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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

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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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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 ℃–24 ℃ 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 ℃–35 ℃ and the nighttime temperature at 22 ℃–25 ℃ 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 ℃ 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

5 μm with a rotary microtome (YD 202, Zhengzhou Nanbei Instrument Equipment, China), and examined under an electron microscope (XSZ-HPR, Chongqing Guangdian Instrument Equipment, China).

Transcriptome analysis

Primary calli from the same scutellums position of at least three individual seeds were collected and frozen immediately in liquid nitrogen for RNA extraction. A total of 3.0 μg RNA, assessed for quantity and quality, were enriched to construct mRNA-sequencing (mRNA-Seq) libraries by sequencing on a HiSeq™ 2000 platform (Illumina, USA).

Raw data (raw reads) in fastq format were first filtered, and the Q20, Q30 and GC contents of the clean data were calculated. All downstream analyses were based on these clean data of high quality. Then, Bowtie v2.2.3 was used to build an index of the reference genome, which was downloaded from the Ensemble (http://plants.ensembl.org/Oryza_sativa/Info/Index.Genome). TopHat v2.0.12 (Trapnell et al, 2012; Kim et al, 2013) was employed to align paired-end clean reads to the reference genome and to generate a database of splice junctions based on the gene model annotation file, and thus provide a better mapping result than other non-splice mapping tools. Furthermore, a series of bioinformatics approaches were exploited for statistical analysis, including HTSeq v0.6.1 (Trapnell et al, 2010), the DEGSeq R package (1.18.0) (Wang et al, 2010), the GOseq R package (Yang et al, 2010), Kobas software (Mao et al, 2005) and OmicShare tools.

Quantitative real-time PCR (qRT-PCR) validation

For qRT-PCR analysis, three independent biological samples were used. Total RNAs were treated according to the manual for the RNA Extraction Kit (Takara, Japan). DNase-treated RNA (5 μg) was reverse transcribed in a total volume of 50 μL using reverse transcriptase (ABI, American) and then stored at -20 ℃. qRT-PCR was performed using 2 ng total RNA in a 20 μL reaction volumes using SYBR Green PCR MasterMix (Thermo) on a One-Step Quantitative PCR system (ABI, American) following the manufacturer's protocols. Each 20 μL sample contained 10 μL SYBR Green Master Mix, 2 μL cDNA and 4 μmol/L forward and reverse primers.

Endogenous hormones determination

Samples were ground in liquid nitrogen for extraction and analysis of endogenous hormones following a previously described method (Stitz et al, 2011). ABA, indole acetic acid (IAA) and SA were extracted with methanol spiked with labeled internal standards, jasmonic acid (JA) and JA-Ile were extracted with ethyl acetate spiked with labeled internal standards ($^{13}C_2$-JA and $^{13}C_6$-JA-Ile), and then ABA, IAA, SA, JA and JA-Ile levels were analyzed by using Shimadzu LC/MS-8040 (Shimadzu, Japan).

RESULTS

Morphological differences in calli induced from MN and IMN

The entire surface and the internal organization of the composition calli from MN and IMN were observed by viewing paraffin-embedded sections under SEM. The images showed that all calli surfaces were covered with distinctive globular nodules containing tightly packed cells, and enlargement and multiplication of the membranous layer that emerged from certain parts of the callus surfaces resulted in simultaneous rupture and extensive striations, which formed an anastomotic network of fibrillar connections (Fig. 1). The most dominant characteristic of somatic embryogenesis of IMN was the irregular surface of the callus, with some smooth and compact portions of the calli partially or completely peeled off (Fig. 1-A), Meanwhile, the somatic embryogenesis from protoplast-derived callus in MN was consisted of white, sheet-shaped structures originating from the yellowish opaque sectors of the callus (Fig. 1-B). It should be pointed out that the diameter of the cellular structures of callus in IMN was greater than MN.

In the internal structure, various types of parenchymatic cells generally divided strongly in a parallel or perpendicular way that caused the callus to swell and the surface to fall apart (Fig. 2). Furthermore, cambial-like tissue inside the large protuberances, vascellum and epidermis-like tissue on the surface of some protuberances was also observed. A band of more densely stained nuclei could be observed in both external and internal layers in the globular embryos of the calli from IMN (Fig. 2-A), and was especially abundant in the peripheral regions in MN (Fig. 2-B), In addition, the number of elongated and highly dissociated cells (non embryogenic) in MN was greater than IMN.

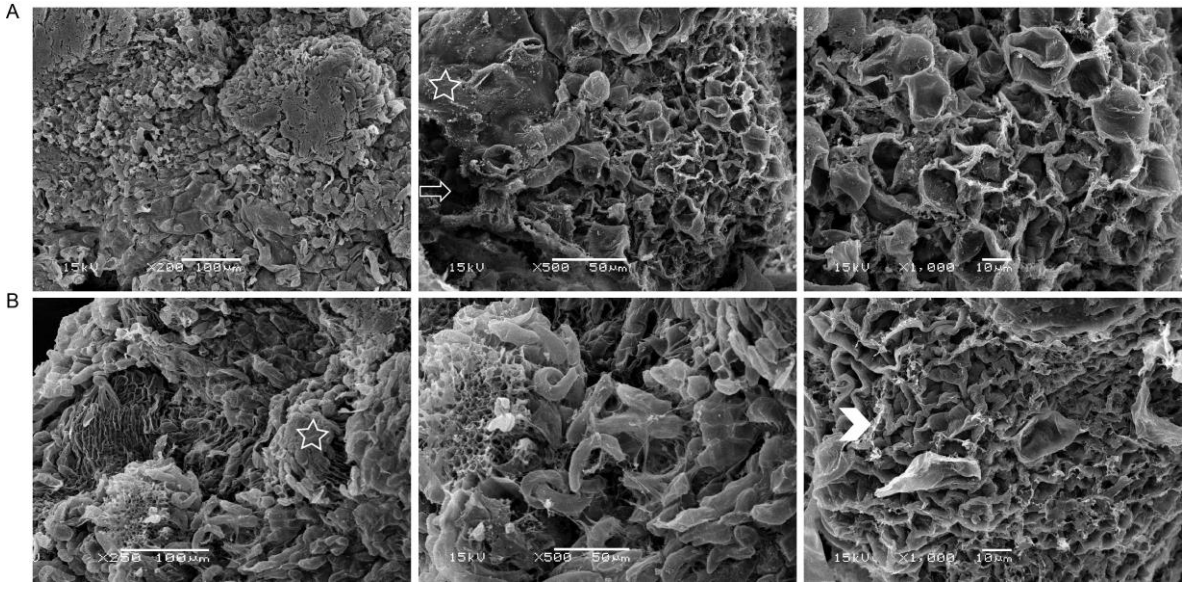

Fig. 1. SEM images of callus surface of IMN (A) and MN (B) after the placement of the callus induction medium for 20 d.

SEM, Scanning electron microscopy; IMN, Immature Nipponbare embryos; MN, Mature Nipponbare seeds.

The asterisks and arrowheads indicate the membrane and fibrillar of the extracellular matrix of the callus respectively. The arrow indicates the membrane structures with holes covering the callus surface.

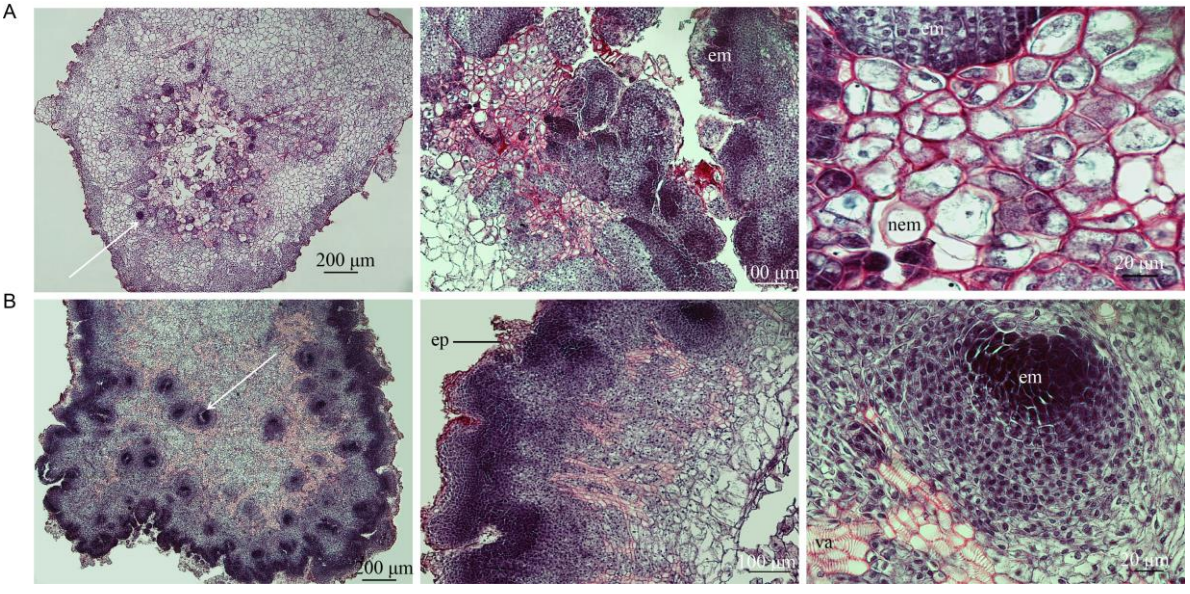

Fig. 2. Histochemical analysis of the somatic embryogenesis of IMN (A) and MN (B) after placement on callus induction medium for 20 d.

IMN, Immature Nipponbare embryos; MN, Mature Nipponbare seeds.

The em, nem and white arrows indicate the embryogenic callus units, nonembryogenic callus and large callus domain/protuberances. The va and ep represent the vascellum and epidermis-like tissue on the surface of some protuberances, respectively.

Hormone signal transduction pathway(s) enabled immature embryos to have high viability

Six RNA-Seq libraries from three biological replicates of MN and IMN were used to systematically investigate the dynamics and differences in the transcriptomes of rice at two growth status. A total of 191 277 258 clean reads were obtained, and each replicate was represented by at least 28.4 million tags, 91% of which had a Phred score ≥ 30 (Table 1). By filtration, we established that 87.11%–89.67% of reads matched the reference sequence database.

In total, 2 173 differentially expressed genes (DEGs) between IMN and MN were identified in each comparison based on a *P*-value < 0.05 and cut-off fold change > 2, which showed that 940 genes were

Table 1. Summary of the fastq file generated by individually sequencing the RNA of all samples.

Sample ID	Total read	GC content (%)	Data with Phred score ≥ 20 (%)	Data with Phred score ≥ 30 (%)	Number of reads aligned to reference genome (%)
MN-1	29 477 712	51.96	96.37	91.27	26 431 504 (89.67)
MN-2	30 345 732	51.95	96.04	90.60	26 884 157 (88.59)
MN-3	31 118 488	52.04	96.08	90.67	27 392 769 (88.03)
IMN-1	33 653 030	51.82	96.29	91.23	30 135 270 (89.55)
IMN-2	37 035 338	53.44	96.03	90.87	32 262 197 (87.11)
IMN-3	29 646 958	51.47	96.14	91.00	26 431 890 (89.16)

IMN, Immature Nipponbare embryos; MN, Mature Nipponbare seeds.

up-regulated and 1 233 genes were down-regulated (Fig. 3-A). To identify the biological pathways that were significantly altered, sequences of DEGs were mapped to reference pathways described in Kyoto Encyclopedia of Genes and Genomes (KEGG). In total, 236 genes mapped to 100 KEGG pathways, and the highest percentage of unigenes (19%) was assigned to plant hormone signal transduction pathway (Fig. 3-B).

qRT-PCR expression analysis of genes related to hormones

To clarify the roles of the biosynthesis, transport, metabolism and signaling pathways of various endogenous hormones, the fragments per kilobase of transcript per million mapped reads (FPKM) based transcript expression pattern was analyzed. In total, 19 genes related to auxin (5), ABA (5), JA (6) and SA (3) were differentially expressed (Fig. 3-C). Their complex expression patterns were further confirmed by qRT-PCR (Fig. 4).

ABA, SA and JA-Ile enrichment in calli of immature embryos

To further determine how the levels and changes of endogenous hormones affect the physiological status of the calli, the endogenous hormones content of calli from IMN and MN were measured. We found that the ABA, SA and JA-Ile levels in IMN were markedly different from those in MN, while there was no significant difference in the incidence between IAA and JA in IMN and MN (Fig. 5). The contents of ABA

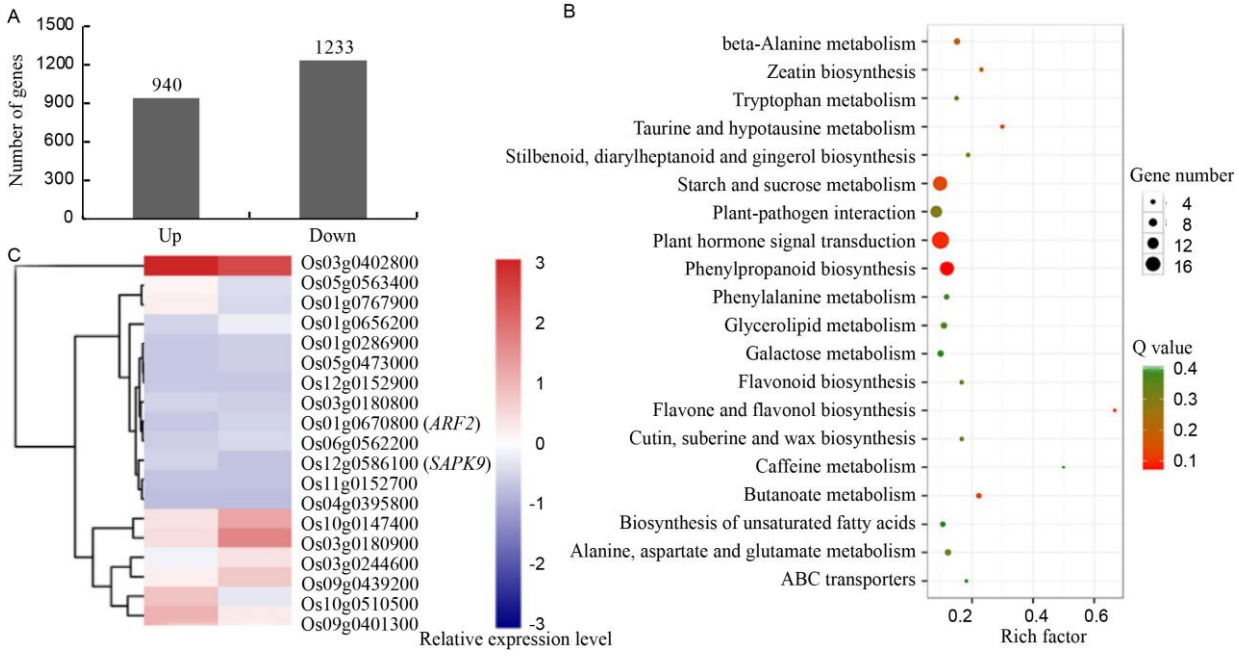

Fig. 3. Global analyze of the mRNA transcriptome data.

A, Differentially expressed genes (DEGs) between immature embryos and mature seeds involved in callus induction. B, Kyoto encyclopedia of genes and genomes (KEGG) pathway abundance of DEGs. A total of 20 pathways were identified in each comparison based on 0 < Q value < 1.0, where the abundance of the pathway is more significant if the value is close to zero. *Y*-axis shows the pathway categories, and *X*-axis shows the richness factor of each pathway. C, The heat map of genes involved in plant hormone signal transduction pathway.

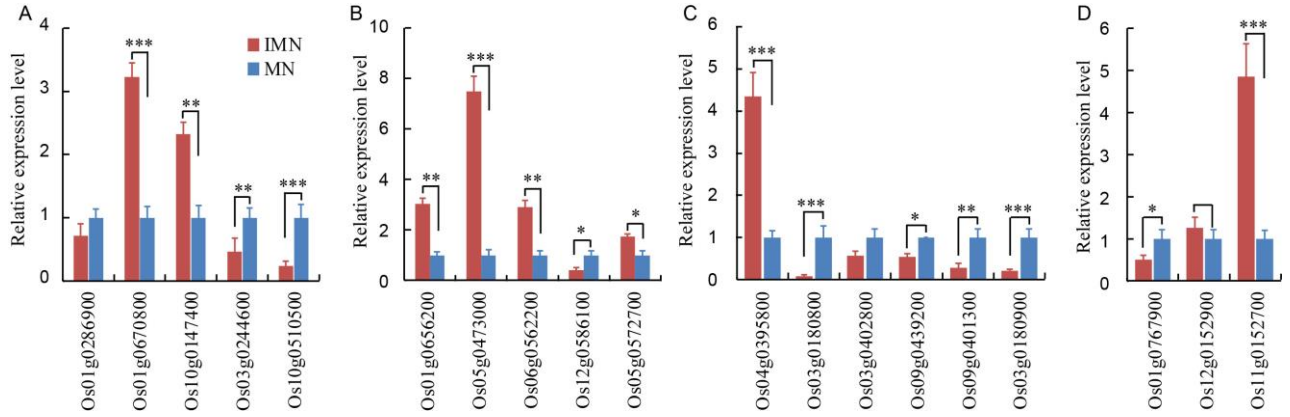

Fig. 4. Relative expression profiles of genes involved in plant hormone signal transduction pathway validated with quantitative real time PCR with the ΔΔCT method.

A, Auxin relative genes; B, Abscisic acid relative genes; C, Jasmonic acid relative genes; D, Salicylic acid relative genes.

IMN, Immature Nipponbare embryos; MN, Mature Nipponbare seeds.

Data represent mean ± SE (n = 5). The significant differences between mean values are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) using the Student's t-test.

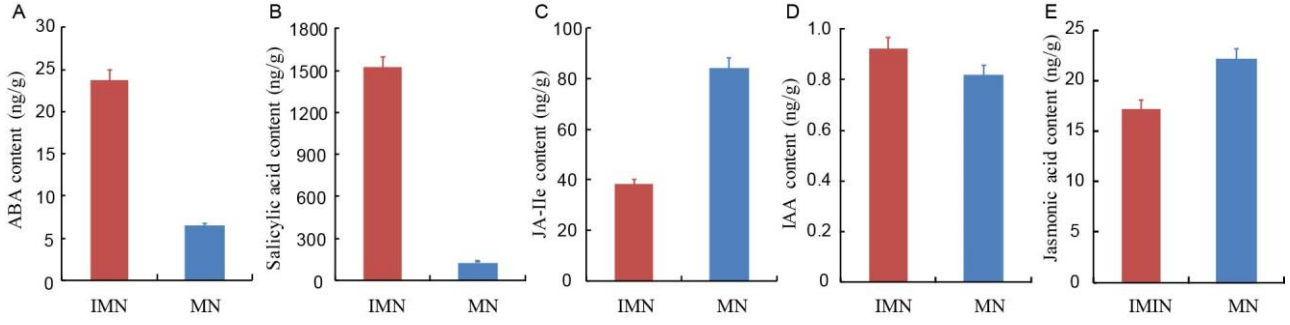

Fig. 5. Abscisic acid (ABA, A), salicylic acid (B), jasmonoyl-isoleucine (JA-Ile, C), indole-3-acetic acid (IAA, D), and jasmonic acid (E) contents of immature Nippobare embryos (IMN) and mature Nippobare seeds (MN).

Data represent mean ± SE (n = 5).

and SA in IMN were about 5-fold and 15-fold higher than those in MN, respectively (Fig. 5-A and -B), whereas the level of JA-Ile was 2-fold lower in IMN than in MN (Fig. 5-C).

DISCUSSION

Callus induction is essential to *Agrobacterium*-mediated transformation. The extremely high callus induction rate has a positive impact on the application of transformation in mature seeds, whereas the immature embryos are less convenient because they cannot be obtained in high quantities and kept viable for a long time on laboratory shelves (Hiei et al, 2008). The recent availability of high-throughput sequencing technologies has provided an unprecedented opportunity to thoroughly explore the molecular mechanism of the phenomenon of calli induced by immature seeds with high regenerative capacities by large-scale expression profile analysis. Gene-annotation analysis and KEGG abundance showed that plant hormone signal transduction and hormone biosynthetic pathways were a key factor to explain why the quantity and quality of calli induced from immature embryos was higher than those of calli induced from mature seeds (Fig. 3). A total of 940 and 1 233 genes showed the dynamic changes in the expression levels by pairwise comparison (Fig. 3-A). And more importantly, according to sequence analysis, the sequences with known function participated in biological processes, including plant hormone signal transduction and phenylpropanoid biosynthesis (Fig. 3-C and -D).

Endogenous ABA levels regulate the synthesis and deposition of storage proteins, lipids and carbohydrates during somatic embryogenesis, and prevent the precocious germination of the maturing embryo (Perez et al, 2015). High levels of ABA may enable IMN to grow rapidly and have evident morphological differences when compared with MN (Figs. 1 and 2).

Auxin is a critical plant growth regulator in cell

division, differentiation and the induction of somatic embryogenesis, which regulates the upstream and downstream mediators of the *ARF-Aux*/*IAA* signaling pathway (Visser et al, 1992; Tiwari et al, 2004). Although explants with higher levels of endogenous auxins may be more responsive to somatic embryogenesis (Jimenez and Thomas, 2006), the content of IAA was almost the same in calli induced by IMN and MN (Fig. 5-D). This phenomenon may be attributed to the fact that endogenous IAA is influenced by many factors (Yang et al, 2012), such as by the high over-expression of *ARF2* in IMN compared with MN (Fig. 3-C and Fig. 4-A). The increased IAA may be used to form dimers of repressive AUX/IAA proteins (Kim et al, 1997).

In recent years, JA and SA have been shown to be significantly involved in the regulation of plant, but their roles in somatic embryogenesis are still unclear (Ahmadi et al, 2014; Yang et al, 2016), most likely due to their effects differing among plant species and explant sources (Rikiishi et al, 2015). For instance, JA inhibits somatic embryo formation in *Medicago sativa* (Rudus et al, 2001), but improves the formation of the protocorn-like body in orchids (da Silva, 2013). The levels of lipid-based hormone signal JA in our study were consistent between IMN and MN (Fig. 5-C), while JA-Ile, as a ligand of JA receptor, was lower in IMN, which indicated that JA may regulates somatic embryogenesis of *japonica* by other derivatives. By contrast, the phenolic derivative SA, which is critical for the regulation of physiological and biochemical processes, was significantly higher in IMN. During the somatic embryogenesis, the utilization of SA may play an essential role, resulting in the complex and redundant regulation. This may have been a contributory factor to the high regenerative capacities of IMN.

In summary, this work revealed that while the calli is induced in the same rice variety, it had significant morphological differences during two distinct stages. We applied RNA-seq analysis to monitor the global transcriptional changes and investigated the expression patterns of DEGs between IMN and MN, founding many tissue-specific genes and DEGs between IMN and MN that may be involved in plant hormone signal transduction and biosynthetic pathways. Moreover, we showed that the levels of ABA, SA and JA-Ile of the calli were evidently different between IMN and MN. Further experimental research is required to define the function of those DEGs associated with plant hormone signal transduction and plant hormone biosynthesis, as well as the exact effect of the three types of hormones, and to illustrate why IMN has high regenerative capacities. Thereafter, a medium can be designed to which the components involved in the embryogenetic process can be added or substituted to improve regenerative capacities of mature seeds, at least to some extent.

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