Growth, yield and shelf-life of isopentenyltransferase (*ipt*)-gene transformed broccoli

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Chan, L. F., Chen, L. F. O., Lu, H. Y., Lin, C. H., Huang, H. C., Ting, M. Y., Chang, Y. M., Lin, C. Y. and Wu, M. T. 2009. **Growth, yield and shelf-life of isopentenyltransferase (*ipt*)-gene transformed broccoli**. Can. J. Plant Sci. **89**: 701–711. Loss of chlorophyll leading to floret yellowing limits the post-harvest lifespan of broccoli (*Brassica oleracea* L. var. *italica* Plenck). Cytokinins are known to delay floral yellowing of plants. A transgene construct pSG766A, which results in the expression of isopentenyltransferase (*ipt*), the key enzyme for cytokinin synthesis, has been developed in broccoli. Expression of the *ipt* transgene is triggered by the senescence-associated gene promoter (SAG-13). Three selfed T_5 lines of *ipt* transformed broccoli (lines 101, 102 and 103) have been obtained through selection for single copy insertion, acceptable horticultural traits and transgene *ipt* activity. These three transgenic inbred lines were evaluated in the field during 2004–2007 to determine their growth, yield and shelf-life after harvest, relative to a non-transgenic inbred line (104) and the parental variety Green King. For most of the vegetative growth parameters measured, year-to-year variability exceeded line-to-line variability. Inbreeding had little impact on the appearance or yield potential of the broccoli lines. Head yields of the transgenic inbred lines 102 and 103 were comparable to the parental variety Green King, but were significantly higher than the non-transgenic inbred line 104, as lines 102 and 103 produced more plants with heavier flower heads. Cytokinin content in the form of isopentenyladenosine was relatively higher in the transgenic lines than in the two non-transgenic controls. When flower heads were stored at $25 \pm 2°C$, the period required to cause 50% floret yellowing was 7.5 and 8.5 d for the transgenic lines 102 and 103, respectively, compared with 5.6 d for the non-transgenic line 104, and 5.1 d for the parental variety Green King. This study showed that the *ipt*-transformed inbred lines of broccoli combined acceptable appearance and yields with enhanced shelf-life.

Key words: *Brassica oleracea* L. var. *italica* Plenck, transgenic broccoli, isopentenyltransferase gene, genetic characterization, shelf-life

Chan, L. F., Chen, L. F. O., Lu, H. Y., Lin, C. H., Huang, H. C., Ting, M. Y., Chang, Y. M., Lin, C. Y. et Wu, M. T. 2009. **Croissance, rendement et durée de conservation du brocoli portant le transgène de l'isopenténylransférase (*ipt*)**. Can. J. Plant Sci. **89**: 701–711. La perte de chlorophylle responsable du jaunissement des fleurons diminue la durée de conservation après récolte du brocoli (*Brassica oleracea* L. var. *italica* Plenck). On sait que les cytokinines retardent le jaunissement des fleurs chez les plantes. Par ailleurs, on a créé le transgène pSG766A, qui exprime l'isopenténylransférase (*ipt*), principal enzyme responsable de la synthèse des cytokinines chez le brocoli. L'expression de ce gène est déclenchée par le promoteur SAG-13, associé à la sénescence. Les auteurs ont obtenu trois lignées T_5 autofécondées de brocoli portant le transgène *ipt* (lignées 101, 102 et 103) en sélectionnant les plants pour l'insertion d'une copie unique du transgène, des caractères horticoles acceptables et l'activité du transgène. Les trois lignées autogames ont ensuite été évaluées au champ de 2004 à 2007 en vue d'en préciser la croissance, le rendement et la durée de conservation après récolte, comparativement à une lignée autogame non transgénique (104) et à la variété parentale Green King. La variabilité de la plupart des paramètres de la croissance végétative mesurés d'une année à l'autre dépasse la variabilité observée entre les lignées. L'autofécondation agit peu sur l'aspect ou le rendement potentiel des lignées de brocoli. Le nombre de pommes des lignées transgéniques autogames 102 et 103 est comparable à celui de la variété parentale Green King, mais il est sensiblement plus élevé que celui de la variété autogame non transgénique 104, les lignées 102 et 103 donnant plus de plants à pomme plus lourde. La concentration de cytokinines sous forme d'isopenténylad énosine est relativement plus élevée chez les lignées transgéniques

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Abbreviations: GK, Green King; ***ipt*,** isopentenyltransferase; **LL,** leaf length; **LN,** leaf number; **LW,** leaf width; **PEL,** petiole length; **PEW,** petiole width; **PLH,** plant height; **PLW,** plant width; **SH,** stem height; **SW,** stem width; **SAG,** senescence associated gene

que chez les témoins non transgéniques. Quand on entrepose les pommes à 25 ± 2°C, il faut respectivement 7,5 et 8,5 jours pour que 50 % des fleurons des lignées transgéniques 102 et 103 jaunissent, contre 5,6 jours pour la lignée non transgénique 104 et 5,1 jours pour la variété parentale Green King. Cette étude révèle que les lignées autogames de brocoli portant le transgène *ipt* of combinent un aspect et un rendement acceptables à une durée de conservation plus longue.

Mots clés: *Brassica oleracea* L. var. *italica* Plenck, brocoli transgénique, gène de l'isopenténytransférase, caractérisation génétique, durée de conservation

Loss of chlorophyll leading to floret yellowing limits the post-harvest lifespan of broccoli (*Brassica oleracea* L. var. *italica* Plenck) (Barth et al. 1992; Coupe et al. 2003; Tsai 2006). Numerous reports (Barth et al. 1993; Tian et al. 1994; Henzi et al. 2000; Artés et al. 2001; Wu and Li 2003; Wang et al. 2004a,b) indicate that increased ethylene synthesis in the harvested florets leads to the destruction of chlorophyll, revealing the underlying yellow pigments. Storage temperature influences the rate of floret yellowing and the corresponding shelf-life of broccoli (Barth et al. 1993; Ku and Willis 1999). A shelf-life of 3 to 4 wk was achieved for flower heads stored at 0°C, but shelf-life was only 2 to 3 d at 20°C (Jiang and Yu 2004).

Cytokinins play an important role in deferring leaf and floret senescence. External application of cytokinin can delay floret yellowing of broccoli (Rushing 1990; Clark et al. 1994). The isopentenyltransferase (*ipt*) gene associated with cytokinin biosynthesis has been cloned from a tumor-inducing plasmid carried by *Agrobacterium tumefaciens*. This gene has been coupled with different promoters to elevate the endogenous cytokinins levels in plant cells (Smigocki and Owens 1988; Smart et al. 1991). *Ipt* transformed plants show altered morphology, stunting, loss of apical dominance, reduction in root initiation, overgrowth of tissues and organs (Li et al. 1992; Ainley et al. 1993), increased tolerance to exogenous auxin and auxin transport inhibitors (Li et al. 1994), and enhanced resistance to insect pests (Smigocki et al. 1993). Gan and Amasino (1995) proposed that regulation of the *ipt*-gene with the senescence associated gene (SAG) promoter could minimize the adverse effects of over-expression of the *ipt*-gene during the vegetative growth phase.

The agrobacterial cytokinin synthetase (isopentenyltransferase, *ipt*) gene driven by the senescence-associated gene promoter (SAG12 or SAG13) has been introduced into broccoli (cv. Green King, a F_1 hybrid variety) via *A. tumefaciens* mediated transformation (Chen et al. 2001). Some of the resulting transgenic lines showed a significant delay in yellowing and senescence of harvested flower heads and leaves (Chen et al. 2001). Since the original transformed parental line was a hybrid, further development of inbred lines and fixation of the transgenes was required. Pure lines of the *ipt*-gene transformants were created by selfing the transgenic lines.

The objective of this study was to evaluate the field performance and shelf-life of selected *ipt*-gene transformed post-T_5 inbred broccoli lines.

MATERIALS AND METHODS

Plant Materials

The original T_0 broccoli plants were obtained by *A. tumefaciens* mediated transformation of the F_1 hybrid variety Green King with pSG766A, a chimeric construct, (a gift of Dr. R. M. Amasino, University of Wisconsin, Madison, WI). The *ipt*-gene was driven by the senescence-associated gene promoter (SAG-13). The kanamycin resistant *npt*II with a 35S promoter was used as a selectable marker (Chen et al. 2001).

This study evaluated the field performance and post-harvest lifespan of three *ipt*-gene transformed inbred lines (101, 102 and 103), one non-transgenic inbred line 104 and the non-transformed parental variety Green King (GK). The three transgenic inbred lines were obtained after five generations of selfing *ipt* transgenic GK, with continuous selection for transgene expression and desirable agronomic characteristics such as plant vigor, acceptable appearance and enhanced shelf-life. Lines 101 and 103 were derived from the same T_1 progeny planted on two separated dates. The non-transgenic inbred line 104 represented the 5th generation of selfed plants from the non-transgenic parental variety GK.

Molecular Identification and Quantification of Transgene Expression

For each transgenic inbred line, at least 20 randomly selected individuals were confirmed by PCR for homozygousity of the *ipt* and *npt*II transgenes. The primer sets for *ipt* and *npt*II and the amplification conditions were as described by Chen et al. (2001). Transgene copy numbers were determined by Southern analyses using a single digestion with a restriction enzyme targeted outside the transgene. For Southern hybridization, 20 μg of total genomic DNA from each plant was digested with either *Eco*RI, *Bam*H1 or *Xba*I (3–4U μg^{-1}DNA) and blotted onto a positively charged nylon membrane (Roche, Manheim, Germany). *Ipt* PCR DIG labeled probe was used in the hybridization (Chen et al. 2001). Transgene copy number was further confirmed by quantitative PCR (data not included). The total genomic DNA was isolated according to Chen et al. (2004).

Expression levels of the *ipt* transgene in broccoli heads stored at 25°C were determined using Northern analysis. The RNeasy Plant Mini Kit (QIAGEN Inc., USA) was used for total RNA extraction from the tissue samples and 10 μg for each sample was loaded for electrophoresis and blotted onto the nylon membrane.

ELISA methods OLCHEMIM Enzyme Immunoassay Kits (Olomouc, Czech Republic) were used to determine the isopentenyladenosine, trans-zeatin riboside and dihydrozeatim riboside content of broccoli florets harvested from the transgenic and non-transgenic control lines. Cytokinins were extracted by grinding the frozen plant tissues to a fine powder and then extracting with ice-cold 80% methanol (10 mL g^{-1} FW) containing sodium diethyldithiocarbamate as an antioxidant (400 μg g^{-1} FW). The (2-^{3}H) cytokinin tracers were added to the extracts to monitor for losses during purification and to validate the chromatographic data. The extracts were then purified over a reversed phase C^{18} column to eliminate chlorophyll and lipids. The extracts were concentrated and diluted with 40 mM ammonium acetate buffer (PH 6.5) containing 5 mM sodium diethyldithiocarbamate and incubated with 0.05 U mL^{-1} wheat germ acid phosphatase for 30 min in the dark (25°C) to dephosphorylate the cytokinin 5′ phosphates. For the immunoassay dilution analysis, elutes were vacuum dried and then re-dissolved in *tris*-buffered saline. Aliquots of these solutions were either analyzed in serial dilutions or mixed with known amounts of cytokinin standards and then analyzed by ELISA. Three replications were used for each line and sampling stage.

Field Experiments

Field experiments were conducted from 2004 to 2007 in an isolated field at Taiwan Agricultural Research Institute (TARI), Wufeng, Taichung, Taiwan. In each year, 3-wk-old greenhouse-grown seedlings with about four to five leaves were transplanted into the field in November. The plot size was 10 m × 1.5 m. There were two rows of each line in each plot, with 60 cm between rows, 45 cm between plants, and 20 plants per row. In each year the three transgenic inbred lines 101, 102 and 103; one non-transgenic inbred line (104); and the parental F_1 hybrid variety GK were evaluated. The five lines were arranged in randomized complete block design (RCBD) with three replicates.

Prior to transplanting, N, P_2O_5 and K_2O fertilizers were applied to the test plot at 270, 150, and 200 kg ha^{-1}, respectively. Insecticides (Mevinphos 10% EC and Methomyl 24% WP; Sinon Corporation Ltd., Shi Chiu, Taichung, Taiwan) were applied at 10-d intervals from the seedling stage to the formation of flower buds to control cabbage worms (*Pieris rapae* L.) and aphids (*Brevicoryne brassicae* L.) (Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture 2000).

Data Collection

At 45 d after transplanting and prior to the formation of flower buds, 10 randomly selected plants from each replicate were evaluated for plant height (PLH) and width (PLW), stem height (SH) and width (SW), petiole length (PEL) and width (PEW), leaf length (LL) and width (LW) and leaf number (LN). The largest leaf on the plant was measured for PEL, PEW, LL and LW. At 12 wk after transplanting, flower heads of 20 randomly selected plants from each plot were harvested and measured for head diameter (FD), head height [with stem, FH(S) or without stem, FH], head weight [with stem, FW(S) or without stem, FW], stem length (FSL) and width (FSW).

Freshly harvested heads were wrapped individually using saran wrap (Nan Ya Plastics Corporation, Taipei, Taiwan) and tested for shelf-life by storing at 25±2°C with 90–95% relative humidity (Artés et al. 2001). Such storage conditions mimic conditions encountered during the retail sale of broccoli. The heads were scored individually for senescence and yellowing of florets using two visual scales: (1) days to reach 10% yellowing of florets and (2) days to reach 50% yellowing of florets.

Statistical Analyses

Data were analyzed separately each year, and then pooled for a combined analysis of variance (ANOVA) to test for year effects and interactions between years and treatments (broccoli lines). Where significant differences occurred, the means were separated by a least significant difference (LSD) test at $P=0.05$ level. All the statistical analyses were conducted using PC Version 9.1 of SAS (SAS Institute, Inc. 2004).

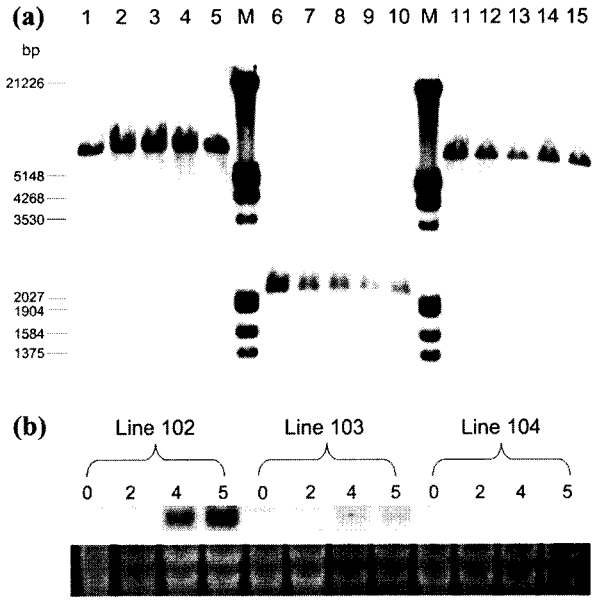

Fig. 1. Molecular characterization of *ipt* transgenic inbred broccoli lines. (a) Southern hybridization of lines 101 (lane 1–5), 102 (lane 6–10) and 103 (lane 11–15) by *Xba* digestion with PCR DIG labeled probe; (b) Northern analysis of lines 102 and 103 and the non-transgenic inbred line 104 at 0, 2, 4 and 5 d after harvest.

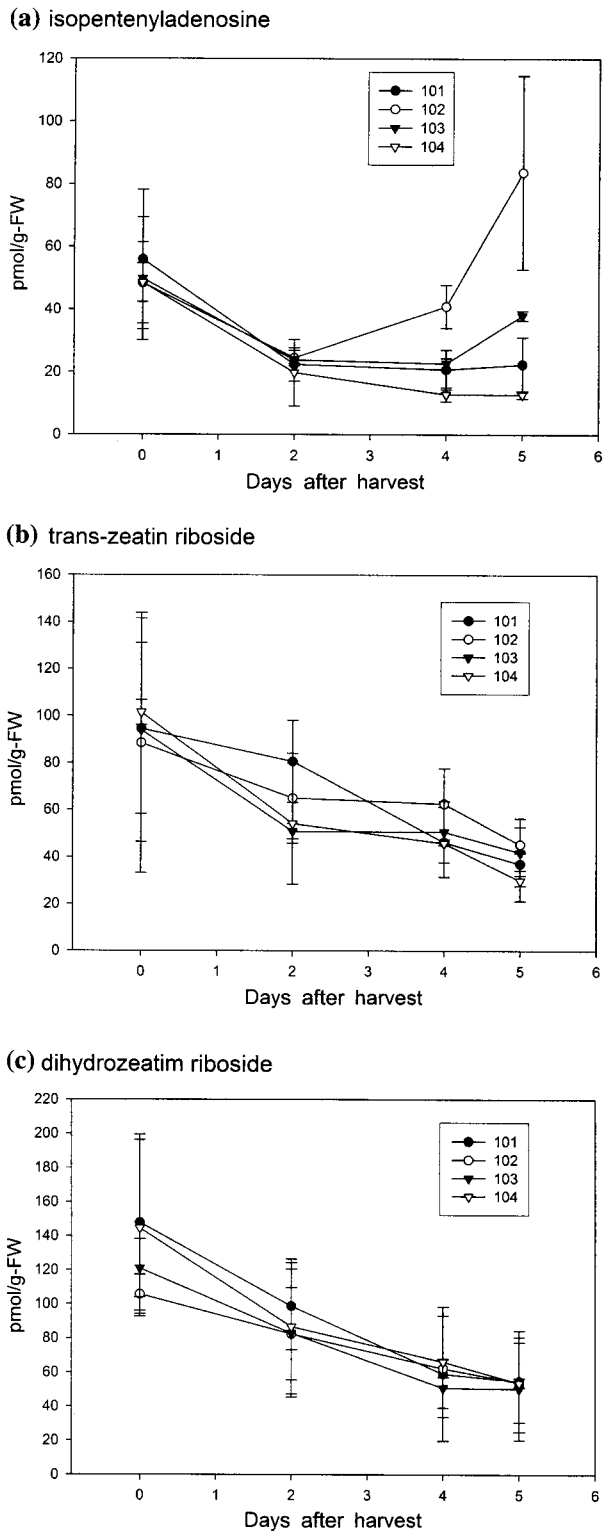

Fig. 2. Determination of cytokinin levels in the florets of *ipt* transgenic inbred broccoli lines at 0, 2, 4 and 5 d after harvest. (a) isopentenyladenosine (b) trans-zeatin riboside and (c) dihydrozeatin riboside. Vertical bars (I) indicate standard errors.

Table 1. Mean squares of ANOVA for morphological traits of three *ipt*-transformed lines, one non-transformed line and the parental variety of broccoli in field trials conducted from 2004 to 2007

		Mean squares							
Source	df	Plant height	Plant width	Stem height	Stem width	Petiole length	Petiole width	Leaf length	Leaf width	Leaf number
Year	3	346.7**	114.2**	351.1**	0.23*	54.1**	0.034*	76.7 **	28.2**	53.9**
Block within years	8	4.8	14.9	0.3	0.04	1.5	0.009	0.6	0.7	0.3
Line	4	105.8*	393.1*	26.0**	0.24	38.0**	0.052	146.9**	30.9**	4.1
Year × Line	12	20.4**	112.3**	1.0	0.12**	7.2**	0.026**	25.1**	5.2**	1.7**
Error	59	6.5	12.6	0.7	0.02	1.2	0.004	1.8	0.3	0.2

*,** Significant F-test at the 0.05 and 0.01 probability levels, respectively.

Table 2. Morphological traits of three *ipt*-transformed lines, one non-transformed line and the parental variety of broccoli in field trials conducted from 2004 to 2007

Year	Line[z]	Plant height (cm)	Plant width (cm)	Stem height (cm)	Stem width (cm)	Petiole length (cm)	Petiole width (cm)	Leaf length (cm)	Leaf width (cm)	Leaf number
2004	101	33.8*abc*	73.5*a*	12.1*c*	3.25*a*	15.0*a*	1.32*a*	41.7*a*	18.8*a*	15.1*a*
	102	40.2*a*	74.4*a*	15.1*a*	2.82*ab*	15.2*a*	1.12*b*	39.1*ab*	18.5*ab*	14.7*a*
	103	28.1*c*	56.5*b*	10.1*d*	2.55*b*	10.1*b*	1.09*b*	32.8*c*	17.4*c*	13.9*b*
	104	36.2*ab*	73.4*a*	13.7*b*	2.64*b*	14.7*a*	1.15*b*	41.7*a*	16.5*d*	14.7*a*
	GK	31.9*bc*	64.0*ab*	12.5*bc*	2.75*b*	12.9*ab*	1.08*b*	38.1*b*	17.8*bc*	13.6*b*
2005	101	42.0*b*	61.6*e*	22.5*cd*	2.90*c*	12.6*c*	1.25*ac*	38.0*c*	17.1*c*	17.0*a*
	102	48.4*a*	78.4*b*	25.3*a*	3.23*a*	14.9*b*	1.30*b*	44.1*b*	21.4*b*	17.2*a*
	103	42.7*b*	67.3*d*	21.8*d*	3.06*b*	12.2 *c*	1.23*c*	40.1*c*	20.9*b*	16.3*b*
	104	45.1*ab*	72.4*c*	23.5*bc*	2.92*c*	15.0*b*	1.17*d*	44.1*b*	17.7*c*	16.2*b*
	GK	48.0*a*	87.8*a*	24.3*ab*	3.32*a*	19.0*a*	1.40*a*	51.2*a*	22.3*a*	16.1*b*
2006	101	33.9*c*	70.4*b*	13.0*c*	2.91*b*	18.7*ab*	1.18*bc*	41.7*bc*	18.7*d*	14.8*a*
	102	38.2*ab*	71.1*b*	16.8*a*	3.02*a*	16.4*cd*	1.15*c*	42.4*b*	21.1*b*	12.6*b*
	103	36.2*abc*	64.4*c*	13.4*bc*	2.96*b*	15.1*d*	1.30*ab*	39.7*c*	20.9*bc*	12.5*bc*
	104	34.9*bc*	74.2*ab*	15.0*ab*	3.02*ab*	18.1*bc*	1.18*bc*	43.4*b*	19.9*c*	11.6*c*
	GK	39.5*a*	76.2*a*	14.4*bc*	3.18*a*	20.0*a*	1.36*a*	49.2*a*	24.4*a*	11.5*c*
2007	101	39.7*bc*	66.2*b*	13.4*b*	2.88*bc*	15.1*a*	1.22*b*	41.4*b*	18.5*d*	12.4*a*
	102	43.8*a*	65.2*b*	16.2*a*	3.06*b*	14.5*ab*	1.19*b*	40.2*b*	19.8*bc*	13.3*a*
	103	32.6*d*	58.3*c*	13.1*b*	2.96*bc*	10.7*c*	1.13*b*	35.1*c*	20.0*b*	12.8*a*
	104	36.3*cd*	68.2*b*	15.7*a*	2.70*c*	12.7*b*	1.09*b*	39.8*b*	18.7*cd*	11.2*b*
	GK	42.7*ab*	79.5*a*	15.5*a*	3.48*a*	15.6*a*	1.43*a*	48.2*a*	23.9*a*	13.1*a*

[z]101–103, transgenic lines; 104, non-transgenic line; GK, parental variety Green King.
a–e Means within a column for each year followed by the same letters are not significantly different at the 0.05 probability level based on LSD test; $N = 3$.

Table 3. Summary of horticultural traits showing significant differences ($P = 0.05$) among the transgenic and/or the non-transgenic controls over the test years (2004–2007)

Compared lines[z]	Horticultural traits[y] showing significant difference in all years	Horticultural traits showing significant difference in 2 or 3 yr
Between non-transgenic lines (104 vs. GK)	LL, LW	PLH, PLW, SW, PEL, PEW, LN
101 vs. 102	SH	PLH, PEL, LW
101 vs. 103	PLW, LW	SW, PEL, LL, LN
102 vs. 103	PLW, SH, LL	PLH, PEL, PEW, LN
101 vs. non-transgenic lines (104 and GK)	–	PEW, LW, LN
102 vs. non-transgenic lines (104 and GK)	–	LW, LN
103 vs. non-transgenic lines (104 and GK)	LL	PLW, SH, PEL, LW

[z]101–103, transgenic lines; 104, non-transgenic line; GK, parental variety Green King.
[y]PLH, plant height; PLW, plant width; SH, stem height; SW, stem width; PEL, petiole length; PEW, petiole width; LL, leaf length; LW, leaf width; LN, leaf number.

RESULTS

Development and Molecular Identification of Transgenic Inbred Lines

Restriction digestion and Southern analyses indicated that lines 101 and 103 have similar hybridized patterns and these patterns are distinct from line 102 (Fig. 1a). Using a single digestion site restriction enzyme such as *Xba*I that operates outside the transgene, we demonstrated that all three of the transgenic inbred lines had a single insertion of the *ipt*-transgene (Fig. 1a). The single site of insertion has been further verified by the quantitative PCR study and flanking sequences analyses (data not shown).

When transgene expression was assayed by Northern hybridization using floral heads after 0, 2, 4 and 5 d of storage at room temperature, hybridized signals were obvious at days 4 and 5 for the transgenic lines 102 and 103, while no signal was noted on the non-transgenic line 104 (Fig. 1b). This suggests that the SAG promoter became active as senescence proceeded, triggering the synthesis of *ipt* mRNA.

When plant cytokinins were monitored during storage of floral head tissues, only the isopentenyladenosine content exhibited distinct differences among the broccoli lines tested (Fig. 2a). At 3 d post-harvest, all three transgenic lines had a higher isopentenyladenosine content than the non-transformed inbred line. Among the three transgenic lines, 102 had a relatively higher isopentenyladenosine content than lines 101 and 103. The increase in isopentenyladenosine content in floret tissues likely reflects *ipt* expression, potentially leading to enhanced shelf life (Fig. 2a). The trans-zeatin and dihydrozeatin levels in the transgenic lines were not significantly different from the non-transgenic line (Fig. 2b and 2c).

Horticultural Traits of *ipt*-transgenic and Non-transgenic Broccoli

Most of the growth characteristics showed significant year, line and line × year effects (Table 1). The effects of environmental conditions (i.e., year) on growth characters were generally greater than the difference between the various lines of transgenic and non-transgenic broccoli. As the line × year effects were significant, the growth characteristics were evaluated on a year-by-year basis. Mean comparisons for the horticultural traits tested in each year, along with differences among transgenic lines, between non-transgenic lines and individual transgenic lines vs. non-transgenic lines are presented in Tables 2 and 3. Significant differences among the three transgenic lines were noted only for PEL in 2 or 3 yr. Line 103 had smaller PLW than lines 101 and 102 in all years. Although lines 101 and 103 were selected from the selfed-progeny of the same T_0 plant, they differed in PLW and LW in all years and were distinct over 2 or 3 yr for SW, PEL, LL and LN. Only the LW and LN of the 102 transgenic line differed from the non-transgenic line, while 101 or 103 showed more than three traits that were distinct from the non-transgenic line. Floret development of the three transgenic lines was normal and the resulting flowers could be used for pollination.

Yield and Quality of Flower Heads of Broccoli

The effects of year, line and the year × line interaction were consistently significant for the head yield traits measured (Table 4). As indicated in Table 5, yields were higher in 2005 than in the other years. In general, head yield of the transgenic lines did not show any consistent variation from the non-transgenic controls (Table 5). Significant differences were found across the years for FSL, while differences in FD and FW were only distinct over 2 or 3 yr among the three transgenic lines (Table 6). Line 102 had the longest FSL followed by lines 103 and 101 (Table 5). Among the transgenic lines, 103 had the highest FW(S) or FW, followed by 102. Mean head yield [FW(S) and FW] of the transgenic lines 102 and 103 were comparable with the commercial F_1 variety GK in all years. The non-transgenic inbred line 104 had the lowest head weight among all the lines tested.

Shelf-life of Flower Heads of Broccoli

Based on time to 10 and 50% yellowing of the flower heads, the transgenic lines 102 and 103 showed a longer

Table 4. Mean squares of ANOVA for quality and yield of flower heads of three *ipt*-transformed lines, one non-transformed line and the parental variety of broccoli in field trials conducted from 2004 to 2007

Source	df	Mean squares						
		Flower head diameter	Flower head height (with stem)	Flower head height (without stem)	Stem length of flower head	Stem width of flower head	Flower head weight (with stem)	Flower head weight (without stem)
Year	3	29.1**	55.1**	11.7**	30.1**	0.58**	83057**	46468**
Block within years	8	0.4	0.6	0.1	0.3	0.04	1145	658
Line	4	62.2**	71.3**	9.3	33.0**	1.26**	142731**	87012 **
Year × Line	12	4.0**	6.3**	3.3**	3.9**	0.06*	5939**	5292**
Error	59	0.7	0.6	0.2	0.3	0.02	1717	1148

*, ** Significant F-test at the 0.05 and 0.01 probability levels, respectively.

shelf-life at 25±2°C than the non-transgenic line 104 and the parental variety GK in all four years of testing (Table 7). On average, lines 102 and 103 of transgenic broccoli had a shelf-life that was 1.5 d longer than the non-transgenic broccoli. The shelf-life extension of line 102 was a little longer (average 0.5 d) and more stable (smaller CV value) than that of line 103. The potential for transformation with *ipt* to extend shelf-life of broccoli is illustrated in Fig. 3. The flower heads of the transgenic lines 101, 102 and 103 remained green after being stored at 25±2°C for 6 d, whereas the flower heads of the non-transgenic line 104 and the parental variety GK had turned yellow.

DISCUSSION

Knowledge of functional genomics and transgenic techniques is useful for molecular farming and crop improvement. In this study, the field performance and post-harvest life span of three *ipt*-transformed lines of broccoli were compared with the original F_1 hybrid variety GK and a non-transgenic line derived from this variety by selfing for five generations. The 4-yr field study was intended to compare the phenotypic and agronomic performance of GM lines relative to the non-transgenic parent.

Transgenic plants with one or two integration sites tend to exhibit high level of transgene expression while higher levels of copy insertion usually result in lower and/or unstable transgene expression and in extreme cases transgene silencing (Flavell 1994; Vaucheret et al. 1998). Results from the Southern analysis and the Q-RT-PCR indicated that the transgenic lines of broccoli used in this project (line 101, 102 and 103) all had a single copy of the transgene. Although the transgenic lines 101 and 103 were derived from the same T_0 plant, they showed significant variations in growth traits such as PLW, LW and FSL. Apparently some divergence had occurred in these lines during the selfed generations. The two non-transgenic controls, F_1 hybrid GK and the selfed inbred line 104, also exhibited distinct differences for a range of growth characteristics. Excluding leaf length (LL), the range of variation in the growth traits for the transgenic lines was within the scope of the variability exhibited by the two non-transgenic controls. This suggests that the *ipt*-transformation did not significantly alter the phenotype of the lines being tested. The results also indicate that inbreeding had minimum impact on the appearance or yield potential of either the transgenic or non-transgenic lines. It was possible to produce inbred lines with vigor and yield characteristics comparable to the hybrid parent. The agronomic traits of both the transgenic lines and the non-transgenic controls showed a degree of year-to-year variability that generally exceeded the effects of either transformation or inbreeding. In a study of heterosis for horticultural traits in broccoli, Hale and Farnham (2007) also identified significant environment effects leading to variations in the plant characteristics and yield traits of both parental and F_1

Table 5. Quality and yield of flower heads of three *ipt*-transformed lines, one non-transformed line and the parental variety of broccoli in field trials conducted from 2004 to 2007

Year	Line[z]	Flower head diameter (cm)	Flower head height (with stem) (cm)	Flower head height (without stem) (cm)	Stem length of flower head (cm)	Stem width of flower head (cm)	Flower head weight (with stem) (g)	Flower head weight (without stem) (g)
2004	101	17 5*a*	20 5*c*	13 9*b*	6 6*c*	3 81*a*	389*a*	303*ab*
	102	15 7*b*	24 5*a*	13 4*bc*	10 9*a*	3 50*b*	421*a*	273*b*
	103	18 5*a*	21 1*c*	12 6*c*	8 6*b*	3 55*b*	452*a*	345*ab*
	104	14 4*b*	23 0*b*	13 9*b*	9 2*b*	2 79*c*	271*b*	182*c*
	GK	17 5*a*	24 4*a*	15.3*a*	9 1*b*	3 42*b*	406*a*	279*b*
2005	101	19 5*b*	18 7*c*	12 7*bc*	6 0*d*	3 95*a*	426*b*	357*c*
	102	19 6*b*	25.8*a*	14 8*a*	11 1*b*	3 87*a*	602*a*	430*b*
	103	23 2*a*	21 6*b*	13 6*b*	8 0*c*	3 86*a*	668*a*	556*a*
	104	15 2*c*	21 1*b*	12 5*c*	8.6*c*	3 17*b*	293*c*	202*d*
	GK	19 1*b*	26 6*a*	13 1*bc*	13 6*a*	3 83*a*	585*a*	387*bc*
2006	101	16 6*b*	17 6*c*	11 3*c*	6 3*d*	3 37*a*	359*c*	285*b*
	102	17 5*ab*	25 1*a*	14 3*a*	10 8*a*	3 61*a*	440*b*	332*ab*
	103	18 2*a*	20 4*b*	11 9*bc*	8 5*bc*	3 48*a*	520*a*	363*a*
	104	11 8*c*	16 5*c*	9 1*d*	7 4*cd*	2 84*b*	208*d*	147*c*
	GK	18 5*a*	21 8*b*	12 2*b*	9 6*b*	3 52*a*	486*ab*	345*ab*
2007	101	14 8*bc*	16 2*c*	11 4*b*	4 8*c*	3 23*b*	246*b*	199*c*
	102	15 8*b*	20 4*a*	13 4*a*	7 1*a*	3 72*a*	371*a*	274*b*
	103	19 6*a*	19 8*a*	13 5*a*	6 3*b*	3 42*b*	430*a*	355*a*
	104	13 4*c*	17 6*b*	11 6*b*	6 1*b*	2 72*c*	218*b*	164*c*
	GK	18 5*a*	20 4*a*	13 5*a*	6 9*a*	3 38*b*	427*a*	325*ab*

[z]101–103, transgenic lines; 104, non-transgenic line; GK, parental variety Green King

a–d Means within a column in the same year followed by the same letters are not significantly different at the 0 05 probability level based on LSD test; $N = 3$

Table 6. Summary of the floral head quality and yield traits showing significant differences ($P = 0.05$) among the transgenic and/or the non-transgenic controls over the test years (2004–2007)

Compared lines[z]	Head yield traits[y] showing significant difference in all years	Head yield traits showing significant difference in 2 or 3 yr
Between non-transgenic lines (104 vs. GK)	FD, FH(S), FSW, FW(S), FW	FH, FSL
101 vs. 102	FH(S), FSL	FD, FSW, FW(S), FW
101 vs. 103	FSL	FD, FH(S), FH, FW(S), FW
102 vs. 103	FSL	FD, FH(S), FH, FW
101 vs. non-transgenic lines(104 and GK)	–	FH(S), FSL, FW(S)
102 vs. non-transgenic lines(104 and GK)	–	FH(S), FH, FSL
103 vs. non-transgenic lines(104 and GK)	–	–

[z]101–103, transgenic lines; 104, non-transgenic line; GK, parental variety Green King.
[y]FD, flower head diameter; FH(S), flower head height(with stem); FH, flower head height (without stem); FSL, stem length of flower head; FSW, stem width of flower head; FW(S), flower head weight (with stem); FW, flower head weight (without stem).

Table 7. Days of storage at 25 ± 2°C to reach 10 and 50% yellowing of heads of three *ipt*-transformed lines, one non-transformed line and the parental variety of broccoli harvested from field trials conducted in 2004–2007

		Days of storage					
Yellowing	Line[z]	2004	2005	2006	2007	Mean (days)	CV(%)
10%	101	3.0*c*	2.7*c*	4.3*ab*	3.4*b*	3.4	21
	102	5.2*a*	4.4*a*	4.2*b*	4.4*a*	4.6	10
	103	4.3*b*	3.2*b*	4.6*a*	4.2*a*	4.1	15
	104	3.2*c*	2.8*c*	3.5*c*	3.4*b*	3.2	8
	GK	2.6*d*	2.3*d*	2.8*d*	2.5*c*	2.6	8
	Mean	3.7	3.1	3.9	3.6	3.6	
50%	101	6.3*c*	5.8*c*	7.5*b*	6.5*c*	6.5	11
	102	8.3*a*	8.6*a*	7.6*b*	7.4*b*	8.0	7
	103	7.4*b*	6.3*b*	8.4*a*	7.9*a*	7.5	12
	104	5.5*d*	5.3*d*	5.7*c*	6.0*d*	5.6	6
	GK	4.9*e*	4.6*e*	5.3*c*	5.4*e*	5.1	7
	Mean	6.5	6.1	6.9	6.7	6.5	

[z]101–103, transgenic lines; 104, non-transgenic line; GK, parental variety Green King.
a–e Means within a column followed by same letters were not significantly different at the 0.05 probability level based on LSD test; $N = 3$.

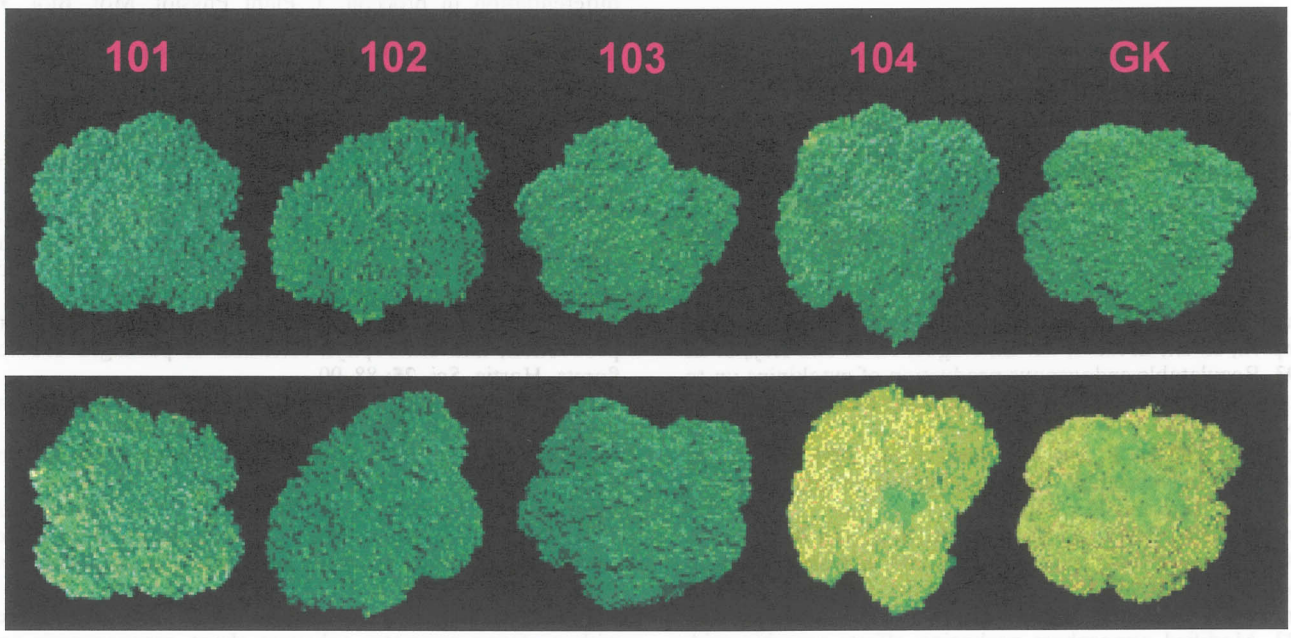

Fig. 3. Color of flower heads of three *ipt*-transgenic lines (101, 102 and 103), one non-transgenic line (104) and the parental variety Green King (GK) of broccoli at harvest (top row) and after 6 d of storage at 25±2°C (bottom row).

lines. Less genotype by environment interaction was noted in the hybrid lines than in the inbred lines.

The cytokinin determination indicated that at 4–5 d post-harvest, *ipt* expression was prominent in the transgenic lines leading to an increase in IP (isopenteny-adenine) type cytokinin content relative to the non-transgenic controls. As cytokinins delay the loss of chlorophyll in senescing tissues, this would explain the observed delay in yellowing of the *ipt*-transformed lines. Levels of trans-zeatin and dihydrozeatin were not influenced by transformation with the *ipt* gene. Studies on microsomal fraction isolation indicated only the IP form of cytokinin was present in cauliflower (*Brassica oleracea* L. var. *botrytis*) (Chen and Leisner 1984; Sakakibara 2006), which is closely related to broccoli (*B. oleracea* L. var. *italica* Plenck). Whether IP type cytokinin is predominant in broccoli or whether trans-zeatin type cytokinin synthesis simply does not occur within the sampling time frame used in this study is not known.

The 50% increase in post-harvest lifespan observed in the transgenic lines only amounted to 1 or 2 d of additional shelf-life when the crop was stored at 25°C. However, a more substantial enhancement of the storage lifespan was observed when the *ipt*-transformed broccoli crop was stored at lower temperatures more typically used in long-term vegetable storage (Chen et al., unpublished).

This study demonstrated that it is possible to delay post-harvest senescence of broccoli through endogenous regulation of cytokinins achieved through transgenic techniques. This improvement in post-harvest performance was achieved without compromising the appearance or agronomic performance of the broccoli crop relative to the parental line.

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