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Transcript Profiling Reveals Abscisic Acid, Salicylic Acid and Jasmonic-Isoleucine Pathways Involved in High Regenerative Capacities of Immature Embryos Compared with Mature Seeds in *japonica* Rice

XIAO Kaizhuan[1, 2, 3], MAO Xiaohui[2, 3], WANG Yingheng[2, 3], WANG Jinlan[2, 3], WEI Yidong[2, 3], CAI Qiuhua[2, 3], XIE Hua'an[1, 2, 3], ZHANG Jianfu[1, 2, 3]

([1]*College of Life Sciences, Fujian Normal University, Fuzhou 350108, China;* [2]*Rice Research Institute, Fujian Academy of Agricultural Sciences, Fuzhou 350019, China;* [3]*Key Laboratory of Germplasm Innovation and Molecular Breeding of Hybrid Rice for South China, Ministry of Agriculture / Incubator of National Key Laboratory of Fujian Germplasm Innovation and Molecular Breeding Between Fujian and Ministry of Sciences & Technology / Fuzhou Branch, National Rice Improvement Center of China / Fujian Engineering Laboratory of Crop Molecular Breeding / Fujian Key Laboratory of Rice Molecular Breeding / Base of South China, State Key Laboratory of Hybrid Rice / National Rice Engineering Laboratory, Fuzhou 350003, China*)

Abstract: Induced pluripotent cell mass plays a role in genetic transformation mediated by *Agrobacterium*. Mature seeds are more recalcitrant to the induction of suitable calli than immature embryos in rice, but the exact molecular mechanisms involved remain elusive. In this study, the morphological structure of calli induced from mature seeds and immature embryos were observed under a scanning electron microscope using a paraffin embedded technique. Meanwhile, a total of 2 173 up- and down-regulated genes were identified in calli induced from mature seeds and immature embryos by RNA-seq technique and furtherly confirmed by quantitative real-time PCR. The results revealed the remarkable morphological differences in calli induced from mature seeds and immature embryos, and plant hormone signal transduction and hormone biosynthesis pathways, such as abscisic acid, salicylic acid and jasmonic-isoleucine, were found to play roles in somatic embryogenesis. This study provided comprehensive gene expression sets for mature seeds and immature embryos that were served as an important platform resource for further functional studies in plant embryogenesis.

Key words: callus; immature embryo; mature seed; *japonica* rice; RNA sequence; hormone

Rice (*Oryza sativa* L.) is one of the most important food crops and feeds more than half of the world's population (Jena et al, 2017). With the increase of population, increasing rice yield will play a pivotal role in maintaining a sufficient food supply. It has been argued that advances in technology can potentially increase agricultural productivity (Mullet et al, 2017). Genetic transformation of rice offers numerous opportunities for the improvement of existing elite varieties and the production of new varieties. Electroporation, polyethylene glycol, protoplasts and micro-projectile bombardment were first used to mediate direct gene transfer in the late 1980s (Toriyama et al, 1988; Yang et al, 1988; Zhang et al, 1988). The soil bacterium *Agrobactriuem tumefaciens* was first employed in plant transformation in the middle of 1990s (Hiei et al, 1994). The advantages of *Agrobacterium*-mediated transformation, including

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Corresponding authors: XIE Hua'an (huaanxie@163.com); ZHANG Jianfu (jianfzhang@163.com)

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high efficiency of transformation, integration of small numbers of copies of transfer DNA into the chromosomes, transfer of relatively large segments of DNA with defined ends and little rearrangement of T-DNA upon transformation, soon ensures that it becomes much more popular in rice than other techniques (Hiei and Komari, 2008; Shen et al, 2017).

Although *Agrobacterium*-mediated transformation has been widely used in *japonica* for more than a quarter of a century (Duan et al, 2012; Zhang et al, 2013; Zhang et al, 2014; Hu et al, 2016), the mechanism of why utilizing immature embryos as the starting material is more efficient than using calli induced from mature seeds (Hoque et al, 2005; Slamet-Loedin et al, 2014; Hofmann, 2016) has been poorly investigated. It is widely accepted that the ability of immature embryos to generate embryogenic calli is greater compared with that of mature seeds (Chu et al, 2016), which are more recalcitrant to induction of suitable calli essential for *Agrobacterium*-mediated transformation. However, the underlying biochemical mechanisms remain obscure.

To better understand and clarify the mechanism underlying the difference regenerative capacities of the two growth stages of the rice seeds *in vitro* culture, in the present study, we identified the remarkable morphological differences in the primary proliferating calli induced by mature Nipponbare seeds (MN) and immature embryos (IMN), and the hormone signal transduction pathway(s) involved in somatic embryogenesis, especially the abscisic acid (ABA), salicylic acid (SA) and jasmonic-isoleucine (JA-Ile) transduction pathways.

MATERIALS AND METHODS

Plant materials and callus induction

Rice variety Nipponbare was supplied from the South Base of the National Key Laboratory of Hybrid Rice of China (Fuzhou City, Fujian Province, China). Mature rice seeds were sown in soil in a greenhouse maintained at temperatures of 18 ºC–24 ºC and photoperiod of 14–16 h during the vegetative growth phase (Hiei et al, 2008). After five weeks, the photoperiod was changed to 12 h, and the daytime temperature was maintained at 28 ºC–35 ºC and the nighttime temperature at 22 ºC–25 ºC for induction of flowering. Subsequently, immature embryos were excised from seeds during 8 to 12 d after post-pollination when embryos were approximately 1.3–1.8 mm in size. Mature seeds were obtained at 20–25 d after pollination.

Seeds with the hulls removed using forceps were surface-sterilized with 70% ethanol for several seconds, and then in 1% sodium hypochlorite solution containing a drop of Tween-20 for 5 min. The immature and mature seeds were then washed with distilled water for several times and patted dry on the sterile filter paper in a sterile petri plate, seeds were then transferred to induction Nutrient Broth (NB) medium. Immature embryos were removed from the seeds with forceps under a stereoscopic dissection microscope (Islam et al, 2014), and transferred to induction NB medium at 26 ºC in the dark. After culture for 20 d, samples were harvested at 16:00–18:00 for further processing.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used for morphological observation of callus tissues (Kumar et al, 2015). For the SEM analysis, primary calli were independently collected for three times, creating three biological replicates for each MN and IMN, and then fixed in formalin-acetic acid fixative for 1 h and dehydrated through a graded series of ethanol (10%, 30%, 50%, 70% and 100%) for 10 min each. Dehydrated tissues were dried in a critical-point dryer (K850, Quorum, Britain). Because callus tissue is normally soft and electrically non-conductive, the samples were then coated with gold in an ion coater (IB-3, Eiko, Japan). Observation of the specimens was performed under a scanning electron microscope (S-4800, Hitachi, Japan) and photomicrographs were taken at different magnifications.

Paraffin-embedded sections

For serial microtome sections, medium-sized primary calli of MN and IMN from three individual seeds were randomly chosen and instantly fixed in formalin-acetic acid fixative for 3 d, rehydrated in 70%, 50% and 25% ethanol, respectively, for 30 min stained with Mayer's hematoxylin 50% ethanol, glacial acetic acid and 2% hematoxylin mixture (1:1:2 by volume) for 2 d. Then, the samples were dehydrated in an ethanol concentration series (30%, 50%, 70%, 85%, 95% and 100%) for 2 h and stained with safranin solution (100% ethanol : 3% safranin as 1:2 by volume) (Liu et al, 2012). Finally, the specimens were embedded in paraffin, sectioned at