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# Cross Talk among Phytohormone Signal and Carbohydrate Metabolism Involving Regenerable Calli Induction under Osmotic Treatment

*Hsiang-Ting Lee and Wen-Lii Huang*

## Abstract

Nonregenerable calli (NRC) derived from immature seeds of japonica rice were inoculated on MS medium containing 10 μM 2,4-D (MSD10). They turned to highly regenerable calli (HRC) when sorbitol was supplemented into the medium. Meanwhile, high levels of endogenous IAA and ABA were accumulated in HRC. Exogenous IAA precursor and ABA in MSD10 have the same effect to enhance regeneration ability. However, there are only partial effects if IAA precursor or ABA was supplemented, respectively. The regeneration ability is prominently decreased from 75% to 25% while an auxin transport inhibitor, 2,3,5-triiodobenzoic acid, was included in the medium. It suggested that endogenous auxin signal and ABA may involve in the induction of HRC. Furthermore, it showed higher contents of glucose, sucrose, and starch and higher expression levels of wall-bound invertase 1, sucrose transporter 1 (OsSUT1), and OsSUT2 genes in HRC than in NRC. The expression levels of PIN-formed 1 and LEA1 were also consistent with the trend of carbohydrate metabolisms. We thus concluded a flowchart for HRC induction by osmotic stress. According to the hypothesis, osmotic stress may regulate endogenous levels of auxin interacting with ABA, then affect carbohydrate metabolism to trigger callus initiation and further shoot regeneration in rice.

**Keywords:** phytohormone, osmotic stress, carbohydrate metabolism, plant regeneration, rice

## 1. Introduction

Totipotency in plant cells allow them to differentiate and regenerate from one single cell into whole plants under conditioned culture [1]. Many factors, i.e., cultivars, carbon sources, phytohormones, and osmotic stress, affect the cell totipotency (**Figure 1**). So far, the regeneration cultures have been successfully established in many plant species including rice, but molecular mechanisms behind this scene are still lots of mist [2–4]. In rice, pluripotent calli were usually induced from seeds or immature embryos cultured on MS medium containing

2,4-D. These calli derived from various cultivars can be classified into two different cell types, nonregenerable callus (NRC) and highly regenerable callus (HRC). After transferred to regeneration medium, HRC can rapidly process shoot organogenesis, but NRC will still retain callus amplification or adventitious root formation [5]. There have been many protocols developed to optimize the induction frequency of NRC, but the shoot regeneration ability still varies among cultivars [6–8].

Previous studies had identified that added osmotic agents like sorbitol or mannitol in callus induction medium can stimulate HRC formation instead of NRC, thus promoting the shoot formation frequency [5, 7, 9–11]. It is still unclear why appropriate osmotic stress during callus induction can promote shoot organogenesis frequency, but there were lots of studies indicating that osmotic stress can stimulate endogenous phytohormone abscisic acid (ABA) accumulation which is also proven to have the function of promoting somatic embryogenesis and shoot organogenesis when it used as an exogenous plant growth regulator in callus culture [11–15]. ABA is widely recognized as a negative plant hormone which mainly functions on stress responses and seed dormancy, but when it is treated in low concentration, ABA could become a positive regulator on root elongation [16]. Although the molecular mechanisms behind this phenomenon are not yet clarified, apparently, ABA has its role in the developmental process.

Other phytohormones, auxin and cytokinin, are also reported to play critical roles in embryogenic callus induction and shoot development [8, 17, 18]. In plants, auxin is considered as a positive growth regulator, which contributes to cell differentiation [19]. High levels of auxin are usually found in shoot and root apical meristems where plants organize their developmental patterns, and so does in HRC [20, 21]. Many studies mentioned that adding different levels of exogenous auxin during callus culture may cause different organogenesis, like low levels of auxin may induce root organ formation while high levels of 2,4-D sustain callus induction [22–24]. Cross talk among auxin and ABA mostly focused on root development or abiotic stress responses [25–30]. Our previous studies had identified that both endogenous auxin and ABA levels were higher in HRC than in NRC and then quickly decreased after transferred to regeneration medium [5, 8]. The interaction and signaling pathway between these two phytohormones to shoot organogenesis are further discussed.
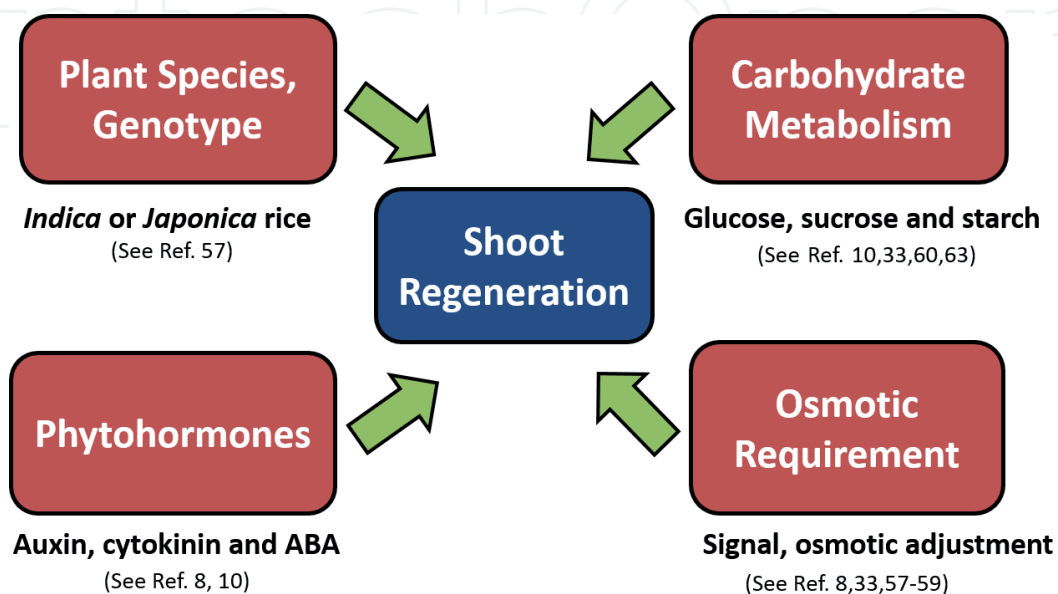


**Figure 1.**
*Factors affecting shoot regeneration in rice.*

Undoubtedly, shoot regeneration is a very complicated process, which is modulated by networks among phytohormones and their downstream metabolic changes in plant cells [31, 32]. In our previous studies, we reported that HRC contained higher levels of glucose, sucrose, and starch, accomplished with higher mRNA transcription levels of cell-wall bound-invertase 1 (CIN1) and SUTs genes [8, 33]. Also, we found that these changes on carbohydrate metabolisms can also be found in osmotic and ABA treated calli, suggesting the possibility of regulation mechanisms on HRC formation [5, 11].

Combined with our findings and previous studies, we proposed a hypothesis for HRC induction in rice callus culture. Osmotic stress may initiate a signal to regulate endogenous auxin to interact with ABA and then stimulate soluble sugars accumulate in callus to trigger embryonic callus formation for shoot regeneration [11].

## 2. Effect of phytohormones on shoot regeneration in rice callus culture

Phytohormones are considered one of the major important factors that control cell fate in callus cultures. Different types and concentrations of auxin treatment may induce different tissue differentiations. Besides, ABA is also considered to play roles in somatic embryogenesis and shoot organogenesis. In this section, we will discuss the functions of auxin and ABA in regenerable callus induction.

### 2.1 Roles of auxin in regenerable callus induction

Auxin is known to play a major role in cell elongation, growth tropisms, and cell fate determination [34]. The main endogenous auxin compound is indole-3-acetic acid (IAA), which could be synthesized via a tryptophan-dependent or -independent pathway [35]. After being synthesized in apical meristems, auxin will be transported to the other tissues through its transporter, PIN-formed proteins (PIN), and AUXIN1/LIKE-AUX1 (AUX/LAX) proteins to deliver the hormonal signal for downstream auxin responses [17, 36]. The distribution of auxin in plants is polarity which means that it is not equally expressed in the whole plant but specifically concentrated in some tissues or limited to cells [36]. Due to this property, scientists are able to monitor the patterns of auxin gradient during plant development [37]. Form the literature, the positions of maximum auxin accumulation were the place processing organ initiation. Therefore, auxin is usually found to have maximum levels in apical meristems or in the developing tissues [38]. During embryogenesis, the expression pattern of PINs dynamically changes within the developmental stages. For example, PIN1 is expressed without polarity until 16-cell stage, but later in 32-cell stage, it will express specifically in the basal part of provascular cells to direct auxin flow to hypophysis. After dividing into transition and early heart embryo stages, the expression of PIN1 will shift to the flank of the apical part, thus accumulating auxin in the edge of apical domain to shape the embryo [39, 40]. Similar developmental patterns can be found during shoot apical meristem (SAM) establishment, where auxin is highly concentrated in leaf primordia and the central region of SAM [18, 41].

In callus, the signal of auxin is mainly located in the central region near callus induction medium. Later, the signal will shift to the surface layer and start SAM establishment for shoot differentiation after transfer into regeneration medium [42]. However, this organogenesis process is blocked when the activity of auxin transporters is inhibited, and so does in auxin sensor *shoot meristemless* (*stm*)

mutants [43]. In our previous studies, we compared endogenous IAA levels between NRC and HRC in rice callus and found that HRC has higher IAA content than NRC, and so does the mRNA expression levels of PIN1, suggesting that HRC has higher auxin sensitivity, which results in higher regeneration ability [5, 8, 11]. Also, there are researches mentioning that overexpression of STM can maintain the somatic embryogenesis frequency even under low concentrations of 2,4-D [44]. Besides, increasing the expression levels of auxin biosynthesis regulator *YUCCA* (*YUC*) genes can also promote shoot organogenesis ability in callus [45]. Similar results can be found in supplement of IAA precursor anthranilic acid (An) in rice callus culture; the shoot regeneration frequency increased by 35% under An treatment, suggesting that a high endogenous auxin level is required for HRC formation [5].

## 2.2 Roles of ABA on regenerable callus induction

ABA is usually considered to play negative roles in plant growth [26, 46]. Except the function of seed dormancy regulation and stress responses, ABA is also reported to have function on root and shoot development [46, 47]. Although there were some genes participating in ABA signaling, which were also reported to involve in the shoot regeneration process, there is still no clarity about the molecular function of endogenous ABA on the shoot organogenesis process [14, 15]. However, some studies, including our previous works, found that ABA was highly accumulated in HRC, but not in NRC [5]. Furthermore, the expression profiles of ABA biosynthesis genes were also upregulated, which match with our results [48].

On the other hand, ABA is reported to stimulate dehydration responses during embryo maturation stages [49]. From the publications, we observed that HRC has less water content and smaller callus size, which is similar to the dehydration phenotype. Thus, it is possible that ABA shared similar regulation mechanisms with embryo maturation during shoot regeneration. Despite the loss of water in HRC, it also showed higher content of soluble sugars [8, 11]. It is already known that high content of sugar may enhance osmotic stress and then stimulate endogenous ABA biosynthesis to ABA responses [33], but the underlying mechanisms to shoot differentiation is still unclarified.

## 3. Cross talk among osmotic stress and phytohormones in callus culture

In cells, osmotic stress could originate from water-deficiency or high salt which caused an imbalance between plastids and apoplast [50]. To achieve tolerant to osmotic stress condition, cells will modulate the content's osmotic adjustments like sugars, potassium ions, or proline to balance its osmotic pressure to environments to avoid collapse. Phytohormone ABA is reported to accumulate under osmotic stress to modulate anion channel *SLOW ANION CHANNEL-ASSOCIATED1* (*SLAC1*) to stomata closure and the potassium transporter KUP to potassium homeostasis [9], while in plant tissue culture, appropriate osmotic stress can help embryonic callus formation [7]. In our case, we found that HRC showed dosage responses to osmotic treatments and has the highest induction frequency under 0.6 M sorbitol treatment. We also noticed that osmotic requirement is various among rice species, some cultivars require higher osmotic stress to induce HRC and some require lower, and even one cultivar can form HRC rapidly without osmotic treatments. We then analyzed the ABA response in those calli and found that HRC does have higher *LATE EMBRYOGENESIS ABOUNDENCE 1* (*LEA1*) gene expressions, which is commonly used as ABA signaling marker. Interestingly, *LEA1* is also upregulated in

the rice cultivar without osmotic stress treatment, indicated the effect of osmotic stress could be on stimulate ABA biosynthesis and its downstream responses during embryonic callus induction [5, 11].

Not only ABA, auxin is also regulated by osmotic stress. In rice, the endogenous levels of auxin are reported to be suppressed under osmotic stress [50, 51]. However, a closer look at the expression levels of different auxin biosynthesis-related genes and the distributions of auxin showed various patterns in the whole plants in different stages, some of them even inconsistent with the overall patterns, suggesting that auxin may function differently among tissues under osmotic stress. As for the patterns of *PINs* under osmotic stress, one of the researches reported that osmotic stress may inhibit the expression of *PIN1* in leaf primordia, thus suppressing shoot development [52]. However, our previous works indicated that *OsPIN1* could be upregulated by 0.6 M sorbitol treatment in HRC, and also in the nonosmotic requirement cultivars [11]. Although there have been many studies performing transcriptomic or proteomic studies of auxin responses under osmotic stress [53, 54], how osmotic stress directs with auxin responses to HRC formation is still less known.

## 4. Roles of carbohydrate metabolisms during HRC induction under osmotic stress treatment

Exogenous carbohydrates are used either as energy sources or osmotic agents in tissue culture. So far, many articles have revealed that different carbon sources may lead to different callus induction abilities [7, 55, 56], but rarely discussed about the carbohydrate metabolisms and signaling pathways in callus cultures. Sucrose is widely used as a main carbon source as well as osmotic agent in rice tissue culture [57, 58]. During the tissue culture, sucrose will be taken up and hydrolyzed into glucose and fructose by CINs, or be transported into cells by SUTs for further application [59]. CINs were already reported to involve in early seeding establishments and grain fillings, and so do the SUTs [60, 61].

According to our studies, HRC induced by osmotic stress seemed to obtain higher contents of glucose, sucrose, and starch than NRC, which also reflected in the higher dry weights [33, 56, 62]. We analyzed the expression patterns of *CIN1* and *SUTs* during rice callus culture. The expression of *CIN1*, *SUT1*, and *SUT2* in HRC was upregulated by osmotic stress, but not in NRC, while in the nonosmotic requirement cultivars, there of these sucrose-uptake genes were expressed earlier than in the cultivar of low-regenerable ability [8, 11]. It is suggested that higher soluble sugars in HRC may be caused from higher sucrose uptake and translocation under stress treatment. Besides, we also observed that osmotic stress induced HRC has lower α-amylase activities and thus increases the content of starches [33], while in nonosmotic requirement cultivar, the callus rather induced ADPG pyrophosphorylase (AGPase) activity to accumulate starches [62]. The results suggested two different regulations on carbohydrate metabolisms to HRC induction. Although there are different ways of starch accumulation in HRC, the accumulated carbohydrates will soon degrade after transplanting the callus onto regeneration medium in 3 days, suggesting that these carbohydrates could be stored as a carbon source in HRC and then used for the developmental process during the regeneration stages [8, 11, 33].

Plants are known to accumulate starch granules specifically in the columella cells [63, 64]. Although the function of these starch granules is mostly reported in root gravity, they could also be markers to point out the stem cell niche, since these starch granules may disappear in the plants with stem cell defect [64]. Our studies also found the accumulation of starch granules in peripheral regions in HRC [62].

High concentration of sorbitol or mannitol will enlarge the distribution of starch granules. It may link to the increase of the shoot organogenesis area [5]. However, the physiological functions and mechanisms of starch accumulation still remained, requiring further studies.

Moreover, the accumulation and metabolism of soluble sugars and starch can be induced by AnA treatment (anthranilic acid and ABA supplemented into the medium together) to replace osmotic stress treatment. High levels of endogenous IAA and ABA at the same time are necessary during HRC induction. Both of them need to decreased suddenly in few days are also an important criteria for further shoot regeneration [5]. To link these metabolic changes with phytohormone regulations, we also introduced auxin transport inhibitor TIBA during callus induction and found that it will inhibit carbohydrate accumulation and result in low shoot regeneration frequency. However, ABA signals seemed to be promoted under TIBA treatment [8, 11]. It is still not clear whether exceeding ABA signals will turn into negative regulator on HRC induction, but these results still indicate that there must be interactions among auxin, ABA, and carbohydrate metabolisms on HRC formation.

## 5. Conclusions

Inducing regenerate tissues from pluripotent cells is a fascinating event. So far, botanists have already shown that they were able to get regeneration plants from callus in many plant species [49, 65–67]. However, why and how plants achieve this process is still unknown, especially in molecular levels. Here, we propose a hypothesis among phytohormone, osmotic stress, and carbohydrate metabolisms on HRC induction based on current knowledge and our findings (**Figure 2**). According to our model, levels of endogenous IAA upregulated by osmotic stress treatment can promote sugar uptake via CIN and SUT, which result in carbohydrate accumulation during callus induction stages. Similar to auxin, endogenous ABA level is also enhanced under osmotic stress, thus modulating starch accumulation during formation of HRC by downregulating $\alpha$-amylase activity. Our studies indicated that exogenous auxin or ABA treatment alone is not sufficient for embryonic or organogenic callus formation, which only increased the plant regeneration rate for 35 and 5%, separately [5]. However, when we combine both ABA and anthranilic acid treatment together, the regeneration frequency can be promoted to 80% similar to osmotic stress treatment, suggesting that there must be some interaction between these two phytohormones. The roles of accumulated carbohydrates in HRC could be used as osmotic agents for further metabolism changes or be consumed as an energy
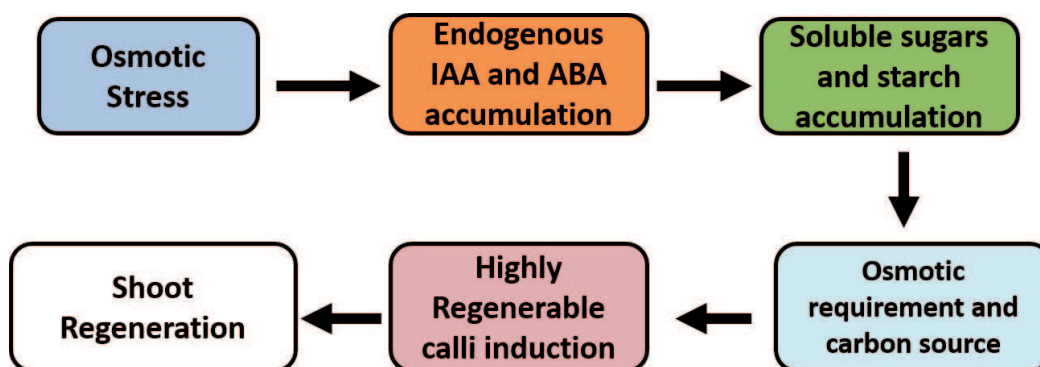


**Figure 2.**
*Working hypothesis of highly regenerable callus induction under osmotic stress treatment in rice.*

source in later regeneration stages. In conclusion, the culture system of shoot regeneration in rice callus is a two-step process. Our studies suggested that induction of highly regenerable callus is more important than different kinds of treatment during the shoot regeneration stage. Besides, osmotic stress triggers a serial of change of endogenous hormone metabolism, sensing, and signal transduction, which leads to increase of sucrose uptake and starch accumulation and provides sufficient carbon source for further shoot regeneration.

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## Conflict of interest

The authors have no conflict of interest.

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