Development of novel plant phenotypes using plant pigment-associated genes©

S.A. Dhekney[1,a], R. Kandel[1], D. Bergey[1], B. Asanakunov[1], V. Sitther[2], Z.T. Li[3] and D.J. Gray[3]

[1]University of Wyoming, Sheridan Research and Extension Center, Sheridan, Wyoming 82801, USA; [2]Morgan State University, Department of Biology, Baltimore, Maryland, USA; [3]University of Florida, Mid-Florida Research and Education Center, Apopka, Florida 32703, USA.

Abstract

A number of flavonoids produced by plants impart specific flower and fruit color. The R2R3-Myb transcription factors are key regulatory genes involved in flavonoid biosynthesis. Such transcription factors can be potentially used in the development of new plant phenotypes via genetic engineering. In the current study, anthocyanin biosynthesis-related genes from *Citrus* (RUBY), grapevine (*VvMybA1*), and maize(leaf color-LC) were isolated and placed along with a NPTII gene under the control of a CaMV35S-derived promoter complex. Embryogenic cultures of *Vitis vinifera* 'Thompson Seedless' were initiated from leaves and floral explants. Somatic embryos at the mid-cotyledonary stage of development were co-cultivated with *Agrobacterium* harboring individual candidate genes to regenerate modified plants. Leaf discs of tobacco cultivar 'Samsun' and petunia cultivar 'Mitcham' were also transformed to produce modified plants. Regenerated plants were transferred to potting mix, hardened under conditions of high humidity and transferred to a greenhouse. Transient anthocyanin expression from various genes was evidenced by bright red spots on explants after 3-5 d of co-cultivation with *Agrobacterium.* Stable gene expression was observed in callus and shoot cultures after 4-8 weeks on regeneration medium. Modified 'Thompson Seedless' plants were recovered after 16 weeks of co-cultivation while 'Samson' and 'Mitcham' produced plants in 4-6 weeks. Regenerated plants exhibited varied patterns and intensity of red pigmentation in mature tissues. While some plant lines exhibited uniform red pigmentation on leaves and shoots, other lines exhibited patchy or interveinal accumulation of the anthocyanin pigment. Normal growth and flowering was observed in all plants. Such plants expressing anthocyanin pigments with varied patterns and intensities could be used as breeding lines for the development of ornamental phenotypes with unique coloration.

INTRODUCTION

Anthocyanins belong to a group of flavonoid compounds that are responsible for a wide array of colors in leaves, flowers and fruit, and function to attract pollinators and seed dispersers (Sakuta, 2014). Anthocyanin pigments also exhibit medicinal properties to mitigate effects of several debilitating diseases such as cancer and cardiovascular disease (de Pascual-Teresa and Sanchez Ballesta, 2008). Anthocyanins are produced from phenylalanine via the phenylpropanoid and flavonoid pathways. The regulation of anthocyanin biosynthesis is regulated by several proteins including the R2R3-Myb transcription factors, basis-helix-loop-helix (bHLH) and WD-repeat (WDR) proteins (Czemmel et al., 2012). The Myb transcription factors are large family of proteins that have key functions in the regulation of the anthocyanin biosynthesis pathway. Several regulatory proteins have been isolated from a number of plant species and expressed in heterologous systems for the constitutive expression of anthocyanin and production of red pigmentation in plant tissues. Anthocyanin-related genes can be potentially used to generate novel colors in plants resulting in the development of new ornamental cultivars. In the current study,

[a]E-mail: sdhekney@uwyo.edu

anthocyanin biosynthesis-related genes were isolated from *Citrus*, *Vitis* and *Zea mays* and inserted in *Vitis vinifera*, *Petunia hybrida* and *Nicotiana tabacum* to produce novel phenotypes of potential value to the ornamental plant industry.

MATERIALS AND METHODS

Plant materials

Embryogenic cultures of *V. vinifera* 'Thompson Seedless' were established as described previously (Dhekney et al., 2009). Briefly, unopened leaves obtained from in vitro micropropagation cultures were placed on NB2 medium (Dhekney et al., 2009) and cultured were placed in the dark for 6-8 weeks. Resulting callus cultures were transferred to light (65 μM m^{-1} s^{-1}) with a 16 h photoperiod at 25°C. Embryogenic callus occurring after 10 weeks of culture was transferred to X6 medium (Dhekney et al., 2009) for the development of somatic embryos. Somatic embryos (SE) at the mid-cotyledonary stage of development were used for gene insertion and plant regeneration.

Seed material for tobacco cultivar Samsun and petunia cultivar Mitcham were surface-sterilized in 50% commercial bleach solution followed by three washes in sterile distilled water. Seeds were then transferred to MS medium and maintained at 25°C and 16-h photoperiod as described above. Leaf discs were obtained from 21-day-old seedlings and used in gene insertion studies.

Vector construction

The coding sequences of the *Citrus* RUBY, *Vitis* MybA1, and maize LC (leaf color) genes were placed along with a npt II gene under the control of a CaMV35S-derived promoter complex. Each gene cassette was placed into a binary vector, which was subsequently transferred to *Agrobacterium* strain 'EHA 105' using the freeze-thaw method (Burow et al., 1990).

Gene insertion and culture development

Agrobacterium cultures harboring the RUBY, MybA1 and LC genes respectively were grown overnight on a rotary shaker at 28°C in MG/L medium (Garfinkle and Nester, 1980). The overnight culture was then centrifuged at 6000 rpm for 8 min to obtain a bacterial pellet. The pellet was resuspended in X2 medium (Li et al., 2006) and grown for an additional 4 h prior to gene insertion experiments.

Co-cultivation of in vitro cultures

Grapevine somatic embryos at the mid-cotyledonary stage of development, and petunia and tobacco leaf discs were used as explants for gene insertion experiments. Explants were transferred to sterile Petri dishes and submerged in *Agrobacterium* solution for 8 minutes. The excess bacterial solution was then removed by pipetting and explants were transferred to a Petri dishes containing two layers of filter paper (Fisherbrand P8) soaked in liquid DM medium (Li et al., 2001). Explants were co-cultivated in the dark for 3 days at 28°C. After 3 days, grapevine somatic embryos were transferred to liquid DMcc medium (DM medium containing 200 mg L^{-1} each of carbenicillin and cefotaxime antibiotics) and transferred to a rotary shaker at 120 rpm to inhibit bacterial growth. After two days of washing in liquid DMcc medium, explants were transferred to Petri dishes containing solid DMcck medium (DM medium containing 200 mg L^{-1} each of carbenicillin and cefotaxime and 100 mg L^{-1} kanamycin) and incubated in the dark at 28°C for 30 days. Resulting callus cultures were then transferred to X6cck70 medium (Li et al., 2006) for development of secondary embryos. Petunia and tobacco explants were blotted on sterile filter paper to remove excess bacterial growth. Explants were then transferred to regeneration medium for shoot proliferation described previously (Dhekney et al., 2011; van der Meer, 2006).

Regeneration of modified plants

Secondary embryos expressing the RUBY, MybA1 and LC genes were identified on the

basis of red pigmentation. Such embryos were transferred to MS1B medium (Li et al., 2006) for embryo germination and plant regeneration.

Modified shoots identified on the basis of red pigmentation were excised from proliferating cultures and transferred to MS medium containing 200 mg L^{-1} each of carbenicillin and cefotaxime and 100 mg L^{-1} kanamycin for rooting. Fully developed plants were transferred to plug trays containing sterile potting mix and maintained under conditions of high humidity for hardening. Fully hardened plants were transferred to a greenhouse.

RESULTS AND DISCUSSION

Transient gene expression in explants was observed as bright red spots after 3 days of co-cultivation (Figure 1A). Transient gene expression decreased in intensity after 8 days of transfer to regeneration medium. Modified callus cultures were evidenced by bright red pigmentation (Figure 1B). Petunia and tobacco explants exhibited multiple shoot proliferation following transfer to regeneration medium Figure 1C) and shoots appeared dark red in color compared to non-transformed proliferating shoot cultures (Figure 1D). Shoot production in petunia and tobacco occurs via indirect organogenesis where co-cultivated leaf discs exhibit callus formation followed by the production of meristemoids. Such meristemoids eventually produce shoots, which may be modified following gene insertion. Modified shoots evidenced by bright red pigmentation produced roots following transfer to MS medium. Similar results were observed with grapevine somatic embryo explants where modified callus cultures produced somatic embryos when transferred to development medium. Secondary embryogenesis is frequently observed in grapevine cultures with secondary embryos arising from surface epidermal cells of the primary embryos (Dhekney et al., 2009; Gray et al., 2005). Thus, grapevine somatic embryos are ideal targets for gene insertion and recovery of stable modified plants via the process of secondary embryogenesis. Plant lines with varying levels and patterns of anthocyanin were observed in the greenhouse (Figure 1E, F). This may be attributed to various factors including copy numbers of inserted genes and their location of insertion in the plant genome. Grapevine plant and tobacco plants exhibiting intense anthocyanin pigmentation showed reduced growth and lack of vigor compared to those with low to moderate levels of anthocyanin pigmentation. This may be attributed to the hyperaccumulation of anthocyanin in the cytoplasm that may cause unintended toxic effects on plant growth and development (Marrs et al., 1995; Marrs, 1996). Varied patterns of anthocyanin pigmentation were observed on the abaxial and adaxial surfaces of plant leaves. Normal flowering was observed in tobacco plant lines expressing the RUBY, MybA1 and LC genes (Figure 1E). The pattern and intensity of floral pigmentation varied among various plant lines.

We are currently studying growth and development of grape and petunia plant lines in the greenhouse. The effects of the constitutive expression of anthocyanin related genes and subsequent anthocyanin pigment accumulation on reproductive development including fruit development and seed viability will be evident as plants mature. Such plants expressing anthocyanin pigments with varied patterns and intensities could be used as breeding lines for the development of ornamental phenotypes with unique coloration. Additionally, they could also serve as a source of red pigment production that could have potential applications in the food industry.

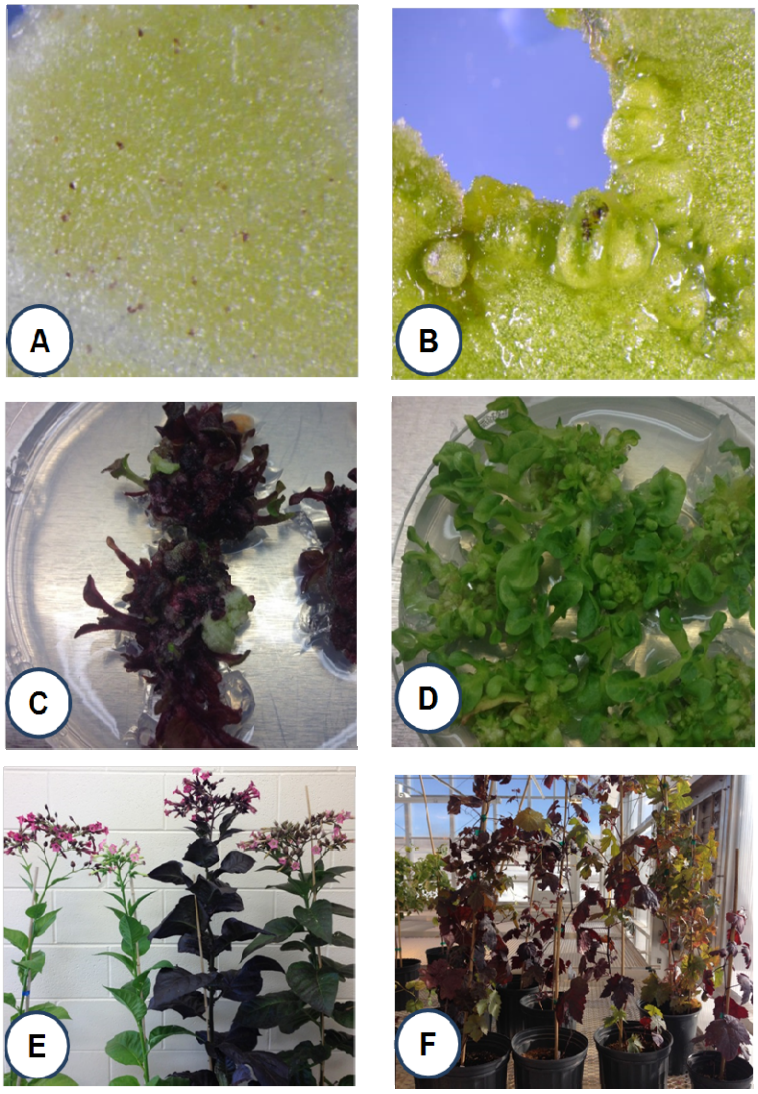

Figure 1. Transient anthocyanin expression (A) in tobacco leaf discs after 3 days of co-cultivation. Stable callus (B) and shoot (C) production on regeneration medium. Note that shoot cultures exhibit dark pigmentation compared to non-transformed control cultures (D). Mature tobacco (E) and grapevine plants (F) exhibiting varied levels and patterns of anthocyanin pigmentation.

ACKNOWLEDGEMENTS

S.A. Dhekney holds the E.A. Whitney Endowed Professorship in the Department of Plant Sciences. The research was supported in part by the Wyoming Agricultural Experiment Station and grants from the USDA/USAID US-Egypt Science and Technology Development Funds Program (grant # 0210-22310-005-24G).

Literature cited

Burow, M.D., Chlan, C.A., Sen, P., Lisca, A., and Murai, N. (1990). High frequency generation of transgenic tobacco plants after modified leaf disk co-cultivation with *Agrobacterium tumefaciens.* Plant Mol. Biol. Report. *8* (*2*), 124–139 http://dx.doi.org/10.1007/BF02669766.

Czemmel, S., Heppel, S.C., and Bogs, J. (2012). R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. Protoplasma *249* (*S2*, *Suppl 2*), S109–S118 http://dx.doi.org/10.1007/s00709-012-0380-z. PubMed

de Pascual-Teresa, S., and Sanchez-Ballesta, M.T. (2008). Anthocyanins: from plant to health. Phytochem. Rev. *7* (*2*), 281–299 http://dx.doi.org/10.1007/s11101-007-9074-0.

Dhekney, S.A., Li, Z.T., Compton, M.E., and Gray, D.J. (2009). Optimizing initiation and maintenance of *Vitis* embryogenic cultures. HortScience *44*, 1400–1406.

Dhekney, S.A., Li, Z.T., and Gray, D.J. (2011). Agrobacterium-mediated transformation and regeneration of transgenic tobacco plants. In Plant Cell Culture, Development and Biotechnology. 3rd edn, R. Trigiano, and D. Gray, eds. (Boca Raton, Florida, USA: Taylor and Francis), p.467–472.

Garfinkel, D.J., and Nester, E.W. (1980). *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. *144* (*2*), 732–743. PubMed

Gray, D.J., Jayasankar, S., and Li, Z.T. (2005). Vitaceae (grape *Vitis* spp.). In Biotechnology of Fruit and Nut Crops, Biotechnology in Agriculture Series No. 29, R.E. Litz ed. (Wallingford, UK: CAB International), p.672–706.

Li, Z., Jayasankar, S., and Gray, D.J. (2001). Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). Plant Sci. *160* (*5*), 877–887 http://dx.doi.org/10.1016/S0168-9452(01)00336-3. PubMed

Li, Z.T., Dhekney, S., Dutt, M., Aman, M., Tattersall, J., Kelley, K.T., and Gray, D.J. (2006). Optimizing *Agrobacterium*-mediated transformation of grapevine. In Vitro Cell. Dev. Biol. Plant *42* (*3*), 220–227 http://dx.doi.org/10.1079/IVP2006770.

Marrs, K.A. (1996). The functions and regulation of glutathione s-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. *47* (*1*), 127–158 http://dx.doi.org/10.1146/annurev.arplant.47.1.127. PubMed

Marrs, K.A., Alfenito, M.R., Lloyd, A.M., and Walbot, V. (1995). A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2.* Nature *375* (*6530*), 397–400 http://dx.doi.org/10.1038/375397a0. PubMed

Sakuta, M. (2014). Diversity of plant red pigments: anthocyanins and betacyanins. Plant Biotechnol. Rep. *8* (*1*), 37–48 http://dx.doi.org/10.1007/s11816-013-0294-z.

van der Meer, I.M. (2006). *Agrobacterium*-mediated transformation of *Petunia* leaf discs. Methods Mol. Biol. *318*, 265–272. PubMed