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Overexpressing rice lesion simulating disease 1-like gene (*OsLOL1*) in *Gossypium hirsutum* promotes somatic embryogenesis and plant regeneration

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Abstract

Background: Cotton somatic embryogenesis is difficult or rarely frequent to present, which has limited gene function identification and biotechnological utility. Here, we employed a rice key somatic embryogenesis-related gene, rice lesion simulating disease 1-like gene (*OsLOL1*), to develop transgenic cotton callus for evaluating its function in ectopic plants.

Results: Overexpressing *OsLOL1* can promote cotton callus to form embryogenic callus, not only shortening time but also increasing transition of somatic callus cells to embryogenic callus cells. And the regenerating plantlets per transgenic *OsLOL1* embryogenic callus were significantly higher than those in the control transformed with empty vector. Analysis of physiological and biochemical showed that *OsLOL1* can repress cotton superoxide dismutase 1 gene (*GhSOD1*) expression, possibly resulting in reactive oxidant species (ROS) accumulation in transgenic callus cells. And *OsLOL1*-overexpressed embryogenic callus exhibited higher α -amylase activity compared with the control, resulting from the promotion of *OsLOL1* to cotton amylase 7 gene (*GhAmy7*) and *GhAmy8* expression.

Conclusion: The data showed that *OsLOL1* could be used as a candidate gene to transform cotton to increase its somatic embryogenesis capacity, facilitating gene function analysis and molecular breeding in cotton.

Keywords: *Gossypium hirsutum*, Somatic embryogenesis, *OsLOL1*, *GhSOD1*, *GhAmy7*

Introduction

Plant somatic embryogenesis is a key process of cell totipotency and is an important type of plant regeneration as well as organogenesis plant regeneration. Cotton somatic embryogenesis generally depends on the genotypes of varieties. Currently, quite a few varieties can take place somatic embryogenesis (Cao et al. 2019; Kumria et al. 2003; Mishra et al. 2003; Sun et al. 2006; Wu et al. 2004; Xiao et al. 2018). Additionally, cotton somatic

embryogenesis and plant regeneration are time- and labor-consuming processes, because it takes a long time to transform somatic callus cells into embryogenic callus cells (somatic-to-embryogenic callus cells) with quite low transition rates (Li et al. 2019). Cotton somatic embryogenesis is a key process in plant regeneration and genetic transformation, which contributes to cotton gene function identification and molecular breeding. However, cotton somatic embryogenesis and plant regeneration are affected by genotypes and environmental factors, of which cotton genotype is a main effector to determine cell fate from somatic callus cells to embryogenic callus cells. The transition of somatic-to-embryogenic cells may be determined by a set of genes' combination or quite a few genes in plants (Cao et al. 2019; Wu et al. 2004; Xiao et al. 2018).

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Currently, the increasing researches showed that some key genes regulate plant somatic embryogenesis and plant regeneration (Borderies et al. 2004; Geshi et al. 2013; Ledwoń and Gaj 2011; Long et al. 2018; Nowak et al. 2015; Pérez-Pérez et al. 2019; Poon et al. 2012; Solís et al. 2016; Stone et al. 2008; Uddenberg et al. 2016; Wang et al. 2005; Zheng et al. 2013). For example, AGAMOUS-Like15 promotes somatic embryogenesis in *Arabidopsis* and soybean (Zheng et al. 2013). LEAFY COTYLEDON1, FUSCA3 regulate somatic embryogenesis induction in *Arabidopsis* under auxin treatment (Ledwoń and Gaj 2011). *PaHAP3A* overexpression can promote ectopic embryo differentiation from maturing somatic embryos of Norway spruce (Uddenberg et al. 2016). *Arabidopsis* ERF022 induces somatic embryogenesis associated with the ethylene-related pathway (Nowak et al. 2015). Arabinogalactan proteins (AGPs) stimulate somatic embryo development in maize (Borderies et al. 2004), *Arabidopsis* (Geshi et al. 2013), cotton (Poon et al. 2012), and *Quercus suber* (Pérez-Pérez et al. 2019). Rice lesion simulating disease (OsLSD1) protein, also named as OsLSD1-like, promotes somatic embryogenesis and plant regeneration (Wang et al. 2005). Recent report showed that miR156-SPL modules regulate induction of somatic embryogenesis in citrus callus (Long et al. 2018). However, the few key genes or proteins have been identified in cotton somatic embryogenesis. Thereby, we investigated whether the somatic embryogenesis-related from other plants can promote transition of cotton somatic-to-embryogenic callus cells.

These key embryogenic genes could regulate the expression of downstream genes to change physiological and biochemical stats of callus cells, facilitating in transition of somatic-to-embryogenic callus cells. These reports indicated that somatic callus cells under abiotic stresses could be easier to transform into embryogenic callus cells (Castander-Olarieta et al. 2019; Mohanapriya et al. 2019). Abiotic stresses usually bring high reactive oxidant species (ROS) accumulation in callus cells, reasoning from expressing repression of antioxidant enzyme genes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and so on (Fehér 2015; Ikeda-Iwai et al. 2003; Pasternak et al. 2002). Recently, starch content in cells was found to determine transition of somatic-to-embryogenic callus cells (Long et al. 2018). The callus cells with high starch levels could develop embryogenic callus cells, but others with low starch levels could not. These researches showed that different physiological and biochemical status in callus cells determined whether these cells could form embryogenic callus cells.

Previous researches showed that OsLOL1 can promote rice somatic embryogenesis and plant regeneration (Wang et al. 2005). Further study of OsLOL1 found that

it repressed *OsSOD1* expression and attenuated SOD activity in rice, leading to ROS accumulation (Wu et al. 2014). In *Arabidopsis*, LSD1, a homolog of LOL1, performs antagonist function with LOL1 in regulating ROS homeostasis in cells (Dietrich et al. 1994). Interestingly, OsLOL1 could promote rice amylase 7 gene (*OsAmy7*) and *OsAmy8* expression, accelerating starch degradation for energy supplying. Thereby, we employed *OsLOL1* as a candidate gene to transform cotton for increasing its embryogenic callus formation capacity.

In this study, we introduced *OsLOL1* gene in *Gossypium hirsutum* cv. Jihe 713 callus cells. The transformed calli were detected by polymerase chain reaction (PCR) analysis, and expression levels were examined by quantitative real-time PCR (qRT-PCR) analysis. The results showed that overexpressed-*OsLOL1* callus cells possessed higher capacity in transition of somatic-to-embryogenic callus cells and plant regeneration compared with the control callus cells transformed with empty vector. Overexpressed-*OsLOL1* conferred cotton callus cell increasing embryogenic capacity mainly due to ROS accumulation and acceleration of energy supplying.

Results

Overexpressing *OsLOL1* increases cotton somatic embryogenesis

Rice LOL1 has been reported to promote somatic embryogenesis and plant regeneration (Wang et al. 2005; Wu et al. 2014). To increase cotton somatic embryogenesis and plant regeneration, we introduced *OsLOL1* gene into the cotton chromosomes through *Agrobacterium*-mediated transformation method. The 35S:OsLOL1 vector was transformed into the cotton hypocotyl fragments as explants. The putative transgenic plantlets with overexpressing *OsLOL1* were generated through callus induction, embryogenic callus formation, somatic embryo emergence and plantlet regeneration. And empty vector was transformed into the explants as the control. About 84.0% calli transformed with 35S:OsLOL1 vector were positive by PCR detection with *OsLOL1* gene as well as positive amplification of *NPT II* gene (Fig. 1a), and the 88.5% control calli were confirmed positive transformants with *NPT II* gene. As shown in Table 1, the number of embryogenic callus with *OsLOL1* was extremely significantly higher than the control at 90 d after transformation, 0.91 vs 0.39 per explant, indicating that OsLOL1 can promote somatic cell embryogenesis. And embryogenic callus with *OsLOL1* can produce much more regenerating plantlets than the control, 11.8 vs 3.6 per embryogenic callus, which showed that OsLOL1 extremely significantly increase plant regeneration rates compared with the control. Moreover, the callus cells with overexpressing *OsLOL1* extremely significantly shortened the time of somatic-to-embryogenesis callus

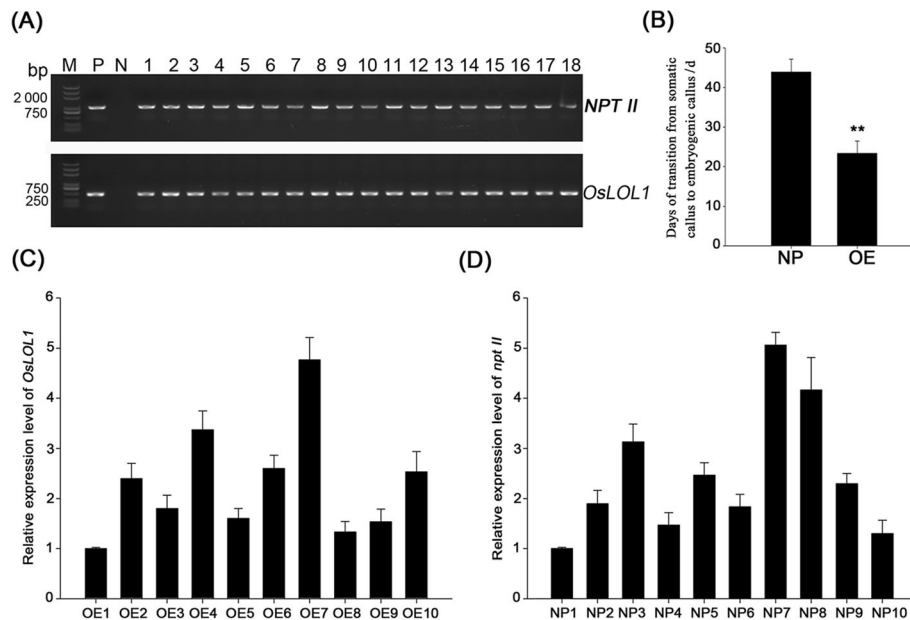


Fig. 1 Development of transgenic cotton embryogenic calli with *OsLLOL1* overexpression and empty vector. **a** PCR detection of transgenic cotton embryogenic calli. M: DNA marker; P: positive control, the plasmid with *OsLLOL1* gene; N: non-transgenic cotton plants; 1–18: various independent embryogenic calli. **b** Days of transition from somatic callus to embryogenic callus. NP: samples with empty vector, OE: those with *OsLLOL1*. Bar values (SD) are calculated through three biological repeats. Double asterisks represent extremely significant differences ($P < 0.01$) by Student's *t*-test. **c** and **d** Relative expression levels of *OsLLOL1* (**c**) and *npt II* (**d**). OE1–10: those with *OsLLOL1* overexpression, NP1–10: the embryogenic calli with empty vector. *GhUbi* gene was used as internal control for normalization. The expression levels of OE1 and NP1 are arbitrarily regarded as "1", respectively. Bars represent SD from biological repeats

by about 21 d compared with the control (Fig. 1b). Together, overexpressing *OsLLOL1* can promote cotton callus embryogenesis and plant regeneration. Additionally, according to qRT-PCR analysis (Fig. 1c and d), at least 10 transformed calli with higher expression level of *OsLLOL1* or *npt II* were selected to subject to gene expression analysis and physiological and biochemical assay.

Overexpressing *OsLLOL1* repressed *GhSOD1* expression

We found lots of transgenic callus cells death around the embryogenic cell granular or somatic embryos although the transgenic *OsLLOL1* calli can form much more embryogenic calli and somatic embryos, indicating that *OsLLOL1* promotes cell death (Fig. 2a). Previous research showed that knockdown of *OsLLOL1* promoted *OsSOD1* expression and attenuated ROS accumulation (Wu et al. 2014). To investigate whether *OsLLOL1* overexpression repressed cotton *SOD1* (JX138573.1)

expression, we conducted qRT-PCR analysis. The result showed that *GhSOD1* expression level in the overexpressing *OsLLOL1* calli was significantly lower compared with the control (Fig. 2b). Consistent with *GhSOD1* expression level, the SOD enzyme activity of *OsLLOL1*-overexpressed calli was extremely significantly lower than that of the control (Fig. 2c). To further measure SOD enzyme activity, we conducted gel nictro-staining assay. The staining intensity of bands loaded with overexpressing *OsLLOL1* callus protein was obviously weaker compared with the control, consistent with quantified results (Fig. 2d). The data showed that overexpressing *OsLLOL1* repressed *GhSOD1* expression and decreased SOD enzyme activity, possibly leading to ROS accumulation in cells. To investigate whether ROS was accumulated in overexpressing *OsLLOL1* calli, the callus tissues were stained with nitrogen blue tetrazole (NBT). The results showed that the colors of the OE calli were darker than that of the control (Fig. 2e), indicating that more ROS in

Table 1 Cotton callus induction, somatic embryogenesis, and regenerating plantlets

Samples	Explants ^a	Induced-calli per explant	Embryogenic calli per explant ^b	Plantlets per embryogenic callus ^c
Overexpressed- <i>OsLLOL1</i>	152.3 ± 18.2	1.76 ± 0.15	0.91 ± 0.07**	11.8 ± 2.2**
Empty vector	156.6 ± 16.5	1.64 ± 0.12	0.39 ± 0.04	3.6 ± 1.1

^a Means ± SD come from 3 experiment repeats. ^b sum of embryogenic calli counted until 90 d after explant transformation. ^c sum of regenerating plantlets for twice subculture of embryogenic callus. ** extremely significant differences ($P < 0.01$) of overexpressed-*OsLLOL1* compared with empty vector by Student's *t*-test

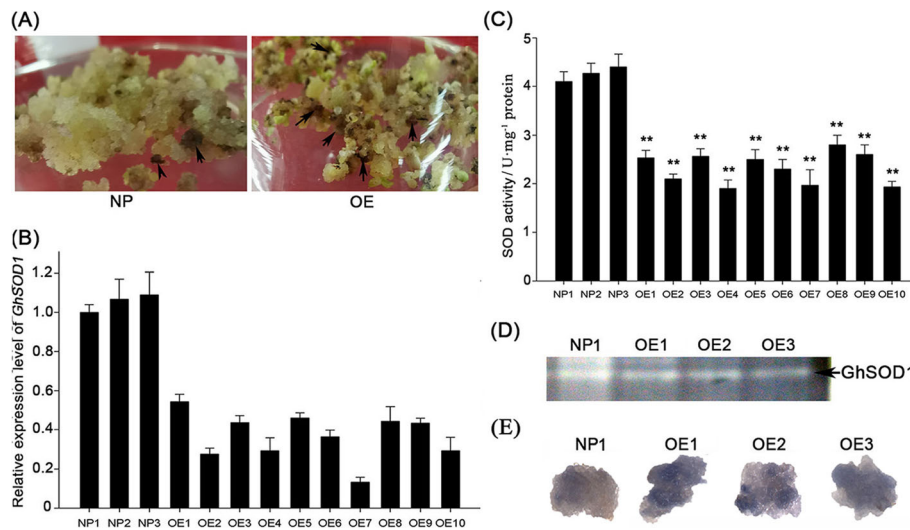


Fig. 2 *OsLLOL1* overexpression represses *GhSOD1* expression and activity. **a** Cell death around embryogenic callus or somatic embryos. Arrows point to the dead cell masses. NP: Calli transformed by empty vector, OE: Calli with *OsLLOL1* overexpression. **b** Relative expression level of *GhSOD1* in embryogenic calli with empty vector or *OsLLOL1* gene. *GhUbi* gene was used as internal control for normalization. The expression levels of OE1 and NP1 are arbitrarily regarded as "1", respectively. **c** SOD activity of embryogenic calli with empty vector or *OsLLOL1* gene. **d** SOD activity by staining analysis in gel in embryogenic calli with empty vector or *OsLLOL1* gene. **e** ROS accumulation of cotton calli by NBT staining analysis. In **(d)** and **(e)**, NP1 is a represent of the embryogenic calli with empty vector. OE1–3 are representatives of the embryogenic calli with *OsLLOL1* overexpression. Bar represents SD from three biological repeats in **(b)** and **(c)**. An asterisk or double asterisks show significant ($P < 0.05$) or extremely significant ($P < 0.01$) differences of OEs compared with NP1 by Student's *t*-test in **(c)** and **(d)**

OE calli was accumulated compared with the control. The results suggested that ROS accumulation by *OsLLOL1* overexpression promotes cotton somatic cell embryogenesis, leading to increase of regenerating plantlets.

Overexpressing *OsLLOL1* increased *GhAMY7* and *GhAmy8* expression

Previous report showed that *OsLLOL1* can promote *OsAmy7* and *OsAmy8* expression to accelerate starch degradation, contributing to seed germination (Wu et al. 2014). To investigate whether overexpressing *OsLLOL1* in cotton calli increased cotton *Amy7* (Ghir_A05G020740.1) and *Amy8* (Ghir_D01G011780.1) expression, we conducted qRT-PCR analysis. The results showed that *GhAmy7* and *GhAmy8* expression levels in transgenic *OsLLOL1* calli were significantly higher by 5 folds compared with the control (Fig. 3a). The α -amylase activity in *OsLLOL1*-overexpressed calli was also significantly higher by 2 folds compared with the control (Fig. 3b). Interestingly, when we measured the starch content, the starch amount in transgenic *OsLLOL1* calli slightly reduced, but not significantly, compared with the control, indicating that cotton calli possess a high potential capacity of starch synthesis. These data suggested that *OsLLOL1* can promote *GhAmy7* and *GhAmy8* expression and increase α -amylase activity, contributing to somatic embryogenesis and plantlet growth.

Discussion

Plant somatic embryogenesis is controlled by single gene or a set of genes (Yang and Zhang 2010). The expression levels of these genes could determine somatic callus cell fate of whether they develop embryogenic callus cells (Yang and Zhang 2010; Rupps et al. 2016). However, cotton somatic embryogenesis highly depends on quite a few genotypes of varieties and is a low rate of transition of somatic-to-embryogenic callus cells (Ge et al. 2015). Here, we introduced a rice somatic embryogenesis-related gene, *OsLLOL1*, into the cotton genome. The results showed that overexpressing *OsLLOL1* can increase cotton somatic embryogenesis capacity and promote plant regeneration.

The *OsLLOL1*-overexpressed embryogenic callus cells had significantly reduced SOD1 activity associated with ROS accumulation compared with the control, possibly leading to increase of somatic embryogenic capacity. The result suggested that high ROS level facilitates somatic embryogenesis in cotton callus cells. This result is similar to ROS accumulation of callus treated by environmental stresses to promote somatic embryogenesis and plant regeneration (Rai and Shekhawat 2011; Rodríguez-Serrano et al. 2012). All in all, high ROS level in callus promotes transition of somatic-to-embryogenic callus cells and plant regeneration.

In this study, *OsLLOL1* overexpression promoted cotton *Amy7* and *Amy8* expression, leading to alpha-amylase activity increase in embryogenic callus cells. High

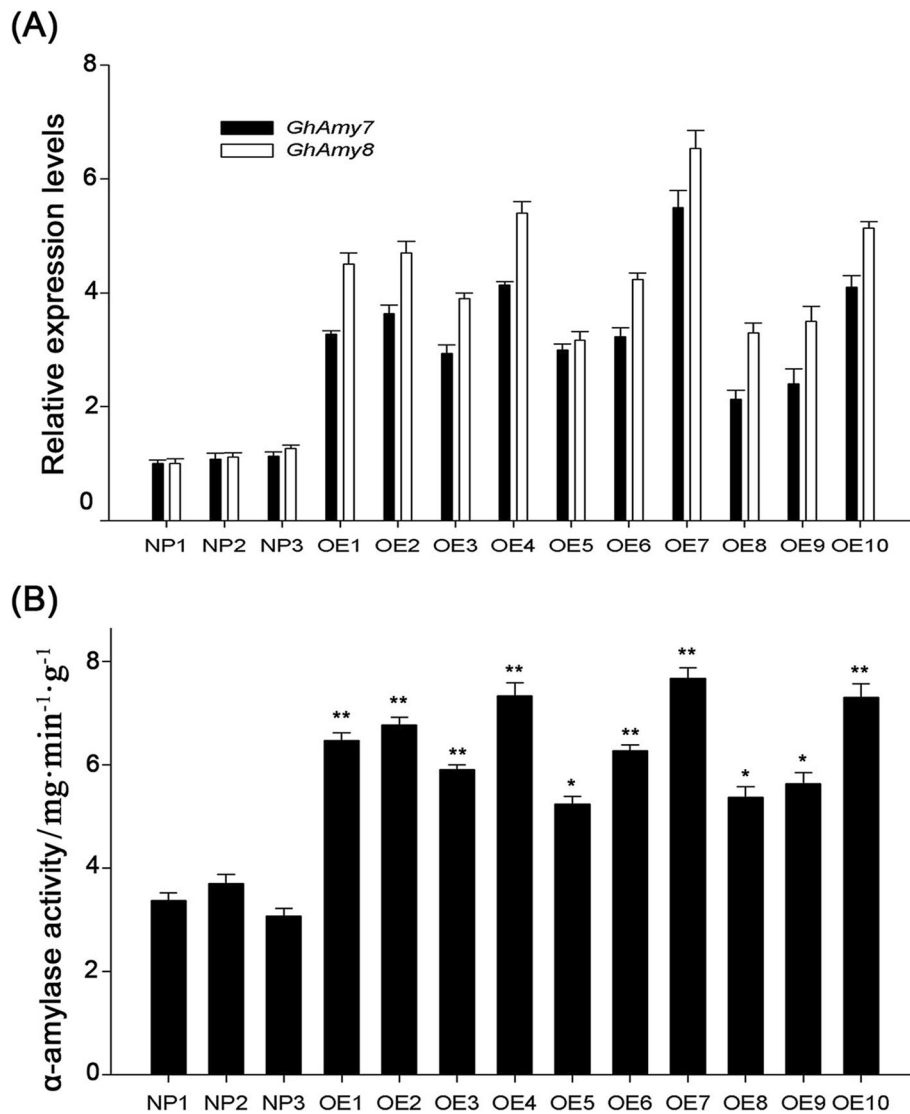


Fig. 3 Overexpressing *OsLLO1* promotes *GhAmy7* and *GhAmy8* expression and α -amylase activity in embryogenic calli. **a** Relative expression levels of *GhAmy7* and *GhAmy8* in the embryogenic calli with *OsLLO1* or empty vector. Bars represent SD from three biological repeats. **b** α -amylase activity of the embryogenic calli with *OsLLO1* or empty vector. Bar represents SD from three biological repeats. One asterisk or double asterisks show significant ($P < 0.05$) or extremely significant ($P < 0.01$) differences of OEs compared with NP1 by Student's *t*-test

amylase level could accelerate starch degradation, providing enough energy to callus cells during transition of somatic-to-embryogenic callus cells. Recently, the report showed that amyloplast in callus cells is important in cell totipotency to determine transition of somatic-to-embryogenic callus cells (Guo et al. 2019). Together, high energy transition and supplying is important in plant somatic embryogenesis and plant regeneration, especially synthesis and degradation of starch.

Materials and methods

Plant materials and transformation

The *G. hirsutum* cv. Jihe 713 were used in this article. The coding sequence (CDS) of *OsLLO1* (GenBank accession

number AY525368) from *Oryza sativa* cv. Nipponbare was amplified and cloned into the pBin438 vector to be driven by CaMV35S promoter. The 35S:*OsLLO1* vector and empty vector were used to transform into Jihe 713 by *Agrobacterium tumefaciens* (strain: LBA4404)-mediated transformation according to previous methods (Wu et al. 2011). The gene cloning primers were listed in Table 2.

DNA extraction

The cotton genomic DNA was isolated and purified from calli by Plant Genome Extraction Kit (TianGen, Beijing, China) according to the instruction manual. The DNA of samples was diluted into $20 \text{ ng} \cdot \mu\text{L}^{-1}$ for PCR reaction. The PCR primers were listed in Table 2.

Table 2 The primers used in PCR analysis

Gene	Forward primer	Reverse primer
<i>OsLLO1</i>	atgccggttccccttctcc	tcagctgctggcttctggtc
<i>npt II</i>	gagccatcaacgggaaac	gaaaaactcatcgagcatcaaatg
<i>GhSOD1</i>	cctcactca atcctgctgg	gagaggaatctgtttgctgac
<i>GhAmy7</i>	ctatgatcactttgttattg	ctcatcaattgctggcaacatac
<i>GhAmy8</i>	gcttatattctaaccatcc	agcccgaatagtaactgtgcttg
<i>GhUbi</i>	cccctgggctggaagatac	ggtgccattactactcgctactg

qRT-PCR reaction

Total RNA of 100 mg embryogenic calli was extracted using the Plant Total RNA Extraction Kit (Sangon Biotech, Shanghai, China). One microgram of total RNA was performed the reverse-transcription reaction to synthesis first-strand cDNA using TransScript First-Strand cDNA Synthesis kit (TransGen). These cDNA samples were further diluted by 20-fold to subject to analysis. The qRT-PCR was employed to check related genes expression levels in embryogenic calli. For qRT-PCR analysis, the SYBRGreen Real-Time PCR Master Mix (TsingKe, Beijing, China) was employed on the CFX96 Touch™ Real-Time PCR detection systems (Bio-Rad, Foster City, United States). The *GhUbi* (*Gh_A09G194200.1*) gene was used as internal control for normalization. All reactions were performed in three biological repeats with three technical repeats. The primers used are shown in Table 2.

SOD activity assay

Total protein was extracted from 100 mg frozen sample in 300 μ L of SOD extraction buffer according to previous reports with minor modification (Van Camp *et al.* 1994). In brief, the resulting homogenate was centrifuged for 5 min at 18 000 *g* to obtain crude protein, whose content was quantified using a protein assay kit (Bio-Rad). The SOD activity was measured according to Beauchamp and Fridovich (Beauchamp and Fridovich 1971). The same experiment was repeated three times.

Forty micrograms protein was subjected to load in 12% non-denaturing polyacrylamide gel electrophoresis (PAGE) and to run at 180 V for 2 h. The gel was stained to determine SOD activity according to the previous methods (Beauchamp and Fridovich 1971; Pan and Yau 1992).

ROS level is monitored by NBT staining in cotton calli according to the operation procedure of the NBT staining kit (Beyotime Biotechnology, Shanghai, China). In brief, the calli were soaked into the BCIP/NBT solution for 20–40 min under dark condition at 37 °C. The reaction was stopped by H₂O, and staining calli were washed with H₂O for 2–3 times. Then, the staining calli were treated with anhydrous alcohol for decolorization and coagulation. Finally, the treated calli were observed under the stereomicroscope and photographed.

Assay of α -amylase activity

The harvested samples were grinded into powder using liquid nitrogen, which were homogenized in a cold PBST buffer and centrifuged at 15 000 $r\ min^{-1}$ for 10 min at 4 °C. To inactivate β -amylase activity, the supernatants were heated to 60 °C for 30 min and centrifuged again to obtain the supernatants for assay of α -amylase activity according to the method of Grosselindemann and Graebe (1991). Determination of α -amylase activity was measured according to the previous reports (Choi *et al.* 1996) with minor modification. In brief, the 2% (m/v) starch solution was used for the assay. An aliquot of enzyme solution was added to a 1.5-mL tube with 0.2 mL of starch solution, 0.2 mL of 0.1 mol·L⁻¹ sodium acetate buffer (pH 5.3) and 0.2 mL of 10 mmol·L⁻¹ CaCl₂. The adjusted 1.2 mL total volume solution was reacted at 37 °C for 10 min and was stopped by 0.4 mL of KI/I₂ solution. The absorbance was measured at 620 nm.

Statistical analysis

Statistical analysis is carried out by two-way ANOVA with Student's *t*-test, and $P < 0.05$ and $P < 0.01$ are considered as significant and extremely significant differences, respectively.

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Authors' contributions

Wu JH and Luo XL initiated the research and designed the experiments; Wang ZA, Wang P, Xiao JL and Zhang AH performed experiment including genetic transformation and biochemical assays; Hu G and Wang ZA carried out molecular analysis and data statistical analysis. Wu JH wrote the manuscript. All authors contributed in the interpretation of results and approved the final manuscript.

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Availability of data and materials

The data and materials for supporting the results of this article are included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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