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ORIGINAL ARTICLE

Kn1 gene overexpression drastically improves genetic transformation efficiencies of citrus cultivars

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Abstract The effects of a maize *knotted1* (*kn1*) gene under the control of the cauliflower mosaic virus 35S promoter on genetic transformation efficiencies of six citrus genotypes were tested. The *kn1* gene construct was used to transform 'Pineapple', 'Hamlin', 'Sucarri' and 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck], 'Carrizo' Citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliate* (L.) Raf.] and Eureka lemon [*Citrus. limon* (L.) Burm.f.] via *Agrobacterium tumefaciens*-mediated infection. Our results demonstrate that expression of the *kn1* gene enhances transformation efficiencies from 3 to 15 fold compared to a control vector, 3–11 fold relative to the highest transformation efficiencies reported for these citrus genotypes. Stable incorporations of T-DNA into the citrus genome have been confirmed with both histochemical staining of GUS activity and molecular analyses. The majority of *kn1* over-expressing citrus plants grow and develop normally at young seedling stages, similar to those of the wild type plants. With all six genotypes of citrus tested including Eureka lemon, a cultivar difficult to be transformed, our results demonstrate that the *kn1* gene may provide an effective molecular tool to enhance genetic transformation efficiencies of various citrus varieties. High transformation efficiency of citrus is of great importance for large scale characterization of gene functions and also cultivar development via transgenic and genome editing technologies.

Keywords *Agrobacterium tumefaciens* · Citrus · Transformation · Regeneration · *kn1*

Abbreviations

CaMV 35S	Cauliflower mosaic virus 35S
kn1	*Knotted1* gene
ipt	Isopentenyl transferase
BAP	Benzylaminopurine
NAA	Naphthaleneacetic acid
IBA	Indole-3-butyric acid
nptII	Neomycin phosphotransferase
MS medium	Musashige and Skoog medium
GUS	β-Glucuronidase
uidA	β-Glucuronidase gene
OD	Optical density
SAUR	Small auxin-up RNA
AS	Acetosyringone
MES	2-(*N*-morpholino) ethanesulfonic acid
X-gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

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CCM Co-cultivation medium
SRM Shoot regeneration medium

Introduction

Citrus is commercially grown over a wide range of climatic conditions and soil types where crops are exposed to a variety of abiotic and biotic stresses that often negatively affect fruit quality and productivity. Important abiotic stresses include excess soil acidity or alkalinity, drought, and temperature extremes, while insects and diseases represent major biotic stresses (Pena et al. 2007). Some diseases, such as Hunglongbing (HLB) and citrus canker, have spread to citrus production areas throughout the world. For instance HLB has caused huge losses to the citrus industries in the US, China and many other countries (Bové 2006; Wang and Trivedi 2013). While no effective HLB control methods or HLB resistance citrus cultivars have been commercialized, transgenic plant technologies have been recognized as a powerful tool to control the HLB disease.

An efficient genetic transformation protocol is a key step for using transgenic technologies to improve citrus quality, productivity, and resistance to abiotic and biotic stresses such as HLB. Several citrus transformation protocols have been previously published. As Donmez et al. (2013) pointed out, however, that "transformation efficiency (for citrus) is generally low and protocols are only effective on certain species or even cultivars". One major limitation associated with existing protocols when used on economically important citrus species is likely low plant regeneration frequencies. Another challenge to using transgenic plant technology in citrus is the long juvenile phase of immature tissues that ranges from 5 to 20 years, which greatly delays analysis of the effects of candidate genes. On the other hand, transgenic plants produced from mature tissue via genetic transformation can drastically shorten flowering and fruiting time and thus speed up field evaluation studies. Cervera et al. (1998) have shown that greenhouse-grown transgenic plants derived from mature tissues can fruit in 14 months. However, efforts have been made to genetically transform mature citrus tissues but the efficiencies are generally extremely low (Marutani-Hert et al. 2012).

The maize homeobox gene *knotted1* (*kn1*) encodes an AP2/ERF transcription factor (Hake et al. 2004; Moon and Hake 2011). It has also been shown that the *kn1* mRNA can be transported bidirectionally via phloem cells in plants (Duan et al. 2014). KN1 may regulate many processes including hormone concentrations and growth and development of floral, shoot meristem and leaf organs (Bolduc et al. 2012; Hake et al. 2004; Hay and Tsiantis 2010; Sano et al. 2005). *Ipt*, a cytokinin biosynthetic gene cloned from *Agrobacterium*, has also been shown to stimulate shoot regeneration (Li et al. 1992; Smigocki and Owens 1988, 1989; Strabala et al. 1989, 1996). Further studies have shown the *ipt* gene may be used as a positive selectable marker for transformation in citrus, tobacco, rice, sweet pepper, aspen and apricot (Ballester et al. 2007, 2008; Ebinuma and Komamine 2001; Ebinuma et al. 1997; Endo et al. 2001, 2002; Kunkel et al. 1999; López-Noguera et al. 2009; Mihálka et al. 2003; Peng et al. 2015). Similar to the *ipt* gene, we have previously reported that the *kn1* gene can be used as a positive selection marker for transgenic tobacco plants (Luo et al. 2006), The utility of the *kn1* gene in genetic transformation of higher plants has also been shown in *Phalaenopsis amabilis* and *Jatropha curcas* (Pei et al. 2010; Semiarti et al. 2007).

Previously, using a sonication-assisted *Agrobacterium*-mediated transformation method combined with vacuum infiltration, Oliveira et al. (2009) reported an 8.4 % transformation efficiencies for juvenile tissues of 'Pineapple' orange. Dutt and Grosser (2009) described an improved protocol for genetic transformation of juvenile explants of 'Carrizo' citrange and 'Hamlin' orange. The authors optimized several parameters including pre-incubation treatment, OD values of *Agrobacterium* cells and *Agrobacterium*-explant co-cultivation durations. They reported optimization conditions for both cultivars and achieved the maximum transgenic plant production, 47 % transformation efficiency for 'Carrizo' and 25 % for 'Hamlin'. However, this type of methods is likely species or cultivar dependent.

The aim of this study was to determine the effects of the *kn1* gene on genetic transformation efficiencies of juvenile citrus explant tissues using six citrus cultivars, 'Carrizo' citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.], 'Pineapple', 'Hamlin', 'Succari', 'Valencia' sweet orange [*Citrus sinensis* (L). Osbeck] and 'Eureka' lemon [*Citrus. limon* (L.) Burm.f.]. Here, we report that transformation efficiencies of the six citrus cultivars can be enhanced drastically from 3 to 15 fold if the *kn1* gene is used. Also, no significant changes in morphology or growth habits have been observed in the *kn1* transgenic citrus plants at seedling stages, which is different from *kn1* overexpressing transgenic plants previously described in the literature. With all citrus cultivars we have tested including a lemon cultivar, we conclude that the *kn1* gene can be useful to enhance genetic transformation efficiencies of various citrus varieties.

Materials and methods

Plant Materials

'Carrizo' Citrange [*Citrus. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] seeds were purchased from TreeSource CitrusNursery (504 N Kaweah Ave, Exeter, CA 93221 U.S.), seeds for 'Pineapple', 'Succari', and 'Hamlin' sweet orange [*Citrus sinensis* (L.) Osbeck] were provided by the University of Florida and Valencia orange [*Citrus sinensis* (L.) Osbeck] and Eureka lemon [*Citrus. limon* (L.) Burm.f.] seeds were purchased from Pearson Ranch California Oranges (1018 W. Teapot Dome Ave. Porterville, CA 93257). External seed coats were removed and seeds were treated with 75 % alcohol for 40–60 s and 1 % sodium hypochlorite for 20 min, and rinsed four times with sterile distilled water. Internal seed coats were then removed under sterile conditions and seeds were cultured in vitro on MS media (Murashige and Skoog 1962) with 30 g/l sucrose, 7 g/l of agar and pH 5.7. Thirty-day-old seedlings were used as a source of explant tissue for transformation. The internodal stem segments about 1 cm in length were harvested from these seedlings and used for *Agrobacterium* infection as reported previously (Dutt and Grosser 2009).

Vector and *Agrobacterium* for transformation

Agrobacterium tumefaciens EHA 105 carrying the binary vector pBin19 with the 35S Promoter::*kn1* (Luo et al. 2006) or the SAUR Promoter::*ipt* (Li et al. 1991, 1992) and 35S-*nptII::uidA* gene plus the vector with only the *35S-nptII::uidA* gene as control vector (Zheng et al. 2007). In these vectors, the *nptII* gene (the kanamycin resistance gene) served as a marker gene for the selection of transgenic plants during transformation. The *uidA* (β-glucuronidase gene) was used as a report gene for the screening of positive transgenic plants.

Agrobacterium tumefaciens EHA 105 strains hosting various pBin 19 vectors were cultured for 40–42 h at 28 °C on LB solid medium containing 100 mg/l kanamycin and 50 mg/l rifampicin. Single colonies were transferred to 2 ml LB liquid medium with 100 mg/l kanamycin and cultivated under 180 rpm, 28 °C for 24 h. After that, 1–2 ml(s) cultivated bacteria was added to 40 ml LB liquid medium with 100 mg/l kanamycin and cultivated to an OD of around 0.6 and then centrifuged at 5000 rpm at room temperatures for 15 min. Prior to plant infection, the bacterial culture was re-suspended in liquid co-cultivation medium (CCM) consisting of MS salts, 0.1 mg/l thiamine hydrochloride, 0.5 mg/l pyridoxine hydrocholoride, 0.5 mg/l nicotinic acid, 2 mg/l glycine, 100 mg/l inositol, 20 mg/l acetosyringone (AS), 30 g/l of sucrose, and 3 mg/l benzylaminopurine (BAP) for 'Carrizo', 'Succari', 'Eureka', and 'Valencia', 3 mg/l BAP, 0.1 mg/l 1-naphthlcetic acid (NAA) and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for 'Hamlin' and 'Pineapple'.

Infection and shoot regeneration

Internodal stem segments (1 cm in length) from 30 day old citrus seedlings were incubated in the *Agrobacterium* cell suspension basically as described by Yang et al. (2011). After being blotted dry on sterilized filter paper, explants were placed horizontally in Petri dishes containing solid CCM and incubated in the dark. Three days after, the internodal stem segments were blotted dry on sterile filter paper and transferred onto shoot regeneration medium (SRM) containing MS salts, 0.1 mg/l thiamine hydrochloride, 0.5 mg/l pyridoxine hydrochloride, 0.5 mg/l nicotinic acid, 2 mg/l glycine, 100 mg/l inositol, 30 g/l of sucrose, 7 g/l of agar. For 'Carrizo', 3 mg/l BAP was added, but 1 mg/l BAP was used for the rest of other four sweet orange cultivars. In the case of 'Eureka' lemon, we used 0.5 mg/l BAP. All SRM were supplemented with 75 mg/l of kanamycin and 150 mg/l of timentin. The explant tissues were transferred onto fresh SRM plates every 3 weeks, and cultured under full spectrum light (45 $\mu Em^{-2}\ s^{-1}$) for 16 h per day at 26 ± 2 °C. GUS positive shoots regenerated from the kanamycin containing medium were grafted on 14 days old 'Carrizo' citrange rootstocks. One to two months later, the established shoots were re-grafted on potted sour orange under greenhouse conditions. We also rooted GUS positive 'Carrizo' shoots in root-inducing medium, which contained half-strength MS salts, 0.1 mg/l thiamine hydrochloride, 0.5 mg/l pyridoxine hydrochloride, 0.5 mg/l nicotinic acid, 2 mg/l glycine, 100 mg/l inositol, 0.5 mg/l NAA, 0.1 mg/l IBA and 0.5 g/l activated charcoal.

GUS histochemical assays and data analysis

Histochemical assays of GUS activity were carried out in a solution consisting of 100 mM potassium phosphate buffer, pH 7.0, 10 mM Na_2EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1 % triton X-100, 1 g/l X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) at 37 °C for 16 h. After 45 days cultured on the shoots induction medium, small leaf slices or entire buds of kanamycin resistant shoots that were longer than 3 mm were used for histochemical GUS staining. The plant tissues were distained in ethanol to remove chlorophylls and other pigments prior to being visually inspected and photographed. Transformation efficiency is defined as the average number of GUS

positive and PCR verified shoots per explant inoculated × 100 %.

All data were analyzed with the SPSS statistical software and Duncan's multiple range test at $P \leq 0.05$.

Molecular confirmation of transgenic plants

Genomic DNA was extracted from leaves of GUS positive and wild type citrus plants. To avoid contaminations of Ti-plasmid DNA from *Agrobacterium* remained in citrus plant tissues, the genomic DNA isolated was fractioned on 0.8 % (w/v) agarose gel with the pBin19-35S::*kn1* Ti-plasmid DNA loaded on the side as a reference. Large-sized-genomic DNAs (about 20–25 kb that is much larger than the Ti-plasmid DNA) were recovered from the agarose gels and used as templates of PCR reactions (Chen et al. 2006). These DNA templates were analyzed by PCR for the *uidA* gene within the T-DNA region and the tetracycline resistance (*tetR*) gene that is outside the T-DNA region of the Ti-plasmid. PCR reaction solution was 20 µl containing 1× PCR buffer (Takara, Japan), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µl e2TAK DNA polymerase (Takara, Japan), 0.25 µM of each primer and 500 ng DNA. The amplification condition started with an initial denaturation step at 98 °C for 5 min, followed by 35 cycles of 98 °C for 10 s, special annealing temperature for 5 s, and 72 °C extension plus a final extension at 72 °C for 10 min. The primers used to amplify the *uidA* gene are gus-F and gus-R, The predicted size of the *uidA* DNA segment amplified is 823 bp. the primers used to amplify the tetracycline gene *tetR* gene are Tet-F and Tet-R, the predicted size of the amplified *tetR* DNA segment is 552 bp. PCR products were separated by electrophoresis on 1 % (w/v) agarose gels. The PCR primers used: gus-F: 5′-ATACCGAAAGGTTG GGC AGG; gus-R 5′-TCACCACGATGCCATGTTCA; tet-F: 5′-GACGAC TGGCGCTCATTTCT; tet-R: 5′-GCA TGAAAAAGCCCGTAGCG.

Expression analysis of *kn1* genes in transgenic plant leaves

Total RNA was isolated from leaves of transgenic and wild type plants using RNeasy Plant Mini Kit (QIAGEN) according to the manual. cDNA synthesis was done with iScript™ cDNA Synthesis Kit (Bio-Rad).

Real time quantitative PCR was performed to analyze the expression levels of the transgenes using SsoFast™ qPCR Supermixes with EvaGreen (Bio-Rad). The reaction system included 20 µl of 1× Supermixes, 0.5 µM each primer for *kn1*, 200 ng cDNA. The qPCR primers used are: qKN1-F: 5′-GAAGCACCATCTCCTGAA; qKN1-R: 5′-CCACCTTCTGAG TCTCTG. Reactions were performed in 40 cycles of 30 s at 95 °C, 5 s at 95 °C, 20 s at 59 °C and 31 s at 72 °C. We used expression of the *ActB* gene as a reference gene for the qPCR assays because Yan et al. (2012) have recommended it as a suitable reference gene to normalize mRNA levels in qPCR analysis of diverse cultivars and tissues based on extensive studies. The qPCR premiers for the *ActB* gene: ACTB-F: 5′-TCTCTTGAAC CTGTCCTTGGA; ACTB-R: 5′-AGTGCC GATACGCT GTCTA. Each sample had three replicates. Relative expression levels were shown as the ratio of the transgenic *kn1* gene to the endogenous *ActB* gene (Yan et al. 2012).

Results and discussion

Kn1 overexpression drastically increases transformation efficiencies of six citrus cultivars tested

KN1 is a transcription factor protein involved in the establishment and maintenance of plant meristems (Bolduc and Hake 2009). The *kn1* gene has been previously used to improve shoot regeneration and transformation in tobacco (Luo et al. 2006; Tamaoki et al. 1997), jatropha (Pei et al. 2010), orchid (Semiarti et al. 2007) and barley (Williams-Carrier et al. 1997). To test the effect of the *kn1* overexpression on genetic transformation of citrus plants, we used *A. tumefaciens* stains hosting a control vector (*uidA* and kanamycin resistance genes), the *kn1* gene plus the *uidA* and kanamycin resistant genes (*nptII*) to transform six cultivars of citrus, 'Carrizo' citrange, 'Pineapple', 'Hamlin', 'Succari', 'Valencia' sweet orange and 'Eureka' lemon. We also used an *ipt* gene plus the *uidA* and *nptII* genes to transform 'Carrizo' citrange, 'Pineapple', 'Hamlin' and 'Succari' orange. We did three to five transformation experiments using hundreds of epicotyl explants for each construct and each citrus cultivar. As shown in Fig. 1a, b, the use of the *kn1* or *ipt* genes drastically increased transformation/shoot regeneration efficiencies from epicotyl segments of the 30 days old seedlings of these citrus cultivars.

Table 1 summarizes the effects of the *kn1* and *ipt* genes and control vector on genetic transformation efficiencies of the six citrus cultivars tested. Transformation efficiencies were calculated by dividing the number of independent GUS positive, PCR confirmed shoots with the total number of explants inoculated and then multiplied by 100 %. Compared to the control vector with a *uidA* gene, we found that the use of the *ipt* gene increased transformation efficiency by 4.8 fold for 'Pineapple' orange, 5.0 fold for 'Hamlin' orange, 4.3 fold for 'Succari' orange and 8.6 fold for 'Carrizo' citrange. Using the *kn1* gene, we observed increases in transformation efficiency for 4.9 fold for 'Pineapple' orange, 5.1 fold for 'Hamlin' orange, 6.4 fold

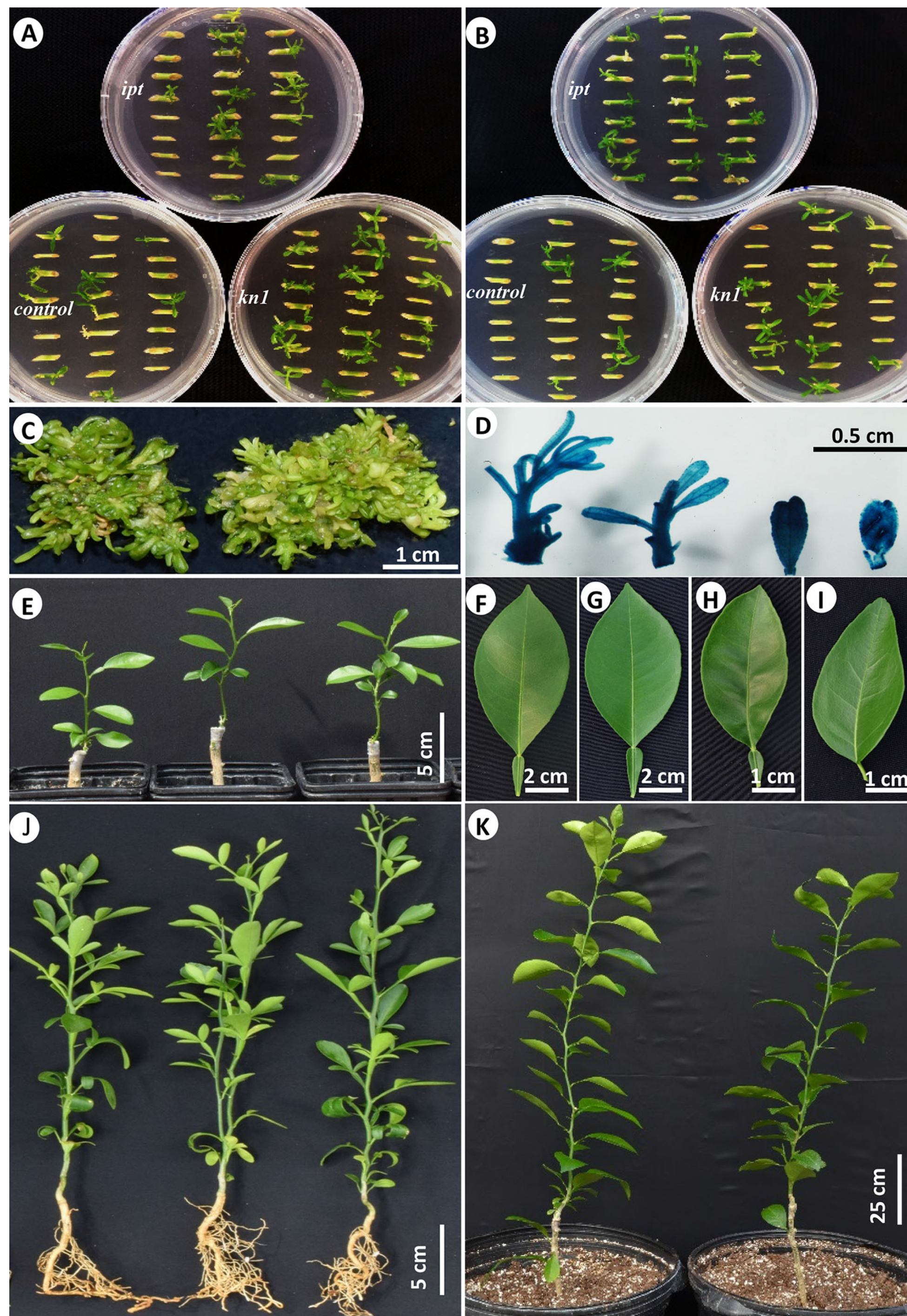

Fig. 1 Effects of *kn1* and *ipt* overexpression on efficiencies of citrus transformation. **a**, **b** Shoots regenerated from the explants infected with *Agrobacterium* containing pBin19-*35S::kn1*, pBin19-*SAUR::ipt* and control vector (**a** 'Pineapple', **b** 'Hamlin'), showing that *kn1* and *ipt* gene enhance citrus transformation efficiencies. **c** Transgenic *SAUR*::*ipt* 'Pineapple' shoots with increase in bud/shoot regeneration efficiency. **d** Histochemical staining of GUS activity in *kn1* transgenic shoots and leaves. **e** Grafted Pineapple wild-type (*the one on the left*) and transgenic 35S::*kn1* 'Pineapple' plants (*the two plants on the right*), these plants were grafted at the same time (*1 month old*). **f** A leaf from wild-type Pineapple citrus. **g** A leaf from 35S::*kn1* Pineapple H10, with no differences observed when compared to wild-type 'Pineapple' leaves. **h** A leaf from *35S::kn1* Pineapple H7 plant, with non-smooth surfaces when compared to the leaves from wild type Pineapple citrus plants. **i** A leaf from *kn1* transgenic Pineapple H2 with no petiole wings. **j** Three month old wild type 'Carrizo' citrus plant (*left*) and *kn1* transgenic 'Carrizo' citrus plants (*middle and right*), showing the *kn1* transgenic 'Carrizo' citrus plants root and grow normally. **k** Eight month old 'Succari' wild type (*left*) and *kn1* transgenic plants (*right*) that were grafted on sour orange rootstocks, showing no significant difference between wild-type and *kn1* transgenic plants

Table 1 Effects of the *kn1* and *ipt* gene on transformation efficiencies of six citrus cultivars

Citrus cultivar	# Explants used[a]	Gene[b]	Transformation efficiency (%)[c]	*kn1*- or *ipt*-mediated increase in transformation efficiency (×, fold)	Previously reported transformation efficiency[d]
Pineapple sweet orange	395	*gus*	19.2b		8.4 % (Oliveira et al. 2009)
	583	*kn1*	95.1a	4.9×	
	502	*ipt*	92.1a	4.8×	
Hamlin sweet orange	345	*gus*	13.5b		25 % (Dutt and Grosser 2009)
	621	*kn1*	69.0a	5.1×	4.7 % (Orbovic et al. 2011)
	307	*ipt*	67.1a	5.0×	3.0 % (Boscariol et al. 2003)
Succari orange	117	*gus*	8.4c		–
	149	*kn1*	54.0a	6.4×	
	168	*ipt*	36.2a	4.3×	
Carrizo citrange	109	*gus*	10.3c		47 % (Dutt and Grosser 2009)
	102	*kn1*	155a	15.0×	41.3 % (Cervera et al. 1998)
	102	*ipt*	88.5b	8.6×	
Valencia orange	68	*gus*	14.3a		23.8 % (Boscariol et al. 2003)
					2 % (Dutt et al. 2009)
	72	*kn1*	60.5b	4.2×	23.3 % (Khan et al. 2012)
Eureka lemon	74	*gus*	8.3a		1.0 % for Bearss Lemon (Dutt et al. 2009)
					1 % for Volkamer Lemon (Dutt et al. 2009)
	76	*kn1*	27.8b	3.3×	3 % for Fino Lemon (Ghorbel et al. 2001)

[a] The total number of explants used from 3 to 5 transformation experiments

[b] pBin19 was the host vector for the genes used

[c] Transformation efficiency (%) was calculated based on the number of transgenic shoots recovered and the number of explants used (# transgenic plants per explant × 100 %). The efficiencies (%) obtained from each of the 3–5 transformation experiments were analyzed with the SPSS statistical software and Duncan's multiple range test at $P \leq 0.05$

[d] The list may be incomplete

for 'Succari' orange, 15.0 fold for 'Carrizo' citrange, 4.2 fold for 'Valencia' orange and 3.3 fold for 'Eureka' lemon. Statistical analyses confirm that the differences in transformation efficiency between the *kn1* or *ipt* vector and the control vector are significant.

In Table 1, we also listed the highest genetic transformation efficiency reported previously in the literature for a comparison. For 'Pineapple' sweet orange, the transformation efficiency for the control vector (pBin19 *35S::nptII*::*uidA* gene) was 19.2 % in our hand and the highest transformation efficiency reported in the literature was 8.4 % (Oliveira et al. 2009). When the *kn1* gene was used, the efficiency was 95 %. In the case of 'Hamlin' sweet orange and 'Carrizo' citrange, we obtained 13.5 and 10.3 % transformation efficiencies, respectively, when the control vector was used. The highest transformation efficiencies reported for these two cultivars were 25 and 47 % (Dutt and Grosser 2009). Using the *kn1* gene, we observed 69 and 155 % transformation efficiencies for these two cultivars, respectively. For 'Valencia' orange, a commercially important cultivar, we observed 14 % transformation efficiency using the control vector and the previously reported transformation efficiencies were 2 % (Dutt et al. 2009) to 24 % (Boscariol et al. 2003; Khan et al. 2012). With the *kn1* gene, we observed 61 % transformation efficiency.

The differences in the transformation efficiencies between ours without the use of the *kn1* or *ipt* genes and those reported by others previously (see Table 1) could be due to differences in vigor for explants and *Agrobacterium* cells or other variations. However, compared with the highest transformation efficiencies reported in the literature and also the control vectors under identical conditions in our own study, the use of the *kn1* gene always drastically enhanced the transformation efficiency of the citrus cultivars tested (Table 1).

Although reasonably high transformation efficiencies had already been achieved previously for some citrus cultivars (see Table 1), genetic transformation for many other citrus species or cultivars including commercially used

Satsuma Mandarin (*Citrus unshiu* Marc.), lemon [*Citrus limon* (L.) Burm. f.], Ponkan (*Citrus reticulata* Blanco) has been difficult (Dutt and Grosser 2010; Dutt et al. 2009; Khawale et al. 2006). For instance, transformation efficiencies of lemon cultivars are normally 1 % to 3 % (Dutt et al. 2009; Ghorbel et al. 2001). With the use of the *kn1* gene, we have showed that the transformation efficiency for Eureka Lemon can be as high as 28 % (Table 1). More recently, Xiao et al. (2014) reported an efficient *Agrobacterium rhizogenes*-mediated transformation method for *Poncirus trifoliate*, with a transformation efficiency of 34 %. Their procedure requires 25 weeks to produce transgenic shoots while our method needs 12 weeks.

Taken all together, our results demonstrate that *kn1* gene is very effective in enhancing genetic transformation efficiencies of all six citrus cultivars tested, with increases in transformation efficiency from 3 to 15 fold from the control vector without the use of the transgenic kn1 gene, or 3–11 fold increase when compared to the highest transformation efficiencies previously published. Further, these six citrus cultivars tested also include a lemon cultivar. Lemon has been generally regarded difficult to be transformed with *Agrobacterium* (Dutt et al. 2009; Ghorbel et al. 2001).

Verification of the stable incorporation of the transgenes into citrus genomes

Because all Ti-plasmid vectors we used in this study contain the *35S::nptII::uidA* fusion gene, in addition to the kanamycin resistance, we used histochemical staining of the GUS activity to confirm stable incorporations of the transgenes into the citrus genome in independently produced shoots of citrus (Fig. 1d). We also used a PCR technique to further verify the stable integration of transgenes into the citrus genome of kanamycin resistant, GUS positive shoots. Since the genomic DNA from T0 generation transgenic citrus plant tissues were used for PCR reactions, it is possible to observe false-positive PCR results because of the Ti-plasmid DNA from residual *Agrobacterium* cells in the plant tissues. Using the method we described previously (Chen et al. 2006), we eliminated possible contaminations of Ti-plasmid DNA from residual *Agrobacterium* cells by fractionating the citrus genomic DNA isolated from T0 transgenic plant tissues on agarose gels with pBin19-*35S::kn1* plasmid DNA as a reference. As shown in Lane 2–9 of Fig. 2a, we were able to separate high molecular weight citrus genomic DNA from the Ti-plasmid DNA on agarose gels. We then recovered high molecular weight citrus genomic DNA with no plasmid T-DNA from the agarose gel and used them as PCR templates. We isolated and purified genomic DNA from randomly selected 12 GUS-positive lines of citrus shoots derived from epicotyl segments infected with *Agrobacterium* strain containing the *kn1* gene. We demonstrated that all 12 GUS-positive lines were PCR positive for the *kn1* gene. Four representative PCR products from these 12 lines were shown in Fig. 2b, demonstrating the presence of the *uidA* gene (Lanes 4, 6, 8 and 10).

To further verify that the presence of transgenes in these GUS positive citrus shoot lines (for examples see Fig. 1d) were not from Ti-plasmid DNA in residual *Agrobacterium* cells in T0 plant tissues, we performed an additional PCR analysis using primers for the tetracycline resistance (*tetR*) gene located in the backbone region of the Ti-plasmid DNA. Our results show that no *tetR* was detected from the same genomic DNA templates for which the *uidA* gene was identified (Fig. 2b shows four representative PCR reactions). These results demonstrate that our technique (Chen et al. 2006) is effective to eliminate the Ti-plasmid DNA from the template DNAs isolated GUS positive citrus tissues, and therefore the *uidA* positive signals observed in the representative plant lines were due to stable incorporations of the transgenes in the citrus genome. Further, some transgenic plants were further confirmed with qPCR for the expression of the *kn1* gene.

Expression of the *Kn1* genes and their effects on citrus plant growth and development

When using the *ipt* gene for citrus transformation, we observed that many of the *ipt* transgenic citrus shoots displayed a bushy phenotype with small shoots or buds (Fig. 1c) and poor root systems (data not shown). We therefore did not characterize the *ipt* citrus plants further. On the other hand, the *kn1* transgenic citrus shoots were normal in growth and development patterns at young seedling stages when compared to the wild type plants. We randomly selected 47 *kn1* transgenic shoots for further analysis. We grafted 42 *kn1* shoots onto rootstocks of sour orange (*Citrus aurantium* L.). Figure 1e shows a wild type shoot grafted onto a sour orange rootstock (left), two *kn1* 'Pineapple' sweet orange shoot grafted onto a rootstock (middle and right). All of these grafted shoots grew normally except two plant lines showing minor alterations in leaf morphology. *Kn1*-Pineapple H7 produced non-smooth leaf surfaces (Fig. 1h) and *Kn1*-Pineapple H2 failed to develop petiole wings (Fig. 1i). Other than these two minor changes, we did not notice any other differences in the *kn1*-Pineapple H2 and *kn1*-Pineapple H7 trees when compared to the rest of the *kn1* transgenic lines or wild type plants. We also rooted five representative *kn1* transgenic shoots of 'Carrizo' citrange and they produced normal roots, indistinguishable from the wild type plants when rooted under the same conditions (Fig. 1j).

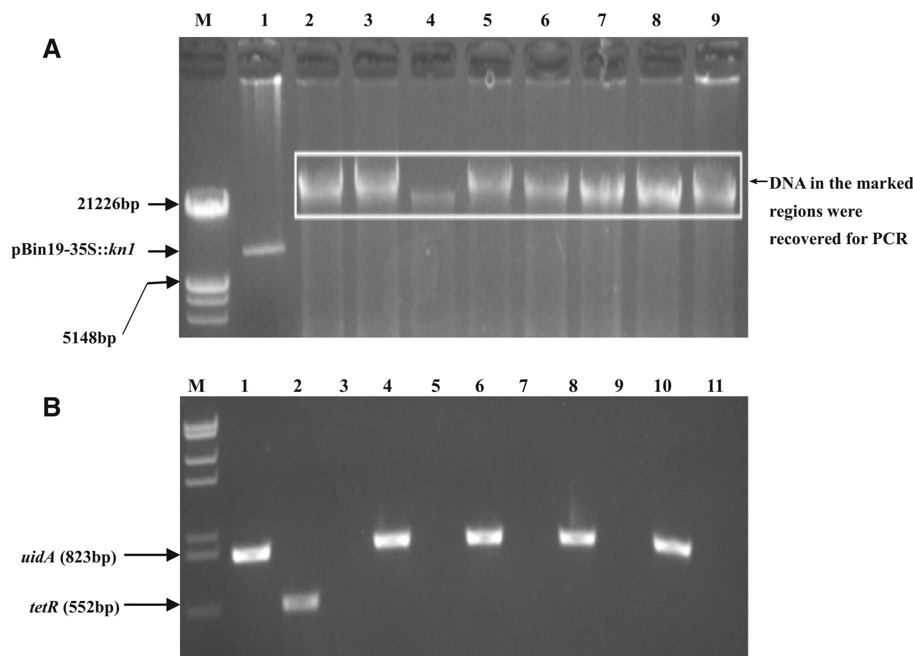

Fig. 2 PCR Confirmation of stable incorporation of transgenes into the citrus genome. **a** Genome DNA isolated from GUS positive citrus tissues were fractioned on an agarose gel. DNA in the marked regions were recovered for PCR reactions. *Lane M* Molecular weight mark of lambda DNA digested with *Eco*RI and *Hind*III, *Lane 1* pBin19-*35S::kn1* plasmid DNA, *Lanes 2–9* Undigested genomic DNA extracted from transgenic plants. **b** PCR reactions were performed as described in "Materials and methods" with primer sequences for the *uidA* gene (within the T-DNA region and should be incorporated into the plant genome) and for the *tetR* gene (outside the T-DNA region, should not be incorporated into the plant genome). *Lane* M: Molecular weight marker. The *lane bands 1, 3, 4, 6, 8 and 10* are for the *uidA* gene, *lane bands 2, 5, 7, 9 and 11* are for the *tetR* gene, *Lanes 1 and 2* Positive control (pBin19-35S::*kn1* plasmid DNA as template) for the *uidA* and *tetR* gene, respectively. *Lane 3*: Negative control (Wild-type Pineapple citrus plant DNA as template) for the *uidA* gene, *Lane 4–10*: GUS positive 'Pineapple' citrus *line 2, 3, 7 and 10* with the presence of the GUS gene and the absence of the *tetR* gene, demonstrating that these plant lines are transgenic

Along with the 42 grafted lines of *kn1* transgenic shoots on sour orange rootstocks, we also grafted the wild type shoots as controls. We did not notice any difference in grafting behavior between the *kn1* transgenic and wild type shoots. Both rooted and grafted *kn1* transgenic plants grew and developed normally when compared to the wild types plants. Except for the non-smooth leaf surfaces observed in *kn1*-Pineapple H7 and missing petiole wings on the *kn1*-Pineapple H2 plants, the 47 *kn1* transgenic citrus plant lines developed and grew normally (Fig. 1j, k). We have not observed any other drastically altered growth and development patterns in all 47 transgenic *kn1* citrus trees that are now 9–12 month old.

We also determined expression levels of the *kn1* gene in 6 representative transgenic citrus plant liness. Table 2 shows that the expression level of the *kn1* gene in *kn1* Pineapple H7 transgenic trees was about 17.5 % of that of the internal reference gene, *ActB* (an *actin* gene in citrus). Yan et al. (2012) examined seven candidate reference genes (*18SrRNA*, *ActB, rpII, UBQI, UBQ10, GAPDH* and *TUB*) and concluded that *ActB* is one of the three best reference genes for normalizing mRNA levels in qPCR analysis of diverse cultivars and tissues of citrus plants.

Table 2 Relative expression levels of the *kn1* gene in representative transgenic citrus lines

Lines	GUS activity[a]	*Relative expression of kn1 gene*[b]
WT	–	Not detectable
Pineapple H2	+++	2.7 %
Pineapple H3	+++	Less than 0.1 %
Pineapple H7	+++	17.5 %
Pineapple H10	+	0.1 %
Carrizo C1	+++	Less than 0.1 %
Carrizo C5	+	Less than 0.1 %

[a] GUS activity is defined based on histochemical staining: "–": no visible blue color; "+": weak but visible blue color; "++": medium strong blue color; "+++": dark (strong) blue color

[b] Relative quantification of the *kn1* gene expression in each sample was based on the expression level of the *kn1* gene versus that of the *ActB* gene. For instance, the *kn1* expression level in transgenic Pineapple Line H2 was 2.7 % of the *ActB* gene

Our qPCR results show that the expression level of the *kn1* gene in Pineapple H2 line was 2.7 % of that of the *ActB* gene. The rest of the four transgenic plant lines had 0.1 % or less than 0.1 % of the *ActB* expression levels (Table 2).

Kn1 Pineapple H7 transgenic tree produced non-smooth surface leaves while Pineapple H2 transgenic tree is petiole wing-less (Fig. 1f–i). The rest of the transgenic tree lines were morphologically indistinguishable from wild type plants. The fact that most *kn1* transgenic plant lines had no changes in growth and developmental patterns also support that *kn1* mediated enhancement of citrus transformation efficiency may not require high expression levels of the *kn1* gene.

It has been well documented that overexpression of the *kn1* gene causes drastic morphological alterations in transgenic plants including loss of apical dominance and increases in adventitious shoot formation, reduction in shoot elongation, and abnormal leaf and floral morphologies, with many of these phenotypes being similar to those observed in *ipt* over-expressing plants (Sinha et al. 1993; Chuck et al. 1996; Tamaoki et al. 1997). When used as a positive selection marker or for enhancing transformation efficiencies in tobacco (Luo et al. 2006; Tamaoki et al. 1997), jatropha (Pei et al. 2010), orchid (Semiarti et al. 2007) or barley (Williams-Carrier et al. 1997), the transgenic *kn1* gene overexpression also caused bushy phenotype, reduced shoot growth, or altered floral and leaf morphology. It is therefore somewhat surprising that overexpression of the *kn1* gene can drastically enhance the transformation efficiencies of all six citrus cultivars tested but does not cause significant alterations in growth and developmental patterns at the early stage of citrus plant growth and development.

The *35S CaMV* gene promoter is a strong and constitutive promoter when used in higher plants (Zheng et al. 2007). However, if the 35S promoter is used to drive an *ipt* gene (a cytokinin biosynthetic gene) expression in tobacco or other plant species, transgenic plants produced have low levels of the *ipt* expression (Smigocki and Owens 1988; Li unpublished data). This is because transgenic cells with high *ipt* expression produced high levels of cytokinins which inhibit shoot elongation. In this study, similar to the effects of the *35S::ipt* gene, high expression levels of the *35S::kn1* gene in citrus cells may also prevent shoot elongation, which may explain why most transgenic citrus plants we produced have relatively low *kn1* expression levels. On the other hand, small increases in transgenic KN1 protein in citrus cells can enhance shoot regeneration upon *Agrobacterium*-mediated transformation but low amounts of the KN1 protein in roots, stems and leaves may not be sufficient to cause morphological changes in vegetative organs of young citrus seedlings.

Although we have not observed drastic changes in growth and development patterns of the 1 year old *kn1* transgenic citrus seedlings, we did observe some changes in leaf morphology because of the expression of the *kn1* transgene, which is consistent with phenotypes observed in some *kn1* mutant or transgenic plants (Moon and Hake 2011). Further, it is not known whether these *kn1* citrus plants will display any alterations in flower and fruit growth and development at the reproductive stage.

To use the *kn1* gene to improve genetic transformation efficiencies of citrus cultivars of interest, one needs to insert the *kn1* gene into the Ti-plasmid vector containing a target gene(s) for citrus transformation. Once transgenic buds/shoots are obtained, the *35S::kn1* gene can be deleted using the gene deletor technology (Luo et al. 2007), or inactivated using CRISPR technologies (Sander and Joung 2014; Xiong et al. 2015). On the other hand, in many cases, removal or inactivation of the *kn1* gene may not be necessary since we have not observed detrimental alterations in plant growth and development in 1 year old *kn1* transgenic seedlings.

In summary, we have demonstrated that the use of the *35S::kn1* gene drastically enhances genetic transformation of juvenile tissues of six citrus cultivars tested, including a lemon cultivar, ranging from 3 to 11 fold increases in efficiency. We have also observed that the *kn1* overexpression in citrus does not lead to significant alterations in leaf morphology, shoot elongation, root development and other developmental and growth processes at early stages of seedling growth. These results demonstrate that *kn1* gene may be an effective tool to enhance genetic transformation of citrus cultivars or species that are difficult to transform as we have demonstrated in the case of lemon. We are currently conducting experiments to determine the effects of the *kn1* overexpression in genetic transformation of mature citrus tissues. Our preliminary results suggest the *kn1* gene can also be effective. If that can be achieved, the *kn1* gene can be used to accelerate candidate gene evaluation or new cultivar development for citrus Huanglongbing and canker disease resistance and also for the enhancement of fruit yield and quality. High transformation efficiency of citrus plants, particularly mature citrus tissues, is crucial for large scale analyses of candidate gene functions and also cultivar development via transgene- and genome editing technologies (Ding et al. 2014; Dutt et al. 2014; Li 2013; van Nocker and Gardiner 2014; Xiong et al. 2015).

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