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Differential response of tomato and tobacco to *Agrobacterium* mediated transformation with c*ytokinin independent -1* (*CKI-1*) gene as influenced by cytokinin levels

J B Mythili[1]*, G V S Saiprasad[3], C Naveena[1], P R Rajeev[1] & K K Upreti[2]

[1]Division of Biotechnology

[2]Division of Plant Physiology and Biochemistry, Indian Institute of Horticultural Research, Hessaraghatta, Bangalore 560089, India

[3]ITC R&D centre, I[st] Phase, Peenya, Bangalore 560058, India

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Cytokinin independent-1 (*CKI-1*) gene was identified through its ability to confer cytokinin independent growth in *Arabidopsis* which has led to this gene being advocated as a selectable marker in plant transformation. Keeping this in view, *CKI-1* gene was assessed as a selectable marker by transformation of tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum* L.). Overexpression of *CKI-1* gene through *Agrobacterium* mediated transformation in tobacco and tomato conferred cytokinin independent shoot regeneration (in media devoid of cytokinin/plant growth regulators) in tobacco, but not in tomato wherein this ability (cytokinin independence) was conferred to T1 explants of *CKI-1* transgenic tomato plant (T0) regenerated on cytokinin medium. Analysis of cytokinin levels revealed that cytokinin independent growth upon transformation with *CKI-1* gene in tobacco (T0) and tomato (T1) was achieved through maintaining/regulating higher endogenous cytokinin levels and *CKI-1* gene expression. Levels of *CKI-1* transcripts assayed through quantitative RT-PCR suggested that there seemed to be a threshold level of endogenous cytokinin level, regulated due to external or internal supply via *CKI-1* gene upto which *CKI-1* gene expression correlated with endogenous cytokinin content and beyond that, either the gene expression was not induced or it remains same. With the incorporation of *CKI-1* gene, it appeared that this threshold level of endogenous cytokinin might be reduced in crops like tomato to support shoot regeneration at lower concentration of cytokinin, but could not be made independent of external supply of cytokinin as in tobacco suggesting that use of *CKI-1* gene as an effective alternate selection marker could not be universally applicable across the species. The results of the present study revealed that *CKI-1* gene in addition to enhancing cytokinin levels, was also involved in contributing to the sensitivity to cytokinin and thus served as a positive regulator of cytokinin signal transduction.

Keywords: *Agrobacterium* mediated transformation, *CKI-1* expression, *CKI-1* gene, Cytokinin levels, Selectable marker

The growing concern on the transfer of antibiotics or herbicide resistance marker genes to wild relatives of crop plants with potential problems to human health and ecosystem has hampered the acceptability of genetically modified crops[1]. To overcome these issues, the production of marker free transgenic plants has become a major objective of plant biotechnologists. To circumvent these problems, several alternate selection systems including oncogenes from *Agrobacterium tumefaciens*, β-glucuronidase (*GUS*) gene and *manA* gene have been developed for plant transformation[2-5]. Additionally, genes involved in promoting explant regeneration have been considered, such as those involved in cytokinin biosynthesis, cytokinin signaling or that influencing shoot apical meristem (SAM) development. Among these systems, *ipt* gene, an oncogene cloned from *A. tumefaciens* encoding the enzyme isopentenyl transferase to catalyze one of the early steps in cytokinin biosynthesis, enables cells to proliferate and differentiate into shoots without exogenous cytokinins[6]. This gene has been used effectively as a positive selectable marker gene in transformation in tobacco, tomato, muskmelon and sweet pepper[7-10]. Another gene viz; maize homeobox gene knotted 1 (*Kn1*) expressed in shoot meristems has also been demonstrated recently as a positive selectable marker gene in transformation of tobacco[11]. However overexpression of *ipt* and *Kn1* gene has been associated with developmental abnormalities associated with elevated levels of cytokinins

*Correspondent author
Telephone: 080-28466420-23
Fax: -080-28466291, E- mail: jbmythili@yahoo.com

especially in case of *ipt* gene. Therefore, efforts on the use of genes involved in the manipulation of cytokinin signal transduction pathway appear to be more efficient and more reliable method for developing marker free transgenic plants[4]. One such gene *cytokinin independent-1* (*CKI-1*) gene involved in cytokinin function was identified as a potential regulator of shoot regeneration. This was identified by its ability to confer cytokinin independent callus growth when overexpressed in *Arabidopsis*. *CKI-1* gene codes for histidine kinase that confers the cell ability to sense endogenous cytokinin triggering shoot organogenesis[12]. *CKI-1* was, therefore, considered as a candidate cytokinin receptor being a histidine kinase and its overexpression caused cytokinin responses. However, no additional data supported *CKI-1* being a cytokinin receptor as it did not get activated by cytokinins unlike other histidine kinases such as *CRE1/WOL/AHK* etc[13]. With so many roles implicated, there is no definite conclusion regarding the wild type function of the protein[14].

Keeping this in view, the use of *CKI-1* gene as a selectable marker was assessed by its overexpression in tobacco and tomato in relation to its expression and cytokinin content.

Materials and Methods

Plant material—Tomato cv. Arka Vikas, procured locally and a local cv of tobacco (*Nicotiana tabacum*) was used for the study.

Transformation—Two gene constructs were used for transformation. *CKI-1* gene present in the binary vector pMON530 under the control of CaMV35S promoter in C58 *Agrobacterium* strain and the other binary vector pCAMBIA2301 without *CKI-1* gene present in LBA4404 *Agrobacterium* strain was used for the experiments. Vector without the *CKI-1* gene but with the reporter (*nptII*) gene in pCAMBIA2301 served as the control vector. In both the constructs, *nptII* gene was used as a selectable marker. The leaf discs of tobacco and cotyledons of tomato from shoots raised *in vitro* and 8-10 days old seedlings were used as explants, respectively. These explants were inoculated with overnight grown culture of *A. tumefaciens* for 2 min followed by co-cultivation for a period of 2 days on Murashige and Skoog[15] medium without antibiotics (kanamycin or cefotaxime) or plant growth regulators (PGR). Following this, one set of explants were transferred to media without PGR (non-inductive/non-regenerating medium) supplemented with or without kanamycin and other set transferred to medium supplemented with PGRs (inductive/regenerating) such as cytokinin viz., benzyl amino purine (BAP 1.0 mgL^{-1}) for tobacco; and BAP (2.0 mgL^{-1}) and indole-3-acetic acid (IAA 0.1 mgL^{-1}) for tomato and kanamycin (100 mgL^{-1} for tomato; 200 mgL^{-1} for tobacco). The two media were also supplemented with cefotaxime 500 mgL^{-1}. Both tobacco and tomato require the inductive medium for shoot regeneration. The medium was gelled with phytagel (0.25%; Sigma Chemical Co; USA) and *p*H was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. The inoculated cultures were incubated in culture racks (white fluorescent tubes; with a light intensity of 30-40 µE m^{-2} s^{-1}) under a 16 h photoperiod in a culture room maintained at 25° ± 2°C.

Raising of T1 seedlings of CKI-1 transformed tomato—The seeds of the primary transformants of tomato with *CKI-1* gene (T0) were grown on medium containing kanamycin to eliminate the non transformant segregants and the explants from the surviving seedlings (T1) were used for *in vitro* regeneration under non-inductive conditions.

Confirmation of the presence of transgene

PCR analysis—DNA was isolated from leaves of control (untransformed), transformed plants and *Agrobacterium* plasmid following CTAB method. Primers specific to *NPTII* gene and *CKI-1* gene were used for PCR amplification. *NPTII* gene: forward primer [(5' GATGGATTGCACGCAGG 3')] and reverse primer [(5' GAAGGCGATAGAAGGCG 3')]; *CKI-1* gene: forward primer [(5' ATGATGGTGAAAGTTACAAAGCTTGTGGCT 3')] and reverse primer [(5' CTAGTGACGTTTGCTTTCGATTTCTCTAATG 3')]

PCR reaction was carried out in 25 µl containing 100ng of sample DNA for *NPTII* and 550-1000 ng of sample DNA for *CKI-1*, 1 µl of 10 m*M* dNTPs mix, 2.5 µl of assay buffer for *Taq* polymerase containing 22 m*M* $MgCl_2$; 0.5 units, *Taq* DNA polymerase; 1 µl, each forward and reverse primers. DNA after initial denaturation at 94°C for 5 minutes was subjected to 35 cycles at 94°C for 1 min, 60°C for 45 sec and 72°C for 1.5 min for *NPTII* gene amplification, while for *CKI-1* gene amplification DNA was subjected to 42 cycles at 94°C for 1 min, 64°C for 45 sec and 72°C for 2.3 min. Amplified DNA fragments were electrophoresed on 1.5% agarose gel and observed under UV light.

Dot blot assay—Genomic DNA (5 µg) from PCR positive transformed plants and DNA of plasmid pMON530 were blotted on to nylon membrane (Hybond N^+ Amersham pharmacia) and hybridized with a labeled *CKI-1* gene probe, washed and detected as per the manufacturer's instructions of AlkPhos direct labeling and detection kit (Amersham Pharmacia Biotech UK Ltd). The *CKI-1* gene probe was prepared by amplifying it from the plasmid using the primers as mentioned above. The PCR product was then labelled and used as a probe.

Cytokinin analysis—The plant samples (1.0 g) in triplicate were extracted in 80% chilled methanol. After filtration, the filtrate was evaporated in vacuo at 35°C. The residue was dissolved in distilled water and *p*H was adjusted to 3.0. The acidic extract was partitioned thrice with chilled diethyl ether, and ether phase was discarded. The *p*H of aqueous phase was adjusted to 8.2 and partitioned thrice against water saturated *n*-butanol. The butanol phase was evaporated in vacuo at 35°C and residue was taken up in 1.0 ml of sample buffer [20 m*M*, tris (*p*H 7.5) containing 5 m*M* $MgCl_2$; and 20 m*M*, NaN_3] for cytokinin (zeatin riboside and dihydrozeatin riboside) estimation by ELISA using laboratory raised polyclonal antibodies.

Gene expression analysis—CKI-1 gene expression was analysed through Relative Quantitative RT-PCR. *CKI-1* gene expression was studied in three transformants of tobacco and tomato plants regenerated on inductive and non-inductive medium upon transformation with *CKI-1* gene. Each plant served as a replicate. It was also studied in three plants regenerated from cotyledon explants of T1 transgenic tomato.

Relative quantitative RT-PCR for gene expression in transformants—Relative RT-PCR provides an estimate of the relative changes in gene expression between samples. The RNA samples (2.5 µg) were reverse transcribed to obtain cDNA using random primers by RETRO script reverse transcription PCR of Ambion as per manufacturers instructions in a final volume of 20 µl. Gene expression analysis for *CKI-1* was carried out through relative RT-PCR (Multiplex RT-PCR) using Quantum RNA 18S internal standards kit provided by Ambion. Relative quantitative RT-PCR for the genes was done using 18S primers: competimers in a 3:7 ratio and *CKI-1* gene specific primers in a multiplex RT-PCR. *CKI-1* specific primers designed for quantitative RT-PCR could amplify a product of 182 bp fragment of the *CKI-1* gene. The bands obtained were quantified using SynGene (A division of Synoptic group, Cambridge, England) software based on the intensity of the ethidium bromide fluorescence. For each sample the signal obtained for the gene specific amplicon was divided by the signal obtained for the 18S amplicon. This yielded a corrected relative value for the gene specific product in each sample. These values have been compared between samples for an estimate of the relative expression of the gene in the various samples.

Statistical analysis—The leaf discs of tobacco and cotyledons of tomato from shoots raised *in vitro* and 8-10 day old seedlings were used as explants respectively for experiments on transformation with reporter gene (control vector) and *CKI-1* gene. Leaf discs (77-92) of tobacco or 58-75 cotyledon explants of tomato were used in different transformations. Leaf disc (4-5) per bottle of tobacco or 6-8 explants of tomato per 50 ml medium served as an experimental unit with 15-20 bottles or 9-10 bottles per experiment for tobacco and tomato, respectively. A group of 4-7 or 3-4 bottles in tobacco or tomato, respectively constituted a replicate. For cytokinin analysis, the plant samples (1.0 g) in triplicate were taken. *CKI-1* gene expression was carried out in three transformants from different treatments with each plant serving as a replicate. The experiments were laid in completely randomized design with 3 replicates. The data were subjected to analysis of variance (ANOVA) after subjecting to two way interaction. Comparison among treatment means were carried out using LSD values and are reported under "CD" at the end of each Table.

Results and Discussion

In an effort to develop marker free transgenic plants, use of *CKI-1* gene as a positive selectable marker has been advocated[4] as the use of *ipt* and *Kn* genes have been associated with developmental abnormalities associated with elevated levels of cytokinins especially in case of *ipt* gene. On the other hand, use of *CKI-1* gene, a histidine kinase molecule implicated in cytokinin signal transduction pathway is thought to be a more efficient and reliable method for developing marker free transgenic plants, as free migration of cytokinin is unlikely to occur for molecules that are specially localized within the cell. *CKI-1* gene has been identified by its ability to confer cytokinin idependent callus growth when

overexpressed in *Arabidopsis*[12]. The phenotype of *CKI-1* overexpressing mutant implicates an important role of *CKI-1* or similar histidine kinase in the regulation of cell proliferation and /or division. *CKI-1* gene was therefore considered as a suitable candidate to evaluate its role as a positive selectable marker for development of transgenic plants through overexpression in tobacco and tomato.

Transformation of tobacco leaf discs with *CKI-1* gene promoted shoot regeneration under non-inductive conditions (in the absence of PGRs or exogenous cytokinins) both in the presence or absence of selectable marker kanamycin with 88% of cultures (2-3 shoots of 0.5-1.0 cm/explant) giving rise to shoot regeneration under kanamycin selection as against complete absence of shoot initiation upon transformation with control vector (reporter gene) under the same conditions (Fig. 1a). Transformation with *CKI-1* gene under inductive conditions resulted in profuse shoot regeneration in 100% of tobacco explants accompanied with regeneration of elongated shoots (2-10 shoots of 0.5-4.0 cm/explant) as against 62% explants showing shoot bud regeneration without any elongation when transformed with control vector (Fig. 1b). Thus, there was significant (5×) enhancement in the transformation frequency when *CKI-1* transformed leaf discs were incubated in the presence of cytokinin (BAP) under inductive conditions (Table 1). The observations were recorded regularly after every 2 weeks of co-cultivation with

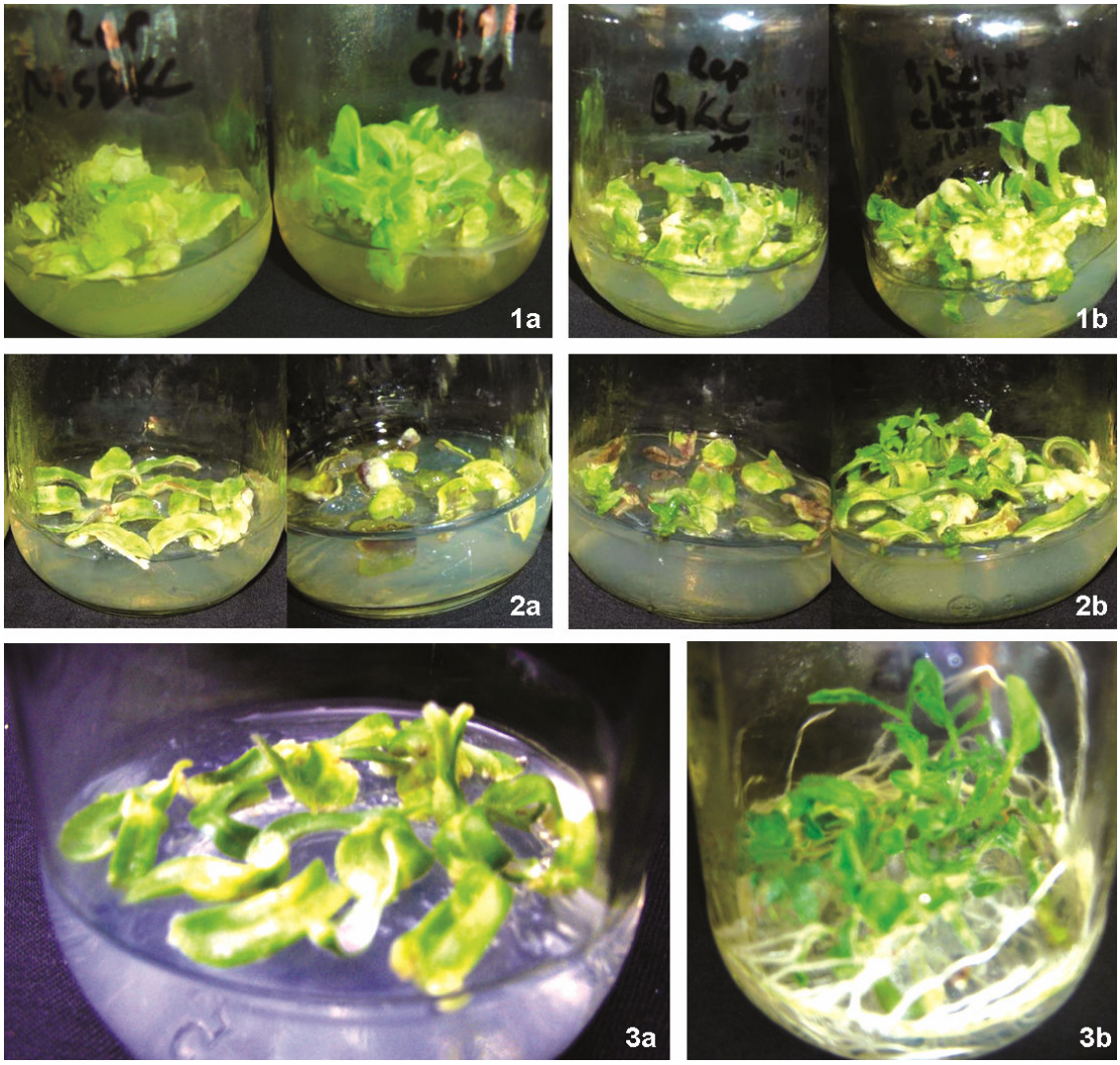

Fig. 1—Influence of reporter gene (LHS bottle) and *CKI-1* gene (RHS bottle) in tobacco on shoot regeneration under (a) non-inductive and (b) inductive (shoot regeneration) conditions. Fig.2—Influence of *CKI-1* gene on tomato shoot regeneration under (a) non-inductive and (b) inductive (shoot regeneration) conditions.Fig. 3—Response of cotyledon explants of (a) untransformed; and (b) T1 transgenic tomato with *CKI-1* gene to shoot regeneration on non inductive medium

the gene. The results suggested that *CKI-1* gene played a role in increasing the shoot regeneration ability as well as to confer cytokinin independent shoot regeneration in tobacco. *CKI-1* gene has been identified, through its ability to confer cytokinin independent callus growth[12] and the results with tobacco transformation confirmed this.

In contrast to these observations, transformation of tomato with *CKI-1* gene or control vector (reporter gene) did not give rise to shoot initiation in any of the

Table 1—Transformation frequency as influenced by *CKI-1* gene in the presence or absence of cytokinins under kanamycin selection

Tobacco (B)									**Mean (A)**
Genes (A)	*Media*	*No of explants co-cultivated*	*No of green explants on selection medium*	*No of explants regenerating shoot buds*	*No of elongated shoots*	*No of PCR positive transformants*	*Transformants (%)*	*No of rooted transfromants*	
*Rep (*npt*II) Control vector	Non inductive medium (No hormones /PGR) (- cytokinin)	84	52	Nil	Nil	Nil	Nil	Nil	
**CKI-1*		92	92	81 (88%)	57 (0.7/explant)	6/10 selected plants	34	10 selected plants	
Rep (*npt*II) Control vector	Inductive medium (with hormones/PGR) (+ cytokinin)	77	64	48 (62%)	69 (1.4/explant)	7/10 selected plants	55	10 selected plants	**Rep 28.7**
CKI-1	do	87	87	87 (100%)	420 (4.8/explant)	8/10 selected plants	294	10 selected plants	***CKI-1* 151**
Mean (B)							**174.5**		
Tomato (B)									
*Rep (*npt*II) Control vector	Non inductive medium No PGR (- cytokinin)	58	22	Nil	Nil	Nil	Nil	Nil	
**CKI-1*	do	62	37	Nil	Nil	Nil	Nil	Nil	
Rep (*npt*II) Control vector	Inductive medium with PGR (+ cytokinin)	85	30	10 (12%)	4	2/2 plants tested	2.4	2	
CKI-1		40	28	21 (53%)	10	3/4 plants tested	8.0	4	
Mean (B)							**5.2**		

*- data not utilized for statistical analysis

Response data of tobacco and tomato explants to transformation by *CKI-1* gene under inductive conditions in the presence of cytokinins under kanamycin selection only has been analysed statistically.

CD ($P \leq 0.05$); **A, 27.3; B, 27.3; AxB, 38.6**.

explants cultured under non-inductive conditions, both in the presence or absence of selectable marker kanamycin (Fig. 2a). However, under inductive conditions in the presence of BAP and IAA transformation with control vector and *CKI-1* gene resulted in regeneration of transformants to the extent of 2.1 and 8.0%, respectively (Fig. 2b and Table 1). As the results were in total contrast to that of tobacco, it was decided to assess the response of the explants from the next generation (T1) *CKI-1* transgenic seedlings. Shoot regeneration from 38-42% cotyledon explants of transgenic T1 seedlings was observed when cultured under non-inductive conditions (in the absence of PGR or exogenous cytokinins) (Fig. 3b).

Evidence for the presence of transgene was confirmed indirectly by amplification of the *NPTII* gene and directly by using primer specific to *CKI-1* gene. Ten randomly chosen putative transformants of tobacco with *CKI-1* gene (regenerated under inductive and non inductive conditions) and control vector with *NPTII* gene (regenerated under inductive condition) were screened and plants that tested positive for *CKI-1* and *NPTII* gene through PCR amplification were used for further analysis (Table 1, Gel pic. not included). In tomato, out of 4 putative transformed plants with *CKI-1* gene tested for *NPTII* amplification, 3 plants showed bands of expected size of 750 bp for the *NPTII* fragment. These three *NPTII* positive plants were confirmed for *CKI-1* gene and all the three amplified 3600 bp for the *CKI-1* gene at the same position as that of the positive control (plasmid DNA), and these transformed shoots tested positive in dot blot assay as well, while there was no amplification in control and non transformed plants (Figs 4-6). Tomato transformants with control vector also tested positive for PCR amplification of *NPTII*.

The results of transformation experiments with *CKI-1* gene in tobacco and tomato demonstrated that the ability to promote cytokinin independent growth varied with the species. Hence, a detailed analysis of the role of *CKI-1* gene in these two crop species was studied in relation to *CKI-1* gene expression and cytokinin content.

In primary tobacco transformants (with *CKI-1* gene) obtained under non-inductive conditions in PGR/cytokinin free medium, *CKI-1* gene expression was 1.4× higher than that of control, while it was 4 × higher than that of control plants under inductive conditions (medium with PGR/cytokinin; Table 2). The cytokinin content in tobacco plants transformed with *CKI-1* gene under non-inductive and inductive conditions was significantly higher (1.6× and 2.6× times, respectively) than that of control. The gene expression patterns when compared with cytokinin content in the control vs transformants revealed a positive correlation as suggested by Sugiyama[16] that the competence for shoot organogenesis may directly reflect the activity level of the *CKI-1* gene. Further, tobacco plants transformed with the control vector under inductive condition recorded equivalent cytokinin (112.53 ng total cytokinin/g tissue) as that of *CKI-1* transformant regenerated in the absence of cytokinin/PGR (110.18; non-inductive medium; Table 3). This further confirmed that *CKI-1* gene does have a role in regulating the cytokinin level and also that *CKI-1* gene conferred the ability on the expressing cells to sense low concentration of endogenous cytokinins that is normally unable to trigger growth and shoot formation[12,16-18]. This ability of the *CKI-1* gene is exhibited through induction of shoot regeneration in non-inductive medium in the absence of cytokinin/PGR in tobacco explants at low

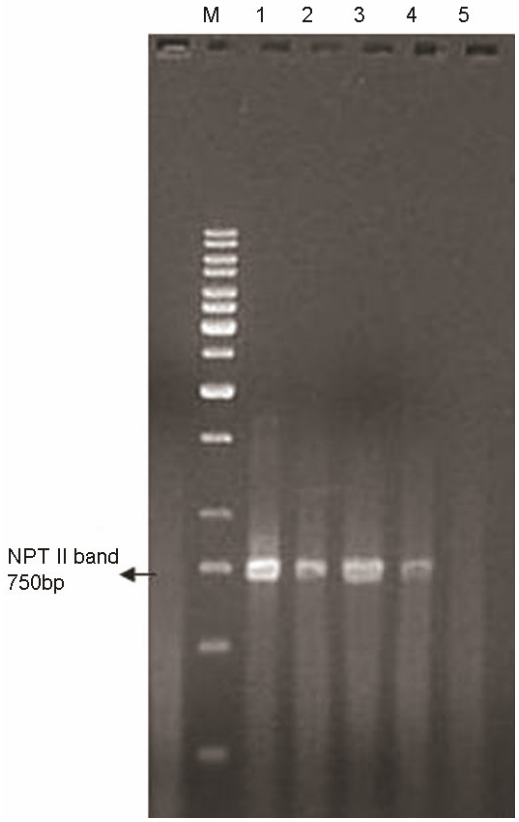

Fig. 4—NPT II gene amplification [Lane 1-positive control; Lane 2-4 transformed plants of tomato; Lane 5- negative control (untransformed plant of tomato); and Lane M- 1 kb ladder]

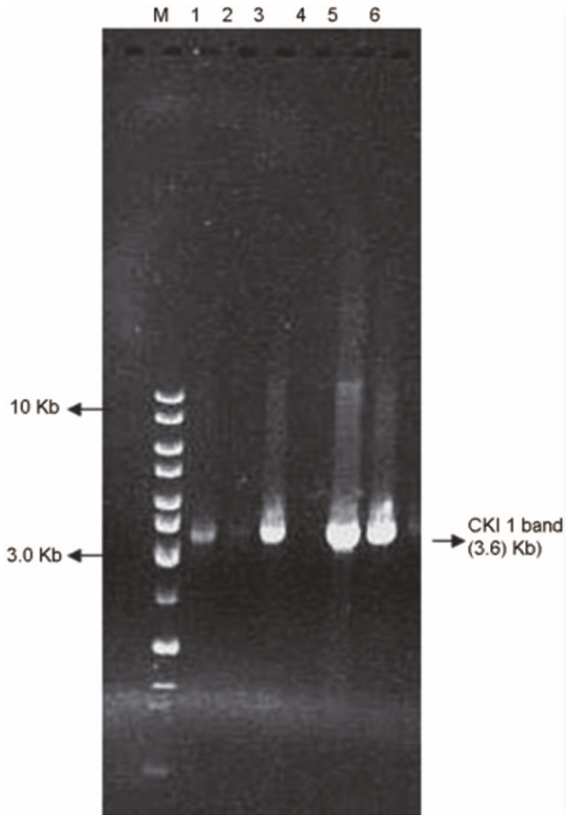

Fig. 5— *CKI-1* gene amplification [Lane 1-positive control; Lane 2- negative control (untransformed plant of tomato); Lane 3-6 transformed plants of tomato; Lane 4- non transformed tomato; and Lane M- 1 kb ladder

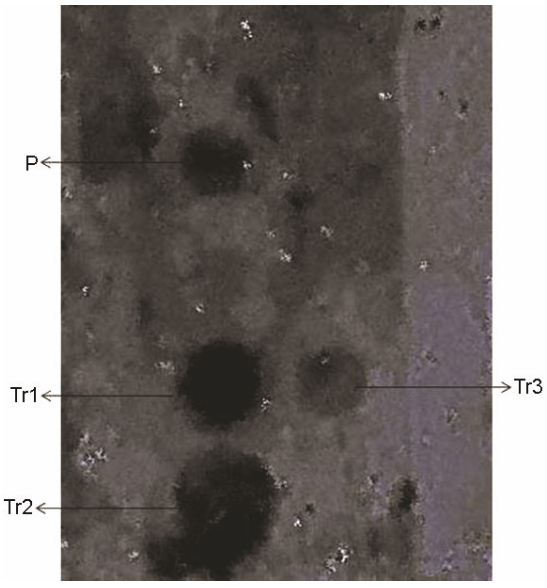

Fig. 6—Dot blot assay of transgenic (Tr1,Tr2,Tr3) tomato plants and plasmid pMON530 (P). Membrane probed with Alk Phos labelled *CKI-1* gene PCR fragment

endogenous concentration of cytokinin. At the same endogenous levels of cytokinin, transformation with control vector was unable to bring about shoot regeneration.

In primary tomato transformants with *CKI-1* gene obtained under inductive conditions (medium with PGR/cytokinin), the gene expression did not vary from that of the control while there was significant increase in the gene expression (1.2×) in the plants derived from T1 seedling cotyledon explants under non-inductive condition (PGR/cytokinin free medium) (Table 2). T0 transformants obtained upon culture in cytokinin medium (inductive conditions) as well as plants derived from T1 seedling cotyledon explants under non-inductive condition exhibited higher cytokinin content (142.22 and 83.34 ng total

Table 2—*CKI-1* gene expression (182 bp fragment) in untransformed control vs transformed tobacco and tomato [values are means of 3 replications]

Plant sample	*CKI-1* gene expression Quantitative Ratio (*CKI-1*/18S)
Untransformed tobacco regenerated on cytokinin BAP (1.0 mg/l) (regenerating/inductive) medium	0.5
T0 Tobacco transformant with *CKI-1* regenerated on MSB cytokinin free (non regenerating/noninductive)medium	0.68
T0 Tobacco transformant with *CKI-1* regenerated on cytokinin BAP (1.0 mg/l) (regenerating/inductive) medium	2.0
Tomato control (untransformed plant) regenerated on cytokinin (BAP 2.0 mg/l and IAA 0.1 mg/l) (regenerating/inductive medium)	0.73
T0 Tomato transformant with *CKI-1* regenerated on cytokinin (BAP 2.0 mg/l and IAA 0.1 mg/l) (regenerating/inductive) medium	0.71
T0 tomato transformant with *CKI-1* regenerated on MSB cytokinin free (non regenerating/noninductive)medium	No regeneration
T1 Tomato transformant with *CKI-1* regenerated on cytokinin (BAP 2.0 mg/l and IAA 0.1 mg/l) (regenerating/inductive) medium	0.84
CD ($P \leq 0.05$); **0.11**	

cytokinin/g tissue) than the untransformed counterparts (63.42 ng/g tissue) suggesting that the *CKI-1* gene might also be regulating the endogenous cytokinin content (Table 3), as it is thought to encode a constitutively active histidine kinase connected to cytokinin signal transduction pathway and thereby, regulating cytokinin levels[17]. However, the cytokinin content of T0 tomato plants derived from cytokinin medium (inductive conditions) was significantly higher (142.22 ng total cytokinin/g tissue) than that of plants regenerating from cotyledon explants of T1 transgenic plants (83.34 ng total cytokinin/g tissue) under non-inductive conditions (cytokinin free medium).

Absence of shoot regeneration in tomato upon transformation with *CKI-1* gene in medium devoid of external cytokinin suggested that *CKI-1* gene might not be able to alter the cytokinin levels to an extent so as to bring about shoot regeneration.

Lack of induction in gene expression in tomato transformants (T0) obtained under inductive conditions (Table 2, medium with PGR/cytokinin) might be due to the feedback inhibition effected by the activation of the negative regulators (i.e. the repressor type of response regulator proteins) in the presence of high endogenous cytokinin content (142.22 ng total cytokinin/g tissue or 2.2× that of control) (Table 3) contributed both by external 2.0 mg/l BAP and endogenous cytokinin[17]. Low cytokinin content of 83.34 ng total cytokinin/g tissue in plants regenerated from T1 seedling cotyledons on non-inductive or cytokinin free medium enhanced the gene expression and enabled to induce shoot regeneration. At same endogenous concentration of cytokinin T1 explants of tomato transformed with control vector was unable to bring about shoot regeneration.

This suggested that unlike in *Arabidopsis*, in tomato the level of cytokinin required to trigger *CKI-1* expression to the desired level was higher (which has to be supplemented by an external source), that is why there was no regeneration in tomato in the absence of cytokinin even upon transformation with *CKI-1* gene. However, lack of *CKI-1* gene expression in T0 transgenic *CKI-1* plants regenerated in the presence of 2.0 mgL^{-1}BAP in the inductive medium might be attributed to the feedback inhibition caused by the high cytokinin content (2.0 mgL^{-1}BAP). This

Table 3—Cytokinin content in tomato (T0 and T1) and tobacco plants transformed with *CKI-1* gene and reporter gene (control vector) under inductive and non-inductive conditions

Treatment	Cytokinin (ng/g tissue)		
	ZR	DHZR	Total
Tobacco control (untransformed plant) regenerated on cytokinin BAP (1.0 mg/l) medium (regenerating/inductive) medium	28.7	39.97	68.67
T0 Tobacco transformant with reporter gene regenerated on MSB cytokinin free (non regenerating/non-inductive) medium	No regeneration		
T0 Tobacco transformant with reporter gene regenerated on cytokinin BAP (1.0 mg/l) medium (regenerating/inductive medium)	52.69	59.84	112.53
T0 Tobacco transformant with *CKI-1* regenerated on MSB cytokinin free (non regenerating/non-inductive) medium	50.57	59.61	110.18
T0 Tobacco transformant with *CKI-1* regenerated on cytokinin BAP (1.0 mg/l) medium (regenerating/inductive medium)	75.0	105.24	180.24
Tomato control (untransformed plant) regenerated on cytokinin (BAP 2.0 mg/l and IAA 0.1 mg/l) (regenerating/inductive medium)	35.97	27.45	63.42
T0 Tomato transformant with *CKI-1* regenerated on MSB cytokinin free (non regenerating/non-inductive) medium	No regeneration		
T0 Tomato transformant with *CKI-1* regenerated on cytokinin (BAP 2.0 mg/l and IAA 0.1 mg/l) (regenerating/inductive) medium	62.56	79.66	142.22
T0 Tomato transformant with reporter gene regenerated on cytokinin (BAP 2.0 mg/l and IAA 0.1 mg/l) (regenerating/inductive) medium	32.56	49.08	81.64
T1 Tomato transformant with reporter gene regenerated on cytokinin (BAP 2.0 mg/l and IAA 0.1 mg/l) (regenerating/inductive) medium	35.63	43.63	79.26
T1 Tomato transformant with *CKI-1* regenerated on MSB cytokinin free (non regenerating/non-inductive) medium	54.89	28.45	83.34
CD ($P \leq 0.05$); 37.42			

ZR-zeatin riboside; DHZR- dihydro zeatin riboside

suggested that there might be a threshold level of cytokinin which might be defined as the concentration of cytokinin beyond which gene expression is not enhanced due to feedback inhibition. However, in the following generation (T1) under cytokinin free medium, once the level of cytokinin below the threshold for feedback inhibition is achieved, regeneration was obtained in T1 *CKI-1* seedling explants. Such a mechanism has been proposed by Glover *et al*[18] who have stated that *CKI-1*, as part of the mechanism for sensing levels of cytokinin, may act in a feedback loop that contributes to maintaining homeostatically the level of active cytokinin. Thus *CKI-1* overexpressing plants are expected to be more sensitive to cytokinin levels and the mechanism of upregulating ctyokinin levels will be activated at lower concentration of cytokinins than in the wild type plants.

Recent advances in cytokinin signaling reveals that every step in signal transduction pathway is executed by components encoded by multigene families with new functions, new feedback loops and connections to other signaling pathways. The quest for determining a common denominator in the seemingly diverse cytokinin responses suggests the possibility of different cytokinin threshold levels required for different responses[18,19].

In conclusion, the results suggested that *CKI-1* gene expression appeared to be influenced by the endogenous cytokinin levels (regulated due to external or internal supply via *CKI-1* gene) within a certain range up to which, the gene expression was induced. The maximum limit in the range has been defined as the threshold level. Beyond this level, the gene expression remained same or reduced. With incorporation of *CKI-1* gene, this threshold level might be reduced in crops like tomato but cannot be made independent of cytokinin as was possible in *Arabidopsis* or tobacco. The present results of the study suggest that *CKI-1* gene cannot be used universally across species as a positive selectable marker gene for *Agrobacterium* mediated transformation as its function does not seem to be independent of cytokinin. Eventhough the role of *CKI-1* gene is not very clear, the results of our study implicate its role both as a cytokinin receptor as well as positive regulator of signal transduction through its role in regulating cytokinin levels and contributing to sensitivity to cytokinin.

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