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Complete List of Authors:	Wei, Chi; Beijing Forestry University, College of Landscape Architecture Cui, Qi; Beijing Forestry University, College of Landscape Architecture Lin, Yuan; Beijing Forestry University, College of Landscape Architecture Jia, Gui xia; Beijing Forestry University, College of Landscape Architecture
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Efficient *Agrobacterium*-mediated transformation of *Lilium* Oriental ‘Sorbonne’ with genes encoding anthocyanin regulators

Chi Wei[†], Qi Cui[†], Lin Yuan, Gui-Xia Jia*

Beijing Key Laboratory of Ornamental Plants Germplasm Innovation & Molecular Breeding, National Engineering Research Center for Floriculture, Beijing Laboratory of Urban and Rural Ecological Environment and College of Landscape Architecture, Beijing Forestry University, Beijing 100083, PR China

* Corresponding author: Gui-Xia Jia (gxjia@bjfu.edu.cn)

[†] These authors contributed equally to this work.

Abstract: Plant anthocyanin biosynthesis is regulated by a combination of transcription factors. Here, exogenous anthocyanin regulator genes *Delila/Rosea1* (*Del/Ros1*) or *Bperu/Colored aleurone1* (*B1/C1*) were introduced to *Lilium* ‘Sorbonne’ using an efficient and optimized *Agrobacterium tumefaciens*-mediated transformation system. During the *B1/C1* transformation, eight types of modified MS medium for bacterial inoculation and co-cultivation were evaluated. Significant enhancement in putative transformation efficiency was observed upon the removal of KH_2PO_4 , NH_4NO_3 and KNO_3 , or with the removal of all the macro elements. Using the optimal medium, 20 s sonication positively affected the transformation, while no significant improvement was observed following heat shock treatments. Replacing sucrose in co-cultivation medium with high concentrations of maltose or glucose also promoted the transformation. Higher transformation efficiencies were observed when using this optimized protocol for the *Del/Ros1* transformation. The *Del/Ros1* and *B1/C1* transformed plantlets appeared light purple during the preliminary regeneration stage, with some deeper purple tissues also observed in *Del/Ros1* plantlets. After the transformation was confirmed by PCR and Southern blot, the color unexpectedly faded into normal green when the transgenic plantlets were rooted, but significant elevations in anthocyanin levels remained in leaves and scales compared with the non-transgenic plantlets. The introduction of these anthocyanin regulator genes may be useful for the modification of *Lilium* flower color.

Key words

Lilium, *Agrobacterium*-mediated transformation, anthocyanin regulators, macro elements, sonication, carbohydrates

Abbreviations

MS, Murashige & Skoog medium;

TDZ, Thidiazuron;

IBA, 4-(3-indolyl) butyric acid;

NAA, naphthaleneacetic acid;

6-BA, 6-benzylaminopurine.

Introduction

Anthocyanins are a major group of pigments involved in a large range of plant functions. They are also key to the consumer appeal of many ornamental, fruit and vegetable products, and are of increasing interest to researchers for their possible dietary benefits to human health (Martin et al. 2011; Davies and Espley 2013). Their biosynthesis, as part of the phenylpropanoid pathway, is well characterized at both the biochemical and molecular level (Grotewold 2006). In addition to DNA sequences being available for most of the biosynthetic enzymes, sequences have also been identified for regulating the biosynthetic gene transcription (Hichri et al. 2011). Of particular note are R2R3-MYB and basic helix-loop-helix (bHLH) type transcription factors (TFs) that form a complex with a WD-Repeat protein (the “MBW” complex) to activate the biosynthetic genes (Gonzalez et al. 2008). This co-action of R2R3-MYB and bHLH proteins in regulating anthocyanin biosynthesis has been consistently found to occur in a range of monocot and eudicot species (Davies et al. 2012).

The bHLH-type TFs *B1* and *Leaf color (Lc)*, as well as MYB-type TFs *C1* and *Purple leaf (Pl)*, were initially reported to play a role in *Zea mays* anthocyanin production (Ludwig et al. 1989, Goff et al. 1990, Cone et al. 1993). There have since been several reports that heterologously expressed these TFs can enhance the anthocyanin content in transgenic plants. For example, the combinatorial effects of *Lc* and *Pl* conferred purple coloration to creeping bentgrass (*Agrostis stolonifera*) (Han et al. 2009), the transgenic plants expressing both *Lc* and *Pl* genes were entirely purple, whereas those expressing *Pl* alone had purple stems and those expressing *Lc* alone lacked purple pigmentation in adult plants. Similarly, Doshi et al. (2007) reported that the transformation of *B1/C1* with embryo-specific promoters in the absence of selective pressure resulted in visibly elevated anthocyanin accumulation in transgenic wheat (*Triticum aestivum*) and triticale ($\times$ *Triticosecale*). The results demonstrate that anthocyanin regulators *B1/C1* could act as visible markers for selection. Furthermore, heterologous expression had been examined in two genes from *Antirrhinum majus*: *Del* (a bHLH-type TF) and

Ros1 (a MYB-type TF). Anthocyanins were accumulated at high levels in transgenic “purple tomato” (*Solanum lycopersicum*) fruit that had strong antioxidant activity and could significantly extend the life expectancy of mice. While anthocyanin level also rose in plants transformed with *Del* or *Ros1* separately, these levels were not as high as those observed when both genes were used together (Butelli et al. 2008).

As the most widely used and well developed technique of genetic transformation, *Agrobacterium*-mediated transformation is featured with simple manipulation, wide range of adaptable plant tissues and generally low copy of foreign genes. However, the transformation of monocots still leaves much to be desired, and the optimization of transformation system still plays a pivotal role in the genetic manipulation of monocots. The removal of some major elements including PO_4^{3-} , NH_4^+ or Ca^{2+} from the co-cultivation medium has been reported to be effective for the transformation efficiency of some plants (Hoshi et al. 2004; Montoro et al. 2000). By adding certain osmoticums into the medium, the osmotic treatment that originally applied to biolistic transformation was also introduced to *Agrobacterium*-mediated transformation of monocots (Cheng et al. 2004). Some sugars used in osmotic treatment, such as glucose, increased the expression of *vir* genes in *Agrobacterium* with acetosyringone (AS) presented (Wise et al. 2005). Besides, several supplementary methods such as sonication, heat and vacuum treatments have also been reported to benefit the transformation of some plant species (Gurel et al. 2009; Liu et al. 2005).

As a monocotyledonous flowering plant with great ornamental values, *Lilium* is one of the most important floricultural crops in the world. Since Mercuri et al. (2003) successfully obtained the first transgenic lily using *Agrobacterium*-mediated transformation, substantial progress has been made on the permanent transformation of *Lilium* spp. (Hoshi et al. 2005; Liu et al. 2011; Ogaki et al. 2008). Notably, favorable results have been reported by Núñez de Cáceres et al. (2011), who used the basal plate discs of *Lilium* ‘Star Gazer’ bulblets as target tissue. However, because genetic engineering of *Lilium* was delayed compared with other plants, the success of transformation systems, especially *Agrobacterium*-mediated systems, is still constrained to

limited genotypes and tissues (Azadi et al. 2013). ‘Sorbonne’, one of the most popular lily cultivars in the market, has been widely cultivated. The *Agrobacterium*-mediated transformation system of ‘Sorbonne’ is still not perfect and different cultivars vary in genetic characteristics. Therefore, the factors affecting the efficiency remain unclear and require further characterization.

Lilium is known to have different warm flower colors, but lacks cool ones such as blue or purple, because it mainly contains anthocyanins and carotenoids as pigments (Nørbæk and Kondo 1999). On the other hand, plants with colored foliage are generally considered to be of distinct ornamental importance. Therefore, several attempts have been made to modify the leaf or flower color of *Lilium*, but satisfying results were hardly received (Azadi et al. 2010b; Qi et al. 2013), mostly due to the low transformation efficiency in monocots and the difficulty in regulating anthocyanin biosynthesis (Tanaka et al. 2012). For the first time, Tanaka et al. (2012) disclosed a method of producing purple-blue flowers of *Lilium* ‘Acapulco’ by the introduction of exogenous *Flavonoid 3', 5'-hydroxylase (F3'5'H)* gene, though the petals were still approximately fuchsia rather than blue.

In this study, we introduced exogenous anthocyanin regulators including *Del/Ros1* and *B1/C1* into *Lilium* ‘Sorbonne’, as the first step to obtain cultivars with novel leaf and flower color. During the *Agrobacterium*-mediated transformation, the protocol was also further optimized to make the genetic modification of *Lilium* more efficient. Our results not only provided a new approach to enhancing the transformation efficiency by the optimization of bacterial inoculation and co-cultivation, but also suggested the potential value of anthocyanin regulators in color modification of *Lilium*.

MATERIALS AND METHODS

Explant preparation and bacterial strain

Aseptic plantlets of *Lilium* oriental ‘Sorbonne’ under dark cultivation were used for study. The bulblets of circumference 30-40 mm with uniform growth were selected after subcultured for 50-

60 d in MS medium supplemented with 60 g L⁻¹ sucrose. The base portions (10 mm × 10 mm) of the scales taken from the outer layers (2-3 layers) of bulblets were excised and used for transformation.

The explants were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 carrying two binary plasmid vectors separately. One vector pJAM1890 harbored *Del* (GenBank No. M84913), *Ros1* (GenBank No. DQ275529) and kanamycin (Kan) resistance gene *nptII* as a selective marker, with constitutive promoter 2×CaMV 35S (Fig. 1A), while the other vector pBAC9075 harbored *B1* (RefSeq: NM_001112236.1), *C1* (RefSeq: NM_001112540.1) and the selective marker *EPSP*, all with promoter 2×CaMV 35S (Fig. 1B).

Optimization of *Agrobacterium*-mediated transformation

Several steps of the optimization experiment were taken successively during the long-term transformation with vector pBAC9075 harboring *B1/C1*. A single colony of *Agrobacterium* was inoculated into 5 mL LB liquid medium containing 50 mg L⁻¹ Kan (Amresco, USA), 50 mg L⁻¹ rifampicin (Rif) (Sigma, USA) and 20 mg L⁻¹ streptomycin (Str) (Solarbio, PR China). After cultivation in a shaking incubator (200 r min⁻¹) for 18 h, 1 mL of the bacterial suspension was transferred into 50 mL fresh LB liquid containing the same antibiotics, and cultured under the same condition until exponential growth. The bacterial suspension was centrifuged at 8000 r min⁻¹ for 8 min and resuspended in the inoculation liquid medium to a final density of OD₆₀₀ = 0.6. The precultured explants were dipped in 20 mL bacterial suspension for inoculation. After 20 min, they were transferred onto the co-cultivation medium, which was the same as the inoculation medium but solidified with 7 g L⁻¹ agar. To optimize the inoculation and co-cultivation medium, we evaluated eight types of MS medium (Murashige and Skoog 1962) modified by removing certain elements (Table 1): standard MS with entire elements (MS₀); MS without KH₂PO₄ (MS₁), CaCl₂ (MS₂), NH₄NO₃ (MS₃), KH₂PO₄/NH₄NO₃/KNO₃ (MS₄), KH₂PO₄/NH₄NO₃/KNO₃/CaCl₂ (MS₅) or H₂PO₄/NH₄NO₃/KNO₃/CaCl₂/MgSO₄ (MS₆); and H₂O only (MS₇), each supplemented with 50 µmol L⁻¹ acetosyringone (AS) and 30 g L⁻¹ sucrose, pH 5.8.

After co-cultured with the bacteria at 25°C in the dark for 3 d, the explants were transferred to the selection medium (MS + 0.01 mg L⁻¹ TDZ + 0.2 mg L⁻¹ NAA + 500 mg L⁻¹ carbenicillin (Carb) (Amresco, USA) + 2.0 mg L⁻¹ glyphosate + 30 g L⁻¹ sucrose + 7 g L⁻¹ agar, pH 5.8) at 25°C under white fluorescent light (40 µmol m⁻² s⁻¹) 16 h per day, subcultured every 2 weeks. When the height of plantlets reached about 10 mm after subcultivation for four times, they were transferred to the rooting medium (1/2 MS + 0.1 mg L⁻¹ IBA + 500 mg L⁻¹ Carb + 2.0 mg L⁻¹ glyphosate + 30 g L⁻¹ sucrose + 7 g L⁻¹ agar, pH 5.8) under the same temperature and light condition. The ratio of rooted plantlets under selective stress to co-cultured scales was calculated as an evaluation index in percentage, namely the putative transformation efficiency.

Based on the results of the experiment above, the effects of some additional treatments for inoculation and co-cultivation were further evaluated. The bacterial suspension with explants was subjected to a combination of additional treatments (shown in Table 2), including 1.5, 3 or 5 min of heat shock and 10, 20 or 30 s of sonication within 20 min of inoculation, along with a control group without these treatments. An Erlenmeyer flask containing the suspension was placed in a 42°C water bath for heat shock, and then in a bath sonicator at 120 W, 40 Hz for sonication. During the co-cultivation with *Agrobacterium*, 30, 50 or 70 g L⁻¹ maltose or glucose were added into the optimized medium instead of 30 g L⁻¹ sucrose, which was used as a control group, as shown in Table 3. The putative transformation efficiency in each test combination was also calculated as above.

The experiments above were based on a completely randomized experimental design with three replications, each replication consisting of about 27 explants cultured in three Petri dishes per treatment. The data collected were subjected to one-way ANOVA or two-way ANOVA (for the experiment involving multiple treatments), and the means were analyzed by Duncan's multiple range tests. Data were represented as mean ± SD. Different lowercase letters represent statistically significant differences (P < 0.05).

The transformation with the other vector, pJAM1890 harboring *Del/RosI*, was then conducted according to the optimized protocol concluded from the experiments above, except that the selective and rooting medium contained 80 mg L⁻¹ Kan instead of glyphosate.

PCR and Southern analysis

For PCR and Southern blot analysis, total DNA was extracted from leaves of the control and putative transgenic plants of *Lilium* according to the protocol associated with the Plant Genomic DNA Kit (DP305, Tiangen, PR China). The PCR amplifications were performed in 25 µL reaction mixture of 0.6 U GoTaq DNA Polymerase (Promega, USA) and 5 µL Ex Taq buffer, 0.2 mmol L⁻¹ each dNTP, 0.5 mmol L⁻¹ each primer, and 50 ng of template DNA, with the forward primer 5'- CGGGGATCCATGGAAAAGAATTGTCGTGGAGT -3', the reverse primer 5'- TCCCCCGGGTTAATTTCCAATTTGTTGGGCCT - for *RosI* in pJAM1890, and the forward primer 5'- TCCACTGACGTAAGGGAT -3', the reverse primer 5'- TGGACCAGAAGAGGGCAT - 3' for 35S/*BI* in pBAC9075. The PCR was carried out by the following thermal cycles: 2min at 95°C for initial denaturation; followed by 35 cycles at 95°C for 1 min (denaturation), 60°C for 30 s (annealing), and 72°C for 1 min (elongation); then a final extension of 10 min at 72°C. The amplification products were detected after electrophoresis on 1% agarose gel.

For Southern blotting hybridization, 15 µg of genomic DNA was digested overnight with HindIII, electrophoresed on a 0.8% agarose gel, and subsequently transferred to a nylon membrane (P36007, Millipore, USA). The probe was prepared from the plasmid DNA of pJAM1890 or pBAC9075 by PCR amplification using the primers for *RosI* or 35S/*BI* listed as above. Probe labeling, hybridization and detection were performed using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Germany) according to the manufacturer's protocol.

Subcultivation and anthocyanin assay

The transgenic lines confirmed were cultured in the subcultivation medium (MS + 0.5 mg L⁻¹ 6-BA + 0.2 mg L⁻¹ NAA + 30 g L⁻¹ sucrose + 7 g L⁻¹ agar, pH 5.8) at 25°C under cool white fluorescent light (40 µmol m⁻² s⁻¹) 16 h per day, subcultured every two month.

The anthocyanin content of the transgenic plantlets was determined as previously described (Ray et al. 2003). Deep-frozen leaves, petioles and scales (1 g of each) of the plantlets subcultured for 6 months were ground in liquid N₂, heated to 55°C for 10 min in 10 mL of 2 mol L⁻¹ HCl, then cooled and incubated overnight in the dark at 24°C for anthocyanin extraction. The extract was clarified by centrifugation, then the absorbance of the supernatant was measured at 523 nm on a spectrophotometer (BioMate 3S, Thermo Scientific, USA), using 2 mol L⁻¹ HCl as a blank. Anthocyanin content of transformed plants was calculated relative to that of non-transformed ones. The experiment was biologically repeated for three times in each transgenic line confirmed. Data combined from all the transgenic lines were represented as mean ± SD. Asterisks (*) indicate statistically significant differences between transformed and non-transformed plantlets by Student's t test (P < 0.05).

RESULTS

Optimization of transformation with *BI/CI*

As shown in Table 1, the full-strength MS medium (MS₀) resulted in a relatively low percentage of resistant regenerants. The putative transformation efficiency of MS₀ was even surprisingly lower than that of H₂O-only medium (MS₇), which contained nothing but acetosyringone (AS) and sucrose. Just removing KH₂PO₄ (MS₁) or CaCl₂ (MS₂) from the MS medium did not contribute to the transformation efficiency either, as both of the medium still contained NH₄NO₃. By contrast, significant effects were acquired upon the removal of NH₄NO₃ (MS₃), and removing NH₄NO₃ with other major mineral components including KH₂PO₄ and KNO₃ (MS₄) further improved the transformation efficiency. A number of *Agrobacterium* colonies could be observed on these NH₄NO₃-free media at 3 d of the co-cultivation, whereas there was no visible bacterial growth on MS₀, MS₁ or MS₂ medium at the same time. On the basis of MS₄ medium, further removing either CaCl₂ alone (MS₅) or with MgSO₄ (MS₆) together

made no significant difference. Removing other elements including micronutrients (Na_2EDTA , FeSO_4 , MnSO_4 , etc.), vitamins and organics—resulting in water-only medium (MS_7)—significantly lowered the percentage of resistant plantlets to the same level as MS_3 did.

According to the results shown in Table 1, we chose MS_6 as the optimized medium, and proceeded to further optimization during the inoculation and co-cultivation respectively. The effects of heat and sonication treatments on the transformation is shown in Table 2. Generally, sonication-assisted inoculation brought more resistant plantlets, especially when the duration was set as 20 s. However, extending its duration to 30 s incurred significantly negative effects compared with other treatments. Two-way ANOVA also revealed extremely significant effects ($P < 0.01$) of sonication on the transformation efficiency, but no significant difference was observed among different heat shock treatments by contrast (Data not shown).

As shown in Table 3, adding 30 g L^{-1} maltose instead of sucrose in MS_6 co-cultivation medium resulted in a slightly elevated transformation efficiency, and significant improvement was observed when its concentration reached 50 g L^{-1} or even higher (70 g L^{-1}). 70 g L^{-1} glucose used as an alternative carbon source also significantly increased the percentage of resistant plantlets, but lower concentrations including 30 and 50 g L^{-1} of it made no significant difference with the control.

Production of transgenic *Lilium* plantlets of *Del/Ros1* and *B1/C1*

After the transformation optimization, 173 glyphosate-resistant plantlets transformed with *B1/C1* were finally obtained from about 1,300 co-cultured scales. Then the transformation with *Del/Ros1* was performed according to the optimized protocol concluded above: MS medium without any macro elements for inoculation and co-cultivation, 20 s sonication and 1.5 min heat shock treatments during inoculation, 50 g L^{-1} maltose instead of sucrose in co-cultivation medium. Finally, 168 Kanamycin-resistant plantlets transformed with *Del/Ros1* were obtained from 510 transformed scales, indicating enhanced efficiency using the optimized system compared with the overall transformation with *B1/C1*.

After confirmed by PCR screening, 14 positive *Del/Ros1* transgenic lines and 16 *BI/CI* lines were obtained. As positive control, *Ros1* and 35S/*BI* were amplified from the plasmid vector pJAM1890 and pBAC9075 respectively. The expected 660 bp *Ros1* or 792 bp 35S/*BI* band was present in all putative transformants and positive controls but was absent from non-transgenic lilies and blank controls (Fig. 2). *Del* and *CI* were also amplified for further confirmation (Fig. S1, Fig. S2). Five lines of each gene were randomly selected for Southern blot analysis. Finally, four *Del/Ros1* lines and one *BI/CI* line were confirmed, indicating that the *Del/Ros1* or *BI/CI* gene had integrated into the genome of ‘Sorbonne’. All the five transgenic lines were confirmed with single-copy gene transformed, since the number of detected bands was expected to reflect the gene copy number. No signal was detected from non-transgenic lilies or blank controls (Fig. 3).

Phenotypes observation and anthocyanin assay

At the early stage of differentiation, some adventitious buds transformed with *Del/Ros1* appeared in light purple, while the *BI/CI* ones only showed slight differences with the controls (Fig. 4A). Then the scales and young leaves of some putative transformed plantlets regenerated, including *Del/Ros1* and *BI/CI* ones, also started to show light purple color (Fig. 4B). Some leaves and scales of *Del/Ros1* plantlets even appeared in a strong purple (Fig. 4C). Unexpectedly, green leaves started to occupy the majority in the Southern-positive lines after months of subcultivation. With continuous subcultivation, the purple in all the transformed plantlets gradually faded into normal green and no visible difference could be observed in comparison with non-transgenic ones after the *Del/Ros1* and *BI/CI* plantlets were rooted (Fig. 4D). By contrast, the color of the control group remained the same throughout the regeneration and subcultivation.

Because the purple plantlets had almost faded completely before reaching the minimum weight for anthocyanin assay, we were only able to take those visibly green tissues of transformed plants as samples for anthocyanin content measurement (Fig. 5). Moderate but significant elevation in anthocyanin levels could be found both in the leaves and scales of

Del/Ros1 and *B1/C1* transgenic plantlets compared with the controls. The relative anthocyanin levels in the petioles of transgenic plantlets were lower than those in other tissues, but we still observed a significant difference between *Del/Ros1* plantlets with non-transformed ones.

DISCUSSION

Macronutrient-free medium recommended for *Lilium* transformation

The efficient delivery of T-DNA to the plant cell plays a pivotal role in genetic transformation. Our study provided an efficient transformation protocol for *Lilium* 'Sorbonne' that significantly improved the lateral transformation of *Del/Ros1*. Major mineral components in the inoculation and co-cultivation medium, especially NH_4NO_3 were found to have negative effects upon the efficiency of *Agrobacterium*-mediated transformation in *Lilium* (Hoshi et al. 2004; Li et al. 2008). Given that receptor adhesion is a prerequisite for *Agrobacterium*-mediated transformation, this reduction in efficiency may be caused by the higher concentrations of elements such as ammonium ions that interfere with receptor-adhesion, thus inhibiting *Agrobacterium* growth. MS medium without NH_4NO_3 has, therefore, become more common for transformation. However, different genotypes and tissues have different responses to the element absence in medium because they may vary in sensitivities to bacterial growth. In a recent report, use of NH_4NO_3 -free medium only resulted in low transformation rates with eight lily cultivars (Wang et al. 2012). Our study suggests that the removal of NH_4NO_3 still plays a positive role in the transformation of *Lilium* 'Sorbonne'.

Removing other macro elements along with NH_4NO_3 further enhanced the transformation efficiency. Although components such as KH_2PO_4 and CaCl_2 did not affect the transformation alone, they did inhibited the transformation in the absence of NH_4NO_3 . The water-only medium decreased the percentage of resistant plantlets, but it was still superior to the standard MS medium. This suggests that while the microelements, vitamins and organics in medium are necessary for the transformation, they could not counteract the inhibitory effects of other macro elements. Azadi et al. (2010a) reported a significant increase in transformation of *Lilium*. × *formolongi* calli with co-cultivation medium lacking macro elements. While our results indicate

that this also applies to ‘Sorbonne’ scales, but unlike the former study, we did not find any promotive effects of MgSO_4 on the transformation. This might result from differences in the explant types or genotypes. Dupré et al. (2000) also reported that the highest transformation efficiency of *Ginkgo biloba* was achieved on the medium without any mineral components. Although the roles of certain components, such as MgSO_4 , needs to be clarified in further study, we recommended that most or all the macro elements be removed from the medium for *Lilium* transformation.

Sonication and carbohydrate treatment also benefited the transformation

Some additional treatments supplementing for the standard *Agrobacterium* inoculation protocol have been reported to improve the transformation efficiency. Sonication assisted *Agrobacterium*-mediated transformation (SAAT) produces thousands of micro wounds that facilitate penetration of *Agrobacterium* into the internal plant tissues and has been effective in improving the transformation efficiency of several plant species (Liu et al. 2005; Santarem et al. 1998). Short heat shock treatment was also reported as a helpful approach in elevating the transformation efficiency, though the mechanism underlying this response remains unclear, partly because of the synthesis of heat-shock protein (Gurel et al. 2009; Patel et al. 2013). To our knowledge, this is the first report in which these two treatments have been combined to study their interaction. Our results suggest that sonication could improve the transformation efficiency significantly, while heat shock treatment in combination with sonication did not significantly promote transformation efficiency. This indicates that sonication might be more useful for *Lilium* ‘Sorbonne’ transformation than heat treatment, though these two treatments did work well in combination. Further studies are required to explain the mechanism underlying the impact of heat treatment on transformation efficiency.

We observed significant improvements in transformation efficiency when a high concentration of maltose was used instead of sucrose. Maltose has been given much importance as an alternative carbon source in medium for better regeneration of some plants (Kumria et al. 2001). It has also been used in the biolistic transformation of *Lilium* (Cohen et al. 2004). To date,

there have been no reports regarding the application of maltose in *Agrobacterium*-mediated transformation of *Lilium*, and its role in co-cultivation medium needs to be characterized further. Patel et al. (2013) previously reported a higher transformation efficiency of rice (*Oryza sativa*) calli with high maltose treatment during the co-cultivation and speculated that maltose worked as an osmoticum. However, our study showed that glucose treatments that endowed the medium with higher osmotic pressure because of their lower molecular weight did not affect the transformation efficiency until the glucose concentration reached 70 g L⁻¹. As a monosaccharide, glucose is expected to induce the expression of *vir* genes in *Agrobacterium* under the presence of AS (Wise et al. 2005), but our study indicates that this might be compromised by the negative effects of glucose itself.

The transformation efficiency increased along with the concentration of carbohydrates, implying that osmotic treatments do positively effect the transformation, though the metabolism of different carbon sources in *Lilium* should also be taken into consideration. It has been reported that maltose is not easily absorbed by *Lilium* bulblets (Haruki et al. 1996) and, thus, stabilizes the initial osmolality in medium. This may partly explain the superiority of maltose in the transformation. Therefore, it might not work to replace the carbon source simply with a higher osmotic one, at least for the transformation of *Lilium* scales, while maltose would find its place for it.

Exogenous regulators induced anthocyanin accumulation in *Lilium*

The genetic modification of color has always been an important research field of ornamental plants. Recent advances in genetic transformation techniques enable the production of desirable and novel flower colors in some ornamental plants. Albert et al. (2010) have demonstrated that anthocyanin pigmentation could be restored in petals of *Cymbidium* cv. JDP by introducing different MYB/bHLH combinations, such as *Dell/CI*, *Lc/Ros1* and *Lc/CI*. Separately over expressed *Ros1* could also enhance the anthocyanin content in *P. hybrida* and *Eustoma grandiflorum* (Schwinn et al. 2014). In addition, specific experiments to accumulate delphinidin-derived anthocyanins by over expression of a *F3'5'H* transgene have been reported for *Dianthus*

caryophyllus (Fukui et al. 2003), *Rosa hybrida* (Katsumoto et al. 2007) and *Chrysanthemum morifolium* (Brugliera et al. 2013), while inhibition of both *Flavonoid 3'-hydroxylase* (*F3'H*) and *F3'5'H* genes have been used to promote cyanidin- and pelargonidin-based pigment accumulation in the genera *Torenia* (Suzuki et al. 2000) and *Osteospermum* (Seitz et al. 2007).

Nevertheless, the introduction of exogenous genes involved in anthocyanin biosynthesis might not necessarily bring about phenotypic difference due to the complexity of the pathway. In this study, *Lilium* 'Sorbonne' was successfully transformed by *Del/Ros1* or *B1/C1* gene for the first time when anthocyanin regulators were introduced into *Lilium*. A distinct but temporary change in color could be observed throughout the regeneration and growth of transformed plants in comparison with the control, though the transformation was confirmed to be permanent rather than transient by southern blot analysis. Azadi et al. (2010b) reported a similar result, in which orange plantlets transformed with carotenoid-related genes finally grew into entirely green plants after months. Even so, such visible color change led by *Del/Ros1* or *B1/C1* could still act as an adequate marker for the preliminary screening of transgenic plantlets.

As the transgenic plantlets were cultured under white light, any coloration resulting from anthocyanin accumulation might have been masked by the chlorophyll such that it could only be observed in new bulblets and leaves where chlorophyll levels are low. Alternatively, the absence of color may be explained by the unclear cell conditions of those plantlets. Anthocyanin such as cyanidin changes its color depending on pH (Goto and Kondo 1991). It shows red in strong acidic conditions and violet in neutral solutions, while it is nearly colorless at pH 4-6 (Starr and Francis 1968). In general, the vacuolar pH of plant cells is weakly acidic, so the anthocyanin synthesized is very unstable in the absence of strong metal chelation or molecular associations (Yoshida et al. 2009). The variation in vacuolar pH during leaf development might cause the loss of purple coloration. Unfortunately, we were unable to determine the anthocyanin content while the purple color in plants was still visible, as insufficient plant material was available for further analyses at that time. However, even after the plant color had faded back to green, the anthocyanin levels in these transgenic plants were still significantly higher than in untransformed

ones. This suggests that in vivo the anthocyanin accumulation, though not yet stable, was actually induced in the transformed lines.

Lilium cannot produce delphinidin, which renders plants blue, because of the lack of F3'5'H in the *Lilium* (Martens et al. 2003). Thus, we can hardly expect to obtain purely blue plants after transformation with anthocyanin regulators instead of the F3'5'H gene itself. However, the introduction of these regulators strongly induced the biosynthesis of anthocyanin, especially the purple pigment cyanidin, in tissues of *Lilium*. This result is similar to the overexpression of *Ros1* in *Antirrhinum majus* (Schwinn et al. 2006) that also lacks F3'5'H (Ishiguro et al. 2012). Other MYB-related transcription factors such as *LhMYB6* and *LhMYB12* have been shown to regulate the anthocyanin synthesis in tepals and tepal spots of *Lilium* cultivars including 'Sorbonne' (Yamagishi et al. 2010). Therefore, we expect that the change in flower color in the transformed plants will be observable even after several years of growth. The cell conditions of tepals might be more suited to maintaining anthocyanin stability, however, so further observation is required to confirm whether flower color changes are stably maintained after transformation.

Our research preliminarily proves that the exogenous transcription factors in the anthocyanin pathway can regulate the pigment biosynthesis and accumulation in *Lilium*, but there is still a long way to go to produce lilies in new leaf color or flower color. Both F3'H (flavonoid 3'-hydroxylase) and F3'5'H utilize the same substrate in the flavonoid biosynthetic pathway (Yoshida et al. 2009). Hence, the strong activity of F3'H in *Lilium* may interrupt the accumulation of delphinidin even if the F3'5'H gene is introduced in (Qi et al. 2013). Tanaka et al. (2012) inhibited the synthesis of endogenous F3'H expression by RNAi and obtained first "blue lily", though the color was not exactly as desired. As exogenous *Del/Ros1* were found to activate the F3'5'H gene if the latter is present (Butelli et al. 2008), our research suggests the cotransformation of F3'5'H and *Del/Ros1* may help obtain "bluer" *Lilium* in further study.

In the present study, we analyzed for the first time in *Lilium* 'Sorbonne' the effects of major mineral components as well as heat, sonication and carbohydrate treatments on *Agrobacterium*-mediated transformation. MS medium without KH₂PO₄, NH₄NO₃ and KNO₃, or without any

macro elements, significantly improved the transformation efficiency when using bacterial inoculation and co-cultivation. Sonication in conjunction with high maltose or glucose treatment also significantly improved the transformation efficiency. Using this optimized transformation system, the percentage of PCR-positive transgenic plants generated was on average 2.7%, this was higher than that obtained in previous studies (Cohen and Meredith, 1992; Tsuchiya et al. 1996; Li et al. 2008). Plantlets transformed with the anthocyanin regulators *Del/Ros1* or *B1/C1* showed obvious color changes during the preliminary stages of development. The introduction of these anthocyanin regulators could act as a visible marker for selection, and may also represent a means of modifying *Lilium* flower color.

Author contribution statement Qi Cui, Lin Yuan and Gui-Xia Jia conceived and designed the experiments; Chi Wei, Qi Cui and Lin Yuan performed the experiments; Chi Wei and Qi Cui analyzed the data; Chi Wei wrote the paper.

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Figure Legends

- Fig 1. Map of vectors. A: pJAM1890 harboring *Del/Ros1*, B: pBAC9075 harboring *B1/CI*.
2×CaMV 35S: Constitutive promoter, nos: Terminator, nptII: Neomycin phosphotransferase II gene, *EPSP*: 5-enolpyruvylshikimate-3-phosphate gene, LB: Left border, RB: Right border.
- Fig 2. PCR analysis in putative transformants. A: *Ros1* in *Del/Ros1* plantlets, B: *35S/B1* in *B1/CI* plantlets. Lane M: DNA molecular size markers, lane 1: Plasmid as positive control, lane 2: ddH₂O as negative control, lane 3: DNA isolated from non-transformed *L. 'Sorbonne'*, lanes 4–: DNA isolated from putative transformants.
- Fig 3. Southern blot analysis of DNA isolated from PCR-positive transformants. A: *Ros1* in *Del/Ros1* plantlets, B: *35S/B1* in *B1/CI* plantlets. CK: Non-transformed '*Sorbonne*', lane DR1–4: *Del/Ros1* transformants, lane BC1: *B1/CI* transformant. Molecular markers are indicated on the left.
- Fig 4. Coloration of transgenic scales and plantlets. A: Transformed and non-transformed scales growing on regeneration medium; arrow indicates the color change. B: Transformed and non-transformed plantlets regenerated from scales after first subcultivation. C: Transformed and non-transformed plantlets after one month's subcultivation. D: The leaf color of transformed and non-transformed plantlets after rooting, growing on rooting medium. CK: Non-transformed *L. 'Sorbonne'*.
- Fig 5. Anthocyanin contents of transgenic plants after rooted. A: Leaves, B: Petioles, C: Scales. CK: Non-transformed *L. 'Sorbonne'*; *Del/Ros1*: Plants transformed with the *Del* and *Ros1* gene; *B1/CI*: Plants transformed with the *B1* and *CI* gene. The relative anthocyanin content was calculated by setting the anthocyanin content of CK to 1. Asterisks (*) indicate statistically significant differences ($P < 0.05$) between transformed and non-transformed plantlets by Student's *t* test at 0.05 probability ($P < 0.05$).

Table 1. Effects of Macro elements on the percentage of resistant plantlets

No. ^a	Medium	Resistant plantlets (%) ^b
MS ₀	MS	7.93±1.30 c
MS ₁	MS – KH ₂ PO ₄	5.95±2.29 c
MS ₂	MS – CaCl ₂	7.45±2.15 c
MS ₃	MS – NH ₄ NO ₃	12.64±2.14 b
MS ₄	MS – (KH ₂ PO ₄ , NH ₄ NO ₃ , KNO ₃)	17.38±2.70 a
MS ₅	MS – (KH ₂ PO ₄ , NH ₄ NO ₃ , KNO ₃ , CaCl ₂)	15.79±2.32 ab
MS ₆	MS – (KH ₂ PO ₄ , NH ₄ NO ₃ , KNO ₃ , CaCl ₂ , MgSO ₄)	18.31±1.71 a
MS ₇	H ₂ O only	13.19±2.18 b

^aThe minuses (–) in MS₁ to MS₆ refer to removing the following elements from MS medium. All the media contained 50 µmol L⁻¹ acetosyringone (AS) and 30 g L⁻¹ sucrose, pH 5.8.

^bDifferent lowercase letters represent significant differences by Duncan's multiple range test at 0.05 probability (P < 0.05).

Table 2. Effects of additional treatments on the percentage of resistant plantlets

No.	Heat shock time (min)	Sonication time (s)	Resistant plantlets (%) ^a
1	1.5	10	21.10±9.85 ab
2	1.5	20	26.80±2.24 a
3	1.5	30	18.52±2.69 b
4	3	10	22.41±6.49 ab
5	3	20	25.08±6.00 a
6	3	30	8.91±2.25 d
7	5	10	15.81±6.98 c
8	5	20	19.70±7.79 b
9	5	30	11.40±3.55 cd
CK	0	0	15.80±4.75 c

^aDifferent lowercase letters represent significant differences by Duncan’s multiple range test at 0.05 probability ($P < 0.05$).

Table 3. Effects of carbon source on the percentage of resistant plantlets

No.	Carbon source	Resistant plantlets (%) ^a
M1	30 g L ⁻¹ maltose	19.22±1.70 ab
M2	50 g L ⁻¹ maltose	22.90±2.03 a
M3	70 g L ⁻¹ maltose	22.27±3.48 a
G1	30 g L ⁻¹ glucose	17.10±2.66 b
G2	50 g L ⁻¹ glucose	17.08±1.64 b
G3	70 g L ⁻¹ glucose	21.41±1.02 a
CK	30 g L ⁻¹ sucrose	18.31±1.71 b

^aDifferent lowercase letters represent significant differences by Duncan's multiple range test at 0.05 probability ($P < 0.05$).

222x41mm (300 x 300 DPI)

141x67mm (300 x 300 DPI)

118x63mm (300 x 300 DPI)

265x360mm (300 x 300 DPI)

483x118mm (96 x 96 DPI)