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Citrus Callus Browning Influences Transformation Efficiency

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ABSTRACT

Our previous experiments on *Agrobacterium*-mediated citrus callus transformation showed that transformant regeneration is often accompanied by callus browning. To further evaluate the effect of citrus callus browning on transformation, embryogenic calli from 13 citrus species/varieties (including *Citrus reticulata*, *C. paradisi*, *Fortunella crassifolia*, *C. sinensis*, etc.) were inoculated with *Agrobacterium* and assessed for their transformation potentials. Transformation results showed that all but one succeeded in regenerating kanamycin resistant tissues, and among them six produced whole transgenic plants. Histochemical β-glucuronidase (GUS) analysis and southern blot hybridization of resistant tissues confirmed transgene integration. In fact, more than 91% of transformed calli were regenerated from browning callus. Moreover, the transformation efficiency was higher in severe browning species/varieties, suggesting that callus browning was closely related to transformation efficiency. A further study showed that the total phenolic content of citrus callus affected callus browning and was positively correlated with transformation efficiency. Taken together, our data indicate that the effect of citrus callus browning on transformation efficiency might correlate with its content of polyphenols.

Keywords: *Agrobacterium*-mediated transformation, browning degree, total phenolic content, transformation efficiency

INTRODUCTION

Agrobacterium tumefaciens possesses the natural ability to deliver its T-DNA fragment into plant cells and is widely used in the genetic transformation of plants. However, many plants still remain recalcitrant to *Agrobacterium* transformation and are transformed at very low efficiency. Factors influencing transformation efficiency have been previously elucidated, and include the *Agrobacterium* strain and plasmid vector (Gutiérrez-E *et al.* 1997), explant types (Franklin *et al.* 2004; Pérez-Clemente *et al.* 2004), physiological state of the explant (Iida *et al.* 1991; Peña *et al.* 2004), co-culture temperature (Blanc *et al.* 2006), co-culture medium (Peña *et al.* 1997; Yu *et al.* 2002; Khanna *et al.* 2004), kind and concentration of the selective agent (Wang *et al.* 2005; Zhu *et al.* 2005) and the addition of inducers like acetosyringone (Li *et al.* 2003; Khanna *et al.* 2004). Plant genotype may affect transformation efficiency significantly (Torregrosa *et al.* 2002; Lin *et al.* 2005). There are great differences in transformation efficiency among citrus species/varieties (Liu *et al.* 2006), however, details about the transformation potentials of most citrus callus and the interaction between citrus genotype and agroinfection are still largely unknown, which needs further study.

Tissue browning or necrosis usually occurred during or after co-cultivation, which resulted in low transformation efficiency. Such donor plants included grape (Pu and Goodman 1992; Perl *et al.* 1996; Das *et al.* 2002), *Arabidopsis thaliana* (Sangwan *et al.* 1992), maize (Hansen 2000), sorghum (Carvalho *et al.* 2004), and citrus callus (unpublished). The possible reasons for low transformation efficiency caused by browning or necrosis were: 1) necrosis or cell death occurred in the same cell layer where T-DNA was transferred (Potrykus 1990); or 2) transgenic cells were imbedded in necrotic/browning tissues which inhibited regeneration (Goodman and Novacky 1994). Nevertheless, GUS expression was also higher in severely necrotic explants (Carvalho *et al.* 2004). In citrus transformation, tissue browning was often observed, but little is known about the browning reactions of different varieties and its relationship with regeneration of transformants.

As previously reported, citrus embryogenic calli were widely used in our lab for protoplast fusion (Guo and Deng 1999; Fu *et al.* 2004; Guo and Grosser 2005; Xu *et al.* 2006; Cai *et al.* 2006; Guo *et al.* 2008) and recently for genetic transformation (Li *et al.* 2002, 2003; Liu *et al.* 2006; Duan *et al.* 2007). In this paper, we reported on transformation of 13 citrus embryogenic calli and elucidated the relationship between citrus callus browning and transformation.

MATERIALS AND METHODS

Regeneration and transformation

Embryogenic calli of 13 tested *Citrus* species/varieties including *Citrus reticulata*, *C. paradisi*, *Fortunella crassifolia*, *C. sinensis*, etc. (**Table 1**) were subcultured once a month on solid MT (Murashige and Tucker 1969) basal medium with 40 g l^{-1} sucrose. *Agrobacterium tumefaciens* strain EHA105 harboring the binary plasmid pROKII (provided by Dr. Peña, IVIA, Valencia, Spain), with 35S-*uidA* and NOS-*nptII* in the T-DNA, was used as the plasmid vector (Peña *et al.* 2001). Newly grown white embryogenic calli were inoculated and co-cultured with *Agrobacterium* according to Duan *et al.* (2007), and were then transferred to embryogenesis medium [basal MT solid medium containing 500 mg/L malt extract or 20% glycerol] supplemented with the selective agents [i.e. 400 mg/L cefotaxime (Cef) and 50 mg/L Km]. Shoot regeneration medium (SRM) was the same as that of Guo *et al.* (2002) [basal MT medium plus 0.5 mg/L BA (6-benzyladenine), 0.5 mg/L Kin (kinetin) and 0.1 mg/L NAA (1-naphthyl acetic acid)]. Transgenic shoots were induced to root on medium containing half strength MT basal medium supplemented with 0.5 mg/L NAA, 0.1 mg/L IBA (3-indole butytic acid), 25 g/L sucrose and 0.5 g/L activated charcoal under the selective pressure of 25 mg/L Km or grafted onto rootstocks *in vitro* to produce whole transgenic plants. Transformation was conducted for 10 Petri plates/ cultivar with three replicates. Transformation efficiency was the mean value calculated

Received: 31 July, 2008. Accepted: 27 November, 2008.

Original Research Paper

Table 1 *Citrus* species/varieties used for transformation.

Citrus types	Cultivars/varieties of *Citrus*		Subculture age (years)
	English name	Latin name	
Tangerines	'Ponkan'	*Citrus reticulata*	7
	'Chazhigan'	*C. reticulata*	7
	'Dancy'	*C. reticulata*	9
	'Bendizao'	*C. reticulata*	7
	'Sunki' tangerine	*Citrus sunki*	10
Sweet oranges	'Valencia'	*Citrus sinensis*	16
	'Anliucheng'	*C. sinensis*	7
	'Jincheng'	*C. sinensis*	7
	'Succari' orange	*C. sinensis*	9
Grapefruit	'Red Marsh'	*Citrus paradisi*	10
Tangerine hybrids	Gailiangcheng × Owari	*C. sinensis* × *C. unshiu*	7
Kumquat	'Hongkong' kumquat	*Fortunella crassifolia*	21
Others	'Microcitrus'	*Microcitrus papauwana*	9

as the ratio of number of the Petri plates with resistant callus clusters to total number of Petri plates of three transformation experiments.

Evaluation of transformants by GUS assay and Southern blotting analysis

Calli selected randomly from resistant and non-transformed callus clusters were immersed in the 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) substrate solution and incubated at 37°C for 12 h according to Jefferson *et al.* (1987). The X-gluc substrate solution was prepared as follows: 90 mg X-gluc was dissolved in 50 ml sodium phosphate buffer (pH = 7.0), by adding 0.1 g / L chloramphenicol, 0.01% (v/v) Triton-100, 20% (v/v) methanol, and was then diluted to 100 ml by sterilized water. DNA from randomly selected 5 resistant callus lines of 'Ponkan' tangerine, 3 lines of 'Red Marsh' grapefruit, 2 lines of 'Chazhigan' tangerine, and 1 line of 'Hongkong' kumquat and non-transformed calli of 'Ponkan' and 'Chazhigan' tangerine was extracted using the CTAB method according to Cheng *et al.* (2003). A total of 15 μg genomic DNA digested with *Hind* III (which has only one restriction site in the plasmid) was separated on a 0.8% agarose gel, and transferred onto Hybond-N^+ blotting membrane under alkaline conditions, according to the manufacturers' instruction. The membrane was probed to specific PCR fragment of *nptII* (gi|19569229) labeled with P^{32}. The *nptII* gene primers were designed as 5′-GACGAG GCAGCGCGGCTAT-3′ and 5′-AAGAAGGCGATAGAAGGCG A-3′ to produce a 596 bp fragment.

Extraction and determination of total phenolic content of citrus callus

The extraction procedure of total phenols was according to Gorinstein *et al.* (2004) with minor modifications. After subculturing for 15 d, each replicate consisting of 0.4 g of non-transformed embryogenic calli was homogenized with 5 ml 40% methanol in a 10 ml plastic tube and incubated at 55°C for 30 min, and then centrifuged at 1000 g for 10 min at 4°C. The supernatant was transferred to a new tube and used for total phenol analysis. Total phenolic content was determined using the Folin-Ciocalteau reagent (Singleton *et al.* 1965; Rocha and Morais 2005). Total phenols in the extract were calculated from a standard curve of chlorogenic acid (0.01–0.1 mg/ml) prepared under identical conditions. Total phenolic content was expressed as μg/g of fresh weight of the citrus callus.

RESULTS

Regeneration of citrus resistant tissues after co-cultivation

Eight weeks after transformation, most citrus calli turned brown on selective medium. However, some browning calli gradually yielded small white calli or green embryos after being cultivated another 30-45 d on embryogenesis induction medium (**Fig. 1A-L**). Resistant calli proliferated quickly on new selection medium after being picked out and cultured alone, which indicated that the selectable marker gene had been introduced into the resistant calli. From 13 citrus species/varieties, 12 succeeded in regenerating a number of resistant callus clusters, six produced whole plants through an embryogenesis pathway, including 'Sunki' tangerine, 'Chazhigan', Gailiangcheng × Owari hybrid, 'Ponkan', 'Succari' orange, and 'Valencia' (**Fig. 1A, 1B, 1G, 1F, 1J, 1K**). Only one resistant callus cluster from non-brown callus was observed on both '*Microcitrus*' and 'Valencia'. Calli of 'Anliucheng' remained unchanged during the selection screening and failed to regenerate new calli in three transformation experiments (**Table 2**).

Various degrees of browning and transformation efficiencies were observed among different citrus calli

Eight weeks after transformation, citrus calli gradually browned depending on citrus species/varieties. This browning process is much rapid than non-transformed citrus calli under the same selective pressure. Among three transformation experiments, 12 tested species/varieties eventually succeeded in obtaining resistant calli, while one ('Anliucheng') remained recalcitrant. More than 91% (11/12) of the resistant callus lines were regenerated from brown calli. Based on the degree of callus browning, these citrus calli were classified into four types (I, II, III, or IV) (**Table 2**). Dark brown calli (type I) were observed in tangerines and their hybrids, including 'Sunki', 'Chazhigan', 'Bendizao', 'Dancy', 'Ponkan' tangerine and Gailiangcheng × Owari hybrid. Calli of 'Red Marsh' and 'Hongkong' kumquat, with moderate brown callus, displayed the type II phenotypes. Calli of '*Microcitrus*' with no brown color present displayed the type IV phenotypes. Sweet oranges, including 'Jincheng', 'Succari' orange, 'Valencia', and 'Anliucheng', varied greatly and were distributed into type II, III (calli with yellow brown), and IV phenotypes (**Fig. 1**).

As is shown in **Table 2**, 'Ponkan' had the highest transformation efficiency with 80.0%, followed by Gailiangcheng × Owari hybrid, 'Sunki' tangerine, and 'Chazhigan' with 60.0, 40.0 and 33.3%, respectively. The mean transformation efficiency of type I calli was as high as 42.2%. Much lower transformation efficiency was achieved in type II (15.5%) and type III species/varieties (13.3%) including 'Succari' orange, 'Hongkong' kumquat, 'Red Marsh', 'Jincheng', and 'Valencia' with eight, seven, six, three, and one resistant callus lines, respectively. The lowest transformation efficiency (3.3%) was observed in type IV species/varieties which included '*Microcitrus*' and 'Anliucheng' with one and zero resistant line, respectively. Based on the results, the transformation efficiency was much higher in intense browning species/varieties, lower in moderate or slight browning ones, and much lower in non-browning ones, which indicated that callus browning was positively correlated with transformation.

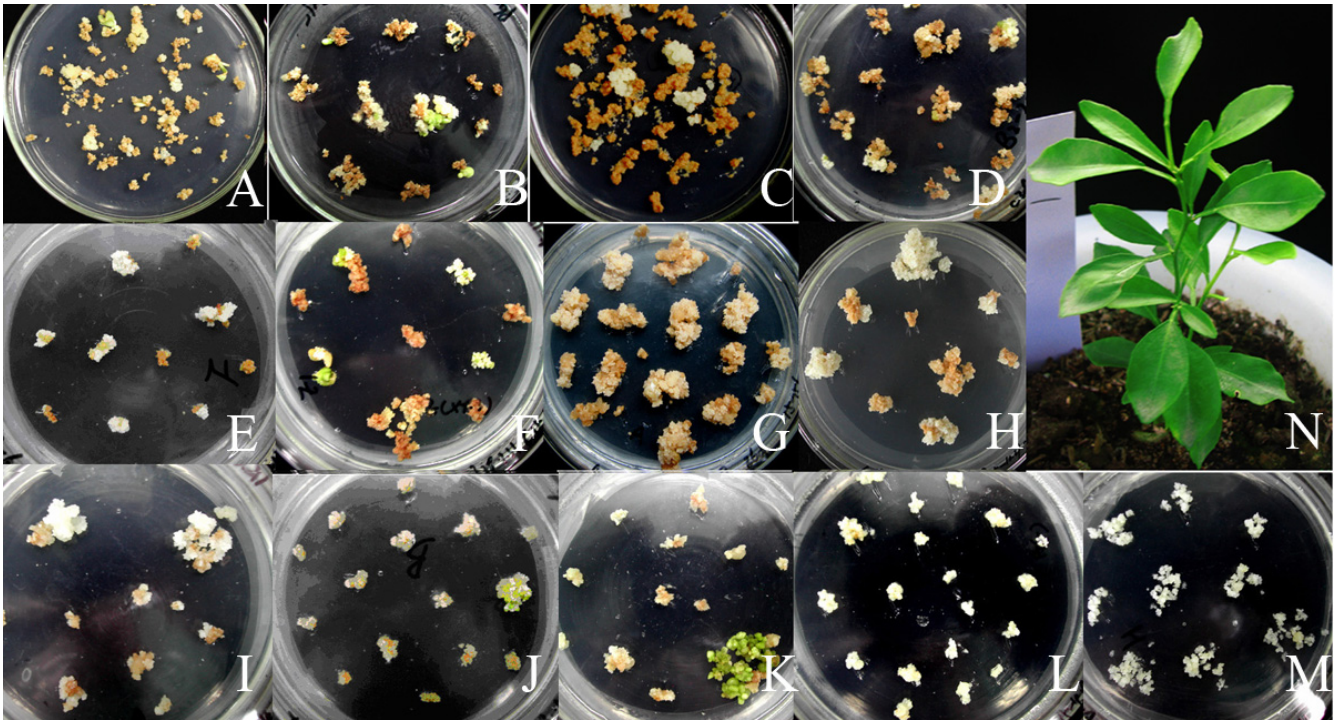

Fig. 1 Browning reactions and regeneration of different citrus callus after agroinfection. (**A**) 'Sunki' tangerine; (**B**) 'Chazhigan' tangerine; (**C**) 'Bendizao' tangerine; (**D**) Gailiangcheng × Weizhang hybrid; (**E**) 'Dancy' tangerine; (**F**) 'Ponkan' tangerine; (**G**) 'Hongkong' kumquat; (**H**) 'Red Marsh' grapefruit; (**I**) 'Jincheng' orange; (**J**) 'Succari' orange; (**K**) 'Valencia' orange; (**L**) '*Microcitrus*'; (**M**) 'Anliucheng' orange; (**N**) Transplanted transgenic 'Ponkan' plants.

Table 2 Browning degrees and transformation efficiencies of different citrus calli.

Types	Species/ varieties	Browning degree *	No. Resistant calluses **			Transformation efficiency *** (%)	Mean value (%)
			1	2	3		
I	'Ponkan'	+++	31	22	32	80.0 (12/15) a	42.2
	Gailiangcheng × Owari	+++	14	12	N	60.0 (6/10) a	
	'Sunki' tangerine	+++	3	3	N	40.0 (4/10) ab	
	'Chazhigan'	+++	3	4	4	33.3 (5/15) bc	
	'Dancy'	+++	0	4	10	20.0 (3/15) bcd	
	'Bendizao'	+++	0	5	1	20.0 (3/15) bcd	
II	'Jincheng'	++	1	2	0	20.0 (3/15) bcd	15.5
	'Hongkong' 'kumquat'	++	5	0	2	20.0 (3/15) bcd	
	'Red Marsh'	++	0	6	0	6.7 (1/15) cd	
III	'Succari' orange	+	0	0	8	20.0 (3/15) bcd	13.3
	'Valencia'	+	0	0	1	6.7 (1/15) cd	
IV	'Microcitrus'	-	0	1	0	6.7 (1/15) cd	3.3
	'Anliucheng'	-	0	0	0	0.0d	

Note: * Browning degree: +++, intense browning; ++, moderate browning; +, slight browning; -, no browning
** No. resistant calli of each experiment; N means no transformation experiment was performed
*** Transformation efficiency was calculated as the mean ratio of the resistant Petri plates to total plates of three transformation experiments; Unlike letters within the same row indicate significant differences are present by Duncan's multiple range test ($P = 0.05$)

GUS analysis and Southern blotting confirmed the integration of T-DNA into resistant calli

More than 91% of randomly selected resistant calli stained dark blue after being washed in the 70% ethanol, which indicated the presence of the *uidA* gene into the citrus genome (**Fig. 2B**). Southern blotting analysis revealed different integration patterns in 10 of 11 randomly selected transgenic calli, with one to four copies at different loci (**Fig. 3**). More than 90% (10/11) of the resistant calli contained the target *nptII* gene, except for R_3 that apparently escaped selection with kanamycin (**Fig. 3**). No hybridization signal was detected in non-transformed control calli (**Fig. 3**, lanes PK and CK).

Total phenolic content affected transformation

In this study, total phenolic contents of the calli were 106.6-567.5 μg/g, depending on different species/varieties. Total phenolic content in tangerines and its hybrids was much higher than that in sweet oranges and that in other species/varieties, which was in accordance with their transformation efficiencies (**Table 3**). From **Table 3**, we can also see that in different citrus categories, the total phenolic content was positively related to transformation efficiencies, while in the same category there was no obvious correlation between them. The results above indicated that total phenolic content of citrus calli was species/variety-dependent and correlated with citrus transformation.

DISCUSSION

In this study, we succeeded in obtaining transgenic materials from 12 of 13 citrus calli via *Agrobacterium*-mediated transformation. The transformation efficiencies among different species/varieties varied greatly, which indicated transformation was genotype-dependent. More than 91% of the resistant calli regenerated from brown calli, which is opposite to previous reports where browning was followed by cell death (Chakrabarty *et al.* 2002; Das *et al.* 2002; Franklin *et al.* 2004). This contradiction might be correlated with the explant morphogenic state, for embryogenic calli used

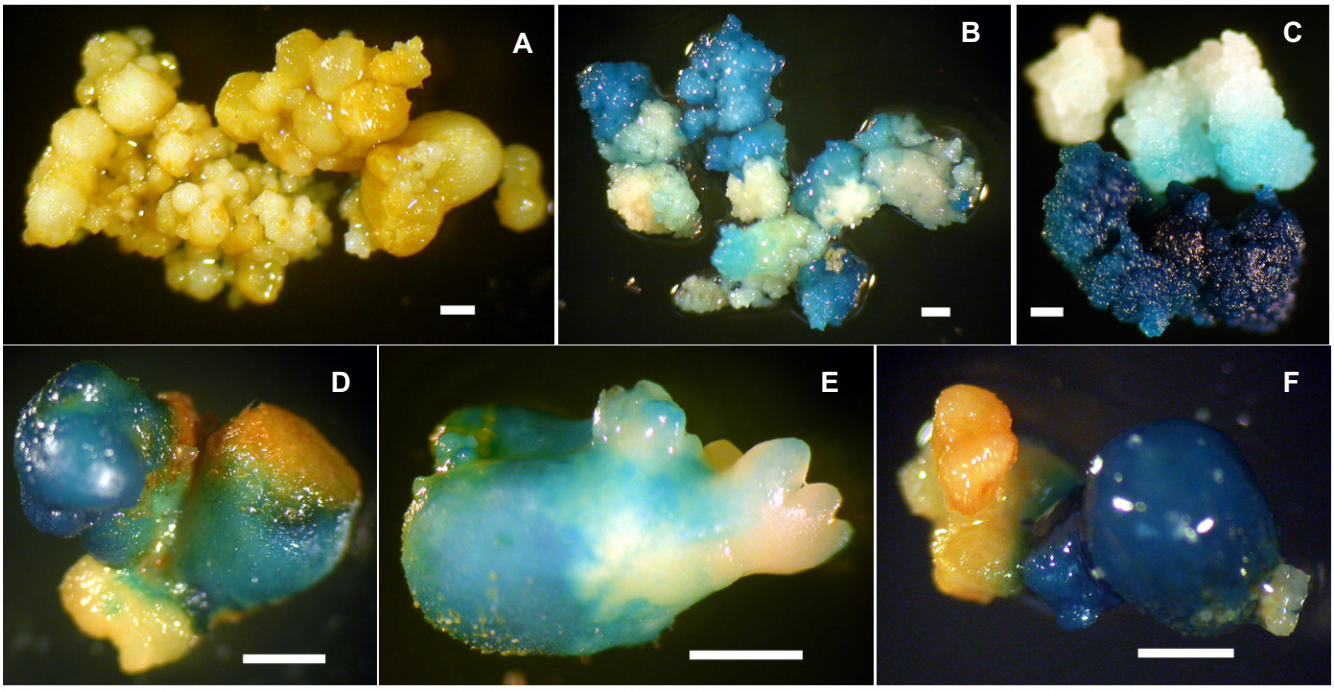

Fig. 2 GUS analysis of resistant citrus calli and embryos. (**A**) GUS-negative non-transformed calli; (**B, C**) GUS-positive calli; (**D-F**) GUS-positive embryos.

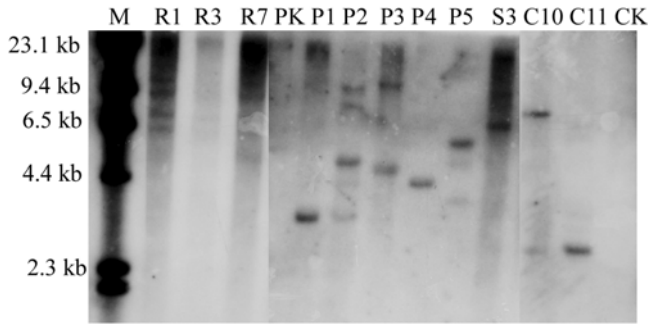

Fig. 3 Southern blot analysis of transformed and non-transformed calli. Genomic DNA was digested with *Hin*dIII and hybridized with a ^{32}P-labelled *npt*II probe. M Lambda DNA/*Hin*dIII marker. R1, R7 transformed callus lines of 'Red Marsh' grapefruit, R3 escapee of 'Red Marsh' grapefruit; PK control calli of 'Ponkan' tangerine, P1-P5 transformed callus lines of 'Ponkan' tangerine; S3 transformed callus line of 'Hongkong' kumquat; C10-C11 transformed callus lines of 'Chazhigan' tangerine, CK control calli of 'Chazhigan' tangerine.

cotyledon, and hypocotyl explants. Transformed tissues regenerated from browning calli after co-cultivation also showed that: 1) browning calli after co-cultivation was not equivalent to dead cells, and 2) transformation preceded callus browning, but time was required for transformed cells to regenerate. The calli of 'Anliucheng' and '*Microcitrus*' without browning after several subcultures on the selection medium indeed stopped growing and died.

Different degrees of callus browning were observed in *Agrobacterium* infected citrus calli from 13 species/varieties, showing that callus browning was genotype-dependent. It is known that oxidation of phenolic substrates by polyphenol oxidase (PPO) is responsible for tissue browning (Asmus and Hanne 1996; Strack and Schliemann 2001). Inhibition of polyphenol biosynthesis or activities of PPO could prevent browning (Hisaminato *et al.* 2001; Murata *et al.* 2001). Our findings herein show: 1) total phenolic content was highly genotype-dependent; 2) callus with high total phenolic content was susceptible to browning. The latter result is consistent with previous reports (Mayer 1987; Luo *et al.* 1999).

It was well known that AS (acetosyringone) is effective for induction of *vir* genes of *Agrobacterium* and it improves transformation efficiency (James *et al.* 1993; Cervera *et al.* 1998; Li *et al.* 2003; Kumar and Rajam 2005). Other phenolic substances, perhaps the phenolic compounds within citrus callus, might also play some roles in *Agrobacterium*-mediated transformation, but this needs further study.

Table 3 Total phenolic content and transformation efficiency of different citrus.

Citrus categories	Cultivars/varieties	Total phenolic content (ug/g)	Mean value (ug/g)	Mean transformation efficiency (%)
Tangerines	'Sunki' tangerine	545.2	322.2	42.2
	'Bendizao'	342.3		
	Gailiangcheng × Owari	282.3		
	'Chazhigan'	276.9		
	'Dancy'	260.1		
	'Ponkan'	226.5		
Sweet oranges	'Anliucheng'	332.5	197.5	11.7%
	'Succari' orange	186.4		
	'Valencia'	162.3		
	'Jincheng'	108.9		
Others	'Red Marsh' grapefruit	115.8	110.1	11.1%
	'Microcitrus'	107.8		
	'Hongkong' kumquat	106.6		

in this study had higher cell division potential than the leaf,

To sum up, successful transformations were performed in several citrus species/varieties and transgenic plants were regenerated from six citrus calli older than 7 years. In *Agrobacterium*-mediated citrus transformation, a close correlation between callus browning and transformation potential was observed. The genotype-dependent total phenolic content contributes to callus browning. This presents evidence that the influence of callus browning on citrus callus transformation is correlated with total phenolic content, and provides important information relevant to the use of recalcitrant species/varieties. However, the mechanism of citrus callus browning before or after agroinfection is still largely unknown. Further studies would be helpful to better understand cell intercommunication, intercellular transport, and the process of DNA repair and recombination during agroinfection.

ACKNOWLEDGEMENTS

This research was financially supported by the national NSF of China, the Ministry of Education of China (Nos. 705037, NCET-04-0734, IRT0548), the National 863 project (2007AA10Z182) and the Department of Science and Technology of Hubei Province. The authors thank Dr Jude Grosser (CREC, University of Florida) for kindly donating several citrus callus lines, and Dr Joseph Smilanick (USDA Agric. Res. Service, Parlier, California) for reviewing the manuscript.

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