanced by long-term sorbitol treatment (>14 days). As well, the enhancement was blocked by TIBA supplemented into the medium. In MSD_{10}-derived calli, *ORR1* showed the highest expression at the 10th days and was quickly reduced, while the expression was inhibited in $MSD_{10}S_6$-derived calli during callus induction (Fig. 5b). In $MSD_{10}S_6T_5$-derived calli, the expression of *ORR1* did not differ during the valuation period. Moreover, the expression of *OsLEA1* in $MSD_{10}S_6$- and $MSD_{10}S_6T_5$-derived calli gradually increased in CIM but was barely detected in MSD_{10} during the whole evaluation period (Fig. 5c). Osmotic stress may trigger endogenous ABA accumulation and induce the expression of *OsLEA1*.

The expression of *OsPIN1* was higher in MSD_{10}- than $MSD_{10}S_6$- and $MSD_{10}S_6T_5$-derived calli at day 1 after transfer to RM (Fig. 5d). The expression was gradually decreased with all media (Fig. 5d). However, the expression of *ORR1* was enhanced during shoot regeneration in $MSD_{10}S_6$- and $MSD_{10}S_6T_5$-derived calli but only temporally enhanced in MSD_{10}-derived calli at day 3 in RM (Fig. 5e). In addition, the expression of *OsLEA1* in MSD_{10}-, $MSD_{10}S_6$-, and $MSD_{10}S_6T_5$-derived calli was very low and did not differ in RM (Fig. 5f). Thus, auxin may have a key role in regenerable calli induction under osmotic stress treatment.

Discussion

We aimed to clarify the possible mechanism of endogenous phytohormone signaling and carbohydrate metabolism

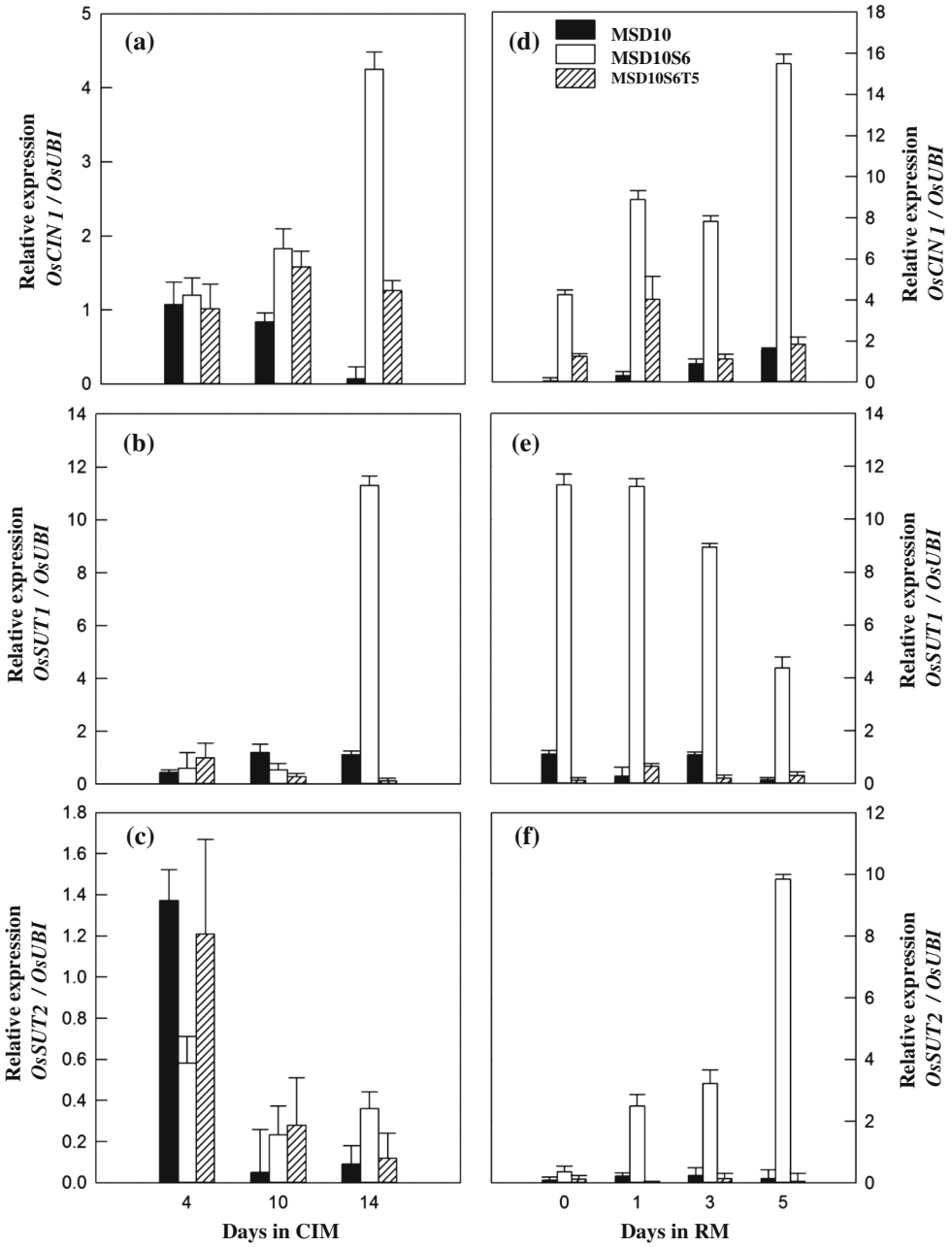

Fig. 4 Real time-PCR analysis of mRNA levels of *OsCIN1*, *OsSUT1* and *OsSUT2* in TNG71 during callus induction and early shoot regeneration. mRNA expression in CIM (**a–c**) and RM (**d–f**). The levels were normalized to that at day 0 in TNG71. *Ubiquitin* level was used as a reference. Data are mean ± SE (n = 9)

during shoot organogenesis induced by osmotic stress in rice callus. Organogenic frequency was increased to 75 % with 0.6 M sorbitol but decreased to 25 % with TIBA supplementation. As well, highly organogenic callus showed high levels of glucose, sucrose, and starch. The expression of *OsCIN1*, *OsSUT1* and *OsSUT2* was increased in sorbitol-treated calli and reduced with non-sorbitol treatment or TIBA supplementation. The changes in expression of *OsPIN1* and *OsLEA1* during culture confirmed the effect of auxin on shoot regeneration. Thus, osmotic stress might regulate endogenous levels of auxins interacting with cytokinin and abscisic acid, then affect carbohydrate metabolism to trigger callus initiation and further shoot regeneration in rice.

Although shoot regeneration systems are well established and applied to produce transgenic plants in rice callus culture, the regeneration frequency is still very low and differs significantly among cultivars (Huang et al. 2002; Khaleda and Al-Forkan 2006; Dabul et al. 2009). Only rarely cultivars of rice can be used for considerable transformant production. Carbohydrates, phytohormones, genotypes and osmotic requirements affect shoot regeneration in rice callus. However, the cross-talk among these factors is still less discussed, especially at the molecular level.

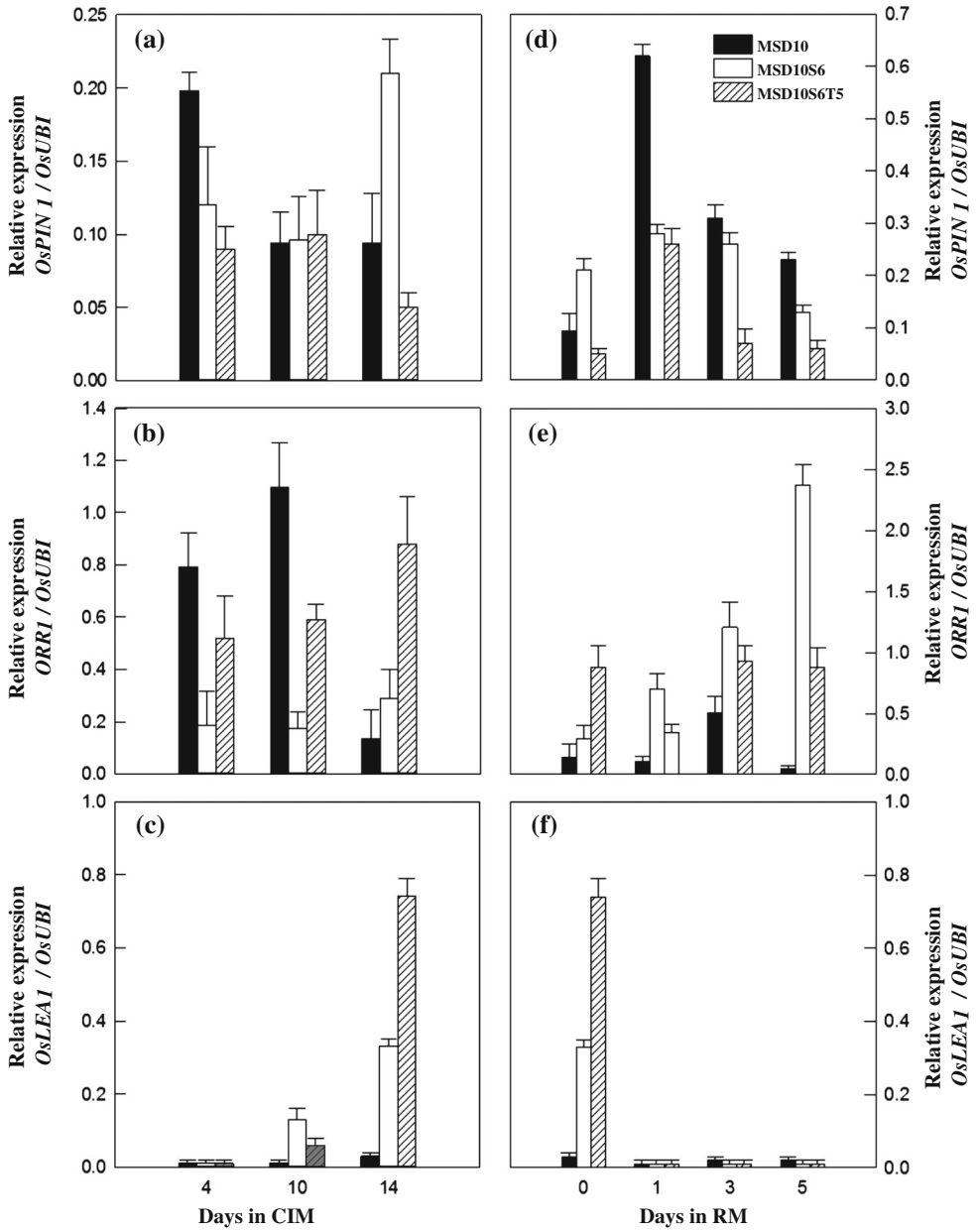

Fig. 5 Real time-PCR analysis of mRNA levels of *OsPIN1*, *ORR1* and *OsLEA1* in TNG71 during callus induction and early shoot regeneration. mRNA expression in CIM (**a–c**) and RM (**d–f**). The levels were normalized to that at day 4 in TNG71. *Ubiquitin* level was used as a reference. Data are mean ± SE (n = 9)

In the past decade, we have endeavored to establish a highly efficient regeneration system and tried to clarify the possible mechanisms of totipotency in rice callus (Huang and Liu 1998; 2002; Huang et al. 2002, 2006, 2012; Lee and Huang 2013). Two cultural steps, embryogenic and/or organogenic callus induction and shoot regeneration, are necessary to enhance the regeneration system from rice explants. Plantlets can be regenerated from rice callus via both somatic embryogenesis and organogenesis but mainly through organogenesis (Huang and Liu 2002; Huang et al. 2006). Exogenous PGRs, especially auxins, applied to induce shoot regeneration, interact with endogenous tissue-specific hormones; thus, the level of endogenous hormones in cultured explants and derived callus are considered the most important factor in shoot regeneration (Valdés et al. 2001; Souza et al. 2003; Zhang et al. 2008; Huang et al. 2012). However, most rice cultivars have low regeneration ability (<5 %) with calli derived from MS basal medium containing 2,4-D alone in CIM (Huang et al. 2002). Exogenous ABA and the IAA precursor anthranilic acid can enhance shoot regeneration frequency to 10 and 35 %, respectively. However, the frequency can be improved to

80 % if ABA and anthranilic acid (AnA) are combined (Huang et al. 2012). More interesting, a high concentration of sorbitol (0.6 M) supplemented in CIM can similarly enhance shoot regeneration with AnA treatment (Huang and Liu 2002; Huang et al. 2002, 2012). Sorbitol used as the osmotic agent and supplemented only in CIM but not included in RM is similar to with AnA treatment. Thus, callus induction stage may be more crucial for final plantlet formation, either cell dedifferentiated or re-differentiated to regenerable calli, than shoot regeneration stage. Our previous studies showed that highly regenerable calli under osmotic stress or AnA treatment possess plentiful starch granules (Huang et al. 2006) and high levels of cellular soluble sugars and starch (Huang and Liu 2002; Fig. 3). Therefore, we were interested in the relation among osmotic stress, plant hormone signals, carbohydrate metabolism, and shoot regeneration in rice callus.

Regenerable callus induction in rice is considered independent of treatment with osmotic stress (Huang and Liu 2002; Huang et al. 2002), exogenous PGRs (Huang et al. 2012), carbon sources (Huang and Liu 1998), and carbohydrate metabolism (Huang et al. 2006). The relationship between osmotic stress and endogenous ABA and IAA levels affecting shoot regeneration has been established (Huang et al. 2012). As well, the effect of osmotic stress on the induction of shoot regeneration through carbohydrate metabolism has been clarified (Huang and Liu 2002). Recently, we constructed the link between endogenous hormones and carbohydrate metabolism during callus induction and shoot regeneration (Lee and Huang 2013). In the present study, the shoot regeneration frequency of TNG71 calli could be greatly enhanced by sorbitol treatment as previously described (Huang and Liu 2002; Huang et al. 2012). As well, the gene expression of *OsPIN1* and *OsLEA1* was induced by osmotic stress treatment in CIM (Fig. 5) and was consistent with levels of endogenous IAA and ABA (Huang et al. 2012). In addition, glucose, sucrose, and starch contents were all significantly higher in $MSD_{10}S_6$- than MSD_{10}-derived calli (Fig. 3) perhaps from the high expression of *OsCIN1* and *OsSUTs* (Fig. 4). Increased CIN activity promoted by osmotic stress has been reported in rice (Huang and Liu 2002), pea (Castrillo 1992), and sweet potato (Wang et al. 1999). The expression of *OsSUT2* was induced by wounding and sucrose treatment (Aoki et al. 2003). However, there are still less known of *OsSUTs* and *OsCIN1* on cell differentiation under osmotic stress treatment. The gene expression of sucrose uptake-related enzymes agrees with the expression patterns of IAA- and ABA-responsive genes. However, the regeneration frequency, soluble sugar and starch contents, and expression of *OsCIN1*, *OsSUTs*, and *OsPIN1* were all inhibited when the auxin transport inhibitor TIBA was supplemented into the $MSD_{10}S_6$ medium. Thus, carbohydrate metabolism and cell differentiation induced by osmotic stress might be triggered by endogenous auxin signaling in rice. Moreover, the auxin signal would interact with ABA and/or cytokinin signals to regulate the downstream physiological and biochemical metabolism. Endogenous phytohormones play a major role in the regulation of morphogenesis. The initiation of regeneration from callus may be related to the balance between auxin and cytokinin. Although the exact nature of these hormonal signals may vary between species, the balance in auxin to cytokinin has a consistent effect on the type of regenerated organs (Charriére et al. 1999; Sugiyama 1999; Fernando and Gamage 2000; Mercier et al. 2003; Jiménez 2005; Zhang et al. 2008). In addition, the effect of added auxins and cytokinins has been related to an interaction with other endogenous hormones such as ABA, thus leading to conspicuous changes in development (Lakshmanan and Taji 2000).

Here, we present a possible working hypothesis for shoot regeneration in rice callus induced by osmotic stress (Fig. 6) according to morphological observations and physiological, biochemical, and molecular determination. According to our proposed scheme, the level of endogenous IAA enhanced by osmotic stress treatment would be the original signal to upregulate sucrose uptake from the medium by cell wall-bound invertase and sucrose transporters for callus formation, which would lead to soluble sugar accumulation. In addition, endogenous ABA level would be high at the late callus induction stage in response to cellular starch accumulation (Huang and Liu 2002) and

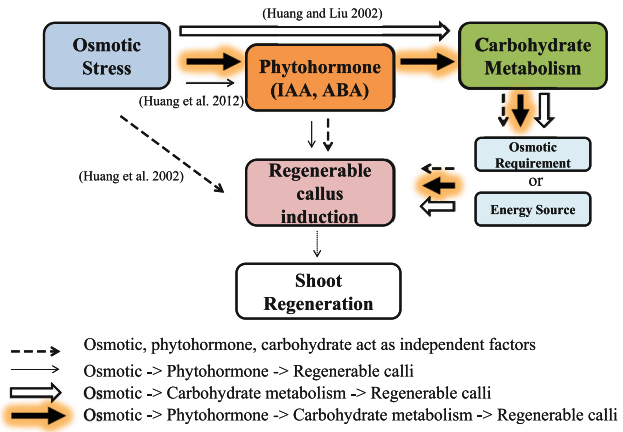

Fig. 6 A possible working hypothesis for shoot regeneration in rice callus induced by osmotic stress. The levels of endogenous IAA and ABA are enhanced by osmotic stress treatment, then sucrose uptake from the medium is increased by cell wall-bound invertase and sucrose transporter, which leads to soluble sugars and starch accumulation. The accumulated carbohydrates would be used as an osmotic signal required for regenerable callus induction and energy sources for further shoot regeneration

regenerable calli differentiation (Jiménez and Bangerth 2001; Nakagawa et al. 2001). The accumulated carbohydrates would be used as an osmotic signal required for regenerable callus induction and energy sources for further shoot regeneration.

In this study, we conclude that auxin might be the key to link the effects of osmotic requirement, carbohydrate metabolism and phytohormone signaling on shoot regeneration. However, the detailed mechanism of how osmotic stress regulates auxin signaling and the role of auxin in carbohydrate metabolism and the other regeneration-related phytohormone signaling still needs to be further studied.

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