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A simple method for transient transformation of pumpkin (*Cucurbita maxima*) seedlings

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Abstract

Currently there are no efficient stable transformation methods for pumpkin. In this work we report an efficient genetic transient transformation method of whole pumpkin (*Cucurbita maxima* cv. Big Max, a dicot) plants in the cotyledonary stage via direct inoculation of *Agrobacterium tumefaciens* and *A. rhizogenes*. A vector harboring a *uidA*-GFP translational fusion and the *Cauliflower mosaic virus* (CaMV) 35S promoter was employed. Transformation with *A. rhizogenes* resulted in the generation of root tumor phenotype within the lower portion of the stem, two months after inoculation. On the other hand, *A. tumefaciens* induces callus tissue also in the lower section of the stem one month after inoculation. Sections from these tissues were confirmed to harbor the transgene through histochemical GUS analysis and quantitative (q) PCR and RT-qPCR. Transgene RNA was also detected in phloem sap exudates, suggesting a via for delivery of foreign-expressed proteins in pumpkin. Plants expressing the maize *KNOTTED1* (*KN1*) genes showed distinct phenotype, consisting in leaf deformations with its known role in determining leaf shape. Transformation efficiency, based on transgene presence and appearance of a phenotype, ranged between 17.6 and 56%. The method described in this work can be used to transiently transform adult plants, and may be applicable to other species recalcitrant to *Agrobacterium*-mediated stable transformation; also, novel traits may be introduced to pumpkin and other plants using this technique.

Keywords: *Agrobacterum rhizogenes*, *Agrobacterium tumefaciens*, Agroinfiltration, Pumpkin, Phloem, Long-distance transport.
Abbreviations: *A. tumefaciens_Agrobacterium tumefaciens*; *A. rhizogenes_Agrobacterium rhizogenes*; qPCR_quantitative PCR; RT-qPCR_Reverse-transcription coupled quantitative PCR; GFP_green fluorescent protein; *KN1_KNOTTED1*.

Introduction

Cucurbits, including pumpkin, are crops of economic importance, cultivated in various parts around the globe (http://www.cucurbit.org/family.html). Furthermore, pumpkin species are models for functional studies of the vascular tissue in plants. The vasculature is composed of two types of tissues, xylem and phloem. The xylem is responsible for transporting mostly mineral nutrients and water, while the phloem transports sugars and photosynthesis products. Also, the phloem contains potential signaling molecules, such as proteins and RNAs (Lough and Lucas, 2006; Lucas, 2010; Lucas et al., 2013). Compared with other plants, the amount of sap that can be obtained and thus analyzed from pumpkin makes it an attractive model to study phloem and xylem long-distance signaling. To date, the sequence of its genome has not been elucidated and its transformation had not been possible. Therefore, the genetic transformation of *C. maxima* may allow the analysis of its genome and assess gene function more effectively. Plant genetic transformation is usually based on the ability of *Agrobacterium* spp disarmed strains to transfer DNA to plant hosts. Stable transformation is used for the generation of plants with a particular trait expressed in all cell types; in principle it could also be accomplished in seedlings giving rise to chimaeras that may help rapid screening of gene function. It may be of particular advantage in the case of plants that may take several years for complete development. Transformation using *Agrobacterium rhizogenes* results in the transfer of a fragment of the Ri plasmid (root-inducing) to plant cells, causing the proliferation of hairy roots. *A. rhizogenes* infects several dicot species, and has been used to obtain plant genetic mosaics in several species (Taylor et al., 2006; Ilina et al., 2012). Other studies (Katavic et al., 1991; Smarrelli et al., 1986) reported difficulties in performing stable transformation of species such as *C. pepo* L. and *C. maxima* Duch., with *A. rhizogenes* strains ATCC 15834, NCPPB 8196 and NCPPB2659. Higher efficiency of transformation of *C. pepo* L., using *A. rhizogenes* strain NCPPB 1855 (Sanitá di Toppi et al., 1997) and *C. moschata* Duch. with *A. tumefaciens* strain EH105 (Nanasato et al., 1997) have been reported. To our knowledge there are no reports of stable or transient transformation of *C. maxima.*

In this work we describe an efficient method for transformation of *C. maxima* cv. Big Max seedlings, employing *A. rhizogenes* K599 and *A. tumefaciens* C58C1. Plantlets grown in pots at the cotyledonary stage were used for this procedure, thus rendering cell culture unnecessary. Hairy roots or calli, respectively, were produced with an efficiency of up to 60%. Two reporter genes, *uidA* (GUS) and *GFP*, as a translational fusion, were introduced into *C. maxima* plants through injection of *Agrobacterium* suspension into the epicotyl. Transgene mRNA was detected in phloem sap exudates of transformed plants. Finally, as proof of principle, the maize *KNOTTED1* (*KN1*) gene under the control of the vascular-specific promoter *rolC* promoter

was introduced into pumpkin to determine whether this technique could be useful for introducing foreign genes producing anatomical changes. Transcripts corresponding to GFP were detected in leaves that arose after the transformation procedure. Additionally, *KN1*-expressing transformants displayed leaf deformations consistent with overexpression of this gene. Thus, this method could be useful for transiently expressing foreign genes in pumpkin and could be applicable in recalcitrant species.

Results

Histochemical analysis of transformed pumpkin plants

Plants were inoculated with *Agrobacterium tumefaciens* or *A. rhizogenes* harboring *GUS-GFP* fusion and subjected to histochemical analysis for GUS. Given the abundance of hairy roots in *A. rhizogenes*-transformed pumpkin, it was assumed that most, if not all, should harbor the transgene, and hence would be GUS-positive. Indeed, most hairy roots emerging from stem were found to stain for GUS (Fig. 1A). A few hairy roots did not show GUS stain (Fig. 1B). Upon closer examination of GUS-positive roots that arose from stem in *A. rhizogenes*-transformed pumpkin, most GUS activity was observed in the root vascular cylinder, and within cells (Fig. S1A and B), discarding that such activity originates in *Agrobacterium*. Apical portions of *A. rhizogenes*-transformed plants showed GUS staining, at varying degrees, in floral buds, stem and in small discrete sectors of leaves. Indeed, strong staining was observed in immature flowers, as well as in lateral buds and stem (Fig. 1C; left and middle; WT control is shown to the right). In leaves discrete blue spots were observed in transformed plants; upon closer examination, these resulted to be stomata; no GUS activity was observed in any other cell type in leaves (Fig. 1D and E). Petiole sections also yielded somewhat weak GUS activity throughout all cell types (Fig. S1C), while GUS activity was detected in non-hairy roots in one case (not shown; see table 1). In general, leaves from plants that were transformed with *A. tumefaciens* showed GUS activity in all leaf blade, although not a very strong one when compared, for example, to hairy roots (Fig. 1F). On the other hand GUS staining was also observed in young flowers in all tissues from this organ (Fig. 1G), as well as in petioles in all cell types (Fig. 2C). In contrast to *A. rhizogenes*-mediated transformation, no GUS staining was observed in roots. The CaMV 35S promoter, used in this study, has been extensively studied, and its expression pattern in model species well defined (Twyman et al., 2003). There is no information regarding the expression pattern of this promoter in pumpkin. However, in *C. moschata*, this promoter drives GFP expression throughout whole leaf tissue, in a manner similar to *A. tumefaciens*-transformed plants (Nanasato et al., 2011), so a similar expression pattern would be expected for pumpkin. In the case of *A. tumefaciens*-mediated transformation, GUS activity showed a similar pattern, except in leaves. No GUS activity was detected in plants inoculated with *A. tumefaciens* or *A. rhizogenes* that did not contain vector (not shown). Total number of results was four, hence the statistical freedom degrees are n_1-1= 3; this value corresponds to the numerator. On the other hand, as a result of the four evaluated tissues by the three aforementioned methods (see Materials and Methods) we obtained twelve sample types (n=12) from which the denominator was calculated $n_2=n_1(n-1)$ resulting in n_2= 33; the latter corresponds to a F-test = 2.83. Likewise, the MSw was calculated to be 0.458, inferred through ANOVA, and used to determine LSD according to the following formula:

$LSD_{A,B}= t_{0.05/2DFW}\sqrt{}MSW\ (1/n_A+1/n_B)$

The results of such analysis are shown in table 3. The differences between the averages was in all cases smaller than the LSD value; thus, there were no statistically significant differences among efficiency means for each treatment, so we can suggest that pumpkin is transformed with the same efficiency using either *A. rhizogenes* or *A. tumefaciens.* Aditionally, regarding the two constructs analyzed in this study, none of them showed to be more efficient in terms of transformation than the other one.

Transgene DNA and mRNAs are detected in transformed plants

In order to determine whether the transgene was present in treated tissues, DNA was obtained from cotyledons and the first true leaves of agroinoculated plants. *GFP* and *KN1* transgenes were detected in several cases (Tables 1 and 2). A quantitative analysis was carried out with these samples to measure transgene DNA content relative to an endogenous gene, *GFP*, *uidA* (GUS) and the CaMV 35S promoter. The values were higher for the transgene than for the endogenous gene (Fig. 1H). To discriminate between the transgene present, either in *Agrobacterium* and that in the plant genome (since no expression of this gene occurs in the bacterium), total RNA was obtained from the first true leaves and trangene mRNA (*GFP* and *KN1*) levels determined through RT-qPCR. No *GFP* RNA was detected in untransformed controls, while levels much higher than a constitutive mRNA, actin (which was employed as an internal control), were observed in transformed plants (Fig. 2A). Also, *KN1* mRNA was detected in transformed plants, but also, at basal levels, in untransformed controls (Fig. 2B). We have previously reported that a *KN1*-like transcript is found in pumpkin phloem sap exudates (Ruiz-Medrano et al., 2007); since the primers used in this work are directed against the homeodomain, and this is quite conserved among members of this gene family, it is possible that in these cases the endogenous transcript was detected.

Transgene mRNAs are also detected in transformed pumpkin phloem sap exudates

Analytical amounts of phloem sap can be obtained from cucurbits, from which different metabolites as well as potential signaling molecules have been isolated, such as diverse proteins and RNAs. Phloem sap exudates were obtained from 5-week old plants, 3 weeks after transformation, in order to determine whether the RNAs encoded by the transgene (*GFP* or *KN1*). Indeed, *GFP* mRNA was detected in phloem exudates from petioles of first true leaves, only in those plants transformed with *A. tumefaciens* or *A. rhizogenes* harboring either pCAMBIA or the pBinrolC plasmids (which contain the *GFP-GUS* and *KN1-GFP* gene fusions) (Fig. 3A). On the other hand, *KN1* mRNAs was also detected in phloem sap exudates; however, some signal was detected in untransformed plants as well as in those transformed with the *GUS-GFP* gene fusion. As mentioned before, the mRNA in untransformed plants could correspond to the endogenous homeodomain-containing transcript (Ruiz-Medrano et al., 2007). These results suggest that expression of transgenes in cucurbits in certain cases

Table 1. Efficiency of transformation by direct inoculation of either *A. rhizogenes* or *A. tumefaciens* onto pumpkin epicotyl. Transformation was monitored in the tumor or in tissues distal to the inoculation sites by PCR, histochemical analysis and confocal microscopy to detect GFP associated fluorescence.

Parameteres	*A. rhizogenes*	*A. tumefaciens*
Total no. of inoculated plants	112	110
Total no. of tumors (hairy root/crown-gall)	9/112	30/110
Total no. of leaves displaying change in morphology	17/112	12/110
Total no. of positive for GFP by PCR	70/112	56/110
No. of positives from hairy roots or crown-gall by PCR	24/38	19/41
No. of positives from leaf by PCR	25/40	15/28
No. of positives from stem by PCR	9/14	8/16
No. of positives from cotyledon by PCR	5/8	5/8
No. of positives from root by PCR	7/12	9/17
Total no. of positives by GUS assay	32/50	28/50
Transformation efficiency based on GUS assay	64%	56%
No. of GUS positives from hairy roots or crown-gall	10/19	7/12
No. of GUS positives from leaf	6/7	5/10
No. of GUS positives from stem	12/18	10/11
No. of GUS positives from cotyledon	3/5	6/10
No. of GUS positives from root	1/1	0/7

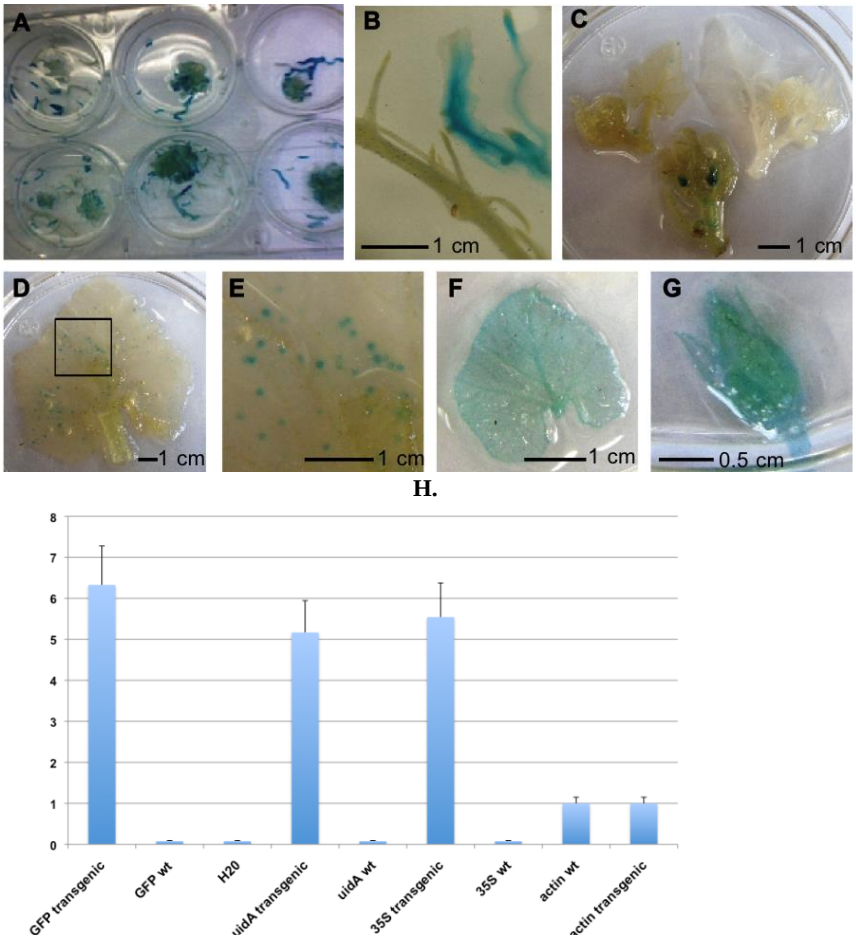


Fig 1. Histochemical analysis of plants inoculated with *A. rhizogenes* (A-D) and *A. tumefaciens* (E-F) harboring a *uidA* (GUS)-GFP fusion directed by the CaMV 35S promoter. A, Hairy roots from six independent *A. rhizogenes*-transformed plants. B, close up of hairy roots from an inoculated plant. C, floral buds displaying GUS activity, stronger in more developed flowers (arrows, middle), compared to younger plants and controls (left and right). Signal was also observed in leaves, stems and petioles (left and middle). D, leaf from an *A. rhizogenes*-inoculated plant showing GUS activity in stomata. E, higher magnification of the region marked in D. F, Leaf from a plant inoculated with *A. tumefaciens* harboring *GUS-GFP* showing GUS activity throughout the whole leaf blade. G, flower bud from a plant inoculated with *A. tumefaciens* harboring *GUS-GFP*. Bars in A-F and G are 1cm in length; bars in G and I are 0.5cm. H. qPCR for transgenes and an endogenous gene in WT and transformed pumpkin plants. DNA was obtained from first true leaves of transformed and WT plants. Actin DNA was used as internal control. The *y* axis indicates the relative DNA levels; actin DNA in WT=1.

may lead to phloem loading of the corresponding mRNAs. This in turn could lead to delivery of mRNAs or proteins of interest to developing tissues, at least in cucurbits.

KN1 expression in pumpkin induces leaf deformation

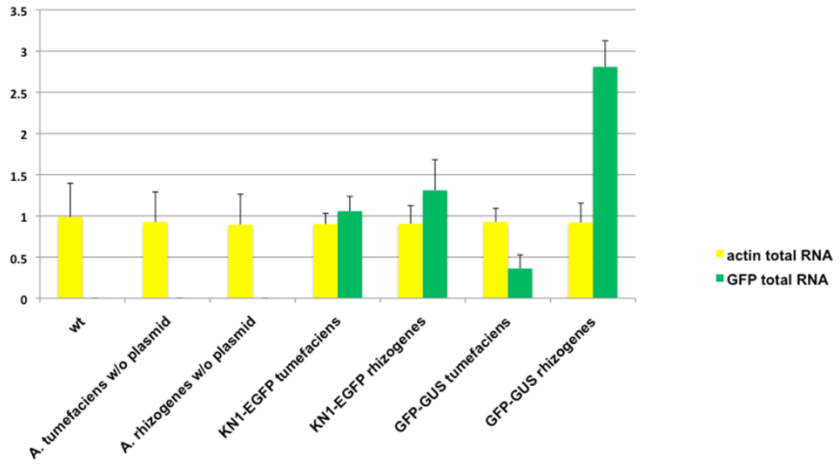
To test whether this transformation method could be used to introduce foreign genes capable of inducing a phenotype in pumpkin, *KN1* from maize was fused in frame to GFP and then cloned into the pBinrolC vector (Kühn et al., 1996) in order to express this gene in vascular tissue. Ectopic expression of *KN1* in maize leads to overgrowth of vascular bundles in leaves, producing the so-called "knotted" phenotype (Vollbrecht et al., 1991). This construct was introduced into *A. tumefaciens* C58C1 and *A. rhizogenes* K599. As mentioned already, *KN1* mRNA was detected in cotyledons and true leaves of several transformed plants (Fig. 6A; Table 2). This mRNA was also detected indirectly by RT-PCR with primers directed to *GFP* (Fig. 2 and 3). One week after inoculation with either *A. tumefaciens* or *A. rhizogenes* harboring the *rolC:KN1* construct, some plants showed outgrowth in the veins of cotyledons (table 2; Fig. 4), suggesting a *KN1*-like overexpression phenotype. As shown in table 2, more plants showed this phenotype when *KN1* was harbored by *A. tumefaciens*. Several transformed plants in later developmental stages (up to two months after transformation) showed more severe phenotypes in true leaves as well, such as leaf crumpling and deformation, consistent with *KN1* expression (data not shown). An additional phenotype was the appearance of lobed leaves, which is also consistent with the overexpression of *KN1* (Fig. 4E-G). All plants displaying a *KN1*-like phenotype were shown to harbor the *KN1* transgene in such leaves as well as in other tissues (Table 2). The transformation method reported was quite reproducible. In terms of efficiency *A. rhizogenes* yielded the highest (64%), compared to *A. tumefaciens* (56%), based on plants that yielded positive for the GUS assay. This was lower in the case of *KN1*; indeed, the proportion of plants showing the knotted phenotype was 52.6% for those in which *A. tumefaciens* was used, and 17.6% in the case of *A. rhizogenes*.

Discussion

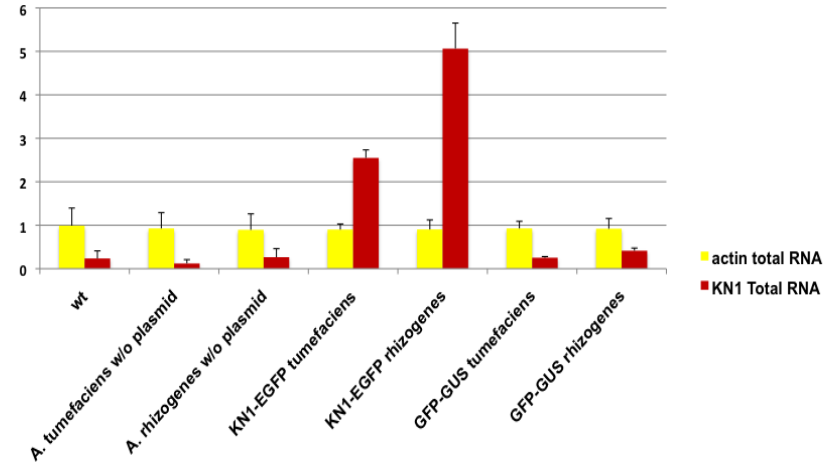
Reproducible genetic transformation methods for plant model experimental systems are essential for the study of gene and genome function; this holds true also for agronomically important crops. It is therefore an indispensable tool in plant molecular biology for the study of gene function and gene regulation from a basic standpoint. Cucurbits, and pumpkin in particular, are excellent models for the study of phloem function, structure and composition, since analytical quantities of phloem sap exudates can be obtained, and several pioneering works have helped elucidate the ultrastructure of phloem (Lough and Lucas, 2006; Lucas, 2010; Lucas et al., 2013). Recently, a role for this tissue in long-distance signaling has been emerging. Some of these long-distance signals are proteins and RNA. This work reports the transformation of young (at the cotyledonary stage) pumpkin plants mediated by *Agrobacterium rhizogenes* and *A. tumefaciens.* Inoculations with *A. rhizogenes* were performed with either sterile water or infiltration medium; in both cases the plants produced hairy roots. Hairy root formation in the absence of hormones suggests that cytokinins and auxins are produced and secreted by the transformed root cells (this work). This could explain the absence of GUS activity in some of these hairy roots (Figure 1B). The presence of the introduced transgenes (CaMV 35S promoter, and the GUS-GFP translational fusion) in the *C. maxima* genome was stable in the transformants even 6 months after transformation, as determined by PCR analysis (not shown). On the other hand, it is not clear why GUS expression in plants inoculated with *A. rhizogenes* is restricted to stomata in leaves, but this suggests that the transgene is integrated only in certain cell types. More work is needed to determine the factors affecting the expression of heterologous promoters using the present system. Aside from the observed phenotype of leaves immediately adjacent to the inoculation site above the cotyledons in some cases, no other morphological changes were detected in transformed plants. Thus, neither the transformation procedure nor the presence of the transgene significantly affected the growth and development of the transformed plants. *KNOTTED1* from maize was the first homeotic gene isolated from plants, and is part of the homeodomain-containing protein superfamily in eukaryotes, the members of which are involved in meristem maintenance and floral organ identity in plants (Hay et al., 2000). *KN1* also induces distinct phenotypes when overexpressed in plants, such as leaf crumpling, changes in leaf shape and size, and shorter internodes (Jackson et al., 1994). Its expression is also associated to cambium, where it controls proliferation (Hake et al., 2004). Maize *kn1* mutations that cause ectopic expression in bundle sheath show a characteristic "knotted" phenotype in leaves, which is similar to the heterologous or constitutive expression of several members of this gene family (Smith et al., 1992). Thus, this gene was introduced into pumpkin to determine whether such phenotype could be observed. It was assumed that expression of this gene under a strong, constitutive promoter such as CaMV 35S, would hinder the development of potentially transformed plants, so the vascular-specific *rolC* promoter from *A. rhizogenes* was used instead. *KN1* is expressed in vascular bundles in maize, so this construct would mimic its expression pattern in its organism of origin (Jackson et al., 1994). Also, this tissue-specific promoter was used to ensure the generation of transformants. Several plants showed a leaf phenotype similar to ones overexpressing *KN1* or *KNOX* genes (Vollbrecht et al., 1991; Hay and Tsiantis, 2010); also some cotyledons displayed knots in veins, thus demonstrating the feasibility of gene transfer in pumpkin using this method. Of note, a higher proportion of plants showed *KN1* overexpression-like phenotype when transformed with *A. tumefaciens* than with *A. rhizogenes*, as mentioned before. Our results support the notion that gene function can be analyzed using this transformation system, without the need of plant regeneration. This could in principle be extrapolated to other plant species, for example those that are more recalcitrant to transformation or perennial species. Given the development of novel technologies that allow for gene editing, this method could be used for these ends, for example, in the analysis of genes involve in phloem function, for which pumpkin may be a suitable model. One such methodology, CRISPR-Cas, has been used recently for introducing mutations in rice genes (Xu et al., 2014). As a proof of principle, in the aforementioned work a herbicide resistance gene was inactivated via CRISPR-Cas editing, leading to a high frequency of mutation (up to 16%). Thus, a similar strategy, in conjunction with the transformation method described in the present work could be used to determine gene function in pumpkin and other cucurbits, especially those that, by analogy or by localization of the corresponding transcript ot protein, may have a role in

Table 2. Effect of *KNOTTED1* expression in pumpkin. Plants were analyzed one to three weeks after inoculation with either *A. tumefaciens* or *A. rhizogenes* harboring maize *KN1* as described before. Transformation was monitored in the tumor or in tissues distal to the inoculation sites by PCR and leaf deformations corresponding to *KN1* overexpression.

Parameters	*A. tumefaciens*	*A. rhizogenes*
Total no. of inoculated plants	78	34
Total no. of tumors (callus/hairy root)	12	4
Total no. of positives by GFP PCR assay	61	27
No. of positives from hairy roots or crown-gall	12	4
No. of positives from leaf	11	10
No. of positives from stem	8	5
No. of positives from cotyledon	26	5
No. of positives from root	4	3
Total no. of positives with knotted phenotype	41	6
Transformation efficiency based on appearance of knotted phenotype	52.6%	17.6%
No. of positives from leaf	25	3
No. of positives from cotyledon	16	3



A



B

Fig 2. Quantification of *GFP* and *KN1* transcripts in leaves from WT and transformed pumpkin expressing *GUS-GFP* or *KN1-GFP* by qRT-PCR. Total RNA was obtained from first true leaves that arose after transformation. A. *GFP* transcript levels in wild type and pumpkin plants transformed with *A. tumefaciens* and *A. rhizogenes* without plasmid, and *A. tumefaciens* and *A. rhizogenes* harboring either *GFP-GUS* or *KN1-GFP*. B. Same as in A, but *KN1* transcript levels were measured instead. In both cases, these were standardized relative to the endogenous actin mRNA levels, used as internal control. The *y* axis indicates the relative transcript levels; actin mRNA in WT=1.

phloem function. Because of its high efficiency, the method described in this work is proposed as a convenient strategy for gene delivery into pumpkin seedlings, considering that transgene mRNAs could be transported through the phloem. More work is needed to determine whether the transgene is transmitted to the next generation progenie.

Materials and Methods

Plant material

C. maxima cv. Big Max plants were obtained from R. H. Shumway´s, Randolph, WI; these were grown and kept in greenhouse conditions, under high humidity (70% RH in air)

Table 3. Results of LSD analysis for different strains of Agrobacterium and the different constructs used.

	GFP-GUS			KNOTTED-GFP		
	A. rhizogenes	*A. tumefaciens*		*A. rhizogenes*	*A. tumefaciens*	
	x1	x2	n	x3	x4	n
Total no. of tumors (hairy root/crown-gall)	0.080357143	0.272727273	1	0.117647059	0.153846154	1
Total no. of leaves displaying change in morphology	0.151785714	0.109090909	2	n/a	n/a	
No. of positives from hairy roots or crown-gall by PCR	0.631578947	0.463414634	3	0.117647059	0.153846154	2
No. of positives from leaf by PCR	0.625	0.535714286	4	0.294117647	0.141025641	3
No. of positives from stem by PCR	0.642857143	0.5625	5	0.147058824	0.102564103	4
No. of positives from cotyledon by PCR	0.625	0.625	6	0.147058824	0.333333333	5
No. of positives from root by PCR	0.583333333	0.529411765	7	0.088235294	0.051282051	6
No. of GUS positives from hairy roots or crown-gall	0.526315789	0.583333333	8	n/a	n/a	
No. of trangene phenotype positives from leaf (GUS expression or Knotted phenotype)	0.857142857	0.5	9	0.088235294	0.320512821	7
No. of trangene phenotype positives from stem	0.666666667	0.909090909	10	n/a	n/a	
No. of trangene phenotype positives from cotyledon	0.6	0.6	11	0.088235294	0.205128205	8
No. of trangene phenotype positives from root	1	0	12	n/a	n/a	
Sum	6.990037594	5.690283109		1.088235295	1.461538462	
Average	0.582503133	0.474190259		0.136029412	0.182692308	
Average of Averages	0.343853778					
MSw	0.458471704					
Degrees of freedom (Numerator)	3					
n2 (Denominator)	33					
Fisher's F	2.83					
LSD	0.782288739					
Calculated Fisher's F for the data	2.142639570					

n/a, not applicable

with day/night temperature of 40/15 ^{o}C and a photoperiod of 12h light/dark; nutrients were applied with irrigation. Between 60 and 150 seeds of per treatment were disinfected for 2 minutes in 70% ethanol with shaking and subsequently immersed in 2 % sodium hypochlorite solution and washed four times with sterile distilled water for 1 minute. The seeds were placed on petri dishes containing moist cotton and kept in dark conditions at 23^{o}C until seed germination (8-10 days). The plantlets were then transferred to pots with cotton as soil and grown for 8 days at 23 ^{o}C (Fig. 2); subsequently these were inoculated with *Agrobacterium*. Eight days post-inoculation the plantlets were transferred into pots containing 18 Kg of sterilized soil, until the plants were used. The plants were watered with B&D solution (Broughton and Dilworth, 1971), and incubated in a growth chamber with a 12h light/12h dark photoperiod at 28ºC.

Vectors

The vector used in this work was pCAMBIA1304, containing a GFP-GUS translational fusion, the expression of which is directed by the CaMV 35S promoter, and also harbors a kanamycin resistance gene. This vector was kindly provided by Dr. Federico Sánchez (IBT UNAM) and has been described elsewhere (Estrada-Navarrete et al., 2007).

Agrobacterium strains

Cucumopine-type *A. rhizogenes* strain K599 (NCPP2659) (Bond and Gresshoff, 1993) and *A. tumefaciens* C58C1 strain (Van Larebeke et al., 1984) were used in this work. pCAMBIA 1304 was introduced into both strains by electroporation. *A. rhizogenes* K599 was grown in Luria-Bertani (LB) medium containing kanamycin (Km) at 50 µg/ml, for 48 hours at 30ºC for inoculation. *A. tumefaciens* C58C1 was grown on YEB medium containing Km at 50 µg/ml, for 48 hours at 30ºC.

Plant material

An average of 60 seeds of *C. maxima* cv. Big Max (R. H. Shumway´s, Randolph, WI) were disinfected for 2 minutes with 70% ethanol using vigorous shaking, and afterwards immersed in 2 % NaOCl and washed four times with sterile distilled water for one minute, placed on petri dishes with cotton, and kept in dark conditions at 23^{o}C until germination (8 to 10 days). The plantlets were subsequently transferred to pots with cotton and grown for 8 days at 23 ^{o}C (Fig. 2) and inoculated with *Agrobacterium* culture. Eight days after inoculation, the plantlets were transferred to pots containing 18 Kg of sterilized soil. The plants were watered with B&D solution (Broughton and Dilworth, 1971) and cultivated in a growth chamber with a 12h light/12h dark photoperiod at 28ºC.

Inoculation with Agrobacterium

A. rhizogenes K599 and *A. tumefaciens* C58C1 were grown on plates, and collected and diluted with 1 ml of infiltration medium (5%W/V sucrose, 0.044 mM benzylaminopurine, 50 mL/L Silwett L-77 (Clough and Bent, 1998), to be used to infect epicotyl and cotyledons. As controls, fresh cultures of *A. rhizogenes* K599 and *A. tumefaciens* C58C1 without plasmid were grown in LB and YEB medium, respectively, without antibiotic. These were then subcultured for 48h and incubated with a final concentration of acetosyringone of 140 µM. *C. maxima* were then inoculated with *A. rhizogenes* K599 and *A. tumefaciens* C58C1 containing the binary vector by injecting the bacterial suspension directly into the cotyledonary nodes with a syringe containing 50 µL of

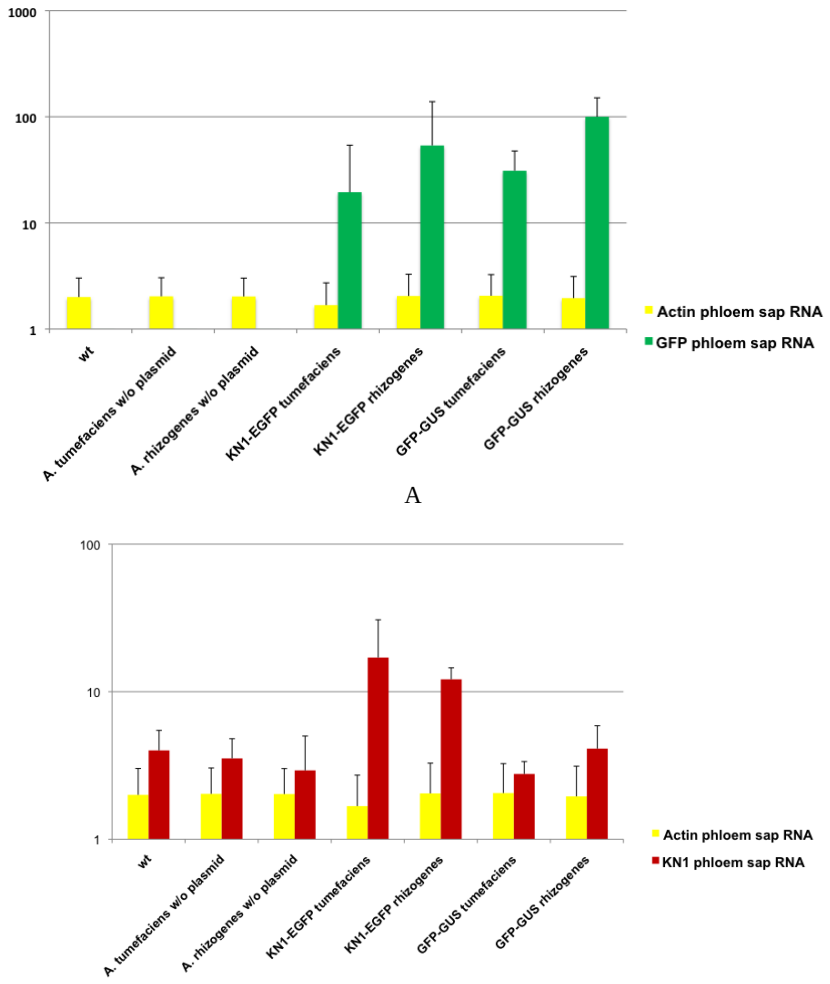


Fig 3. Detection of *GFP* and *KN1* transcripts in phloem sap exudates from WT and transformed pumpkin expressing *GUS-GFP* or *KN1-GFP* by qRT-PCR. A. *GFP* transcript levels in wild type and pumpkin plants transformed with *A. tumefaciens* and *A. rhizogenes* without plasmid, and *A. tumefaciens* and *A. rhizogenes* harboring either *GFP-GUS* or *KN1-GFP*. B. Same as in A, but *KN1* transcript levels were measured. The *y* axis indicates the relative transcript levels; actin mRNA in WT=1.

inoculum (*Agrobacterium*). Plants were covered with a transparent plastic bag to maintain humidity at 90%. Inoculated plants were maintained in a growth chamber (12h light/12h dark at 28ºC) for 15 days; afterwards the bag was removed. Wild type plants were control transformed with *A. rhizogenes* K599 and *A. tumefaciens* C58C1 with no plasmid. The plants infected by *A. rhizogenes* and *A. tumefaciens* started to show hairy roots or calli, respectively, approximately one month post-inoculation.

GUS assay

GUS activity was analyzed by the histochemical assay described by Jefferson et al. (1987). These assays were performed on plant systemic tissue two months after inoculation with *Agrobacterium*. Tissue was vacuum-infiltrated for 30 min with GUS-staining solution [100 mM potassium phosphate buffer, pH 7.0; 10 mM EDTA; 0.1% Tween 20; 5 mM potassium ferricyanide; 5 mM potassium ferrocyanide; 2 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-Gluc; Research Organics; Cleveland, OH)]. The tissues were incubated overnight at 37°C in darkness. After staining, tissues were washed to remove chlorophyll; first, with an ethanol: acetic acid 3:1 mixture, and then with a methanol: acetone mixture 3:1. Afterwards, tissue was clarified with 45% glycerol, 20% phenol and 20% lactic acid. Samples were analyzed by light stereomicroscopy and fluorescence microscopy (Axiolab HBO 50/AC Zeiss Filter Set 15; Zeiss, Germany).

PCR analysis and cloning

Genomic DNA from stems and cotyledons was extracted with the DNeasy Plant kit following the manufacturer's recommendations (Qiagen, Hilden, Germany). The presence of T or R-DNA in hairy roots and callus was determined by amplification of the *GFP* open reading frame from total DNA. The primers used for GFP (which amplifies the complete open reading frame): GFP Forward 5′-ATGGTGAGCAAGGGCGGAGGAGCTG-3′ and GFP Reverse 5′-TTACTTCTACAGCTCGTCCATGCCGAG-3′. The primers for the CaMV 35S promoter were 35S FOR 5'-AGATTAGCCTTTTCAATTTCAGAAAGAATG-3', and 35S REV 5'-CGTGTTCTCTCCAAATGAAATGAACTT-CC-3', which amplifies the complete promoters (845 bp). For genomic DNA amplification, samples were heated to 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 s, 62ºC for 45 s, and 72ºC for 1 min with a T-1000 Professional Basic Gradient Thermocycler (Biometra, Germany). PCR products were separated by agarose gel electrophoresis, stained with

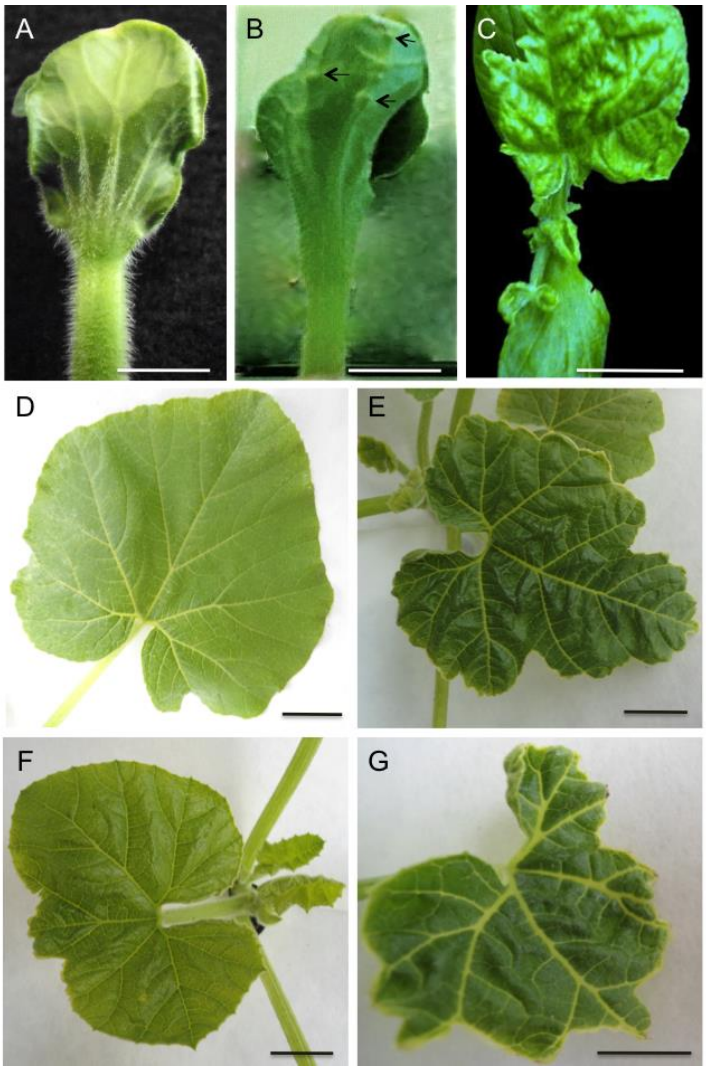


Fig 4. Leaves from *KN1*-expressing pumpkin plants show distinct phenotype. A. Cotyledon of a wild type plant 7 days after mock inoculation. B. Cotyledon of a plant 7 days after inoculation with *A. tumefaciens* harboring *KN1-GFP*. Arrows indicate deformations in veins. C. First true leaf of a plant four weeks after transformation with *KN1-GFP*. D. First true leaf from mock-inoculated plant four weeks after inoculation. E-G, First true leaves from three independent plants four weeks after transformation with *KN1-GFP* harbored by *A. rhizogenes*. Bars in A and B are 1 cm; bars in C-G are 2 cm.

ethidium bromide and visualized under UV light. DNA concentration was measured with a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington DE). The maize *KNOTTED1* ORF was obtained by digesting the pKOC plasmid (kindly provided by Prof. David Jackson, Cold Spring Harbor Laboratory) with *Eco*RI and *Age*I, which releases the ORF without its stop codon, and was cloned into pBluescript KS+ (Invitrogen); then EGFP (Clontech) was inserted in frame in the *Bam*HI site, and the whole cassette excised with *Sac*II and *Kpn*I, blunt-ended and inserted into the *Bam*HI site of the pBinrolC binary vector (Bevan, 1984), which consists of the *rolC* promoter from *A. rhizogenes* inserted in pBin19 (Kühn et al., 1996). The resulting plasmid was introduced to *A. tumefaciens* C58C1 and *A. rhizogenes* K599 by electroporation as described.

Real-Time PCR and RT-PCR

The primers used for *uidA* (which amplifies 200 bp from the 3' end of the open reading frame) were GUS FOR 5'-ATGTTACGTCCTGTAGAAACCCCAACC-3', and GUS REV 5'- TCATTGTTTGCCTCCCTGCTGCCGGTTT-3'. GFP ORF and a fragment of the *uidA* (GUS) gene were amplified from genomic DNA using a GreenER qPCR Super Mix (Invitrogen) following the manufacturer's recommendations, using the conditions described in the previous section.

Messenger RNA levels for *GFP* and *KN1* in total RNA and phloem sap exudates were determined by quantitative RT-PCR with the Express One-Step Sybr GreenER qPCR Super Mix (Invitrogen) kit according to the manufacturer's instructions. Phloem sap exudates were obtained essentially as described before (Ruiz-Medrano et al., 2007). Actin primers used were the following: CmACT5deg forward: 5´-AAYTTGGAYGAYATGGARAAR-3´ and CmACT3deg reverse:RAANGTYTCRAACATDATYTGNGTCAT, which amplified a 200 bp fragment. For maize *KNOTTED1* mRNA detection, the following primers were used: KN1 forward, 5'GGCCTTACCCCTCAGAGACTCAGAAGG 3'; KN1 reverse, 5' CTAGCCGAGCCGGTACAGCCCGCCGTC 3'; the expected size of the amplicon being 230 bp. 1µL of RNA (500 ng/µL) and 50 pM of each primer were used in a 25 µL reaction mix. The Real Time RT-PCR reactions were incubated in a Rotor Gene 3000 apparatus (Corbett Research,

Australia). Conditions for amplification were 40 min at 42°C for first strand synthesis, followed by 50 cycles: 40 sec at 94°C, 45 sec at 60°C, and 50 sec at 72°C. RT-PCR reactions were performed by triplicate for each treatment. *GFP* transcripts were detected essentially as described above. To ensure that no dimers were amplified in the reaction, a dissociation curve was generated through progressive heating of the samples (60-95°C), in which only one curve was observed. Relative quantification of transcript accumulation was performed according to the method described by Livak and Schmittgen (2001), the 2-ΔCT´ method was used for RNA quantification. Three repeats were analyzed per treatment.

Statistical analysis

Fisher´s Least Significant Difference (LSD) was used to determine whether there were significant differences in transformation efficiency using different strains and different genetic constructs (Meier, 2006; Williams and Abdi, 2010). The procedure compares the means of two or more groups; thus, an analysis of one global null hypothesis was performed, which in this case is: There is no significant difference in transformation efficiency among all treatments and the several strains used. Briefly, LSD was calculated to know the maximum limit allowed between each treatment´s mean in order considering that these belong to the same population. Afterwards the real difference was calculated between the populations' average compared to the LSD. The four populations considered in this study were plants transformed with 1) *A. rhizogenes* harboring *GFP-GUS* (x_1), 2) *A. tumefaciens* harboring *GFP-GUS* (x_2), 3) *A. rhizogenes* harboring *KNOTTED-GFP* (x_3) and 4) *A. tumefaciens* harboring *KNOTTED-GFP* (x_4). Transformation efficiency was evaluated through PCR assays, analysis of phenotypic changes and the direct effect on transgene expression; the latter was performed in several tissues such as leaves, cotyledons, stem and roots.

Conclusion

An efficient method for transient transformation of whole *C. maxima* plants at the cotyledonary stage was established. *Agrobacterium rhizogenes* strain K599 and *Agrobacterium tumefaciens* strain C58C1 could both efficiently transform this plant and formed chimaeras. Also, the hairy root tumor could produce hormones for its growth and of the surrounding cells. The expression of the maize *KN1* gene led to well-defined phenotypes. This method may prove particularly useful for the transient expression of plants recalcitrant to *Agrobacterium*-mediated transformation, and of perennial plants. Similarly, this method could be used for the delivery of proteins of interest to distant tissues in agroinfiltrated plants, in the case of cucurbits (in which phloem flow pressure is high); in other species this could be accomplished by fusing the protein to endogenous supracellular movement proteins, such as CmPP16 (Xoconostle-Cázares et al., 1999).

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

RR-M and BX-C designed the experiments, coordinated the work, carried out plant transformation experiments, and wrote the manuscript; FAR-O and BX-C carried out plant transformation assays, Real Time PCR and RT-PCR, and analysis of transformed and control splants. RT-M carried out analysis of transformed and control plants. All authors read and approved the final version of the manuscript.

References

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12: 8711-872.

Bond JE, Gresshoff PM (1993) Soybean transformation to study molecular physiology. In: Plant Responses to the Environment. Edited by PM Gresshoff. CRC Press, London pp. 25–44.

Broughton WJ, Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J. 125: 1075-1080.

Clough SJ, Bent A (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735-743.

Estrada-Navarrete G, Alvarado-Affantranger X, Olivares JE, Guillén G, Díaz-Camino C, Campos F, Quinto C, Gresshoff PM, Sanchez F (2007) Fast, efficient and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium rhizogenes*. Nat Protoc. 2: 1819-1824.

Hake S, Smith HMS, Holtan H, Magnani Enrico, Mele G, Ramirez J (2004) The Role of knox in Plant Development. Annu Rev Cell Dev Biol. 20: 125-151.

Hay A, Tsiantis M (2010) KNOX genes: versatile regulators of plant development and diversity. Development. 137: 3153-3165.

Ilina EL, Logachov AA, Laplaze L, Demchenko NP, Pawlowski K, Demchenko KN (2012) Composite *Cucurbita pepo* plants with transgenic roots as a tool to study root development. Ann Bot. 110: 479-489.

Jackson D, Veit B, Hake S (1994) Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development. 120: 405-413.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Katavic V, Jelaska S, Bakran-Petricioli T, David C (1991) Host tissue differences in transformation of pumpkin (*Cucurbita pepo* L.) by *Agrobacterium rhizogenes*. Plant Cell Tiss Org Cult. 24: 473-497.

Kühn C, Quick WP, Schulz A, Riesmeier JW, Sonnewald U, Frommer WB (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. Plant Cell Environ. 19: 1115-1123.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 25: 402-408.

Lough TJ, Lucas WJ (2006) Integrative plant biology: role of phloem long distance macromolecular trafficking. Annu Rev Plant Biol. 57: 203-232.

Lucas WJ (2010) Plant vascular biology and agriculture. J Integr Plant Biol. 52: 4-7.

Lucas WJ, Groover A, Lichtenberger R, Furuta K, Yadav SR, Helariutta Y, He XQ, Fukuda H, Kang J, Brady SM, Patrick JW, Sperry J, Yoshida A, López-Millán AF, Grusak MA, Kachroo P. (2013) The plant vascular system: evolution, development and functions. J Integr Plant Biol. 55: 294-388.

Nanasato Y, Konagaya K, Okuzaki A, Tsuda M, Tabei Y (2011). *Agrobacterium*-mediated transformation of kabocha squash (Cucurbita moschata Duch) induced by wounding with aluminum borate whiskers. Plant Cell Rep. 30: 1455-1464.

Ruiz-Medrano R, Hinojosa-Moya JJ, Xoconostle-Cázares B, Lucas WJ (2007) Influence of Cucumber mosaic virus infection on the mRNA population present in the phloem translocation stream of pumpkin plants. Functional Plant Biol. 34: 292-301.

Ruuska SA, Girke T, Benning C, Ohlrogge JB (2002) Contrapuntal Networks of Gene Expression during *Arabidopsis* Seed Filling. Plant Cell. 14: 1191–1206.

Sanitá di Toppi L, Pecchioni N, Durante M (1997) *Cucurbita pepo* L. can be transformed by *Agrobacterium rhizogenes*. Plant Cell Tissue Organ Cult. 51: 89-93.

Smith L G, Greene B, Veit B, Hake S (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. Development. 116: 21-30.

Smarrelli J, Watters MT, Diba LH (1986) Response of various Cucurbits to infection by plasmids harboring strains of *Agrobacterium*. Plant Physiol. 82: 622-624.

Taylor CG, Fuchs B, Collier R, Lutke WK (2006) Generation of composite plants using *Agrobacterium rhizogenes*. Methods Mol Biol. 343: 155-167.

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol. 21: 570-578.

Van Larebeke, N, Engler G, Holsters M, Van den Elsacker I, Zaenen S, Schilperoort RA, Schell J (1974) Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing ability. Nature. 252: 169-170.

Vollbrecht E, Veit B, Sinha N, Hake S (1991) The developmental gene *Knotted-1* is a member of a maize homeobox gene family. Nature. 350: 241-243.

Xoconostle-Cázares B, Xiang Y, Ruiz-Medrano R, Hong-Li W, Monzer J, Byung-Chun Y, McFarland KC, Franceschi VR, Lucas WJ (1999) Plant Paralog to Viral Movement Protein that Potentiates Transport of mRNA into the Phloem. Science. 283: 94-98.

Xu RF, Li H, Qin RY, Wang L, Li L, Wei PC, Yang JB (2014) Gene targeting using the *Agrobacterium tumefaciens*-mediated CRISPR-Cas system in rice. Rice. 7: 5.