

Overexpression of maize anthocyanin regulatory gene *Lc* affects rice fertility

Yuan Li · Tao Zhang · Zhong-Wei Shen ·
Yu Xu · Jian-Yue Li

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Abstract Seventeen independent transgenic rice plants with the maize anthocyanin regulatory gene *Lc* under control of the CaMV 35S promoter were obtained and verified by molecular identification. Ten plants showed red spikelets during early development of florets, and the degenerate florets were still red after heading. Additionally, these plants exhibited intense pigmentation on the surface of the anther and the bottom of the ovary. They were unable to properly bloom and were completely sterile. Following pollination with normal pollen, these plants yielded red caryopses but did not mature normally. QRT-PCR analysis indicated that mRNA accumulation of the CHS-like gene encoding a chalcone synthase-related

protein was increased significantly in the sterile plant. This is the first report to suggest that upregulation of the CHS gene expression may result in rice sterility and affect the normal development of rice seeds.

Keywords Anthocyanin regulatory gene (*Lc*) · Chalcone synthase-like gene expression · Sterility · Transgenic rice

Introduction

The maize anthocyanin regulatory gene, *Lc*, is a member of the *R* gene family of MYC-like transcription factors, which is responsible for the expression of anthocyanins in coleoptiles, leaf blade joints (auricles), roots (weak expression), nodes, silks, pericarp and leaf blades. Lloyd et al. (1992) first introduced the *Lc* into tobacco and *Arabidopsis*. They found that transgenic tobacco lines expressing *Lc* show intense red pigmentation in the corolla tube, corolla collar, and anther filaments, and also found that transgenic *Arabidopsis* lines expressing *Lc* produce higher than normal amounts of anthocyanins in leaf, stem, sepal, and pistil (stigma, style and ovary) tissues containing chlorophyll. Thus far, transgenic tomatoes (Goldsborough et al. 1996), alfalfa (Ray et al. 2003), apple (Li et al. 2007) and creeping bentgrass (Han et al. 2009) containing *Lc* have been obtained. Apple calli (Li et al. 2007) and creeping bentgrass calli (Han et al. 2009) transformed by *Lc* alone become red/purple.

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Y. Li · T. Zhang · Z.-W. Shen · Y. Xu · J.-Y. Li (✉)
College of Life and Environmental Sciences,
Shanghai Normal University, Shanghai 200234,
People's Republic of China
e-mail: lijianyue01@yahoo.com.cn

Y. Li
e-mail: liyuannkc@gmail.com

T. Zhang
e-mail: 18817594883@163.com

Z.-W. Shen
e-mail: nbu.shenzhongwei@163.com

Y. Xu
e-mail: violethsu@126.com

Because plants transformed with *Lc* may show obvious and visible phenotypes, *Lc* could be used as a valuable reporter gene in biological engineering. Growing public concern regarding the presence of antibiotic resistance genes in transgenic crops has limited consumer acceptance of genetically-modified plants. Rice is an important crop and a major food source. If a visual marker gene could be substituted for an antibiotic-resistance gene in an expression vector, then the transformed rice calli might produce intense colour pigmentation, allowing selection of these calli for further regeneration culturing.

To test this theory, we investigated whether rice calli transformed with *Lc* change colour. Although there was no visible colour change in the transformed rice calli, we found that *Lc* transformation affected rice fertility: most transgenic rice plants were completely sterile. Additionally, the reproductive organs of the sterile transgenic plants exhibited unique traits, and mRNA expression levels of the *Chsl* gene encoding a chalcone synthase-related protein (Qu et al. 1997), were increased significantly in the sterile transgenic plant.

Materials and methods

Plasmid vector and rice transformation

An expression vector pCAMBIA1301-35S-*Lc* (Supplementary Fig. 1) with the anthocyanin regulator gene *Lc* cDNA under control of the CaMV35S promoter was constructed in our laboratory (Wei et al. 2005). Calli were produced from mature embryos of rice cultivar “*Chao2-10*” (*Oryza sativa* L. *japonica* cv. “*Chao2-10*”) seeds and infected with *Agrobacterium tumefaciens* with p1301-35S-*Lc*. The transformation method employed has been described previously (Li et al. 2008a).

PCR analysis of genomic DNA from transgenic plants

The primers LC1 (5'-GCCGGCTCTCTGTCGCCGGA-3') and LC3 (5'-GTCTGCTGCGCCTCGCCGTCTC-3') were designed to amplify a *Lc* fragment (GenBank:M26227) under the following conditions: 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, 62 °C for 40 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min.

Southern blot analysis of transgenic plants

The primers ZH16 (5'-GCGTCTGCTGCTCCATACAA-3') and ZH17 (5'-TGACATTGGGGAGTTTAGCG-3') were designed according to the sequence of the hygromycin-resistance gene (GenBank:AF234297) in the vector p1301-35S-*Lc*. A 506-bp fragment was amplified and was used as the probe in Southern blot analysis. Total DNA from the transgenic plants was digested with *EcoRI* at 37 °C overnight. The Southern blotting procedure was carried out in accordance with the protocol of the Amersham ECL direct nucleic acid labelling kit.

Lc and CHS-like gene (*Chsl*) expression analyses of transgenic plants

Total RNA was extracted from florets of the non-transgenic rice cultivar “*Chao2-10*” and the transgenic plants, and then treated with DNase I to eliminate genomic DNA contamination. The *Lc* mRNA transcriptions of transgenic plants were identified by RT-PCR using LC1 and LC3 primers.

Using β -actin as an internal standard, the mRNA expression levels of the *Chsl* was analysed by QRT-PCR (Bio-Rad, CFX96). Based on the β -actin mRNA sequences (GenBank:X16280.1), the primers Actin-F (5'-TGGCATCTCTCAGCACATTCC-3') and Actin-R (5'-TGCACAATGGATGGGCCAGA-3') were designed. According to the *Chsl* mRNA sequences (GenBank:X91811.1), specific primers CHSL-F (5'-GTCCCTTCCTAGAGCTTCAGTT-3') and CHSL-R (5'-GTCCC TTCCTAGAGCTTCAGTT-3') were designed. 20 μ l reaction contained 10 μ l iQ SYBR Green SuperMix (Bio-Rad), 0.5 μ l forward primer (10 nM), 0.5 μ l reverse primer (10 nM) and 1 μ l template and 8 μ l DEPC water. The mixture was incubated in a 96-well plate at 94 °C for 5 min, followed by 45 cycles at 15 s at 94 °C, 20 s at 55 °C, 20 s at 72 °C and 5 s at 85 °C then melting curve program (65–95 °C), with a heating rate of 0.5 °C/s and a continuous measurement. The experiments were repeated two times.

Results

Production and molecular identification of transgenic rice lines

Through differentiation and regeneration of the selected calli, 28 independent transgenic seedlings

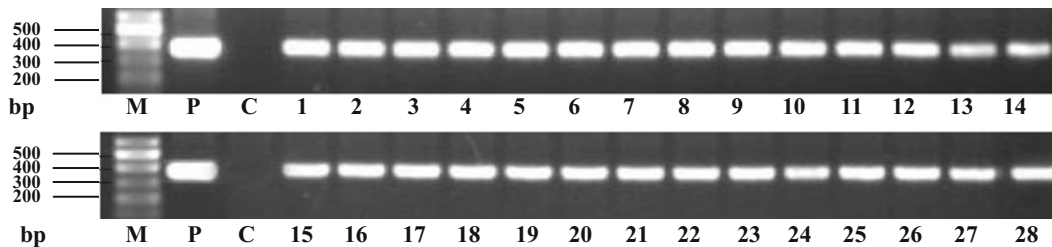


Fig. 1 Agarose gel electrophoresis of PCR product to confirm the *Lc* in genomic DNA of the transgenic rice. A size of 369 bp were amplified from the genomic DNA of all of the transgenic

plants and the vector p1301-35S-*Lc*, whereas no bands were obtained for the wild type plant. *M* marker DNA, *P*, p1301-35S-*Lc*, *C*, wild type; *Lanes 1–28* transgenic plants

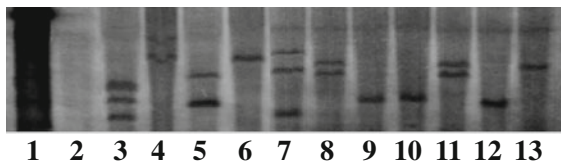


Fig. 2 Some of the T_0 transgenic plants were identified by Southern blot. The results indicated that the copy number and the integration sites of the foreign genes in the transgenic plants can be estimated based on the pattern of the hybridisation bands, suggesting that most of the transgenic plants integrated one to three copies of p1301-35S-*Lc* T-DNA. *Lane 1* p1301-35S-*Lc*, *Lane 2* wild type, *Lane 3–13* transgenic plants

transgenic plants were able to open. Regardless of anthesis, the transgenic plants were completely sterile.

The anthers of the non-transgenic plant were light yellow after heading (Supplementary Fig. 3a). After stripping away the lemma and palea of the transgenic plant florets after heading, the anthers were pink. There was intense red pigmentation between anther cells (Supplementary Fig. 3b and 3c), and the redness on the anther surface and between anther cells gradually darkened with the development of the florets (Supplementary Fig. 3c). Pigmentation phenomena were also observed out the bottom of the ovaries (Supplementary Fig. 4).

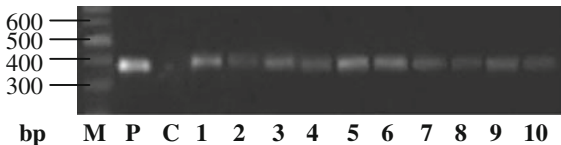


Fig. 3 RT-PCR testing of some T_0 transgenic plants. The result suggested that the *Lc* was transcribed normally in transgenic plants. *M* marker DNA, *P* p1301-35S-*Lc*, *C* wild type, *Lanes 1–10* transgenic plants

were produced, and 17 transgenic plants survived. Integration of the *Lc* in plant genome was confirmed by PCR (Fig. 1) and Southern blot (Fig. 2). To check whether the *Lc* mRNA could be transcribed, RT-PCR of T_0 transgenic plants were analysed (Fig. 3).

Phenotypic observation of transgenic plants

Ten of the 17 surviving transgenic rice lines showed red spikelets during early growth of the rice (Supplementary Fig. 2b). Most of the florets were green after heading, but some degenerate florets were still red (Supplementary Fig. 2d). These plants were able to elongate their filaments but could not bloom normally. Very few florets from these

Hybridisation experiments

We pollinated the completely sterile transgenic plants with fertile pollen from non-transgenic rice via conventional hybridisation. As a control, we covered non-pollinated panicles of the same transgenic plants with paper bags immediately following removal of half of their hulls. The ovaries of the completely sterile transgenic plants were able to undergo intumescence and elongation and to develop normally during the first week after pollination. The caryopses were red (Supplementary Fig. 5). The normal growth of the transgenic rice caryopses began to decline approximately 1 week later, and the caryopses became dehydrated and dry 2 weeks later, so the hybrid seeds did not mature normally. We repeated this hybridisation experiment several times, and the results were consistent. There were no seeds on the non-pollinated control panicles. We also used a cytoplasmic male sterility (CMS) line as a female parent and pollinated it with pollen from the transgenic plants. We harvested four F_1 seeds, and their caryopsis colour was normal, but there were no PCR-amplified *Lc* gene bands found for these F_1 plants.

Table 1 Comparison of the *Chsl* expression levels between the transgenic plant and “*Chao2-10*” control before heading and after heading by QRT-PCR analysis

Transgenic and wild type plants	Relative expression ratio before heading \pm Standard deviation	Relative expression ratio after heading \pm Standard deviation
Transgenic plant	0.66 \pm 0.06	1.2 \pm 0.01
“ <i>Chao2-10</i> ” control	0.1 \pm 0	0.18 \pm 0.02
Transgenic plant/ <i>Chao2-10</i> control	6.9	6.5

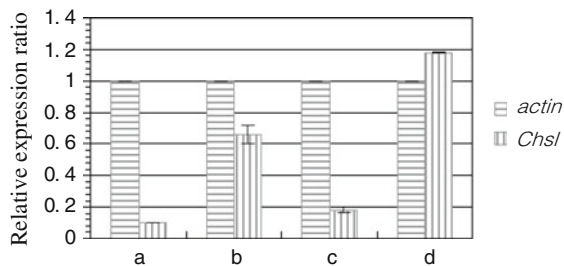


Fig. 4 *Chsl* expression analysis on the florets of the transgenic plant and wild type before heading and after heading by QRT-PCR. The results showed that the mRNA levels of the *Chsl* in the transgenic plant were higher than the control plant in both periods. Error bars represent standard deviation. a wild type florets before heading, b transgenic plant florets before heading, c wild type florets after heading, d transgenic plant florets after heading; *actin*: Internal standard; *Chsl*: a chalcone synthase-related protein gene

QRT-PCR analysis of the *Chsl* expression

Florets from the non-transgenic rice cultivar “*Chao2-10*” and the sterile transgenic plant collected before heading and after heading were used as the material for QRT-PCR analysis. Using the β -*actin* gene as an internal standard, the mRNA accumulation levels of the *Chsl* was analysed to determine whether the *Lc* might regulate the expression of *Chsl* in the sterile transgenic plants (Table 1, Fig. 4).

Discussion

Prior to this study, we had transformed the p1301-35S-*Lc* vector into tobacco. The transgenic tobacco had shown intense red pigmentation in the corolla tube and corolla collar, and the filaments of the transgenic plants were pink (Li et al. 2008b). In this study, the filaments of transgenic rice with the *Lc* remained colourless, and the transformed calli also did not exhibit a colour change. Schijlen et al. (2004) have reported that the introduction of *Lc* alone may be sufficient to enhance anthocyanins in tissues that would normally

accumulate flavonoids, while in tissues that cannot accumulate flavonoids, both *Lc* and *Cl* (another type of MYB transcription factor in maize) are necessary to increase the amounts of anthocyanin pigments or flavonols. Our research indicates that the transcription factor of the MYB family might not be expressed in filaments and calli in rice plants, which differs from what has been observed for tobacco filaments, apple calli (Li et al. 2007) and creeping bentgrass calli (Han et al. 2009).

Chalcone synthase (CHS) is one of the key enzymes regulating flavonoid metabolism. A lack of chalcone synthase activity affects the germination of pollen and the growth of pollen tubes in maize resulting in self-sterility (Taylor and Jorgensen 1992). Napoli et al. (1999) have found that petunias with a mutation resulting in white anthers, designated *wha*, are functionally male sterile and that transgenic complementation with functional chalcone synthase A (CHSA) cDNA suggests that the genetic lesion responsible for the *wha* phenotype is in *Chs*. Qu et al. cloned a *D5* cDNA from rice anther, and confirmed that *D5* gene was an anther-specific gene encoding a chalcone synthase-related protein, and suggested that it represents a novel member of CHS gene family (Qu et al. 1997). Zheng et al. (2000) introduced the anti-sense strands of the *D5* gene guided by the rice gene *actin1* promoter into rice, and found that the performance of pollen tubes in plants with the anti-*D5* gene was abnormal. A decrease in the expression level of the chalcone synthase gene might thus impact on pollen development. Schijlen et al. (2007) proposed a new strategy to obtain parthenocarpic tomatoes through downregulation of the flavonoid biosynthesis pathway and all of their strong *Chs*-silenced tomato lines were observed to develop parthenocarpic fruits. Research in *Raphanus sativus* by Yang et al. (2008) has suggested that the expression of *Chs* was strongly inhibited in the development of functional pollen not only in nuclear-dependent male sterility but also in cytoplasmic male sterility. Thus far, no reports have suggested that overexpression of the *Chs* related to flavonoid

biosynthesis might lead to rice sterility. Based on the results of our hybridisation experiments, we suggest that rice sterility may be caused by *Lc* affecting male gametophyte development; however, we believe that the introduction of *Lc* might impact the normal development of rice seeds.

Li et al. (2008b), using *Lc*, obtained 24 independent transgenic tobacco plants that produced T₁ seeds that could germinate, grow and bear T₂ seeds. This demonstrates that *Lc* guided by a constitutive promoter did not cause male sterility or impair seed development in tobacco. In rice, however, *Lc* can lead to abnormal development of male gametophytes and rice seeds and to self-sterility. It is inferred that there might be differences between dicots (tobacco) and monocots (rice) regarding the mechanism underlying the sterility caused by enzymes involved in flavonoid biosynthesis.

The *D5 (Chsl)* gene was expressed specifically in tapetum cells and in the peripheral cells of the vascular bundle of rice anthers (Zheng et al. 2000). It is inferred that the *Chsl* expression had increased significantly in those cells of the transgenic plants transformed by *Lc* in this research. CHS is the first key enzyme catalyzing dedicated reaction in flavonoid biosynthesis pathway. Anomalous expression of the CHS gene might affect the subsequent flavonoid synthesis reaction. According to previous studies (Zheng et al. 2000) and our study, we suggest that there may be a very close relationship between flavonoids accumulation and male gametophyte development in rice. Perturbation of a balanced flavonoid accumulation by decreased or increased *Chs* expression might affect rice fertility.

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