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Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene

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Abstract

The cytokinin gene, isopentenyl transferase (*ipt*), was placed under the control of a heat-inducible promoter from the *Drosophila melanogaster hsp70* gene and introduced into *Nicotiana plumbaginifolia* by cocultivation with *Agrobacterium tumefaciens*. Transformants were analyzed for organ-specific expression, cytokinin levels and effects on plant development before and after heat induction. The *ipt* gene transcripts were detected in leaves and stems but not roots of transgenic plants following a 2 hour, 45 °C treatment. Maximum mRNA levels observed occurred 2 hours after heat treatment and 46 hours later were detected only in leaves. Zeatin and zeatinriboside concentrations 2 hours after heat shock ranged from over 900 to 2000 pmol/g, representing a greater than 140- to 200-fold increase over uninduced levels. After 46 hours, approximately 50% of the cytokinins are still present in the leaves as opposed to much reduced levels in the stems. Transgenic plants were greener, shorter, had an underdeveloped root system, reduced leaf width, and increased growth of axillary buds. After a single heat treatment, plants exhibited a darker green pigment and continued growth of lateral buds. Transient accumulations of endogenous cytokinins following thermal induction did not appear to alter the plant's preprogrammed pattern of differentiation.

Introduction

Phytohormones have long been recognized as mediators of growth and differentiation in plants. Early studies on tobacco callus culture [30] indicated that auxins and cytokinins, the two major classes of phytohormones, could be used to manipulate morphogenesis. However, it was soon realized that many tissue-cultured cells would not undergo morphogenesis even under a multitude of different culture conditions. Over the years, methods for regenerating specific plants have been developed, but often the knowledge gained from successful manipulation of one species or cultivar is not applicable to other species or cultivars even if the plants are closely related genetically. One of the major blocks to regeneration of recalcitrant plant species is the lack of understanding of fundamental regulatory mechanisms

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of morphogenesis. The determination of endogenous phytohormone concentrations during growth and differentiation may help to elucidate their role in various patterns of differentiation. However, since endogenous pools of phytohormones are very low, they are difficult to measure and relate to specific physiological processes. On the other hand, external applications of hormones are subject to problems associated with uptake, transport, and metabolism. The availability of phytohormone-specifying genes from *Agrobacterium tumefaciens* provides an alternative genetic approach to the study of morphogenesis through the *in vivo* manipulation of hormone ratios [20, 25, 31].

The T-DNA region of the Ti plasmid of *A. tumefaciens* encodes genes involved in biosynthesis of cytokinin and auxin. Inactivation of any phytohormone gene by transposon insertion leads to altered cytokinin-to-auxin balances in tobacco crown galls incited by such mutagenized T-DNA and to altered tumor morphology [1, 10]. Cells transformed with the functional auxin genes (*tms1* and *tms2*) and inactivated cytokinin biosynthetic gene (*ipt*) form roots on the resulting tumors whereas those transformed with the functional cytokinin biosynthetic gene and inactivated auxin genes have a shooty phenotype [27]. Function of these phytohormone-specifying genes in controlling morphogenesis has been examined further by cloning individual genes from the T-DNA region and introducing them into plant cells [3, 20, 31]. These studies established that a gene specifying the production of a single phytohormone can alter the morphogenic potential of the recipient cell.

The shooty tumor response is encoded for by the isopentenyl transferase (*ipt*) gene in the T-DNA and has been shown conclusively to be involved in cytokinin biosynthesis [2, 4, 6]. Isopentenyl transferase catalyzes the condensation of AMP and isopentenyl pyrophosphate to form isopentenyl AMP, a precursor to all other cytokinins [23]. Molecular manipulations of the *ipt* gene for expression in transgenic plants have revealed interesting phenomena. Overexpression of the gene product in *Nicotiana* uniformly incited shoots more frequently, rapidly and profusely than did the unmodified gene, and in *Cucumis sativa* (cucumber) it uniquely induced shoots [31]. This is in contrast to studies with petunias where shoots could not be regenerated [19]. One of the major drawbacks to constitutive overexpression of cytokinin is the almost complete suppression of root formation, which may be overcome by inducible and localized expression of cytokinins. Recently, Medford *et al.* [25] regenerated plants from shoots transformed by the *ipt* gene under control of the heat-inducible maize *hsp70* promoter. Although the plants exhibited characteristics associated with cytokinin overproduction, they concluded that the plant's programmed pattern of differentiation was not affected following thermal induction of the cytokinin gene.

In the present study, transient expression of the *ipt* gene is examined in transgenic plants to gain a better understanding of its function in growth and differentiation. The gene was linked to a *Drosophila hsp70* promoter, introduced into *N. plumbaginifolia* cells, and regenerated plants analyzed for cytokinin accumulation and tissue distribution prior to and after thermal induction.

Methods

Vector construction

An *Xba* I/*Xmn* I DNA fragment of 456 bp from the *Drosophila melanogaster hsp70* gene, containing the promoter and 199 nucleotides of untranslated leader sequence, was fused through its 5′-untranslated region to the coding region of the isopentenyl transferase gene from pTiB6S3 (Fig. 1A [17, 31, 36]). The source of the promoter was plasmid p229-1 obtained from Carl Wu (National Institutes of Health, Bethesda, Maryland). The truncated *ipt* gene carries its own transcription terminator and polyadenylation signals. An *Eco* RI/*Hin*d III hs-*ipt* fragment was then subcloned into binary plant transformation vectors, pKYLX71, derived from pKYLX7, and pBin19, and mobilized into two different *A. tumefaciens* strains, EHA101 (pEHA101) and

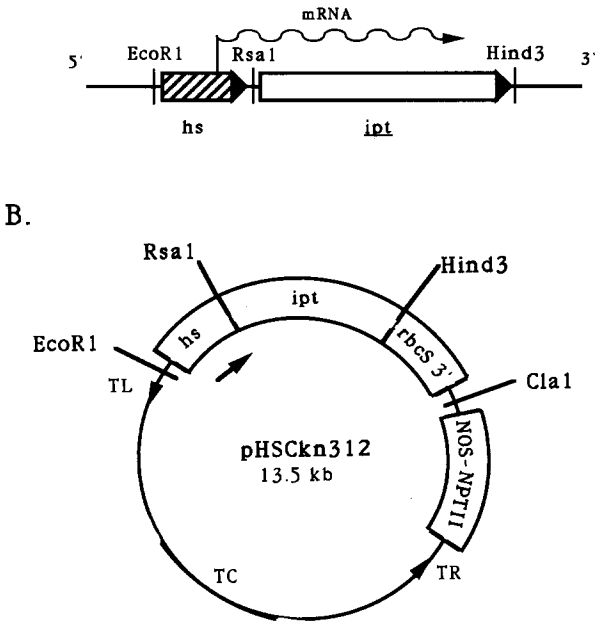

Fig. 1. Construction of a chimeric hs-*ipt* gene.
A. Structure of the chimeric *ipt* gene. The *Drosophila melanogaster hsp70* promoter (hs) was fused to the pTiB6S3 plasmid *ipt* coding region. The 456-bp fragment contains the promoter region plus 199 nucleotides of untranslated region. B. Plant transformation vector, pHSCkn312. The vector contains the T-DNA right (TR) and left (TL) border sequences from pTiT37 for integration into the plant genome. The NPTII gene is used as a selectable marker for kanamycin-resistant plant cells. The tetracycline resistance gene (TC) allows selection in bacteria. Transcription terminator (rbcS 3') is from the pea *rbcS-E9* gene.

LBA4404 (pAL4404), respectively (Table 1) [5, 14, 15, 27, 28]. The pKYLX71 vector in which *Sph*I site has been removed from the multiple cloning site to eliminate a potentially interfering ATG codon was obtained from Arthur G. Hunt (University of Kentucky, Lexington, Kentucky). Strain EHA101 (pEHA101) is a disarmed version of the hypervirulent L,L-succinamopine strain A281. It has been demonstrated that the hypervirulence of A281 is encoded in a region outside of the pTiBo542 T-DNA. Strain LBA4404 carries the disarmed octopine Ti plasmid, pAL4404, derived from pTiAch5.

Bacterial strain and growth conditions

The *A. tumefaciens* strains, plasmids and the corresponding *ipt* gene constructs used are listed in Table 1. The chromosomal background of all strains is C58 (a Ti plasmid-cured nopaline strain) except for the LBA4404 strains which have an Ach5 chromosomal background [14, 27, 37]. *Escherichia coli* strain JM83 was used in cloning experiments and strain MM294 (pRK2013) was used as the helper strain in triparental matings [9]. All *E. coli* and *A. tumefaciens* strains were grown as previously described at 37 °C or 30 °C, respectively [15, 31]. Media were supplemented with appropriate antibiotics at the following concentrations: kanamycin 50 μg/ml, tetracycline 12.5 μg/ml, and ampicillin 50–100 μg/ml.

Plant transformations

The *ipt*-gene constructs were transferred to the plant genome by cocultivation of *A. tumefaciens* bacteria with leaf pieces from *N. plumbaginifolia* 43A plants as previously described with the exception that cut leaf pieces were preincubated on agar media for 1–2 days prior to infection with the bacteria to enhance transformation [16, 24, 31]. After a 24 hour cocultivation, leaf pieces were washed with media containing Cefotaxime (200 μg/ml, Calbiochem), carbenicillin (200 μg/ml, Sigma) and plated on selective agar media which contained kanamycin (100 μg/ml) in addition to the Cefotaxime and carbenicillin. Regenerated shoots were transferred to fresh media and subsequently rooted on 1-naphthaleneacetic acid (NAA; 0.5 μg/ml) in the presence of kanamycin sulfate (50 μg/ml). Leaf pieces from the putative transformants were tested for callus formation and ability to regenerate shoots on media containing 200 or 100 μg kanamycin sulfate per ml, respectively. Seeds from primary transformants were germinated on kanamycin (100 μg/ml) and gibberellic acid (GA, 0.1 mM) after an overnight incubation in 1000 μg/ml GA [33]. The GA breaks the dormancy and improves the germination frequency of the seeds. Segre-

Table 1. A. tumefaciens strains and plasmids.

Strain	Relevant plasmid(s)	Plasmid characteristics		
		ipt gene construct[a]	Marker[b]	Reference
ACS101	pEHA101	–	KM	This work
	pHSCkn312	hs-*ipt*	TC	
ACS102	pEHA101	–	KM	This work
	pCkn116	t-*ipt*	TC	
ACS103	pAL4404	–	–	This work
	pHSCkn45	hs-*ipt*	KM	
ACS104	pAL4404	–	–	This work
	pCkn22	t-*ipt*	KM	
EHA101	pEHA101	–	KM	Hood *et al.*, [15]
LBA4404	pAL4404	–	–	Ooms *et al.*, [27]
A281	pTiBo542	native *ipt*/Ti	–	Sciaky *et al.*, [29]

[a] hs-*ipt*, *hsp70* promoter fused to the *ipt* gene; t-*ipt*, truncated *ipt* gene; Ti, Ti plasmid.
[b] KM, kanamycin; TC, tetracycline.

gation ratios of kanamycin-resistant (green) to kanamycin-sensitive (bleached) seedlings were calculated and associated with chromosomal insertion events in the plant cells.

Nucleic acid preparations and blot hybridizations

E. coli and *A. tumefaciens* plasmid DNAs were isolated by the rapid alkaline lysis method described by Maniatis *et al.* [22]. High molecular weight plant DNA was prepared by the method of Dellaporta *et al.* (1984) with the following modification. The lysate was treated with RNase A (100 μg/ml, Sigma) for 30 min at 37 °C after the first DNA precipitation step followed by extraction with phenol/chloroform, 1 : 1 (vol/vol), and chloroform. Total RNA was isolated by adaptation of a method by Chirgwin *et al.* [7].

RNA (20 μg) was separated on 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose membranes [22]. Prehybridizations and hybridizations were done in 6 × SSC/0.25% (wt/vol) dry milk/0.1 mg of salmon sperm DNA per ml/50% (vol/vol) formamide at 42 °C. As probe, an *Rsa* I/*Hind* III *ipt* gene fragment was labeled to a specific activity of 2–4 × 10^8 cpm/μg using a random primer kit and was added in excess to the hybridization solution (Fig. 1A; Boehringer Mannheim). Filters were washed at moderate stringency (1 × SSC, 1 × SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) as well as at high stringency (0.1 × SSC) and exposed to X-AR5 film at −70 °C with two intensifying screens (Cronex Lightning Plus, DuPont). Autoradiographs were scanned on a densitometer to determine gene transcript levels.

Heat shock treatments

Plants and tissues were exposed to 45 °C for 2 h in a controlled environmental growth chamber with relative humidity of 70% [25]. They were preconditioned at 45 °C for 15 min prior to the

beginning of the 2 h treatment and returned to their normal growing conditions of 25 °C and 40% relative humidity after the heat treatment. At time of heat shock, plants were 9 to 10 weeks old and were in the vegetative stage of growth. They were 14 to 23 cm tall and had 8 to 12 fully expanded leaves.

Zeatin/zeatinriboside analysis

The amounts of zeatin (Z) and N^9-substituted derivatives of Z in plant tissues were determined using an analytical kit (De Danske Sukkerfabrikker, Copenhagen). Plant extracts were purified on columns packed with anti-zeatinriboside (ZR) antibodies. Eluted Z and ZR were quantified by ELISA. The plant tissues used in the analysis were frozen in liquid nitrogen prior to extraction in 80% methanol.

Results

*Plant transformation with the hs-*ipt *gene*

Transgenic plants were obtained using both the supervirulent L,L-succinamopine and less virulent octopine *Agrobacterium* strains, each carrying either the hs-*ipt* or the truncated *ipt* (t-*ipt*) (Table 1). However, with the supervirulent strains, ACS101 (pHSCkn312, pEH101) and ACS102 (pCkn116, pEH101) more than twice as many shoots were incited on the edge of the infected leaf piece on selective media than with the less virulent strains, ACS103 (pHSCkn45, pAL4404) and ACS104 (pCkn22, pAL4404). Shoots arising from all infections exhibited similar frequencies of survival when replated on kanamycin-containing media. Transgenic (RO) plants representative of all infections were grown to maturity and their progeny (Rl) screened for transformation by germination on kanamycin-containing media. Seeds exhibiting a 3 : 1 segregation pattern of resistant to sensitive seedlings, indicative of insertion at a single locus in the plant genome, were used in further analyses. The copy number of the *ipt* gene was confirmed by Southern blot analysis (data not shown).

Analysis of ipt *gene transcription*

Transformed axenic cell cultures were established from leaves removed from kanamycin-resistant shoots and screened for *ipt* gene transcripts. Cell lines in which the *ipt* message was clearly present 2 hours after a 45 °C heat shock treatment were used in subsequent experiments (Fig. 2). More than 40 independently transformed transgenic plants (R0) were derived from these cultures reducing the possibility of regenerating chimeric plants containing normal and transformed cells.

Progeny of 3 independently transformed R0 plants carrying the hs-*ipt* gene construct were heat-shocked *in vivo* for 2 hours at 45 °C and analyzed for accumulation of the *ipt* gene transcripts. Tissues from two different plants of each independent transformant were analyzed before heat shock and at different times after heat treatment. All the leaves and the whole stem and root mass were used for analysis at each point to minimize variability. Greatest increases in transcripts levels were always detected in leaves and stems 2 hours after the end of the heat treatment (Fig. 3). Transcription rates fluctuated somewhat from plant to

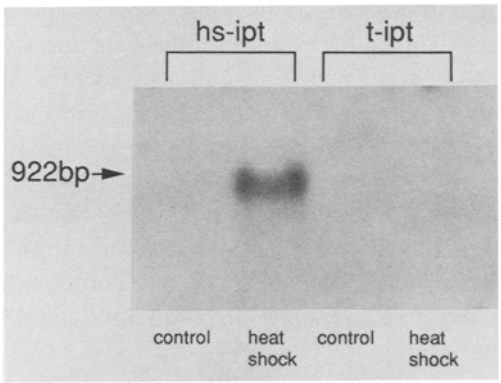

Fig. 2. Induction of *ipt* transcripts in axenic tissue cultures. *Nicotiana* cultured tissues transformed with the hs-*ipt* and the t-*ipt* was heat-shocked at 45 °C for 2 hours. Total RNA was isolated 2 hours after heat treatment and the transcript levels compared at control and heat shock temperatures. The chimeric *ipt* gene mRNA is indicated by an arrow.

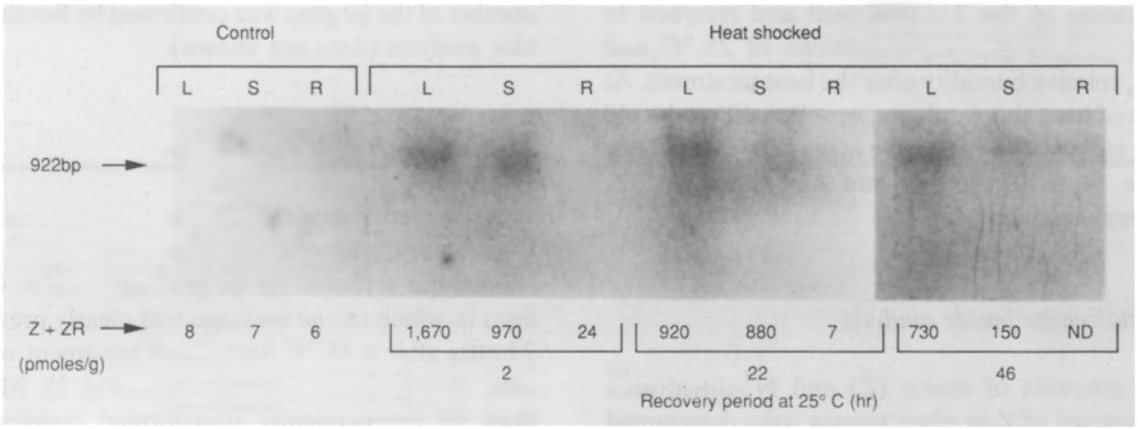

Fig. 3. Analysis of *ipt* transcript levels and zeatin/zeatinriboside concentrations in transgenic plants. Transcript levels of the *ipt* gene in leaves (L), stems (S) and roots (R) of thermally uninduced (control) transgenic plants and ones exposed to 45 °C for 2 hours (heat shocked). The expected 922-bp chimeric *ipt* gene mRNA is indicated. The corresponding Z and ZR concentrations in pmoles/g fresh tissue are indicated below each transcript and represent an average of 6 samples from 3 independent transformants. The analysis was done before and 2, 22 and 46 hours after heat induction. ND indicates that the concentration was not determined.

plant, but in general, the amount of *ipt* RNA in leaves was twice that observed in stems and did not change significantly at 6 hours (data not shown). After 22 hours, the relative transcript levels were approximately the same in leaves and reduced by an average of 20% in stems when compared to those at 2 hours. On the average, only 6% of the message was detected in the leaves after 46 hours and the corresponding RNA in stems was reduced to uninduced levels (Fig. 3). Transcripts were not observed in any tissues prior to heat shock or in roots after heat induction. Since the same *hsp70* promoter fused to a different gene had highest levels of transcription and enzyme activity in *N. tabacum* roots and stems after exposure to 37 or 40 °C for 45 minutes, the hs-*ipt* transformed plants were treated in a similar fashion and their soil temperature was monitored [35]. Such treatments still failed to induce detectable *ipt* gene messages in the roots (data not shown).

At no time were *ipt* gene transcripts detected in plants and tissues transformed with the truncated *ipt* gene (Fig. 2).

Cytokinin levels in transgenic plants

Tissues from the heat-shocked transgenic plants and cultures were also analyzed for accumulation of the cytokinins zeatin (Z) and zeatinriboside (ZR) since previous reports have demonstrated that these are the major cytokinins in *ipt*-transformed shoots and crown galls [1, 25, 31, 32]. In order to estimate the percent recovery of these cytokinins frm plant tissues, known quantity of ZR was added to untreated transformed tissues at time of extraction and quantitatively analyzed by ELISA following purification on columns packed with antizeatinriboside antibodies. The percent recovery ranged from 64 to 98, with an average of 74%. In addition, to validate the ELISA results, few of the extracts were prepared with ^{15}N-Z internal standard, and using GC-MS-selective ion monitoring, the concentration of Z and ZR permethyl derivatives determined (G. Buta, unpublished).

The Z and ZR levels were considerably elevated in leaves and stems of hs-*ipt*-transformed plants 2 hours into the recovery period after the heat treatment (Fig. 3). In general, an increase of over 200-fold as observed in leaves and about 140-fold in stems. By 22 hours, the cytokinin

levels were reduced by about 50% in leaves and only slightly in stems. But by 46 hours, most of the cytokinins were still detectable in leaves and not stems. Z and ZR levels were elevated over 4-fold in roots 2 hours after heat shock even though no corresponding increase in *ipt* gene transcript levels was ever observed.

Transformed tissue-cultured cells from which transgenic plants carrying the hs-*ipt* gene were regenerated had lower levels of cytokinins after heat induction when compared to those in leaves and stems of whole plants. Cytokinin levels of three such cultured lines were determined (lines 45, 47, and 43; Table 2). Immediately after a 2 hour heat shock, the Z and ZR levels averaged 100 pmol/g of tissue and corresponded to a 4- to greater than 50-fold increase in cytokinin. Cytokinin levels 2 hours into the recovery period were in the range of 105 to 380 pmol/g compared to the average 1 320 pmol/g in leaves and stems. After 22 hours, two of the lines (43 and 47) had slightly higher levels of cytokinin and one line (45) was reduced. Only line 47 was determined to have multiple *ipt* gene insertions which may be responsible for the increase. In general in culture lines, an average increase of 23- and 28-fold was observed 2 and 22 hours into the recovery period, respectively, whereas in leaves and stems of transgenic plants, the increases were greater than 140-fold 2 hours after the end of the heat treatment. This reduction in cytokinin concentration is probably not caused by the dilution effect from mixed populations of normal and transformed cells since cultured lines were established from leaves taken from kanamycin-resistant shoots.

No significant increases in Z and ZR were observed in heat-shocked cells transformed with the truncated *ipt* (lines 10 and 37) or in normal plant tissues (Table 2).

Table 2. Zeatin (Z) and zeatinriboside (ZR) levels in transformed tissue-cultured plant cells.

ipt gene construct[a]	Callus line	Z/ZR levels (pmoles/g)[b]			
		Control	Recovery period at 25 °C (h)[c]		
			0	2	22
hs-*ipt*	45	3	155	105	70
	47	7	70	210	310
	43	20	80	380	450
t-*ipt*	37	13	40	5	10
	10	4	1	10	3
	normal	2	4	1	ND

[a] See Table 1.
[b] Fresh weight.
[c] Hours after a 2 hour, 45 °C heat shock treatment.

Effect of elevated cytokinins on plant phenotypes

Transgenic plants were examined for effects induced by the hs-*ipt* gene. When plants containing the gene were cultured *in vitro*, they were shorter than the plants transformed with the truncated *ipt* gene or normal plants (Fig. 4A and 4B). At time of heat shocking, the R1 plants were on the average 40% shorter than the controls. This reduction in height appeared to be caused by a decrease in internode length, however, a decrease in the number of nodes could not be totally ruled out. In many cases the plants' stems were also bent which caused them to lean to one side. The roots were less developed (Fig. 4C), leaves were narrower, and the plants were greener than controls or normal plants. All of these effects were noted in plants not exposed to a heat treatment. When transferred to a greenhouse or an environmental growth chamber, the plants no longer were significantly shorter or greener (Fig. 4E), but regained their darker green pigment after thermal induction of the *ipt* gene. An increase in growth of axillary buds was also observed prior to and especially after heat treatment (Fig. 4F and G).

Discussion

The importance in plant development of the relative levels of two major classes of phytohormones, the cytokinins and auxins, has long been recognized mainly from studies utilizing exogenous hormone applications. Most recently, however, effects of endogenous manipulations of hormone

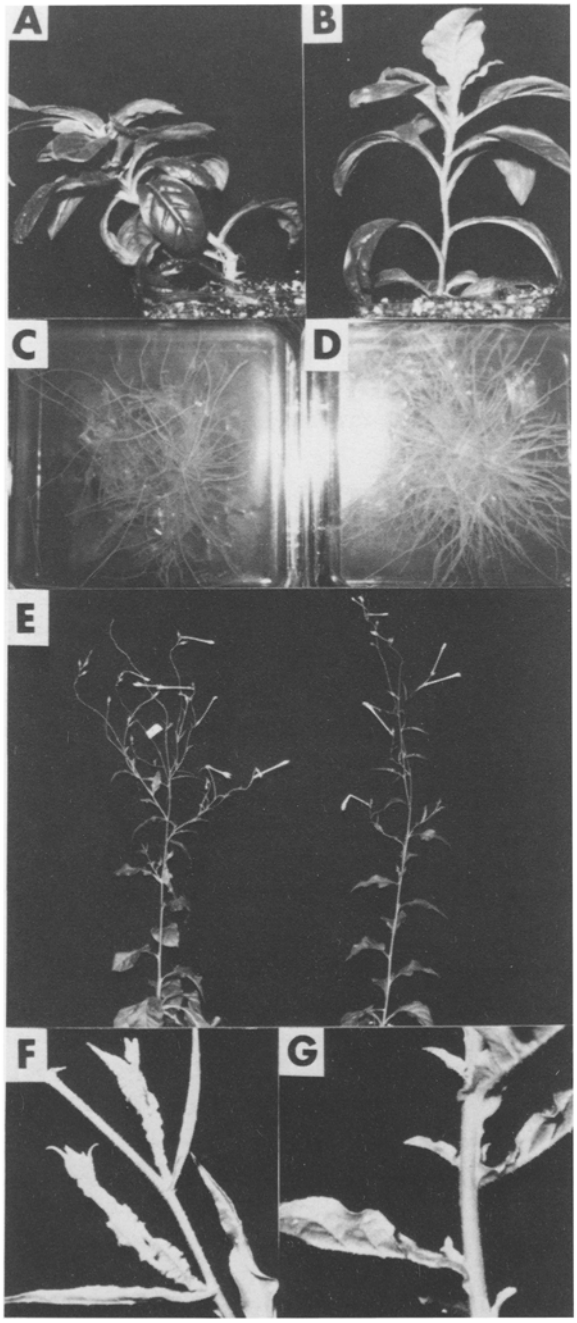

Fig. 4. Comparison of phenotypes of plants transformed with the hs-*ipt* gene and controls.
(A) Plant transformed with the hs-*ipt* gene and propagated *in vitro*.
(B) Plant transformed with the t-*ipt* gene.
(C) Underdeveloped root system of an hs-*ipt* transformant and root system of a control plant (D).
(E) Plant transformed with the hs-*ipt* gene and grown in the greenhouse (left) and control plant (right).
(F) Release of axillary buds caused by hs-*ipt*.
(G) Axillary buds on control plants.

levels on growth and differentiation have been reported. In this paper, the effect of a single gene involved in cytokinin biosynthesis is evaluated in transgenic plants for tissue-specific and transient expression. In order to control the levels of endogenous cytokinin, the *ipt* gene from *A. tumefaciens* was fused to a *Drosophila melanogaster hsp70* promoter. This promoter's functional features have been shown to be conserved after transfer into a plant genome [35]. Following heat induction, increased accumulation of the *ipt* transcripts was readily detected in leaves and stems but not roots of tobacco plants and in cultured tissues transformed with the hs-*ipt* gene. Increases in transcript levels were directly correlated with accumulation of Z and ZR cytokinins in the corresponding tissues.

Transgenic plants exhibited phenotypes associated with excess cytokinin even when they were not thermally induced. They had less developed root systems, exhibited increased growth of axillary buds, and when grown *in vitro* were shorter and greener. Medford *et al.* [25] have reported a 3-fold increase in ZR in leaves from similarly transformed uninduced plants and speculated that small increases in cytokinin production over a period of time could result in elevated cytokinin levels and account for the observed phenotype. However that seems unlikely based on the data presented in Fig. 3 and Table 2 since Z and ZR levels in uninduced transformants were not significantly higher except after heat treatment. It is well established that the *hsp70* genes in plants are induced by a variety of diverse stresses in addition to heat shock. Physical trauma such as wounding, water stress, exposure to hormones and various chemicals activate the *hsp70* genes of plants [11, 13, 21]. Therefore, the stress of initial tissue culturing necessary to obtain transgenic plants may have been sufficient to induce the *hsp70* promoter linked to the *ipt* gene at a very early stage of plant cell differentiation causing a heritable alteration in the programmed pattern of development. The observed phenotype may also be the result of expression of the *ipt* gene in tissues, cells, or organs other than leaves, stems, and roots which are more responsive to alterations of endogenous cytokinins.

The transient accumulation of endogenous cytokinins following thermal induction did not negatively affect the growth and development of transgenic plants. Maximum Z and ZR levels observed in leaves and stems following heat treatment represent only a third to a half of those previously reported for *N. plumbaginifolia* tissues transformed with the 35S-*ipt* gene construct [31]. This constitutive overproduction of cytokinins within plant cells was shown to enhance the cells' ability to undergo shoot organogenesis and was correlated with increased cytokinin-to-auxin ratios [32]. The 35S-*ipt*-transformed shoots exhibited characteristic loss of apical dominance and inhibition of root formation whereas transient increases in cytokinin levels in leaves and stems of hs-*ipt* transformed plants did not alter preprogrammed plant cell development. Possibilities exist that the increase in active cytokinins did not persist long enough for a phenotypic response to be observed. In addition, since it has been reported that expression from the 35S promoter is constitutive and occurs in a wide variety of organs and cell types, cytokinin production from the hs-*ipt* gene may not be occurring at an appropriate stage of cell development or be localized in specific cells, tissues, or organs to affect differentiation [38].

Tissue-specific differences in response to heat shock have been reported. Maize leaves show a typical heat shock response as do elongating portions of the primary root, but the more mature portions of the root exhibit a reduced ability to synthesize heat shock proteins [8]. In this report, no *ipt* gene transcripts were detected in the roots before or after heat treatment and only a 4-fold increase in Z and ZR was detected after heat induction. The determined cytokinin concentration for the roots would be grossly underrepresented if only the root tips were being fully induced by the heat treatment. However based on the work of Spena and Schell [35], maximum expression of the neomycin phosphotransferase (NPTII) gene driven by this same *hsp70* promoter was observed in the roots of *N. tabacum* plants, and lowest levels were found in leaves. Therefore, the steady-state levels of the *ipt* and NPTII transcripts appear to be regulated differently in the roots suggesting that factors other than the *hsp70* promoter may be involved in modulating tissue-specific and temporal expression. Mechanisms may exist in the roots which are specific for degrading excess *ipt* messages or inhibiting *ipt* gene transcription regardless of the source of the promoter. Metabolic enzymes involved in cytokinin breakdown may also be involved in controlling the levels of cytokinins in roots. Since cytokinins are primarily synthesized in the roots and transported up through the xylem to the shoot apex, it would be favorable for a plant to have root-specific, post-transcriptional mechanisms regulating the production of a hormone whose levels appear to be critically important in normal shoot development. Such mechanisms may be lacking or not be fully functional in leaves and stems since they are not the primary sites of cytokinin synthesis. Signal receptors for high levels of cytokinins may be lacking as well. Others have observed disruption of cell organization in roots as a result of exogenous applications of cytokinin or in transgenic plants expressing the cytokinin gene, also suggesting that the root system indeed may be more sensitive to alterations in cytokinin levels than the shoot system or other tissues [12, 18, 25].

Development in plant cells is composed of two processes, that of cell differentiation and growth. This study supports the view that cytokinins, in combination with auxins, delineate differentiation pathways in plant cells. Plants transformed with the heat-inducible cytokinin gene were developmentally affected as evidenced by their altered phenotype even when thermally not induced. This change in preprogrammed pattern of differentiation appears to occur early in development and is not altered further on subsequent exposure to transient elevations of cytokinins. The other process, that of plant cell growth, also seems to be affected, however, it is primarily observed in whole plants exposed to transient increases in cytokinin concentrations.

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