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Communication

Cytokinin-to-Auxin Ratios and Morphology of Shoots and Tissues Transformed by a Chimeric Isopentenyl Transferase Gene

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ABSTRACT

Tissues transformed with the isopentenyl transferase (*ipt*) gene cloned from the T-DNA region of the Ti plasmid or with the *ipt* gene placed under the control of the cauliflower mosaic virus 35S promoter (35S-*ipt*) were analyzed for auxin and cytokinin. Free and total indole-3-acetic acid (IAA) levels in 35S-*ipt*-transformed *Nicotiana tabacum* and cucumber cells were reduced by 12 to 78% in comparison to untransformed tissues. In contrast, free IAA concentrations in 35S-*ipt*-transformed *Nicotiana plumbaginifolia* were almost three times those of untransformed tissues, while total IAA levels were not significantly affected. Cytokinin levels in these antibodies were elevated an average of 300-fold resulting in a 24- to over 2000-fold increase in the cytokinin-to-auxin ratios. High cytokinin-to-auxin ratios correlated with the shooty phenotype of transformed tissues propagated *in vitro* in the absence of added growth hormones. We conclude that increased endogenous cytokinin-to-auxin ratios can induce cells to undergo morphogenesis and that elevated cytokinin levels can also induce auxin-autonomous growth of transformed tissues without causing a corresponding increase in endogenous IAA levels.

The importance in plant development of the relative levels of two major classes of phytohormones, the cytokinins and auxins, has long been recognized. Determination of endogenous phytohormone concentrations during growth and development may help define the role of cytokinins and auxins in various patterns of differentiation. However, because of their low endogenous pools such determinations have been difficult to obtain. In an alternate approach, experiments using exogenous phytohormones established that regeneration of shoots or roots from many tissue cultured cells can be induced by increasing or decreasing, respectively, the cytokinin-to-auxin ratio of the growth medium. Since not all crop species respond to such a treatment, this may reflect problems of uptake, compartmentalization, or metabolism of exogenously supplied phytohormones. The ability to manipulate the endogenous hormone levels *in vivo* alleviates such problems.

Endogenous hormone levels have been reported for normal plant tissues and ones transformed with the T-DNA from wild-type and mutant Ti plasmids of *Agrobacterium tumefaciens* (1, 7, 9, 11, 12, 15, 20). The T-DNA region encodes genes involved in cytokinin and auxin biosynthesis and imparts the trait of hormone-autonomous growth on transformed cells (16). Elevated levels of cytokinins (Z[1] and ZR) and auxin (IAA) have been found in galls and tumors. Ratios of these two classes of hormones are speculated to control gall and tumor morphology (1). However in some of these studies, the reported phytohormone concentrations may have been confounded by the presence of phytohormone-producing *A. tumefaciens* cells present within the galls (1). Also, possible effects of other T-DNA genes on the expression of plant phytohormone genes or on other plant cell processes cannot be excluded (2, 14, 18).

To eliminate possible effects of other T-DNA genes, we cloned the gene encoding the first enzyme in the cytokinin biosynthetic pathway, isopentenyl transferase (*ipt*), from the T-DNA region (17). By replacing the native promoter on this gene with the 35S promoter from cauliflower mosaic virus, the level of *ipt* gene transcription was increased more than 10-fold in transformed tobacco and cucumber tissues. As we have reported, this correlated with more than a 100-fold increase in cytokinin content and an enhanced ability of the cells to undergo shoot organogenesis presumably due to increased cytokinin-to-auxin ratios (17). We report here the auxin levels for these transformed tissues and both cytokinin and auxin contents of additional samples not previously analyzed in order to study the effects of *in vivo* phytohormone ratios on the cells' morphogenic potential.

MATERIALS AND METHODS

Bacterial Strains

Strains of *Agrobacterium tumefaciens* used have been described previously (17). Briefly, in one strain, a truncated *ipt* gene cloned from pTiB6S3 was placed under the control of the 35S promoter from cauliflower mosaic virus, and the chimeric gene construct, termed 35S-*ipt*, was inserted into a binary plasmid, pBin19. Another strain carried the native Ti *ipt* gene.

[1] Abbreviations: Z, zeatin; ZR, zeatinriboside.

Shoot and Tumor Induction

Protocols used for infection of *Nicotiana* plants and cucumber hypocotyls and for leaf piece transformation have been published (17). Transformed tissues were propagated on MS agar medium with B_5 vitamins and kanamycin (100 μg/mL) (5, 13). Tissues were frozen in liquid N_2 10 d after passage and stored at −80°C until analyzed.

IAA Analysis

Analyses were done as previously described (3, 4). Plant material (2 g fresh weight) was ground in liquid N_2 and transferred into 8 mL of 65% isopropanol/35% imidazole buffer (pH 7.0). After adding [$^{13}C_6$]IAA as an internal standard (0.1–1 μg/g sample) and approximately 50,000 dpm of [^{3}H] IAA as a radiotracer (22.5 Ci/mmol, Amersham) the extract was equilibrated for 1 h at 4°C, centrifuged at 10,000*g* for 5 min, and the supernatant divided into two parts, one for analysis of free IAA and one for total IAA (free and conjugated forms) as described previously (3). Both the internal standard and radiotracer were provided by Jerry Cohen.

To determine free IAA content, the extract was applied to an amino anion exchange mini column (PrepSep, Fisher) conditioned by sequentially washing with hexane, ethyl acetate, acetonitrile, and methanol. IAA was eluted with methanol containing 2% acetic acid. The eluent was evaporated to dryness and resuspended in 50% methanol for HPLC purification on a C_{18} column packed with 5 μm Whatman ODS-3 resin. Radioactive fractions were methylated and analyzed by GC-MS selected ion monitoring as previously described (3).

For determination of total IAA (free and conjugated), the sample was hydrolyzed in sealed Teflon vials purged with water saturated nitrogen gas in the presence of 7 N NaOH for 3 h at 100°C after removal of the extraction medium by evaporation. The hydrolysate was diluted and titrated with 2 N HCl to pH 2.5 at 0°C, desalted on a conditioned C_{18} column, purified by HPLC, and analyzed by GC-MS selected ion monitoring as described above for free IAA.

Z and ZR Analysis

Frozen plant material (1–2 g fresh weight) was ground in liquid N_2, extracted and purified on an immunoaffinity column. Z and N^9-substituted derivatives were quantitatively analyzed by an ELISA technique using a kit provided by DDS[2] (De Danske Sukkerfabrikker, Copenhagen) as previously reported (17).

RESULTS

IAA Analysis

The 35S-*ipt* gene construct appeared to negatively affect the free and total IAA levels in two *N. tabacum* cultivars, Xanthi and MD609, and in cucumber. Free IAA levels were reduced by an average of 66% and total IAA by 29% when compared to normal tissues (Table I). However, in *N. plumbaginifolia* tissues transformed with the 35S-*ipt* gene, an average 2.8-fold increase in free IAA was observed, while the total IAA was not significantly elevated. In *N. plumbaginifolia* tissues transformed with the native Ti *ipt* gene, free IAA was elevated an average of 1.4-fold when compared to normal tissues but was only half the levels found in the 35S-*ipt*-transformed antibodies.

Z Content

Z and ZR levels determined for additional 35S-*ipt*-transformed tissues were similar to those previously reported by us (17) and are included in Table I for comparison purposes. Taken together, the Z and ZR levels in these shoots and tissues averaged about 300-fold higher than in normal tissues (Table I). Highest increases in Z and ZR were in *N. tabacum* cv Xanthi. Multiple copies of the 35S-*ipt* gene integrated into the genome of two of these lines (giving 8,017 and 14,831 pmol *Z + ZR*/g fresh weight), as well as cucumber (data not shown) (17) may be contributing to the higher levels of Z and ZR observed. In contrast to the 35S-*ipt*-transformed tissues, only a sevenfold increase over normal levels was observed in tissues transformed with the native Ti gene.

Table I. *Phytohormone Concentrations in Tissues Transformed by T-DNA Cytokinin Gene under Control of 35S Promoter or Its Native Ti Plasmid Promoter*

Tabulated are the Z, ZR, and IAA concentrations.

Tissue	*ipt* gene	Z + ZR	IAA[a]	Z + ZR[b] / IAA
			pmol/g fresh wt	
N. plumbaginifolia	35S-*ipt*	3548	48 (266)	74 (13)
		2416[c]	50 (244)	48 (10)
	Native Ti	239	29 (161)	8.2 (1.5)
		391	22 (160)	18 (2.4)
	Endogenous	36[c]	18 (229)	2.0 (0.16)
N. tabacum cv Xanthi	35S-*ipt*	8017	20 (254)	401 (32)
		14831	21 (259)	706 (57)
		6667	ND[d]	317 (26)[e]
		2236	ND	106 (8.7)[e]
	Endogenous	15[c]	53 (295)	0.3 (0.05)
N. tabacum cv MD609	35S-*ipt*	2478[c]	24 (181)	103 (14)
		3019	33 (98)	91 (31)
		4337	ND	150 (31)[e]
		6891	ND	238 (49)[e]
	Endogenous	23[c]	84 (236)	0.3 (0.1)
Cucumber	35S-*ipt*	5340[c]	11 (80)	485 (67)
	Native Ti	83[c]	10 (64)	8.3 (1.3)
	Endogenous	31[c]	49 (123)	0.6 (0.25)

[a] This value represents free IAA and the number in parentheses total IAA (free and conjugated forms). [b] Calculated for free IAA and in parentheses for total IAA. [c] Values previously reported (ref. 17). [d] Not determined. [e] Calculated using the average IAA concentrations determined for that particular genotype and *ipt* construct.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of the other products that may also be suitable.

Comparison of Phytohormone Ratios to Tissue Morphology

The ratios of cytokinin-to-auxin were greatly elevated in antibodies transformed with the 35S-*ipt* gene construct whether calculated on the basis of free or total IAA (Table I). The Z + ZR/IAA ratios for *Nicotiana* tissues ranged from 48 to 706 for free IAA and 8.7 to 57 for total IAA (Table II). Corresponding values for tissues transformed with the native *ipt* gene were 8.2 to 18 and 1.5 to 2.4 for free and total IAA, respectively. These values represent an average 710-fold increase in cytokinin-to-auxin ratio in tissues transformed with the 35S-*ipt* gene and a sevenfold increase in those transformed with the native gene. The comparable increases in ratios using total IAA were 387- and 12-fold.

The elevated cytokinin-to-auxin ratios were associated with a shooty phenotype of transformed tissues. Such teratomas were characterized by loss of apical dominance and inhibition of root formation (Table II) (17). In a few cases, however, tissues were composed mainly of unorganized cells which had the highest Z + ZR/IAA ratios obtained for a particular genotype (Table II). This phenomenon was observed with certain *N. tabacum* cv Xanthi, MD609 and cucumber tissues transformed with the 35S-*ipt* gene. These calli exhibited dark green areas corresponding to shoot buds which never developed further.

DISCUSSION

In this study we examined the effects of a single introduced gene, isopentenyl transferase, on cytokinin-to-auxin ratios in transgenic plant material. All of the transformed tissues examined were characterized by high Z + ZR/IAA ratios due mainly, but not entirely, to greatly elevated levels of Z + ZR. To our knowledge, all previous reports of both cytokinin and auxin concentrations were on tissues transformed with wild-type and mutated Ti plasmids carrying the unaltered *ipt* gene (1, 7, 15). In those studies, the cytokinin content appeared to be elevated to some extent over levels in normal plants, however, considerable variation was observed. The varieties of tobacco and alternate analytical methods employed may have contributed to the differences observed. In addition, other T-DNA genes introduced with the *ipt* gene may have had modulating effects in the transformed tissues. This is depicted by the recent reports which implicate regulatory roles for the TL-DNA from the Ri plasmid in transgenic tobacco plants and for the *tml* locus from the Ti plasmid in soybean tumors (14, 18).

We observed a reduction in free and total IAA levels in two genotypes of tobacco and in cucumber transformed with the 35S-*ipt* gene. Free IAA levels in these plants were reduced to an even greater extent than total IAA. These findings are contrary to numerous reports of enhanced IAA levels resulting from exogenous applications of cytokinins (6, 8, 10, 19) or from transformation of tobacco and carrot with the native *ipt* gene carried on a mutated Ti plasmid (7). We only observed a significant increase in free IAA in *N. plumbaginifolia* tissues transformed with the 35S-*ipt* gene or, to a lesser degree, with the native gene. However, total IAA levels in *N. plumbaginifolia* as well as cucumber transformed with the native Ti gene were lower.

Surprisingly, all of the *ipt*-transformed *Nicotiana* and cucumber tissues exhibited auxin-autonomy. Since, in general, a corresponding increase in IAA levels was not observed, we speculate that cytokinin-producing cells may be providing a signal for inducing auxin-autonomous growth. Recently, Binns *et al.* (2) have shown that *N. glutinosa* cells carrying the Ti *ipt* gene also exhibit auxin-autonomous growth.

Elevated ratios of cytokinin-to-auxin in 35S-*ipt* transformed tissues correlated with the more rapid and prolific induction of shoots observed on stems infected with this construct. Some transformed shoots, however, began to proliferate mainly as undifferentiated cells. Interestingly, the cytokinin concentrations and the cytokinin-to-auxin ratios in these transformed unorganized tissues were the highest observed within a particular group of infected tissues. From these observations it seems possible that plant cells are induced to undergo morphogenesis with increasing endogenous cytokinin-to-auxin ratios; however at a certain point the effect may become detrimental to the cells' ability to differentiate and/or survive. This may explain why many of our tissue cultured shoots failed to survive on hormone-free medium. Although initially assumed to be untransformed, in retrospect it seems likely that at least some of these shoots were lost because of unfavorably high cytokinin levels.

Table II. *Comparison of Z + ZR/IAA Ratios and Phenotypes of Nicotiana and Cucumber Tissues Transformed with the ipt Gene Constructs*

ipt Gene Construct	Z + ZR/IAA[a]	Morphology
Endogenous	0.3–2.0	Normal leaves
Native Ti	8.2–18	Teratomas
35S-*ipt*		
N. plumbaginifolia	48–74	Teratomas
N. tabacum cv	106–401	Teratomas
Xanthi	706	Callus
N. tabacum cv	91–103	Teratomas
MD609	150–238	Callus
Cucumber	485	Callus

[a] Ratios taken from Table I.

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