出國報告（出國類別：出席會議）

「2013 水稻功能性基因體國際研討會」出國報告

服務機關：國立嘉義大學
姓名職稱：黃文理　副教授兼系主任
派赴國家：印度
出國期間：中華民國 102 年 11 月 19 至 11 月 24 日
報告日期：中華民國 102 年 12 月 10 日

目次

一、摘要

「水稻功能性基因體國際研討會」（ISRFG）自 2003 年首度於中國上海召開大會以來，目前已成爲全世界水稻研究領域最重要的國際會議，固定於每年 11 月份左右舉辦，今年（2013 年）是第 11 屆大會，於 11 月 20 日至 23 日於印度新德里舉辦，大會主題爲「永續食品與營養安全」，會中針對非生物性逆境、構造與發育、生物性逆境、比較與演化基因體學、表觀基因體學、食品與營養、生物資訊學與系統生物、分子育種、微小 RNA、及轉譯基因體學等十大領域。大會特別邀請 23 位專家發表專題演講，各領域也有 62 篇論文獲選進行口頭宣讀，207 篇論文以海報展示方式發表研究成果，會議同時向『印度綠色革命之父』美名之 Swaminathan 博士致敬，並邀請他發表專題演講。此次會議國內共有來自中研院、台大與嘉大等共 12 位學者專家研究生出席。參加本次國際研討會收穫豐碩，除接收到來自全世界水稻各領域專家最新研究成果資訊外，也與各國與會學者有密切互動，也攜回會議書面資料一份，也對印度風土民情與最新水稻相關研究現況有進一步了解

二、出國目的：

本次出國目的主要是參加第 11 屆水稻功能性基因體國際研討會，自 2003 年於中國上海召開第一屆水稻基因體國際研討會以來，固定每年舉辦一次會議，由世界各國輪流主辦，已成爲全世界水稻界最重要的國際會議與年度盛事，台灣也曾於 2011 年於中央研究院主辦過，今年（2013 年）於印度新德里舉辦，本人執行國科會與農委會計畫多年，將於會中發表研究成果論文，並與世界相關專長領域之專家共同研討，有助於日後相關計畫之執行與教學。

三、出國行程

日期	行程規劃	地點	備註
102.11.19	去程	台灣桃園→印度新德里	
102.11.20-23	報到與參加會議	印度新德里	
102.11.24	回程	印度新德里→台灣桃園	

四、會議過程

(一) 國內出席人員

本次會議國內共有來自中央研究院 3 人（由植微所邢禹依研究員帶領）、台灣大學 8 人與嘉義大學 1 人（本人）等共 12 人與會。

(二) 會議內容

1. 開幕式：

印度是世界人口第二多的大國，稻米是印度人民最重要的主食，雖然到目前爲止，仍有許多印度人民處於糧食與物資不足的飢餓狀態，但隨著經濟逐漸發展，印度政府在農業生產與科技研發部分也投入愈來愈多的心力與經費，目標就是希望能夠提供印度人民基本的糧食需求。因此，藉由承辦本次這場國際研討會，於開幕式時，一方面提升其國內稻作研發科技水平，另外，也特別向印度國內非常受到尊崇，有『印度綠色革命之父』美名之 MS Swaminathan 博士致敬，並邀請他發表專題演講，從第一次綠色革命談到印度目前之糧食與研發概況，另外也一一介紹此次會議來自世界各國主要的聯絡人與專家。

2. 專題演講部分

今年大會主題爲「永續食品與營養安全」(Sustaining Food and Nutritional Security)，

會中針對 Abiotic Stress, Architecture and Development, Biotic Stress, Comparative and Evolutionary Genomics, Epigenomics, Food and Nutrition, Informatics and Systems, Molecular Breeding, Small RNA, and Translational Genomics 等領域安排許多頂尖專家演講與論文發表，其中大會特別邀請 23 位專家發表專題演講，各領域也有 62 篇論文獲選進行口頭宣讀。因應近年來氣候變遷的議題，非生物性逆境與分子育種技術仍是最受矚目的單元，聽眾總是擠滿了演講廳，另外，近年來特別重視根部發育之研究，如果說 20 世紀糧食的增產是因爲地上部（千粒重、穗數等產量構成要素）品種改良的結果，已有許多科學家們預言，21 世紀的糧食增產關鍵會是在根部性狀的改良，這也是目前最熱門的研究主題之一。

除了這些領域之外，因應近年來龐大的 DNA 序列資訊分析，比較生物資訊學與演化生物學研究最重要的工具就是生物資訊軟體分析技術，也成爲目前水稻功能性基因體研究的主流項目，爲增加參與人員對常用的生物資訊軟體的熟悉度，此次研討會特別安排專家導引參與者實際上網操作練習，也是個窩心的安排。

各領域比較受到注意的講題與摘要簡述如下：

(1) 美國 Prof. R.A. Wing: 繼水稻基因體解序計畫之後，由美國主導世界各國共同參與的利用不同基因組成之各野生稻（AA, BB, FF）與栽培稻（亞洲型與非洲型）雜交與全基因體比較，目的在於了解水稻的起源與演化，並探索許多演化過程中被人爲淘汰或消失的有用基因，例如抗逆境與抗病蟲害等等基因，國內由中研院爲代表也參與此計畫，目前已開發出許多生物資訊方法（如 Annotation Edit Distance; AED）並找到新的 SNP 分子標誌。

(2) 中國張啓發院士（Dr. Q. F. Zhang）: 主要介紹利用秈粳雜交高不稔性的特性，了解水稻稔性之機制，不親和的研究從早期以 S loci 相同或相異的概念著手，接著以分子生物學方法進行 S5 QTL 定位，並細部定位到此基因座之基因可能爲 Aspartic protease 酵素，具有多個 ORFs，藉此具有多種不同的辨識與調節功能，非常複雜的機制，大陸針對此不稔性特性深入研究主要是爲了 F1 雜交種子製種上遇到的困難，投資的人物力之大讓人印象深刻與佩服。

(3) 日本 Prof. M. Yano: 藉由根部形態不同的 IR64（淺根系）與菲律賓 Patong 品種（深根系）兩水稻品種雜交分離後代進行深根性 QTL 探勘，結果在第九條染色體上發現 Deeper Rooting 1 基因座（Dro1），進一步鑑定出其爲受 Auxin 負向調控之基因，除了影響根部發育外也會影響根部的形態，Yano 團隊並利用分子標誌輔助選種技術將此基因座轉移至 IR64 品種中，發現除改善根部結構與深根性外，對乾旱逆境耐受性有顯著提高，在嚴重乾旱田間條件下，仍維持一定產量，正進行新品種命名中。

(4) C4 rice project: 由 Prof. W. P. Quick 領軍接受 Gates Foundation 補助跨國的大計劃，除由 Prof. Quick 親自介紹此團隊的理念與策略外，也有團隊成員介紹近來的成果，除了從各國水稻突變庫中篩選維管束鞘組織間葉肉細胞數變少的突變體外，也利用與水稻近緣物種（*Setoria*）雜交，目的在找尋調控維管束鞘發育之基因，目前已有特定候選基因，也利用基因轉殖過量表現策略，驗證基因功能。

3. 海報展示部分

今年度共有 207 篇論文以海報展示方式發表研究成果，因爲論文數太多，配合今年度的主軸，共分成八大領域、兩梯次進行成果展示，本人發表的論文「Cross talk between endogenous hormone signals and carbohydrate metabolism for inducing regenerable callus by osmotic stress treatment」探討滲透壓處理影響水稻癒合組織植株再生的機制可能是透過內生植物荷爾蒙調控到碳水化合物之代謝進一步影響植株再生，參加第二分組「Architecture and Development」之成果展示，由於水稻癒合組織植株再生是水稻基因轉殖成功與否最基本且重要的關鍵步驟，本研究探討植株再生的機制引起許多學者的興趣，也有深入之討論。

4. 會議成果

參加本次國際研討會收穫豐碩，除接收到來自全世界水稻各領域專家最新研究成果資訊外，也與各國與會學者有密切互動，如日本東京大學研究鐵元素轉

運的 Prof. Nishizawa、NARS 研究根系發育與分子標誌輔助選種的 Prof. Yano、韓國水稻功能性基因體大師 Prof. G. An、中國大陸張啓發院士、美國的 Prof. R. Wing 等等，都是本次大會邀請的貴賓，此外，也攜回會議書面資料一份，也對印度風土民情與最新水稻相關研究現況有進一步了解。

五、出國心得

此次到印度出席國際研討會，對許多同行台灣專家而言是既期待又怕受傷害，主要是因爲都是初次到訪印度，總是件興奮的事，不過出國前來自親朋好友們都再三的警告要注意衛生與飲食，尤其是飲用水與絕對不要生食，不然一定很慘，所以同行者有許多人行李都打包了台灣的礦泉水，到了印度後才發現來自各國的學者都有一樣的憂慮，雖然研討會在五星級 The New Delhi Grand Hotel 舉行，三餐食宿都在飯店中，不過還是有些擔心。此行除研討會本身非常精彩外，令人印象深刻的還包括印度料理，幾乎餐餐都有各式各樣的咖哩，不過最讓人懷念的應該算是印度現烤餅皮，很有嚼勁且安全。此外不得不提的是印度空氣污染非常嚴重，人民生活水平貧富落差非常大，隨處可見人畜共處，非常髒亂，交通也非常紊亂，想想自己生活在台灣，還真是一件幸福的事。

另一方面，每每參加國際研討會都會覺得台灣對科技研發真的不太重視，尤其是農業科技，世界各國爲增加糧食生產，近年來無不積極投入大量人力物力進行分子育種或科技研發，組成各式研究團隊，反觀國內始終是單兵作戰，且農業科技研發經費越來越難申請到，農委會經費一大半都拿去作爲救助金補助款，國科會的計畫能真正落實到產業的更是寥寥可數，尤其看到中國大陸的進步更是讓人心驚膽跳，原本水稻科技研發是我們的強項，現在我們在國際上幾乎已被邊緣化了，再不積極奮起，以後可能連最重要的水稻我們都要靠進口了，希望不會有那麼一天才好。

六、建議事項

1. 非常希望政府能夠補助經費辦理大型國際研討會，增加國際能見度。
2. 積極籌組研究團隊，畢竟團結力量大，不然很難在現今潮流中冒出頭來。

附錄一　會議議程

<table>
<tr><td colspan="4">Day 1: November 20, 2013; Wednesday</td></tr>
<tr><td colspan="2">8:00 Onwards</td><td colspan="2">REGISTRATION: Grand Ballroom (GBR) Prereception area</td></tr>
<tr><td colspan="2">09:30 - 11:00</td><td colspan="2">OPENING CEREMONY & KEYNOTE ADDRESS (GBR) Chair Person: Manju Sharma Chief Guest: MS Swaminathan</td></tr>
<tr><td colspan="4">Welcome: Akhilesh K Tyagi Guests of Honour: Qifa Zhang, Dinesh Singh, S Ayyapan Introduction of the keynote speaker and remarks: Manju Sharma Keynote address: MS Swaminathan Vote of Thanks: Jitendra P Khurana</td></tr>
<tr><td colspan="2">11:00 - 11:30</td><td colspan="2">TEA/COFFEE BREAK (GBR Lawns)</td></tr>
<tr><td colspan="2">11:30 - 13:30</td><td colspan="2">PLENARY LECTURES (GBR) Chair Persons: Sudhir Sopory, Rod Wing</td></tr>
<tr><td colspan="4">Rod Wing, USA Qifa Zhang, China Masahiro Yano, Japan Usha Vijayraghavan, India</td></tr>
<tr><td colspan="2">13:30 - 14:30</td><td colspan="2">LUNCH (GBR Lawns)</td></tr>
<tr><td>14:30 - 16:00</td><td>Concurrent Session I (GBR 1) Abiotic Stress I Chair: Anil Grover, San Segundo Blanca</td><td>Concurrent Session II (GBR 2) Architecture & Development Chair: Narayan Upadhyaya, R Srinivasan</td><td>Concurrent Session III (GBR 3) Comparative and Evolutionary Genomics Chair: Antonio Costa de Oliveira, RP Sharma</td></tr>
<tr><td>Anil Grover, India Dibyendu N Sengupta, India Tiago Lourenco, Portugal R Venkategowda, USA</td><td colspan="2">Yongzhong Xing, China Ajay Kohli, Philippines Sanjay Kapoor, India Yang-Seok Lee, Korea</td><td>N K Singh, India Pankaj Jaiswal, USA Jitendra K Thakur, India Ramanjulu Sunkar, USA</td></tr>
<tr><td colspan="2">16:00 - 16:30</td><td colspan="2">TEA/COFFEE BREAK (GBR Lawns)</td></tr>
<tr><td>16:30 - 18:00</td><td>Ajay Parida, India Niranjan Chakraborty, India Sneh-Lata Singla-Pareek, India Girdhar K Pandey, India</td><td>Changyin Wu, China Gauravi Deshpande, India Letian Chen, China</td><td>Yesheng Zhang, China Rachel S Meyer, USA Masahiko Kumagai, Japan Jorge Duitama, USA</td></tr>
<tr><td colspan="2">18:00 - 19:00</td><td colspan="2">Discussion & Personal Interaction</td></tr>
<tr><td colspan="2">19:00</td><td colspan="2">WELCOME DINNER (GBR Lawns)</td></tr>
</table>

Day 2: November 21, 2013; Thursday

09:00 - 10:30		PLENARY LECTURES (GBR) Chair Persons: Masahiro Yano, Jitendra P Khurana	
Lizhong Xiong, China Christophe Perin, France Naoka Nishizawa, Japan			
10:30 - 11:00		TEA/COFFEE BREAK (GBR Lawns)	
11:00 - 13:00		PLENARY LECTURES (GBR)	
William Paul Quick, Philippines Peter Westhoff, Germany Jie Luo, China Gynheung An, Korea			
13:30 - 15:30		LUNCH (GBR Lawns) & POSTER SESSIONS I & II (Brix) (Co-ordinators: Sanjay Kapoor, Alok Sinha, Saurabh Raghuvanshi, Mukesh Jain)	
15:30 - 16:30	Concurrent Session IV (GBR 1) Abiotic Stress 2 Chair:Naoko Nishizawa, Arun Lahiri Majumder	Concurrent session V (GBR 2) Small RNA and Epigenomics Chair:Blake Meyers, M Udaykumar	Concurrent session VI (GBR 3) Informatics and Systems Chair: Bin Han, Paramjit Khurana
Arun L Majumder, India Nelson Saibo, Portugal	**Yoshiki Habu, Japan Dao-Xiu Zhou, France**	**Antonio C de Oliveira, Brazil Sushma Naithani, USA**	
16:30 - 17:00		TEA/COFFEE BREAK (GBR Lawns)	
17:00 - 18:20	M K Reddy, India Alok K Sinha, India Ashwani Pareek, India Ming-Der Shih, Taiwan	Meenu Kapoor, India Saurabh Raghuvanshi, India Sarah Anderson, USA	Bijayalaxmi Mohanty, Singapore Olivia Wilkins, USA Sunil Archak, India
19:00		BUFFET DINNER (GBR Lawns)	

Day 3: November 22, 2013; Friday

09:00 - 10:30		PLENARY LECTURES (GBR) Chair Persons: Gynheung An, RB Singh	
Blake Meyers, USA Dabing Zhang, China Venkatesan Sundaresan, USA			
10:30 - 11:00		TEA/COFFEE BREAK (GBR Lawns)	
11:00 - 13:00		PLENARY LECTURES (GBR)	
Trilochan Mohapatra, India Bin Han, China Olivier Panaud, France Yue-le Caroline Hsing, Taiwan			
13:00 - 14:00		LUNCH (GBR Lawns)	
14:00 - 15:30	Concurrent session VII (GBR 1) **SHIMAMOTO session** (Biotic Stress): Chair: Hiroshi Takatsuji, Bharat B Chattoo	Concurrent session VIII (GBR 2) Molecular Breeding Chair: HS Gupta, Yue-Ie Hsing	Concurrent session IX (GBR 3) Translational Genomics Chair: Venkatesan Sundaresan, Rakesh Tuli
Shiping Wang, China Tilak R Sharma, India Sampa Das, India Yoji Kawano, Japan	**Yaoguang Liu, China** Arvind Kumar, Philippines Darshan S Brar, India Endang M Septiningsih, Philippines	**Karuppannan Veluthambi, India** Ashok K Singh, India Hiroaki Saika, Japan Karabi Datta, India	
15:30 - 16:00		TEA/COFFEE BREAK (GBR Lawns)	
16:00 - 17:30	**Masaki Mori, Japan** Indranil Dasgupta, India RM Sundaram, India	**Shunsuke Adachi, Japan** Kshirod Jena, Philippines Dak Deborah, India Vandna Rai, India	**Bharat B Chattoo, India** Amol Samant, India Paras Yadav, ILS, India AK Bhattacharya, Leica
17:30 - 18:30		Meeting of the International Organizing Committee (Board room)	
18:30 - 19:30		CULTURAL EVENING (GBR Lawns)	
19:30		BANQUET DINNER (GBR Lawns)	

Day 4: November 23, 2013; Saturday	
09:00 - 10:30	PLENARY LECTURES: (GBR) Chair Persons: Ebrahimali A Siddiq, Peter Westhoff
Blanca San Segundo, Spain Hiroshi Takatsuji, Japan Ramesh Sonti, India	
10:30 - 11:00	TEA/COFFEE BREAK (GBR Lawns)
11:00 - 12:30	PLENARY LECTURES: (GBR)
Daniel Zilberman, USA Akhilesh Tyagi, India Andy Pereira, USA	
12:30 - 15:00	LUNCH (GBR Lawns) & POSTER SESSIONS III—VIII (Brix) (Co-ordinators: Sanjay Kapoor, Alok Sinha, Saurabh Raghuvanshi, Mukesh Jain)
15:00 - 16:30	PANEL DISCUSSION on "Rice Functional Genomics in Sustaining Food & Nutritional Security" Chair Persons: K Vijayraghavan, Swapan K Datta (GBR)
K Vijayraghavan, Swapan K Datta, Rod Wing, Qifa Zhang, Kailash C Bansal, KK Narayanan, Usha Zehr	
16:30 - 17:00	Announcement of ISRFG 2014 Closing ceremony (GBR)
17:00 - 17:30	High Tea (GBR Lawns)

附錄二　發表論文全文

Cross talk between endogenous hormone signals and carbohydrate metabolism for inducing regenerable callus by osmotic stress treatment

Wen-Lii Huang* and Shiang-Ting Lee

Department of Agronomy, National Chiayi University, Chiayi City 600, Taiwan, R.O.C
(* requests for offprints: E-mail: wlhuang@mail.ncyu.edu.tw)

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 (NRC) 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.

Key words: *Oryza sativa* L., Plant hormones, Shoot organogenesis, Sucrose metabolism.

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 regeneraton in plant tissue culture: genotype (Huang et al. 2002; Glowacha et al. 2010), exogenous and endogenous hormones (Jimenez 2005; Barreto et al. 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 such as abscisic acid (ABA), free amino acids, and soluble sugars (Wang et al. 1999; Huang and Liu 2002; Jimenez 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; Wang et al. 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 (Jimenez 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; Pernisova 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; Petrasek and Friml 2009; Radmacher 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 (Petrasek 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 (Muller and Sheen 2007) and the gene expression can be recognized at the 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; Harting and Beck 2006; Pernisova 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. 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 3 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 acetic acid (IAA) transportation. 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). Both culture stages were maintained at 27-28 ℃ and 200 μM photons m^{-2} s^{-1} with a 12-h light/12-h dark photoperiod. Because calli less than 7 days old are too small and difficult to collect, they were harvested and weighed as fresh weight only at days 10 and 14 on CIM. The collected calli were dried in a ventilating oven at 80℃ for 48 h to constant weight. Water content (%) determination and shoot organogenesis frequency (%) evaluation were according to our previous studies (Huang et al. 2012; Lee and Huang 2013). The results were obtained from at least 3 independent experiments.

Extraction and determination of sucrose, glucose and starch

Samples were harvested and weighed after inoculation for 4, 7, 10, 14 days in CIM and 1, 3, 5 and 7 days in 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 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-strand 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-strand 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 ℃ for 5 min, 40 cycles of 95 ℃ for 10 s, 60 ℃ for 30 s, then, 95 ℃ for 15 s and 55 ℃ 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. 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 3 replicates of amplification with 3 independent batches of total RNA samples.

Statistical analysis

Results are shown as mean ± SE from at least 3 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 callus started to initiate from immature seeds on MSD_{10} medium at day 4 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 day 10. The mean fresh weight of each callus at day 14 was approximately to 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 day 14 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 days 10 and 14 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 days 10 and 14 (Fig. 1b).

$MSD_{10}S_6$ – derived calli at 14 days old showed green spots and shoot primordia emerging at days 10 to 14, with multiple shoots seen at day 28 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 day 14 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). 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, 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 might be based on 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-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 7 days (Fig. 3d-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-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 day 3 and later (Fig. 3d-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 due to the increase of sucrose uptake from the medium resulting from cell wall-bound invertase activity. 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 was 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 day 14. 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

was still low and did not significantly fluctuate. However, levels of the 3 genes were greatly enhanced in $MSD_{10}S_6$ – derived calli in RM. *OsCIN1* and *OsSUT2* were upregulated early 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 in 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 result in cellular sucrose, glucose, and starch accumulation.

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 ribosides in highly regenerable rice calli induced by osmotic stress that quickly decreased 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 day 4 in MSD_{10} medium then gradually decreased, but the opposite was observed in $MSD_{10}S_6$ – derived 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 day 10 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 period evaluated. 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 on regenerable calli induction under osmotic stress treatment.

Discussion

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

carbohydrate metabolism 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 auxin 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; Khleda et al. 2006; Dabul et al. 2009). Only rarely can cultivars of rice 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.

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; Huang et al. 2006; Huang et al. 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 embrygenesis and organogenesis but mainly through organogenesis (Huang and Liu 2002; Huang et al. 2006; Jiang 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 (Valdes 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 or the IAA precursor anthranilic acid can enhance shoot regeneration frequency to 10% or 35%, respectively. However, the frequency can be increased 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 as 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 inducing 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 was 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 a balance between different endogenous phytohormones. 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 (Charriere et al. 1999; Sugiyama 1999; Fernando and Gamage 2000; Mercier et al. 2003; Jimenez, 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 regenerable calli differentiation (Jimenez 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 effect 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.

Acknowledgements

This work was supported by Council of Agriculture, Taiwan.

Legends to figures

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). Scale bar = 5 mm. Bars with different lowercase letters indicate significant difference ($P < 0.05$).

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 ($P < 0.05$).

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).

Fig. 4 Real time RT-PCR analysis of mRNA levels of *OsCIN1*, *OsSUT1* and *OsSUT2* in TNG71 during callus induction and early shoot regeneration. mRNA expression in (a-c) CIM and (d-f) RM. The levels were normalized to that at day 0 in TNG71. *Ubiquitin* level was used as a reference. Data are mean ± SE (n=3).

Fig. 5 Real time RT-PCR analysis of mRNA levels of *OsPIN1*, *ORR1* and *OsLEA1* in TNG71 during callus induction and early shoot regeneration. mRNA expression in (a-c) CIM and (d-f) RM. The levels were normalized to that at day 4 in TNG71. *Ubiquitin* level was used as a reference. Data are mean ± SE (n=3).

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.

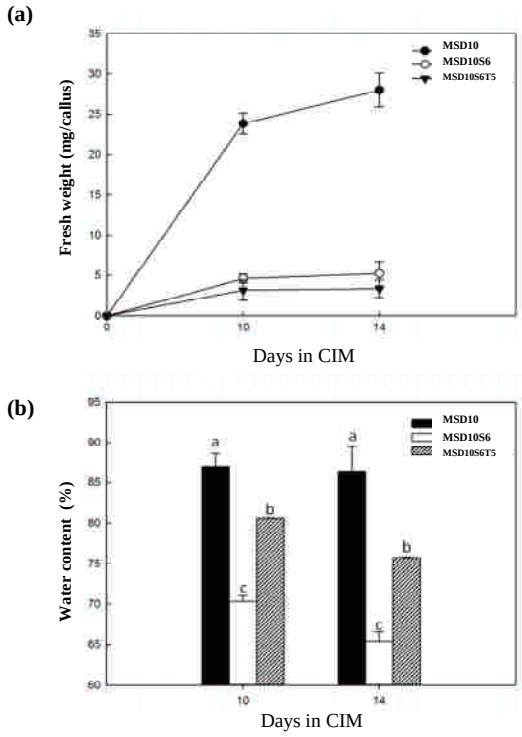

Fig. 1

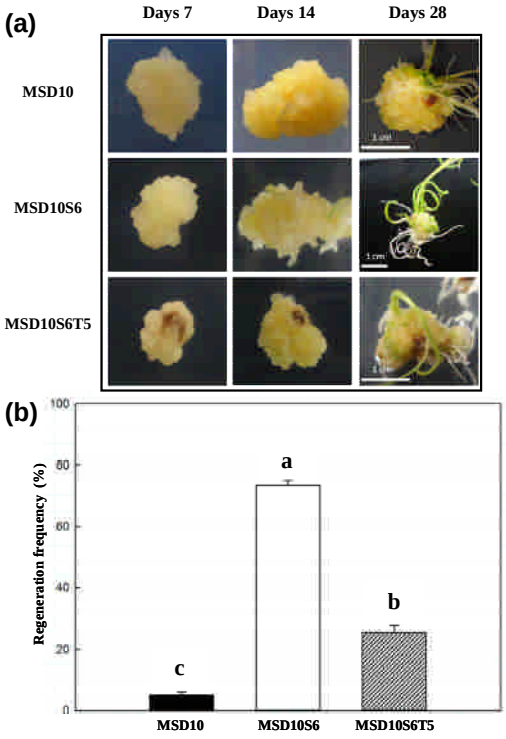

Fig. 2

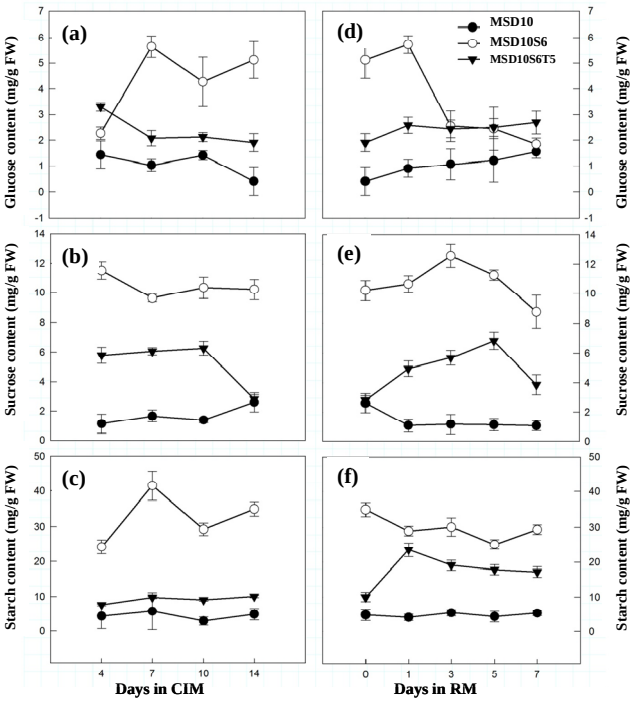

Fig. 3

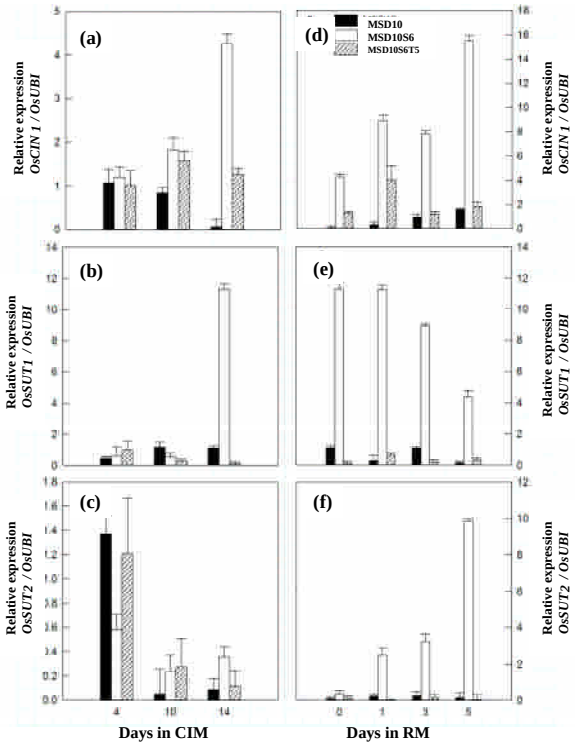

Fig. 4

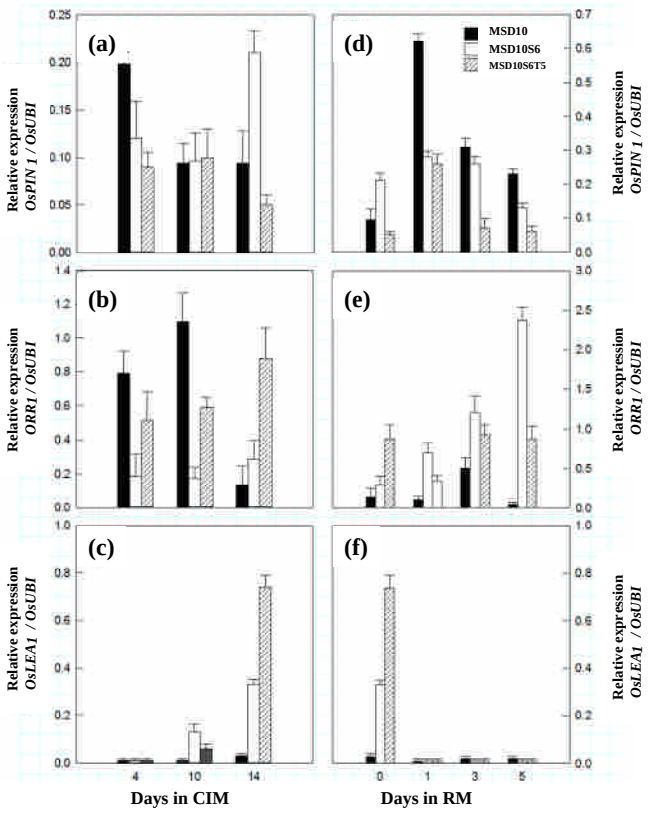
(a)
(b)
(c)
(d)
(e)
(f)
MSD10
MSD10S6
MSD10S6T5
Relative expression OsPIN 1 / OsUBI
Relative expression ORR1 / OsUBI
Relative expression OsLEA1 / OsUBI
Days in CIM
Days in RM

Fig. 5

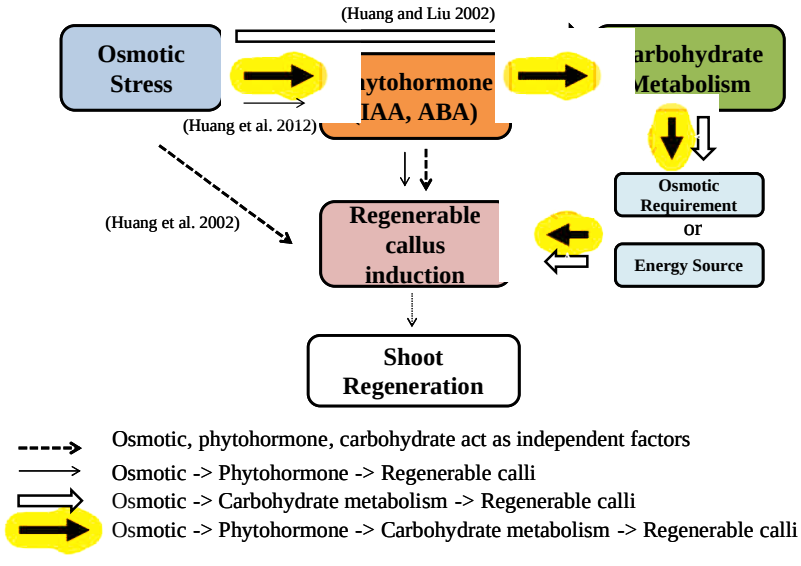
(Huang and Liu 2002)
Osmotic Stress
(Huang et al. 2012)
(Huang et al. 2002)
Regenerable callus induction
Shoot Regeneration
Osmotic Requirement
or
Energy Source
Osmotic, phytohormone, carbohydrate act as independent factors
Osmotic -> Phytohormone -> Regenerable calli
Osmotic -> Carbohydrate metabolism -> Regenerable calli
Osmotic -> Phytohormone -> Carbohydrate metabolism -> Regenerable calli

Fig. 6