Citrus Section / Refereed Manuscript

Proc. Fla. State Hort. Soc. 126:xxx–xxx. 2013.

Transgenic Expression in Citrus of *Vitis* MybA1 from a Bidirectional Promoter Resulted in Variable Anthocyanin Expression and Was Not Suitable as a Screenable Marker without Antibiotic Selection

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Additional index words. citrus breeding, disease resistance, tolerance, huanglongbing

Transgenic strategies offer potential solutions for major problems facing Florida citrus. Intragenics, in which all transgene components are from the target species' gene pool, may alleviate consumer concerns and might also facilitate deregulation, providing growers with consumer-accepted transgenic solutions more quickly. Resistance to antibiotics is typically used as a selectable marker for plant transformation and this project was initiated as a proof of concept to test a plant gene as a visual screenable marker alternative to antibiotic-resistance. Transformation with *Vitis* MybA1 has been demonstrated to produce purple shoots in grape and was tested as a surrogate for a citrus Myb that might be used as an intragenic plant-pigmentation marker in citrus transformation. 'Hamlin' and Carrizo epicotyls segments were exposed to *A. tumefaciens* EHA105 containing the DAT or DEAT vector with *Vitis MybA1* driven by the D35S promoter and neomycin phosphotransferase II (*NptII*, conferring kanamycin resistance, originally isolated from *E. coli*), driven by the Nos promoter. Transformation was compared with and without kanamycin (100 mg·L^{-1} = 0.00083 lb/gal) in the shoot regeneration medium with the DEAT vector. In sum, over 16 separate experiments, more than 1300 explants each of Carrizo and 'Hamlin' were treated. In all cases, 6–20 fold more shoots resulted when kanamycin was excluded from the medium, since there was no negative selection against non-transformed shoots, but no shoots with red pigmentation were recovered. When kanamycin was included, 28% of resulting 'Hamlin' shoots (6 out of 21) and 57% of Carrizo shoots (321/405) displayed anthocyanins. Phenotypes recovered included plants with blotchy reddish leaves and plants with cupped leaves. Pigmented 'Hamlin' shoots were very weak and subsequently died. Several deep red Carrizo transformants resulted with potential as research tools and ornamentals.

Genetically engineered (GE) cultivars have been deregulated for commercial use in 25 different agricultural crops (ISAAA, 2013). GE crops are grown on 11% of global arable land with four field crops (soybean = *Glycine max* (L.) Merr., maize = *Zea mays* L., cotton = *Gossypium hirsutum* L., and canola = *Brassica napus* L.) responsible for 99% of the area planted to GE crops (Nature Publishing Group, 2013). Several GE horticultural crops are being produced commercially since they provide solutions to otherwise intractable threats, much as huanglongbing (primarily associated with *Candidatus* Liberibacter asiaticus Jagoueix) appears to be for citrus. Virus-resistant transgenic summer squash (*Cucurbita pepo* L.) is widely grown in the southeastern U.S. (NCFP, 2008) and virus-resistant transgenic papaya (*Carica papaya* L.) accounts for ~70% of Hawaii's papaya production (Hudson and Gunn, 2008). Many expect use of GE crops to expand, providing solutions to many agricultural problems. However, no GE fruits or vegetables are currently permitted into the European Union (GMO Compass, 2013) and consumer concerns about GE crops remain substantial. There is much greater skepticism in European consumers compared to those in North America, Asia, and Australia/New Zealand (Frewer et al., 2013); however, GE skeptics are widespread. Laws requiring labeling of GE foods are being implemented in many countries (e.g., Viljoen and Marx. 2013), will likely increase awareness of GE foodstuffs, and may prejudice consumer selection.

Intragenics (in which all transgene components are from the target species' gene pool but can be from diverse related species and may be from different regions in the genome) or cisgenics (in which all or most transgene components are from the target species' gene pool and are in their original genomic orientation), reportedly reduce consumer concerns and might also facilitate deregulation or may even be exempt from regulation (reviewed in Holme et al., 2013). In a survey of European consumers, 55% supported cisgenic crops vs. only 22% in support of conventional transgenic crops (Podevin et al., 2012) , and cisgenic apple (*Malus* ×*domestica* Borkh.) and canola cultivars are close to commercialization. Therefore, it may be expedient to develop intragenic or cisgenic methodology for citrus.

Resistance to antibiotics using bacterial genes is typically used as a selectable marker for plant transformation and is not permissible within current intragenic or cisgenic definitions. Methods have been used to produce marker-free plants expressing intragenes or cisgenes, but efficiency is reduced (reviewed in Holme et al., 2013). Efficiency of citrus transformation is already

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low, so this project was initiated to test a plant gene as a visual screenable marker as a model for an intragenic alternative to antibiotic resistance marker genes. The sweetpotato [*Ipomoea batatas* (L.) Lam.] gene *IbMyb1* was transiently expressed in tobacco and proposed as a possible intragenic screenable marker (Kim et al., 2010), but was not tested in a system for stable transformation. Transformation with *Vitis MybA1* has been demonstrated to produce purple shoots in grape and in this report was tested as a proof of concept for use of plant-pigmentation markers in citrus transformation. If successful, a citrus Myb would then be used as a stand-alone intragenic screenable marker, which could provide growers with consumer-accepted transgenic solutions more quickly.

Materials and Methods

'Hamlin' [*Citrus sinensis* (L.) Osbeck] and Carrizo [*C. sinensis* × *Poncirus trifoliata* (L.) Raf.] epicotyls segments were exposed to *Agrobacterium tumefaciens* [(Smith and Townsend) Conn] EHA105 containing the DAT or DEAT binary vector with the *Vitis vinifera* L. *MybA1* (*VvMybA1*) driven by a double enhanced CaMV35S promoter and neomycin phosphotransferase II [*NptII*, conferring kanamycin resistance, originally isolated from *Escherichia coli* (Migula) Castellani & Chalmers], driven by the Nos promoter. Details of these constructs and vectors are outlined in a previous publication (Li et al., 2011). DEAT harbors a bidirectional promoter complex which resulted in markedly greater anthocyanin expression than DAT in transgenic grapevine (*Vitis vinifera*) and tobacco (*Nicotiana tabacum* L.) (Li et al., 2011). Transgenic shoots were developed according to the method of Orbovic and Grosser (2006) with modifications as noted below. Transformation with DEAT was compared with and without kanamycin (100 mgL^{-1} = 0.00083 lb/gal) in the shoot regeneration medium, while DAT was only used with kanamycin selection. In sum, over 16 separate experiments, 2300 explants of Carrizo and1300 explants of 'Hamlin' were treated, using 65–300 explants per experimental run. Regenerating shoots were counted on each plate and each shoot visually classified as deep red, intermediate red, or green based on anthocyanin expression. After reaching 1 cm (0.4 inch) in height, all red shoots and a subset of green shoots from the regeneration medium of 'Hamlin' were micrografted onto Carrizo seedlings while shoots of Carrizo were micrografted onto Volkamer lemon (*C. volkameriana* V. Ten. & Pasq.) seedlings, all in soilless mix. Micrografted shoots were covered with plastic bags and placed in a growth chamber at 27 °C (81 °F) and 60% humidity for 2 weeks. Plants were transferred to a greenhouse and exposure to ambient humidity was achieved by clipping away the protective bag over a 2-week period with intermittent misting. Presence of the *VvMybA1* transgene was determined using the PCR primers specified in Li et al. (2011). DNA from two leaf samples per plant were extracted using REDExtract-N-Amp kit (Sigma-Aldrich Co., St. Louis, MO) and two PCR reactions were run on each extracted sample. PCR reactions were carried out using a PTC-100 thermalcycler (MJ Research Inc. St. Bruno, Quebec) in a 22-µL (5.8×10^{-6} gal) reaction mixture containing 2× REDExtract-N-Amp PCR Readymix (Sigma-Aldrich Co.) and 0.5 µM of each primer. Thermalcycling conditions were as follows: one cycle at 95 °C (203 °F) for 3 min; 35 cycles transitioning between 94 °C (201 °F) for 1 min, 56 °C (133 °F) for 45 s, and 72 °C (162 °F) for 45 s; followed by 1 cycle at 72 °C (162 °F) for 10 min.

Shoots were evaluated at intervals of 3 to 4 months after micrografting for color and other phenotypic traits. For four *VvMybA1* positive Carrizo plants with diverse color, tissue was assessed using a chroma meter (model CR-400, Minolta Camera Corp., Ramsey, NJ) on three dates (15 Sept. 2011, 7 Oct. 2011, and 3 Feb. 2012). Three leaves were assessed on each plant on each date and where possible the same leaves were sampled on the second and third dates. In all cases, data presented are means of three measurements. When shoots reached 25 cm (10 inches) in height, several of the most distinctive transformants were propagated by micrografting onto rootstocks.

Results and Discussion

Initially, recovery of pigmented shoots from DEAT was greater than that from use of DAT, so the DEAT vector was used for all subsequent experiments. In all cases, 6–20 fold more shoots resulted when kanamycin was excluded from the medium (Table 1; Fig. 1), presumably since there was no negative selection against non-transformed shoots. However, no shoots with red pigmentation were recovered when kanamycin was not in the regeneration medium. Evaluation for the transgene using PCR resulted in no positives for the green shoots tested from the kanamycin-free medium (Table 2). Ballester et al. (2008) reported effectiveness of several screening systems in Carrizo and 'Pineapple' sweet orange transformation, including exclusion of selective antibiotics and evaluation for beta-glucuronidase expression in regenerated shoots: they observed 2.4- to 20-fold more shoots/explants without a selective marker and 1% to 7% of resulting shoots were transformed without kanamycin vs. 7% to 69% with kanamycin present. When kanamycin was included in medium for our study, 28% of resulting 'Hamlin' shoots (6 out of 21) and 57% of Carrizo shoots (321/405) displayed anthocyanins ranging from fairly uniform dark purple to blotchy red, and with many appearing to have sectors of normal green tissue (Fig. 2). When shoots from medium containing kanamycin were tested for the *VvMybA1* transgene, 15% of green shoots were positive from one of the two leaf samples (Table 2). When blotchy red shoots were tested, 55% of the blotchy shoots were positive in both leaf samples, 36% were negative, and 9% were positive in one of the two leaf samples (Table 2). This is consistent with some blotchy shoots being chimeral for the transgene but this was not unequivocally verified. Chimeral shoots from citrus transformation have been previously reported (Peña et al., 1997). Using *VvMybA1* as a selectable marker in grapevine, some red shoots were recovered on medium without kanamycin but a high proportion was observed to be chimeral (Gray, unpublished).

Phenotypes recovered included plants with blotchy reddish leaves and plants with cupped leaves. Pigmented 'Hamlin' shoots were very weak and subsequently died as did all of the initially dark red Carrizo plants. The most darkly pigmented survivor (tree #45) was scored as green when initially micrografted. When transgenically introduced into tobacco , *VvMybA1* initially resulted in red shoots, but within 2 to 3 weeks red color became patchy and then leaves became almost uniformly green with red veins apparent under some conditions, but with red color being evident in reproductive tissues (Li et al., 2011). In grapevine, plants transformed with the DEAT construct accumulated high levels of anthocyanin but displayed developmental delays and abnormalities and all plantlets died soon after transplanting (Li et al., 2011). Another research group has evaluated citrus expres-

Table 1. Shoot regeneration on medium with and without kanamycin in Carrizo and 'Hamlin' explants co-cultivated with *A. tumefaciens* carrying the vectors DEAT or DAT , both containing the *Vitis vinifera MybA1* gene (associated with anthocyanin overexpression) and NptII (kanamycin resistance).

Citrus cultivar	Construct	Co-culture date	Selection medium	Explants (no.)	Green shoots (no.)	Deep red shoots (no.)	Blotchy red shoots (no.)
Hamlin	DEAT	11/16/2010	MSB1AB	117	7	0	5
		1/25/2011		81	1	0	0
		2/11/2011		155	1	0	0
		2/17/2011		203	4	0	0
		2/18/2011		180	1	0	0
		2/25/2011		83	0	0	0
		3/26/2013		65	1	0	1
		2/11/2011	MSB1 No Kan	149	25	0	0
		2/17/2011		154	21	0	0
		2/25/2011		85	26	0	0
		3/26/2013		65	13	0	0
Carrizo	DAT	12/16/2010	MSB1AB	297	164	2	15
Carrizo	DEAT	11/12/2010	MSB1AB	257	63	25	56
		12/21/2010		134	39	2	3
		4/28/2011		164	19	13	18
		5/3/2011		136	12	8	21
		3/14/2013		65	4	5	6
		3/22/2013		275	27	29	34
		4/3/2013		220	10	3	8
		4/28/2011	MSB1 No Kan	200	171	0	0
		5/3/2011		80	129	0	0
		3/22/2013		250	668	0	0
		3/14/2013		65	142	0	0
		4/3/2013		220	211	0	0

Fig. 1. Regeneration plates of Carrizo explants exposed to *A. tumefaciens* with the DEAT vector containing *VvMybA1* and *NptII* (kanamycin resistance). Two top plates do not contain kanamycin. Two bottom plates contain 100 mg·L^{-1} (0.00083 lb/gal) kanamycin.

sion of *VvMybA1* with a unidirectional promoter, and reported that some strongly pigmented plants were lacking in vigor, while others appeared normal (Dutt and Grosser, personal comm.).

Chroma meter data indicated changes in leaf coloration over time and between leaves of different developmental stages (Table 3). Only *a** axis data are presented, as changes between green and red were most striking. Negative values of *a** indicate green color while positive values indicate magenta. Considerable variability was seen between leaf color in the plants evaluated, which were representative of the phenotypic range produced. Most trees were blotchy red and displayed substantial green color with negative *a**. In three of the four plants tested, there tended to be a greater greenish cast to the leaves in Feb. 2012 than in Oct. 2011. Tree #45 was quite red/purple in color, though new flush leaves were somewhat greener than more mature leaves.

In grapevine the regulatory gene *VvMybA1* specifically activates expression of UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT) (Matus et al., 2008), which catalyzes addition of a glucosyl moiety to anthocyanidin producing stable and highly pigmented anthocyanin (Heller and Forkmann, 1993). The *VlMybA1* (from *Vitis labrusca* L.) also shows great specificity and uniformity for enhanced anthocyanin production when overexpressed in the closely related *V. vinifera* (Cutanda-Perez et al., 2009) and was shown to activate genes regulated as a cluster in the last steps of anthocyanin synthesis. In the study reported here, this specificity and uniformity was not apparent in Carrizo, with great diversity in pigmentation and even some leaf morphology changes that have

Table 2. Results of PCR test for *VvMybA1* in green and blotchy red shoots from regeneration on medium with and without kanamycin in Carrizo and 'Hamlin' explants co-cultivated with *A. tumefaciens* carrying vectors containing the *Vitis vinifera MybA1* gene (associated with anthocyanin overexpression) and NptII (kanamycin resistance). Results are interpreted as indicating chimeral shoots when shoot color and MybA1 presence were not consistent.

Shoot color	Kanamycin in medium	All MybA1+ (%)	All MybA1– (%)	MybA1+/MybA1– (%)
Green	no	0	100	0
Green	yes	0	85	15
Blotchy red	yes	55	36	9

Fig. 2. Diversity of pigmentation in Carrizo shoots regenerated on medium containing kanamycin following transformed with *VvMybA1*.

proven stable through clonal propagation (Fig. 3). Appearance of a phenotype in a single transformation event might be explained by disruption of structural genes from transgene insertion, but occurrence of the same phenotypes repeatedly (as seen with blotchy red or cupped leaves in this study) suggests function of the transgene itself.

Fig. 3. Diversity of phenotype in Carrizo transformed with *VvMybA1*. Plant #45 at top, plant #34 in middle, and most extreme leaf cupping phenotype at bottom.

Table 3. Chromameter tests documenting diversity and developmental changes in pigmentation of Carrizo shoots transformed with *Vitis vinifera MybA1* gene.

Plant	Date	Leaf 1	Leaf 2	Leaf 3	Comments
#19	9/15/2011	–6.3	–5.5	–15.1	Three random fully expanded leaves
#31		–4.6	–8.1	–3.8	
#34		–7.1	–4.4	–4.2	
#45		1.6	–1.0	–0.4	
		Top leaf	Middle leaf	Bottom leaf	
#19	10/7/2011	–15.2	–8.8	–10.3	
#31		–19.9	–11.0	–4.1	
#34		–3.8	–5.6	–5.7	
#45		–2.2	1.4	2.1	
#19	2/3/2012	–6.6	–0.6	–11.5	Same middle and bottom leaf as 10/7/11
#31		–12.4	–14.2	–7.6	Same middle and bottom leaf as 10/7/11
#34		–8.9	–10.4	–5.8	Same top and middle leaf as 10/7/11
#45		–4.2	0.3	–0.8	Same middle and bottom leaf as 10/7/11

In Arabidopsis [*A. thaliana* (L.) Heynh.] a complex network of positive and negative feedback mechanisms regulate anthocyanin synthesis (Petroni and Tonelli, 2011), and it appears that *VvMybA1* generally upregulates anthocyanin expression in citrus but does not totally dominate the network of factors involved. Myb transcription factors are numerous even within individual organisms (339 in *A. thaliana*, 269 in *V. vinifera*, 36 in *Homo sapiens* L.) and often interact in a combinatorial manner to regulate gene expression (Feller et al., 2010). *VvMybA1* is in a subgroup of Myb transcription factors associated primarily with proanthocyanidin and anthocyanin expression in diverse plant species (Feller et al., 2010), but perhaps *VvMybA1* also interacts in other networks within the Carrizo genome.

Conclusions

In the experiments with citrus reported here, *VvMybA1* delivered by the DEAT construct does not appear to be an effective screenable marker when used without the kanamycin selection system, since no visibly red shoots were recovered when kanamycin was excluded from the medium.

Variability in red color expression between individual transformants was much greater than expected and may reflect diversity of response from use of a regulatory transgene from a heterologous system. Several deep red Carrizo transformants resulted with potential as research tools and ornamentals.

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