Studies on Embryogenic Competence and Regeneration Potential with Relation to Anthocyanin Biosynthesis in Cotton (G. hirsutum)

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Abstract

Varietal genotype is very important in determining which varieties of cotton will produce embryos because most of the commercial varieties are recalcitrant to regeneration. Identification of genes involved in the induction of competence for embryogenesis and subsequent embryo development presents a challenge for modern molecular biology. Certain varieties of cotton, especially Coker 310 exhibit anthocyanin pigmentation during embryogenic calli induction and form somatic embryos readily, while other genotypes MCU 12 and KC3 does not exhibit anthocyanin pigmentation and are recalcitrant to regeneration. To find out the relationship between anthocyanin synthesis and somatic embryogenesis if any, candidate genes for anthocyanin synthesis and somatic embryogenesis were tested for its presence through PCR analysis in genomic DNA samples of Coker 310, MCU 12 and KC3. The results of this study revealed that the candidate gene sequences for the enzymes involved in anthocyanin biosynthesis (chalcone synthase, flavanone 3 hydroxylase, dihydroflavanol reductase, leuconanthocyanidin deoxygenase and anthocyanidin synthase) and transcription factors that regulate the expression of anthocyanin biosynthesis genes (bHLH and MYB) and the candidate gene sequences encoding the proteins associated with somatic embryogenesis (the arabinogalactan proteins, lipid transfer proteins and high mobility group proteins) were found in all the samples of Coker 310 while none of them were present in the samples of MCU 12 and KC 3. It may be inferred from this study that apart from the genes associated with somatic embryogenesis, genes associated with anthocyanin biosynthesis and regulation may also be involved in somatic embryogenesis and regeneration in cotton. Also, these genes can be used as reliable markers to screen cotton varieties for their embryogenic potential.

Keywords

Somatic embryogenesis; Anthocyanin synthesis; Embryogenic callus; The candidate Genes; Embryogenic potential

Background

Cotton (Gossypium hirsutum) is a recalcitrant crop to regenerate from in vitro tissue cultures. Compared with many other crops, it is more difficult to obtain somatic embryogenesis and plant regeneration in cotton. Price and Smith (1979) were the first to report somatic embryogenesis in cotton (G. klotzschianum), although complete plant could not be regenerated. Davidonis and Hamilton (1983) subsequently described plantlet regeneration from Coker 310. Since then, in vitro plant regeneration via somatic embryogenesis in cotton has been reported in several laboratories using various strategies (Finer 1988; Gawel and Robacker, 1990; Leelavathi et al., 2004; Khan et al., 2010). But only a limited number of varieties can be induced to produce somatic embryos and regenerated plants. The most responsive lines are Coker lines that are no longer cultivated. Genotype-dependence is one of the important factors that restrict somatic embryogenesis and plant regeneration. It is therefore imperative to improve the embryogenic competence and regenerability of a wider range of cotton cultivars to accelerate the production of transgenic cotton varieties. To screen the varieties for their embryogenic potential or to develop highly regenerable varieties would be greatly facilitated, if a reliable marker could be identified. As somatic embryogenesis is the preferred plant regeneration technique for cotton transformation, identification of vital genes of somatic embryogenesis are of great importance for improving the embryogenic competence and regenerability of a
wider range of cotton cultivars and thus accelerating the production of transgenic cotton varieties.

**Importance of anthocyanins in induction of cotton somatic embryogenesis**

During the process of somatic embryogenesis in cotton, Mishra et al. (2003) and Haq and Zafar (2004) observed significant amount of red pigmentation in the embryogenic callus resulting from the accumulation of small amounts of anthocyanins and reported that the anthocyanin induction in embryogenic callus is a good indicator of somatic embryogenesis. The major focus of this study therefore was to find out the relationship between anthocyanin synthesis and somatic embryogenesis in cotton.

**Anthocyanin biosynthesis**

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom (Kong et al., 2003). In the plants, anthocyanidins can be classified to six categories according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus, including pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin. However, in nature, the most commonly occurring anthocyanidin is cyanidin (Tahara, 2007). Anthocyanins are biosynthesized through the phenylpropanoid metabolic pathway and flavonoid metabolic pathway. The key enzymes involved in the biosynthesis of anthocyanin includes cyanidin-3-glucoside, chalcone synthase, flavanone-3-hydroxylase, dihydroflavanol reductase, leucoanthocyanidin deoxygenase and anthocyanidin synthase (Xiao et al., 2007). Wang et al. (2010) reported that the three enzymes viz., chalcone synthase, flavanone 3 hydroxylase and leucoanthocyanidin deoxygenase involved in secondary metabolism pathways were up-regulated at mature-cotyledon embryo stages in longan which suggests a role of flavonoid compounds in embryo development.

A number of genes implicated in the regulation of anthocyanin related pathways encode proteins belonging to the MYB family of transcription factors (Jin et al., 2000). R2R3-type proteins form the largest class of MYB factors in plants which are involved in activation of anthocyanin pigmentation in various plant species (Jin et al., 2000). The large size of the MYB family in plants indicates their importance in the control of plantspecific processes including the regulation of secondary metabolism, control of cell morphogenesis, regulation of meristem formation, floral and seed development and the control of the cell cycle (Du et al., 2009). Several plant bHLH-type proteins act as transcriptional regulators involved in anthocyanin biosynthesis, phytochrome signalling, fruit dehiscence, carpel and epidermal development, as well as stress response. The anthocyanin pathway has been shown to be activated by similar MYB and bHLH proteins in a wide variety of species, indicating that their function is well conserved (Koes et al., 2005). Ozeki et al (2000) reported that in cultured carrot cells the transcription factor Dcmybl4 was found to be involved not only in the induction of anthocyanin synthesis and metabolic differentiation, but also in the induction of embryogenesis, and morphological differentiation.

**Key proteins and their role in somatic embryogenesis**

Arabinogalactan proteins (AGPs), Lipid transfer proteins (LTPs), and High mobility group proteins (HMGPs) play a vital role in somatic embryogenesis. Characterization of extracellular protein markers for somatic embryogenesis offers the possibility of determining embryogenic potential of plant cells in culture long before any morphological changes have taken place, and many extracellular protein markers for embryogenic potential have been described.

**Arabinogalactan proteins (AGPs)**

Arabinogalactan proteins (AGPs) represent a heterogeneous group of proteoglycans which are commonly found in the cell membrane, cell matrix, cell walls, intercellular spaces, and certain cytoplasmic vesicles and are rich in hydroxyproline, alanine, glycine and serine. AGPs are thought to be involved in different aspects associated with plant growth and development, such as fertilization, somatic embryogenesis, xylem differentiation, cell division, expansion and death, cell adhesion, fibre development and signaling cascade (Yang and Zhang, 2010). Hengel et al. (2001) presented evidence that AGP side chains with intact arabinogalactan carbohydrate moieties are essential for the effect on somatic embryogenesis, whereas hydrolytic activation with
endochitinases appears essential for full embryo-forming activity of the AGPs. Poon et al. (2004) reported that cotton cells undergoing somatic embryogenesis produce specific AGPs that are not produced by non-embryogenic cells and is consistent with the hypothesis that AGPs play a role in somatic embryogenesis.

**Lipid transfer proteins (LTPs)**

Nonspecific lipid transfer proteins (LTPs) represent a protein family that is ubiquitous in plants. These proteins are characterized by their ability to transfer phospholipids between membranes and to bind fatty acids in vitro. The ltp gene has been implicated as a well-known early marker of somatic embryogenesis induction in carrot (Sterk et al., 1991) being that it is linked to the protoderm layer formation, which exerts a regulatory role in controlling cell expansion during embryo development in developing somatic and zygotic embryos. LTP genes were found to be necessary for normal somatic embryogenesis to occur. Under and overexpression of a putative LTP gene affect sequential developmental stages during somatic embryogenesis by changing the morphology and occurrence frequency of somatic embryos. Zeng et al (2006) found that the increased activity of LTPs may facilitate membrane biosynthesis, cell expansion and polar differentiation during SE period and suggested that lipid-transfer protein (LTP) genes might have relevant roles during cotton somatic embryogenesis.

**High mobility group proteins (HMG)**

The HMG proteins are abundant and highly mobile proteins in the cell nucleus that influence chromatin structure and enhance the accessibility of binding sites to regulatory factors. In plants, the chromosomal high mobility group (HMG) proteins of the HMGB family typically contain a central HMG-box DNA-binding domain that is flanked by a basic N-terminal and an acidic C-terminal domain. Due to their remarkable DNA bending activity, HMGB proteins can increase the structural flexibility of DNA, promoting the assembly of nucleoprotein complexes that control DNA-dependent processes including transcription. Therefore, members of the HMGB family act as versatile modulators of chromatin function (Wu et al., 2009). Zeng et al (2006) found one of the cDNA encoding high mobility group protein (HMGB) in their subtractive cDNA library was associated with cotton somatic embryo development. Hu et al (2010) used RNA interference (RNAi) to down-regulate the expression of GhHmgB3 during cotton somatic embryogenesis (SE) of Gossypium hirsutum cv Coker 201 by transforming both hypocotyl and embryogenic calli (ECs) via Agrobacterium tumefaciens.

This study was therefore undertaken to study the regeneration potential of the Ghirsutum genotypes Coker 310, MCU 12 and KC 3 with specific markers for the genes associated with anthocyanin synthesis and regulation and somatic embryogenesis

1 Results

PCR analysis performed with the genomic DNA samples of control young plants of Coker 310, MCU 12 and KC 3, 150 days old calli of MCU 12 and KC 3, 150, 180, 210 and 240 days old embryogenic calli, globular, torpedo and cotyledonary stage somatic embryos and 30 and 60 days old regenerated plantlets of Coker 310 using specific primers of the genes associated anthocyanin biosynthesis and regulation and somatic embryogenesis revealed that the candidate gene sequences for the enzymes involved in anthocyanin biosynthesis (chalcone synthase, flavanone 3 hydroxylase, dihydroflavanol reductase, leucoanthocyanidin deoxygenase and anthocyanidin synthase) and transcription factors that regulate the expression of anthocyanin biosynthesis genes (bHLH and MYB) and the candidate gene sequences encoding the proteins associated with somatic embryogenesis (the arabinogalactan proteins, lipid transfer proteins and high mobility group proteins) were found in all the samples of Coker 310 while none of them were present in the samples of MCU 12 while none of them were present in the samples of MCU 12 and KC 3 (Plate 15-17) (Figure 1).

2 Discussion

During the period of subculture for the induction of embryogenic calli in the medium supplemented with 1.9 g L⁻¹ KNO₃, 170 - 180 days old embryogenic callus showed anthocyanin pigmentation only in Coker 310 while it was not observed in the genotypes MCU 12 and KC 3. Of the three genotypes, the embryogenic calli and somatic embryos was induced only from Coker
Figure 1 PCR amplification of candidate genes encodig the enzymes associated with anthocyanin biosynthesis (A-E), transcription factors associated with regulation of anthocyanin biosynthesis (F-G) and the proteins associated with somatic embryogenesis (H-J)

Note: M: 100 bp ladder; Lane 1-3: Genomic DNA of Coker 310, MCU12 and KC 3 (Control plants) respectively; Lane 4-7: Callus cultures of Coker 310 (150 d,180 d,210 d, 240 d old callus) respectively; Lane 8-10: Somatic embryos of Coker 310 (globular, torpedo and cotyledon) respectively; Lane 11-12: Callus cultures of MCU 12 and KC 3 respectively; Lane 13-14: Regenerated plantlets of Coker 310 (30 and 60 days old) respectively; A: Chalcone synthase (CHS); B: Flavanone 3 hydroxylase; C: Dihydroflavanol reductase (DFR); D: Leucoanthocyanidin deoxygenase (LDOX); E: Anthocyanidin synthase (ANS); F: bHLH Transcription factor; G: MYB Transcription factor; H: Arbinogalactan proteins (ARBs); I: Lipid transfer proteins (LTPs); J: High mobility group proteins (HMG)

310, while it was not observed in MCU 12 and KC3. Hence, to find out the relationship between anthocyanin synthesis and somatic embryogenesis if any, PCR analysis was performed with the genomic DNA samples of young control plants and callus at different ages of all the three varieties (Coker 310, MCU 12 and KC 3) and somatic embryos at different stages of development and young regenerating plantlets of Coker 310 using specific primers of the genes associated with anthocyanin biosynthesis and somatic embryogenesis. The results indicated that the genes encoding anthocyanin biosynthesis and associated transcription factors and somatic embryogenesis were found only in Coker 310 and not in MCU 12 and KC 3 genotypes. Wang et al (2010) reported that the enzymes viz., chalcone synthase, flavanone 3 hydroxylase and leucoanthocyanidin deoxygenase involved in secondary metabolism pathways were up-regulated at mature-cotyledon embryo stages in longan, which suggested a role of flavonoid compounds in longan embryo development. The presence of the different candidate genes of anthocyanin pigmentation in Coker 310 which responded for the regeneration of somatic embryos indicates that there may be relationship between anthocyanin synthesis and somatic embryogenesis in cotton.

In the current study, it was found that, of the three genotypes Coker 310 was found to have high potential for invitro regeneration as the candidate gene
sequences encoding LTPs, AGPs and HMGPs were present in all samples of Coker 310 while the two genotypes MCU 12 and KC 3 were found to be regeneration non-responsive. These results are evident from the works of Hengel et al (2001) that the embryogenic AGPs can be successfully used to promote the regeneration of whole cotton plants, Pedroso and Pais (1995) that in Camellia leaf cultures, during induction of somatic embryogenesis, LTP genes were found to be necessary for normal somatic embryogenesis and Hu et al (2010) that Gossypium hirsutum high mobility group protein (GhHmgB3) regulated the proliferation and differentiation of cotton SE.

It may be inferred from this study that apart from the genes associated with somatic embryogenesis, genes associated with anthocyanin biosynthesis and regulation may also be involved in somatic embryogenesis and regeneration in cotton. Also, these genes can be used as reliable markers to screen cotton varieties for their embryogenic potential. In order to determine the precise role of these genes in the embryogenesis process, further studies on the regulation of the expression of these genes offer opportunities for understanding the mechanism controlling the differentiation of somatic cells and the detailed steps by which these genes direct somatic embryogenesis. The recognition of various aspects orchestrating this process will provide deeper insight into understanding the enigmatic reprogramming of cells in higher plants and will provide novel target genes for the improvement of somatic embryogenesis ability. This will accelerate the production of transgenic plants in industrial and agricultural applications.

3 Materials and Methods
3.1 Induction of embryogenic calli
Delinted seeds of the cotton (Ghirsutum) cultivars Coker 310, MCU 12 and KC 3 were obtained from the Department of Cotton, Tamil Nadu Agricultural University, Coimbatore. The seeds were surface sterilized by rinsing with 70% ethanol for 1 min and washed three times with sterile distilled water prior to a 10 min treatment with 0.1% HgCl₂. After thorough washing with the sterile distilled water they were germinated on a half strength MS basal medium supplemented with 15 g/L sucrose and 9 g/L agar. The germination bottles were then incubated for one week in the culture room at (25±2)°C under 16/8 hr photoperiod. Cotyledon explants from seven day old seedlings of Coker 310, MCU 12 and KC 3 were cultured on MS medium supplemented with 0.1 mg/L 2, 4-D and 0.5 mg/L kinetin for the induction of callus. After one month of culture, the callus initiated was separated from the explant and subcultured in the same medium for further one month for callus proliferation. Highly proliferating 60 days old callus was then transferred to the full strength MS medium for callus maturation. After maturation of callus for about two months (age of the callus 120 days) in full strength MS medium, the callus from the three genotypes were transferred on to MS medium supplemented with 1.9 g/L KNO₃ for the induction of somatic embryogenesis. After one month of culture, the callus was subcultured in the same fresh medium where during this period of subculture, 17~180 days old embryogenic callus showed red pigmentation. Of the three genotypes embryogenic calli and somatic embryos was induced only from the Coker 310, while it was not observed in MCU 12 and KC3. Embryogenic calli obtained only from the genotype Coker 310 was maintained by periodical subcultures to obtain the regenerants.

3.2 Genomic DNA extraction
Total genomic DNA was extracted from the young leaves (30 d old) of Coker 310, MCU 12 and KC3, embryogenic callus (150 d, 180 d, 210 d and 240 d old) of Coker 310, globular, torpedo and cotyledonary embryos of Coker 310, 150 days old callus of MCU 12 and KC3, one and two month old regenerated plantlet of Coker 310 by the CTAB method as described by Paterson et al (1993) and quantified using nanodrop by measuring the absorbance at 260 nm. The genomic DNA was then diluted by 0.1×TE buffer to 50~100 ng/µL and used as template for PCR amplification.

3.3 PCR amplification
The candidate gene sequences for the enzymes involved in anthocyanin bio-synthesis (Chalcone synthase (CHS), Flavanone 3 hydroxylase (F3H), Dihydroflavanol reductase (DHR), Leucoanthocyanidin
deoxygenase (LDOX) and Anthocyanidin synthase (ANS) and transcription factors controlling the expression of anthocyanin synthesis (MYB and BHLH transcription factors) and somatic embryogenesis (Arabinogalactan proteins, Lipid transfer proteins, High mobility group proteins) were obtained from the National Centre for Biotechnology Information (NCBI) database and used for PCR analysis for surveying the regeneration responsive and regeneration non responsive lines.

The PCR was performed in 5 μL reaction volumes with final concentrations of 5 ng of DNA, 1.5 mM MgCl₂, 0.1 mM of dNTPs, 1X PCR buffer, 0.6 pM of primers and 0.2 U of Taq DNA polymerase (SibEnzyme Ltd., Russia) in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, USA) with the following cyclic conditions: initial denaturation at 94°C for 5 min then 10 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s (temperature reduced by 1°C for each cycle) and extension at 72°C for 30 s. This was followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 30 s and extension at 72°C for 30 s with the final extension of 10 min at 72°C. The PCR products were then resolved on 2% agarose gel and documented using alpha imager.

Authors contributions
There is a paucity of research investigations in the life of people and plants. I have made a very small and humble attempt to reduce a fraction of it. I wish to share my contributions as an author of this research report. Cotton is a recalcitrant crop to regenerate from in vitro tissue cultures. During the invitro regeneration of cotton, the 170-180 days old calli exhibited anthocyanin pigmentation which resulted in somatic embryo formation and good regeneration response. Anthocyanin induction in embryogenic callus is reported to be a good indicator of somatic embryogenesis. Therefore the focus of this study was to find out the relationship between anthocyanin synthesis and somatic embryogenesis in cotton. A thorough study was made regarding the biosynthesis of anthocyanin and the molecular aspects of somatic embryogenesis and the gene specific primers were designed for anthocyanin biosynthesis and somatic embryogenesis with the aid of the bioinformatics tool available in NCBI database. Good results were obtained which have been interpreted in this research report. I also express the depth of my sense of gratitude to my respectable chairman for his indefatigable guidance, explicit and unaccountable help rendered for this investigation.

References
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