Identification and expression pattern analysis of YUCCA and ARF gene families during somatic embryogenesis of Lilium spp.

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Abstract

Auxin is a key phytohormone in plant somatic embryogenesis (SE) and YUCCA and AUXIN RESPONSE FACTORS (ARFs) are two key genes involved in auxin biosynthesis and auxin signaling pathways, respectively. They have been reported in participating to the catalytic production of endogenous indole-3-acetic acid (IAA; a natural auxin) and regulating the transcription of auxin-responsive genes. To explore the structural characteristics of the YUCCA and ARF Families of Lilium spp. L. and its expression pattern during SE induction and development processes, 6 YUCCA genes and 12 ARF genes were screened from the transcriptome database, and their nucleotides and encoded proteins were analyzed. At the same time, the expression patterns of YUCCA and ARF genes were analyzed by reverse transcription quantitative PCR, and endogenous IAA content was measured. Results show that the members of Lilium indole-3-pyruvate monooxygenase YUCCA (LiYUCs) are structurally conserved among Lilium spp., Oryza sativa, and Arabidopsis thaliana. The LiARFs are classified into six groups, most LiARFs have a closer affinity to the monocotyledon Oryza sativa. All the 12 LiARFs are involved in the SE induction and development, but their expression patterns differed. The LiYUC2/4 and LiARF5/7/21 had expression profiles corresponding with IAA content during the SE induction periods. The LiYUC4/10 and LiARF7/17/18/20/21/22 showed a similar downward trend with IAA content during the progress of the SE development. The results provide a basis for further research on the functions of YUCCA and ARF genes during somatic embryogenesis of Lilium spp.

Additional key words: indole-3-acetic acid biosynthesis and signaling pathways, in vitro culture, phylogenetic analysis.

Introduction

Auxin plays critical regulatory role in plant growth and development, especially in the plant morphogenesis including organogenesis and somatic embryogenesis (SE) (Su et al. 2009, Zhang et al. 2016, Wójcikowska and Gaj 2017). Exogenous auxins, including α-naphthalene acetic acid, 2,4-dichlorophenoxyacetic acid (2,4-D), picloram, etc., has been utilized alone or in combination with other plant growth regulators to induce callus of Lilium spp. (Bakhshaie et al. 2016). But how exogenous auxin affects the embryogenic callus (EC) induction through changes in endogenous auxin has not been reported in Lilium spp.

Auxin activity can be achieved at different ways, including auxin biosynthesis and signaling pathways (Wang et al. 2017). There are many parallel synthesis pathways of IAA in plants, whereas indole-3-pyruvic acid is currently considered as the main pathway (Mano and Nemoto 2012) and has been found in Arabidopsis and rice (Mashiguchi et al. 2011, Won et al. 2011, Abu-Zaitoon et al. 2012). YUCCAs, encoding flavin monooxygenase-like enzymes (FMOs), key enzymes in auxin biosynthesis, are highly conserved in plant (Liu et al. 2014). The homologous YUCCA genes have been cloned in various plants such as rice (Yamamoto et al. 2007, Yi et al. 2013), tomato (Kim et al. 2013), and petunia (Tobeña-Santamaria et al. 2002). For example, in maize, the monocot-specific YUCCA gene SPARSE INFLORESCENCE (SPI) is the key gene in the formation of axillary meristem and lateral organs (Gallavotti et al. 2008). The ZmYUC1 is critical for maize endosperm development (Bernardi et al. 2012). YUCCA genes are required for SE induction and the
development in Arabidopsis (Bai et al. 2013). Bai et al. (2013) also reported that ethylene down-regulates auxin biosynthesis in Arabidopsis by inhibiting the expression of YUCCA genes. The quadruple mutations of YUCs in yuc1yuc4yuc10yuc11 disturb local auxin distribution, which causes abnormalities in embryonic development such as the absence of hypocotyls or root meristem. Previous studies indicated that YUCCA-mediated auxin local synthesis is essential for Arabidopsis SE and participates in the formation and local distribution of auxin (Wójcikowska et al. 2013).

AUXIN RESPONSE FACTORS (ARFs), transcription factors that influence the expression of auxin-regulated genes, can specifically bind to auxin response elements in promoters of auxin-responsive genes to activate or inhibit the expression of downstream genes (Ulmasov et al. 1999, Hagen and Guilfoyle 2002, Shen et al. 2015). ARFs have been reported to have important functions in growth and development, including embryogenesis, vegetative organ development, flower or fruit development, vascular bundle formation, and so on (Carey and Krogan, 2017, Peng et al. 2017, Ren et al. 2017, Liu et al. 2018). Until now, the analysis of ARF gene family has been carried out in various plants such as Arabidopsis (Remington et al. 2004), rice (Wang et al. 2007), alfalfa (Shen et al. 2015), tomato (Kumar et al. 2011), and other plants. In addition, the function of ARF genes in plant regeneration also has been reported. Lee and Seo (2017) proved that high temperature promotion of callus formation requires the BIN2-ARF7/19-LBD in Arabidopsis. Roodbarkelari et al. (2015) reported that the ability of AGO10 to maintain shoot meristem cells during Arabidopsis embryogenesis is connected with down-regulating ARF2-mediated auxin response. Lin et al. (2015) presumed that the expression of ARF10, ARF16, and ARF17 negatively regulating auxin signals during the SE processes of Dimocarpus longan indicate the importance of ARF genes in plant regeneration.

Lilium spp., monocotyledonous bulbous plants belonging to Liliaceae family, have large flowers, bright colors, and strong adaptability, and thus they are utilized as important fresh cut flowers and potted flowers. More and more scientists have carried out research on their physiology and molecular biology (Bakhshaie et al. 2016). China is one of the world’s major genetic resources and distribution countries of Lilium spp. From the 115 species of Lilium, 55 species and 18 cultivars are originated in China. Thus it has great theoretical and also economic significance to carry out research on somatic embryogenes (SE) of Lilium spp.

The role of ARFs and YUCCAs in SE of Lilium spp. are still unclear. Therefore, the aim of this work was to analyze ARF and YUCCA families in Lilium by using bioinformatic methods. Based on the information from Lilium spp. transcriptome database, 12 ARF genes and 6 YUCCA genes containing intact coding sequence (CDS) regions we detected in the Lilium cultivar ConcaD’Or and their characteristic sequences annotated. Reverse transcription quantitative PCR method was conducted to analyze their expression patterns during the SE induction and developmental stages.

Materials and methods

Plants and cultivation: Seedling roots of the Lilium spp. L. Oriental × Trumpet hybrid called ConcaD’Or were used as explants. Roots approximately 1.0 cm in length, including the root tips, were inoculated on a Murashige and Skoog medium supplemented with 1.0 mg dm⁻³ picloram and cultured in the dark and a temperature of 25 ± 2 °C to induce embryogenic calli (Zhou et al. 2013). Root explants were sampled after 0 d (control), 20 d (embryogenic callus induction1, ECI1), 40 d (ECI2), and 55 d (ECI3) on culture medium to represent different stages of ECI (Chen et al. 2019).

Then, embryogenic callus was inoculated on a Murashige and Skoog medium supplemented with 0.5 mg dm⁻³ abscisic acid (ABA) to observe its growth and development. Callus, globular embryo, shield-shaped embryo, cotyledonal shaped embryo, and mature embryos were sampled at 0 d (callus), 10 d (A1), 20 d (A2), 30 d (A3), and 40 d (A4), respectively (Fig. 1). Each development stage explants were sampled 3 times, each sample contained more than 10 explants. Materials were immediately frozen in liquid nitrogen and stored at -80 °C until total RNA was extracted.

Determination of IAA content: Root explants induced at 0, 20, 40, and 55 d, and embryos cultured at 0, 10, 20, 30, and 40 d were sampled and frozen in liquid nitrogen to detect the content of IAA. The sample preparation was conducted according to Pan et al. (2010). The IAA measurement was carried out high performance liquid chromatography mass spectrometry method as previously described (Liu et al. 2013). The biological samples were repeated more than three times.

Gene identification of the ARF and YUCCA family: The CDS sequences of the LiARFs and LiYUCs were derived from the transcriptome data of Lilium (SRP098955), Arabidopsis thaliana, and Oryza sativa sequencerences were derived from the NCBI database. The conserved domain of the LiARFs and LiYUCs were analyzed using SMART (http://smart.embl-heidelberg.de/) and NCBI database. The molecular mass of the ARF and YUCCA proteins, the isoelectric point of the proteins as well as their affinity/hydrophobicity were predicted using the ExPASy (http://web.expasy.org/protparam/) tool. The Netphos 3.0 serve (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict the protein phosphorylation sites. To exhibit the structural divergence of LiARF and LiYUC proteins, the conserved motifs were performed with multiple expectation maximization for motif elicitation (MEME v. 4.11.2) online program (Bailey et al. 2015).

Evolutionary tree construction: Homology comparisons of the multi-sequence amino acid were performed using the ClustalW program with the default parameters, and phylogenetic trees were constructed by using the MEGA-X software with the neighbor-joining method.

Reverse transcription quantitative PCR: Total RNA
was extracted from the samples using a RNeasy Plant Mini kit (Omega, Norcross, GA, USA) according to the instructions. First-strand cDNA synthesis was performed using the Primer-Script TM RT reagent kit (TaKaRa, Dalian, China).

SYBR® Premix Ex TaqII (TaKaRa) was used to conduct the reverse transcription quantitative PCR reactions. The PCR reaction system consisted of 2 mm$^3$ of cDNA (1:10), 10 mm$^3$ of 2 × SYBR Premix Dimer Eraser, and 1 mm$^3$ of each primer (Table 1 Suppl.), synthesized by Shanghai Shenggong Bioengineering Technology Service Co.. Results were normalized using the internal reference genes EIF (EUKARYOTIC INITIATION FACTOR 1α; acc. No. KP861874) and EF1 (ELONGATION FACTOR 1-α; acc. No. KJ543461). PCR reactions were performed in a light cycler 480II (Roche, Penzberg, Germany) under the following conditions: an initial incubation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s, and cooling at 40° C for 30 s. The gene-expressions were calculated over three biological replicates by Genorm software.

Statistical analysis was conducted by Excel 2010 and SPSS 17.0 software. In order to assess the statistical significance of the differences among sampling stages, a one-way analysis of variance followed by the Duncan’s multiple range test ($P \leq 0.05$ and 0.01) was employed. Fold change were compared to the control period during the induction stages or callus period during the development stages.

Results

A total of 12 LiARF genes were identified by BLASTP analysis, each containing a plant-specific PRONE domain. For example, the DNA binding domain (DBD) motif was found in LiARF5/7/9/11/12/15/17/19/20/21, the C-terminal domain (CTD) motif was found in LiARF5/7/9/11/12, the ARF motif was found in LiARF5/7/9/11/12/15/17/19/21 (Fig. 1 Suppl.). The amino acid length ranges from 250 to 1 009, the molecular mass ranges from 27 142.88 to 112 309.86 and the isoelectric point ranges from 4.95 to 9.74. The specific characteristics are shown in Table 2 Suppl.

A total of 6 LiYUC genes were identified by BLASTP analysis, each containing flavin adenine dinucleotide-binding motif, GC motif, ATG-containing motif1, ATG-containing motif2, FMO-identifying sequence, and NADPH-binding motif (Fig. 2 Suppl.). The amino acid length ranges from 251 to 429, the molecular mass ranges from 27 854.23 to 48 347.92 and the isoelectric point ranges from 5.93 to 8.97. The specific characteristics are shown in Table 2 Suppl. The gene structure analysis of LiARFs and LiYUCs according to the phylogenetic relationship are shown in Fig. 3 Suppl.

To investigate the evolutionary relationship between LiARFs and those in other species such as Arabidopsis thaliana and Oryza sativa, a neighbor-joining tree was generated based on alignments of the amino acid sequences of 12 LiARFs, 22 AtARFs and 23 OsARFs. The results showed that all the ARFs could be grouped into six major clusters (I, II, III, IV, V, and VI) based on their phylogenetic relationships (Fig. 2A). Twelve LiARFs were distributed among the six clusters. For example, LiARF16/22 were classified into OsARF16/LiARF17/19/20/21 were classified into OsARF8/10/13/18/20 and AtARF5/10/16/17, LiARF12 was classified into OsARF6/12/17/25, LiARF5 was classified into OsARF2/3/14/15 and AtARF3/4, LiARF7/9/11 was classified into OsARF1/7/4/23/9/23 and

![Fig. 1. Different stages of somatic embryogenesis of Lilium spp. cv. Conca D’Or callus: embryogenic callus (A1); callus transferred to a Murashige and Skoog medium supplemented with 0.5 mg·dm$^{-3}$ abscisic and grown for 10 d (A2), 20 d (A3), and 40 d (A4). Bars are 0.2 cm.](image-url)
Fig. 2. Phylogenetic analysis of AUXIN RESPONSE FACTOR (ARF) and YUCCA proteins in *Lilium* spp., *Arabidopsis thaliana*, and *Oryza sativa* constructed by using the MEGA-X software with the neighbor-joining method.
showed a trend, which peaked at A4 period, the remaining B displayed up-regulation, displayed a distinct up-regulation during A2/A4 periods, cluster IV included A, ≤ 0.01. A showed an increased expression at the ECI2 stage and then its decreased expression at ECI3 stage. AtARF1/9/11/18/13/5/7/8/9-a showed a decreased expression at the ECI1 stage and then its decreased expression at ECI3 stage. LiYUCs were distributed among the four clusters. For example, cluster I included LiYUC4, AtYUC1, AtYUC4, OsYUC1 4-a, OsYUC1 4-b, OsYUC1 4-c; cluster II included LiYUC10, AtYUC10, AtYUC11, OsYUC10 11-a, OsYUC10 11-b, OsYUC10 11-c; cluster III included LiYUC2, LiYUC6, AtYUC2, AtYUC6, OsYUC2 6-a, OsYUC2 6-b; cluster IV included LiYUC5, LiYUC9, AtYUC3, AtYUC5, AtYUC7, AtYUC8, OsYUC3 5 7 8 9-a, OsYUC3 5 7 8 9-b. Root explants induced at 0, 20, 40, and 55 d, and somatic embryos cultured at 0, 10, 20, 30, and 40 d were sampled to detect their IAA content. Results showed that all the IAA content decreased during A1 period, increased during A2 period, and decreased during A3 and A4 periods (Fig. 3B). The expression patterns of LiARFs were significantly different at different ECI stages (Fig. 4A). LiARF5, LiARF7, and LiARF21 displayed a distinct up-regulation during the embryogenic callus induction stages. LiARF17/20/22 showed an increased expression at the EC12 stage and decreased expression at EC13 stage. LiARF11 showed a decreased expression at the EC11 stage and then its expression increased at EC12 and EC13 stages. LiARF12/18 showed a decreased expression at the EC11 stage and then its expression increased to a maximum at EC12 stage. The expression patterns of LiYUCs also changed at different ECI stages. LiYUC2 and LiYUC4 showed a significantly increased expressions at the EC13 stage, while LiYUC5, LiYUC6, LiYUC9, and LiYUC10 showed high expressions at 0 d (control) and the expressions decreased significantly at EC11, EC12, and EC13 stages (Fig. 5A). The content of IAA increased with the induction time of embryogenic callus which is consistent with the expression pattern of LiYUC2 and LiYUC4.

The development of SE underwent different ontogenetic stages, i.e., globular, shield-shaped, and cotyledonal shaped, on a medium supplemented with 0.5 mg dm⁻³ abscisic acid and 10 d (A1), 20 d (A2), 30 d (A3), and 40 d (A4), respectively. Means ± SEs, n = 3, ** - significant differences between treatments at P ≤ 0.01.
embryos. The content of endogenous IAA decreased with the development of SEs which was consistent with the expression trend of LiYUC4 and LiYUC10, indicating that they may play a positive role in the development of somatic embryos.

**Discussion**

The importance of auxin in determining cell fates in regenerating callus, tissues, or organs by activating specific developmental programs has been shown (Ikeuchi...
The present study revealed the main structural features of the YUCCA and ARF gene families based on the transcriptome database of SE induction in Lilium spp. The full length of CDS for 6 members in YUCCA family and 12 members in ARF family were successfully isolated. The LiYUCs and LiARFs were named based on their phylogenetic relationships with Arabidopsis homologs.

The YUCCA genes were highly conserved among Lilium spp., Oryza sativa, and Arabidopsis thaliana according to the analysis of results. YUCCA4 proteins can be grouped into 4 groups: YUC1-4, YUC2-6, YUC3-5-7-8-9, and YUC10-11 (Poulet and Kriechbaumer 2017). In the present study, LiYUC4 was classified into the AtYUC1-4/OsaYUC1-4 group and LiYUC10 was classified into the AtYUC10-11/OsaYUC10-11 group, which were referred to their function in embryogenesis (Stone et al. 2008, Bai et al. 2013). LiYUC2 and LiYUC6 were classified into the AtYUC2-6/OsaYUC2-6 group, which were the main YUCs expressed in shoots; LiYUC5 and LiYUC9, the main YUCs expressed in roots (Chen et al. 2014), were classified into the AtYUC3-5-7-8-9/OsaYUC 3-5-7-8-9 group. In addition, the protein domain analysis of LiYUCs showed that they also contain the conserved motifs (flavin adenine dinucleotide binding motif, GC motif, ATG-containing motif, FMO-identifying sequence, and NADPH-binding motif).
motif). The function of YUCCA genes in somatic embryogenesis has been reported. Many plant embryonic regulators such as LEAFY COTYLEDON 1 (LEC1) and LEC2 appear to promote somatic embryogenesis by modulating auxin biosynthesis and signaling (Ikeuchi et al. 2019). For example, LEC1 can induce auxin biosynthesis via induction of YUC10, LEC2 can activates YUC2 and YUC4 in Arabidopsis (Stone et al. 2008). In addition, Bai et al. (2013) reported that yuc1yuc4yuc10yuc11 quadruple mutations showed abnormalities in embryonic development including absence of hypocotyls or root meristem. In our research, the expressions of LiYUC2, LiYUC4, and LiYUC10 were also significantly changed during SE induction and development, which was consistent with the above conclusions. In addition, expressions of LiYUC6, LiYUC5, and LiYUC9 were also changed which may play a role in the SE induction and development.

Different from the YUCCA genes, ARF protein has been reported to have three characteristic regions, including DBD, ARF, and CTD domains (Guilfoyle and Hagen 2007). The present study of LiARF protein showed that most LiARFs have the characteristic region, except LiARF16 and LiARF22 which do not have the DBD motif, ARF motif, and CTD motif. Phylogenetic analysis indicated that the most LiARFs were classified into OsARF group, indicating the closer relationship with OsARFs than AtARFs.

In general, ARFs are classified as activators or repressors during the gene transcription, AtARF5/6/7/8/12 are reported as activators and AtARF1/2/3/9 as repressors of the target genes transcription (Tiwari et al. 2001). In addition, ARF7/19 also play key roles in callus induction in Arabidopsis root explants (Fan et al. 2012). In our research, the expressions of LiARF5/7/21 were up-regulated during the induction stage and LiARF7/11/12/13/16/17/19/20/21/22 showed a trend of increasing during the first SE development stage, indicating that the LiARF s play significant roles. The ARF functions in somatic embryogenesis has been reported in many plants such as Arabidopsis, Dimocarpus longan, and Gossypium hirsutum (Lin et al. 2015, Wojcikowska and Gaj 2017, Sun et al. 2019). However, the ARF functions in somatic embryogenesis of Lilium spp. still need more research to be conducted.

The induction and regeneration of plant SEs is a complex process. In this study, the embryogenic callus induction on root explants requires the addition of picloram, a herbicide with auxin-like properties similar to 2,4-D (Kelford and Caso 1966). The presence of picloram can induce the biosynthesis of IAA in our research, which is consistent with other reports (Vondráková et al. 2011, Pescador et al. 2012, Ayil-Gutiérrez et al. 2013, Song et al. 2020). The relationship between IAA biosynthesis and development of carrot callus has been reported (Michalczyk et al. 1992). In carrot, most of the initial cell clusters developed into somatic embryos after initial 2 weeks of culturing in medium without 2,4-D, and at least a 10-fold decreasing IAA content was detected (Michalczyk et al. 1992). Similar results were found in Arabidopsis where correct WUSCHEL expression was induced by removal of exogenous auxin (Su et al. 2009). In Lilium spp., the removal of auxin from the medium and addition of ABA can promote the development of embryogenic callus into somatic embryos and IAA content decreased during this processes (Fig. 3B).

Abscistic acid is one of the commonly used hormones for inducing embryogenic callus. For example, exogenous ABA can promote the formation of embryogenic callus and SE maturation in Glycine max, Cocos nucifera, and Vitis vinifera (Fernando et al. 2000, Tian et al. 2000, Prado et al. 2014). Especially in some woody plants, ABA can assist the proembryo formation (Zavattieri et al. 2010, Jain et al. 2013, Prado et al. 2014, Zhang et al. 2014, Zhou et al. 2016). In our study, the addition of ABA also promote the SE development of Lilium spp., which provide reference on SE development in bulbous plants.

Taken together, we identified 6 YUCCA genes and 12 ARF genes in Lilium spp. In addition, the IAA content and the expressions of LiYUCs and LiARFs were detected to search the key genes during SE induction and development. According to the results, LiYUC2/4 and LiARF5/7/21 had the similar expression profile with IAA content pattern during the SE induction periods. LiYUC4/10 and LiARF7/17/18/20/21/22 expressions showed similar downward trend as the IAA content during the SE development stages. These genes seems to be engaged in the Lilium spp. somatic embryogenesis. Results of this study provided important information to reveal the functions of IAA in somatic embryogenesis of Lilium spp.

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