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Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene

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Abstract

The bacterial isopentenyl transferase (*ipt*) gene involved in cytokinin biosynthesis was fused with a promoter from the proteinase inhibitor II (PI-IIK) gene and introduced into *Nicotiana plumbaginifolia*. Transcripts of the *ipt* gene were wound-inducible in leaves of transgenic PI-II-*ipt* plants. In leaf disks excised from fully expanded leaves, transcript levels increased 25- to 35-fold within 24 h and by 48 h were reduced by about 50%. In flowering plants, message levels were 2- to 5-fold higher than in preflowering plants. These plants were used to test for defensive properties of cytokinins against insects. *Manduca sexta* larvae consumed up to 70% less of the PI-II-*ipt* leaf material on flowering plants than larvae feeding on controls. Normal development of *Myzus persicae* nymphs was also delayed. Approximately half as many nymphs reached adulthood on PI-II-*ipt* leaves than on controls. Zeatin and zeatinriboside levels in leaves remaining on PI-II-*ipt* plants after hornworm feeding were elevated by about 70-fold and the chlorophyll *a*/*b* content was double that of controls. Exogenous applications of zeatin to the PI-II-*ipt* leaves enhanced the level of resistance to the tobacco hornworm and almost completely inhibited normal development of the green peach aphid nymphs. Transcript levels of an acidic chitinase gene were low and minimally inducible in PI-II-*ipt* leaves. The mode of action of the cytokinin gene product on enhanced insect resistance is not clear but may involve the products of secondary metabolic pathways.

Introduction

Phytohormones are known to have pivotal roles in promoting normal growth and development of plants and may also contribute to the mechanisms of defense [9, 11, 24, 25]. Cytokinins are among the most active plant substances discovered and have been implicated in the physiological and biochemical processes with marked effects on flowering, fruit set and ripening, leaf

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senescence, seed germinatin, and stomatal function [9, 28]. Exogenously applied cytokinins have been shown to suppress the induction of hypersensitive necrosis by viruses [3, 4]. High endogenous cytokinin levels in non-rooting tobacco shoot lines (T-cyt) transformed with a gene involved in cytokinin biosynthesis caused an increase in the expression of defense-related mRNAs [22]. A group of pathogenesis related proteins encoded by these genes is coordinately induced by wounding and pathogenic infections [7, 37].

Cytokinins as well as other plant hormones have commercial applications as bioregulators and in combination with endogenous hormones may protect plants from pests and pathogens by inducing physiological changes in the plants [13, 14, 15, 34]. Cytokinins have been shown to influence secondary metabolic pathways whose products exhibit insecticidal properties [6, 25, 33]. Utilization of numerous secondary metabolites in crop protection, either by conventional plant breeding or by genetic engineering, is being evaluated [12, 13]. Since current interests are focusing on combined effects of natural defense mechanisms of plants and biotechnology for crop improvement, we evaluated the role cytokinin may play in insect resistance [23]. To avoid the uptake and metabolism associated with exogenous hormone applications, tobacco plants were genetically engineered with a wound-inducible cytokinin biosynthesis gene. The isopentenyl transferase (*ipt*) gene from *Agrobacterium tumefaciens* was fused with a promoter from the proteinase inhibitor II (PI-IIK) gene known to be induced in the leaves of transgenic plants by mechanical wounding and insect chewing [19, 20]. We are reporting on the enhanced resistance of these plants to the tobacco hornworm (*Manduca sexta*) and the green peach aphid (*Myzus persicae*).

Materials and methods

*Construction of the PI-II-*ipt *gene*

A chimeric cytokinin gene was constructed by fusing the bacterial isopentenyl transferase (*ipt*) gene [30] to the 5′ regulatory region of the potato proteinase inhibitor II-K (PI-II) gene [36]. A 0.8 kb *Eco* RI/*Bam* HI DNA fragment from plasmid pRT24 which contains the PI-II gene promoter was fused through its 5′-untranslated region to the coding region of the *ipt* gene from pTiB6S3 [5, 29]. The truncated *ipt* gene carries its own transcription terminator and polyadenylation signals. Within the reconstructed gene, a transcription initiation site is located 101 bp upstream of the *ipt* gene start codon. An *Eco* RI/*Hin*d III PI-II-*ipt* fragment was subcloned into a binary plant transformation vector and mobilized into *A. tumefaciens* strain EHA101 (pEHA101) for infection and transformation of *Nicotiana plumbaginifolia* leaf disks as previously described [17, 18, 29]. A binary vector which carries a truncated *ipt* (t-*ipt*) gene without a functional promoter was used as a negative control for transformation experiments [29].

Plant material

Reconstructed *ipt* genes were transferred to the plant genome by cocultivation of *A. tumefaciens* bacteria with *N. plumbaginifolia* 43A leaf disks [29]. Ten kanamycin-resistant, independently transformed shoots were regenerated. Seeds from the primary transformants were germinated on kanamycin-containing medium (100 μg/ml) and resistant progeny screened for wound-inducible expression of the *ipt* gene. Three 2.5 cm incisions were made on each half of a fully expanded leaf and 24 h later RNA was extracted and analyzed as described below. Homozygous kanamycin-resistant R2 progeny were selected from R1 plants segregating 3:1 for kanamycin resistance.

Insect feeding experiments

Fully expanded leaves on PI-II-*ipt* plants were used in leaf disk and whole leaf assays. Leaf disks of 1.45 cm^2 were cut with a cork borer and placed in a 60 mm × 15 mm Petri dish on filter paper wet with water or water plus the cytokinin zeatin at

10 and 20 μg/ml in 1 or 2% methanol, respectively (Sigma). Petioles of detached leaves were submerged in sealed vials containing water or water plus zeatin as above and placed in large Petri dishes lined with filter paper. Surface areas of leaf disks and leaves were measured before and after insect feeding with a surface area meter (LI-COR, Lincoln, NE). Data in Figs. 2 and 3 were analyzed by analysis of variance and means were compared using the least significant difference test.

M. sexta larvae (tobacco hornworms) were maintained on an meridic diet prior to the feeding trials. For each experiment, a weighed neonate or a third-instar larvae was placed in a Petri dish with either 5 leaf disks or a whole leaf. The larvae were allowed to feed for 48 or 72 h. When whole plants were infested, a single neonate larvae was used per plant and plants were wrapped during the last instar with cheesecloth to prevent escape. Larval weights were recorded weekly. Three to five plants per treatment were infested in 3 replicated experiments.

M. persicae nymphs were propagated in a rearing chamber for feeding experiments. A nymph less than 12 h old was placed on a single leaf disk. After 8 days we determined whether the nymph was alive or dead, the stage of development (nymph or adult female), and whether or not the adult female reproduced.

Nucleic acid preparations and blot hybridizations

A rapid alkaline lysis method was used for preparation of plasmid DNAs [21]. High-molecular-weight plant DNA was prepared by the method of Dellaporta *et al.* [10] and treated with RNase A (100 μg/ml, Sigma) as previously described [29]. Total RNA was isolated from leaf material using a guanidium isothiocyanate method of Chirgwin *et al.* [8]. DNA (10 μg) was cut with restriction enzymes and electrophoresed in 1.0% agarose. RNA (20 μg) was fractionated on 1.2% agarose-formaldehyde gels. For Southern analysis, gels were soaked in 0.25 M HCl for 10 min and blotted onto Zeta-probe nylon membrane (Bio-Rad, Richmond, CA) using 0.4 M NaOH. For northern analysis, RNA gels were blotted in 20 mM Sodium-acetate, pH 5.0. Blots were hybridized with either a 0.6 kb *Ava* II/*Hinc* II internal fragment of the *ipt* gene from *A. tumefaciens* or a 0.9 kb *Eco* RI fragment of acidic chitinase (PR-Q) [26]. Probes were labelled by random priming and used in hybridization reactions at 65 °C for 24 h in 0.5 M sodium phosphate buffer pH 7.0, 1 mM EDTA and 7% (w/v) SDS. Blots were washed at 65 °C with 40 mM sodium phosphate pH 7, 1 mM EDTA and 5% SDS and exposed to X-AR5 film at −80 °C. Northern blot message quantitations were obtained on a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). Blots were stripped and reprobed with a *Pst* I/*Pvu* I 1.3 kb fragment coding for an 18S rRNA from *Xenopus laevis* in order to adjust for any differences in RNA loading and transfer to the nylon membranes [32].

Chlorophyll analysis

Chlorophyll was extracted by homogenizing 0.1 g leaf tissue in liquid N_2 and resuspending in 5 ml of cold 80% acetone. After overnight incubation at −20 °C, the extracts were cleared by centrifugation and the absorbance measured at 645 and 665 nm to determine chlorophyll *a* and *b* concentrations [16]. Averages of duplicate samples are reported.

Zeatin analysis

The concentration of zeatin and N^9-substituted zeatin derivatives were determined using analytical kits as previously reported [29, 30, 31]; De Danske Sukkerfabrikker, Copenhagen; IDETEK, San Bruno, CA). Plant tissues were extracted in 80% methanol overnight at −80 °C. All extracts were purified on columns packed with antizeatin riboside antibodies and eluted cytokinins quantified by ELISA using both kits. To determine the percent recovery, control samples were spiked with 1000 to 2000 pmol of zeatin

riboside or zeatin (Sigma). For each plant, 3 to 4 samples were analyzed.

Results

Analysis of transgenic tobacco plants for wound-induced expression of the ipt *gene*

A cytokinin gene whose expression is activated by mechanical wounding and insect chewing was constructed and introduced into *N. plumbaginifolia* genomes. The chimeric PI-II-*ipt* gene codes for the first enzyme in the cytokinin biosynthetic pathway and its expression is regulated by the promoter from the PI-IIK gene [19, 30, 36]. Fusions with the β-glucuronidase or chloramphenicol acetyl transferase (CAT) reporter genes indicated that the PI-IIK promoter directs gene expression in tissues preferentially consumed by insects and that CAT gene expression in leaves of transgenic tobacco plants had no effect on the extent of leaf consumption by various insects [20, 35, 36]. Ten kanamycin-resistant, independently transformated shoots were chosen and grown to maturity. Some distinguishable alterations in transgenic plant phenotypes were observed (A. Smigocki, unpublished). R1 and R2 PI-II-*ipt* plants were slightly taller and had bigger leaves with specific leaf weight (g/cm_2) reduced by 40% in comparison to controls.

Primary transformants and R1 progeny were screened for wound inducible expression of the *ipt* gene since is was initially reported that the 3′ end of the PI-IIK gene was required to confer wound inducibility on a reporter gene fused to the same promoter [36]. However upon further analysis using a more sensitive system, the expression of the reporter gene with a different 3′ end was found also to be wound-inducible and leaf specific in all transgenic tobacco plants [2]. We also demonstrate that the expression of the *ipt* gene which carries its own transcription and polyadenylation signals is wound inducible in leaves of PI-II-*ipt* transgenic plants (Fig. 1A).

For insect feeding studies, R2 PI-II-*ipt* plants homozygous for kanamycin resistance were selected from seeds of R1 plants segregating 3:1 on kanamycin-containing media. The *ipt* gene transcript levels in disks excised from fully expanded leaves of preflowering and flowering PI-II-*ipt* plants increased 25- to 35-fold in 24 h. About 50% of the transcripts were still detected after 48 h (Fig. 1B). At all time points analyzed, the *ipt* message levels in flowering plants were 2- to 5-fold higher than in preflowering plants.

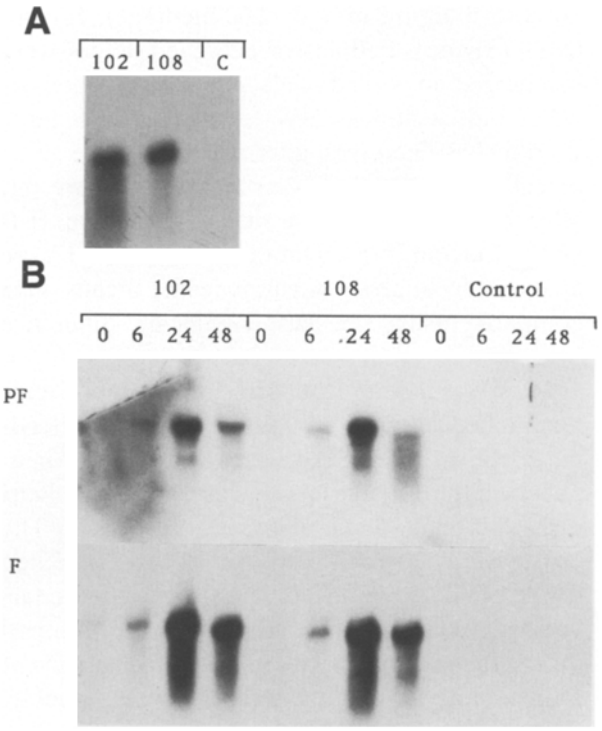

Fig. 1. A. Induction of *ipt* transcripts by wounding. Northern blot of total leaf RNA from wounded R1 PI-II-*ipt* (102, 108) and transgenic control (C) tobacco plants. B. Analysis of *ipt* transcript levels in leaf disks at 6, 24, and 48 h after excision from fully expanded leaves on preflowering (PF) and flowering (F) homozygous R2 PI-II-*ipt* plants (102, 108) and control plants. The 0 time corresponds to RNA from tissues collected immediately after excision.

*Analysis of insect feeding on transgenic PI-II-*ipt *plants*

In leaf disk tests, *M. sexta* larvae were fed leaf material from flowering PI-II-*ipt* plants (Fig. 2A). Larvae consumed only 8 and 13% of the leaf

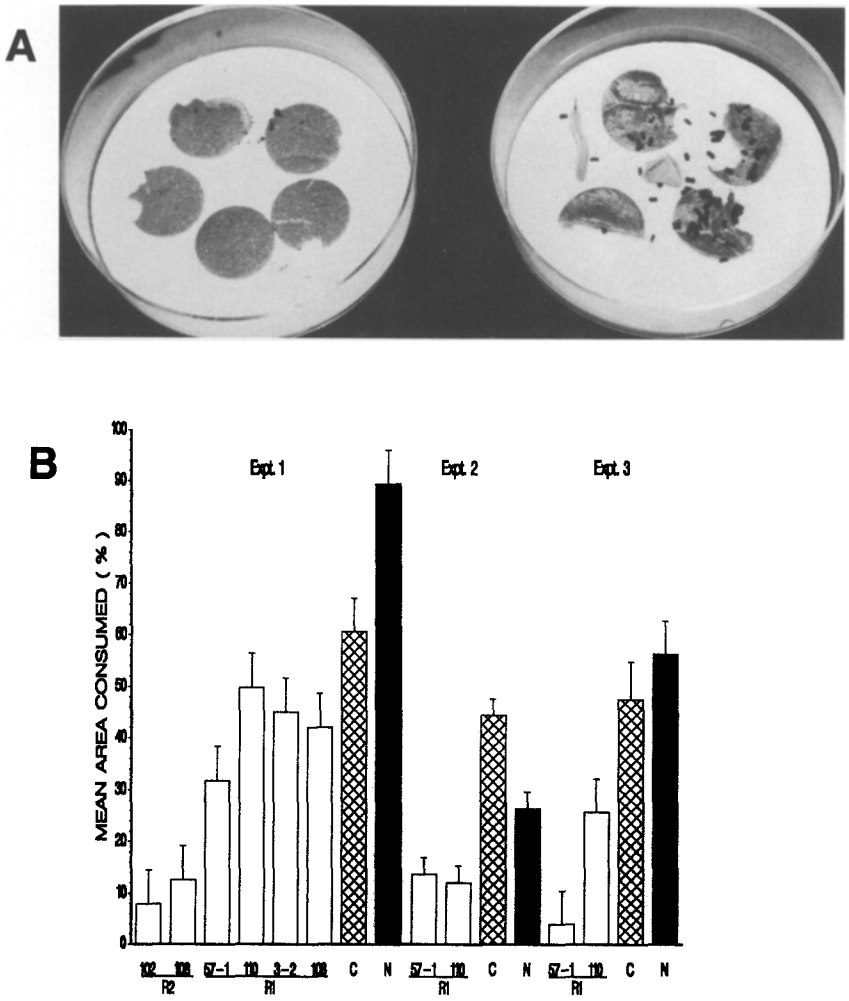

Fig. 2. A. Consumption of leaf disks from PI-II-*ipt* transformant 110 (left) and from normal untransformed plant (right) 24 h after infestation with third-instar *M. sexta* larvae. B. Proportion of mean leaf disk area consumed by larvae feeding on PI-II-*ipt*, transgenic control (C), and normal (N) plants in 48 h. Disks were excised from homozygous R2 (102 and 108) and heterozygous R1 (57-1, 110, 3-2 and 108) PI-II-*ipt* plants. Three plates per treatment were used in Exp. 1 and 5 plates in Exp. 2 and 3.

disks as measured by area when feeding on homozygous transformants 102 and 108, respectively (Fig. 2B, Exp. 1). In comparison, larvae fed disks excised from transgenic control and untransformed plants consumed 5 to 11 times more of the tissues. Insects feeding on disks from heterozygous R1 plants (57-1, 11, 3-2, 108) consumed 20 to 50% less than those feeding on controls. Two additional experiments with heterozygous plants 57-1 and 110 confirmed a significant decrease in leaf disk area consumption when compared to the controls (Fig. 2B, Exp. 2 and 3). An overall reduction in all disks consumed by the larva in Exp. 2 and 3 corresponds to less feeding by neonates vs. third instar larvae used in Exp. 1. Since we determined that the specific leaf weight (g/cm^2) of the PI-II-*ipt* plants is reduced by about 40%, the magnitude of the observed enhanced resistance is that much more significant.

Homozygous R2 progeny were further analyzed by an excised leaf assay (Fig. 3A). Third-instar tobacco hornworm larvae feeding on leaves from flowering PI-II-*ipt* plants (102 and 108) consumed only about a third to a half of what was consumed by larvae feeding on control leaves

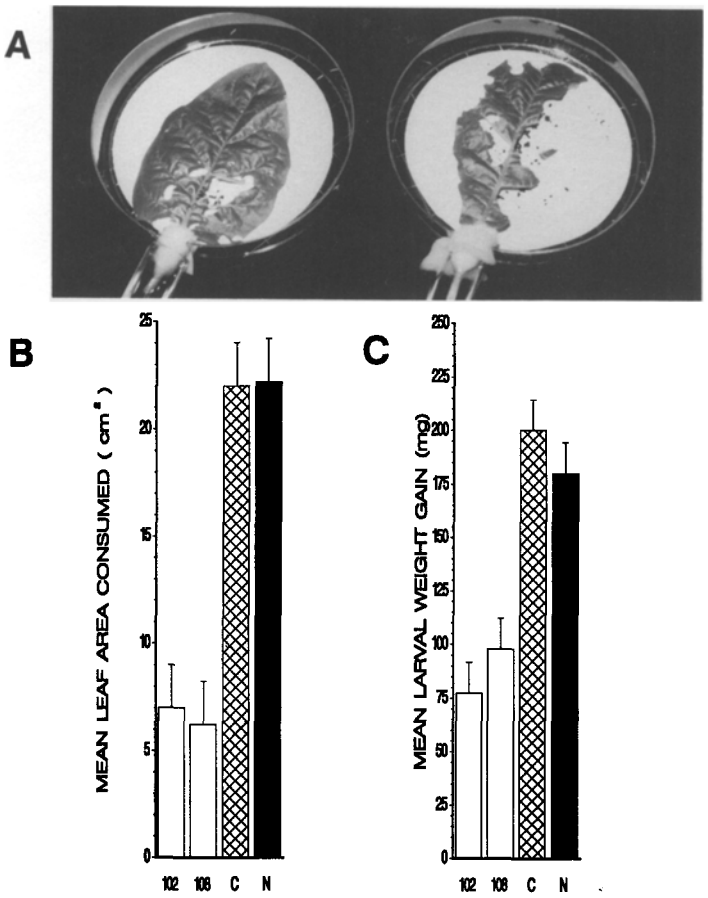

Fig. 3. Whole leaf assay for insect resistance. A. Detached leaves from flowering, homozygous PI-II-*ipt* plant 102 (left) and transgenic control plant (right) 72 h after infestation with third instar *M. sexta* larvae. B. Mean leaf area consumed by larvae after 72 h of feeding on flowering PI-II-*ipt* (102, 108) control (C) and normal (N) leaves. Ten leaves per treatment were used. C. Mean weight gain of larvae feeding on the same leaves.

(Fig. 3B; Table 1). Mean larval weight gain was reduced by 30 to 60% in comparison to the controls (Fig. 3C, Table 1).

Resistance at the whole plant level was evaluated by infesting homozygous PI-II-*ipt* flowering plants with a single neonate hornworm larvae.

Table 1. Resistance of homozygous R2 PI-II-*ipt* plants (102, 108) to the tobacco hornworm larvae. Effects of zeatin (Z; 10 μg/ml) uptake by leaves from preflowering and flowering PI-II-*ipt* plants were determined by calculating the mean leaf area consumed and larval weight gain after 3 days of feeding. Each number represents an average of six replicates.

Plants	Mean leaf area consumed (cm^2)				Mean larval weight gain (g)			
	preflowering		flowering		preflowering		flowering	
	–	Z	–	Z	–	Z	–	Z
102	25	9	18	5	0.23	0.22	0.17	0.10
108	24	14	14	11	0.25	0.23	0.23	0.17
Normal	24	23	34	29	0.29	0.32	0.32	0.30

About 19 days later, the larvae pupated, however, the consumption of the PI-II-*ipt* plants was greatly reduced in comparison to the control plants (Fig. 4). In general, all the insects preferred the newly emerging and younger leaves and only when these were exhausted they fed on the older leaves [35]. During the course of the experiment weights of larvae feeding on PI-II-*ipt* or control plants did not differ significantly and all emerging adults appeared normal.

A leaf disk assay was used to evaluate the resistance of PI-II-*ipt* plants to the green peach aphid. After 8 days of feeding on disks from flowering PI-II-*ipt* plants (102 and 108), 30 to 40% of the nymphs developed into adult females, and those 50 to 80% reproduced (Table 2). On control and normal tissues, on the average, 74% of the nymphs reached adulthood and 93% reproduced.

Effect of zeatin applications on insect feeding

Feeding zeatin through petioles of leaves excised from PI-II-*ipt* plant prior to flower development, caused a 40 to 60% reduction in mean area consumed by the tobacco hornworm larvae and a 30% reduction of mean larval weight gain as compared to normal controls (Table 1). Zeatin uptake by leaves from flowering PI-II-*ipt* plants boosted the level of insect resistance normally observed in these plants by up to 36% based on mean area consumed. A similar response to zeatin was not observed with any of the leaves from normal, untransformed plants. In control experiments with 1% methanol alone, no negative effects on the hornworm feeding were observed.

Placing leaf disks from PI-II-*ipt* or control and normal plants on filter paper wet with zeatin at 10 or 20 μg/ml delayed the development of the green peach aphid nymphs (Table 2). Of the surviving nymphs, most were immature and did not reproduce within the 8 day test. Nymphs feeding on control leaf disks placed in 1 or 2% methanol developed normally.

Fig. 4. Enhanced resistance of PI-II-*ipt* plants (left) to *M. sexta* larvae as compared to transgenic control plants (right). After 19 days of feeding, the larvae consumed much less of the leaf material on flowering, homozygous 108 plants in comparison to control plants.

Characterization of transgenic plants

At the end of the whole plant experiment, leaves remaining on PI-II-*ipt* plants that were either not consumed or partially fed on by the hornworm were markedly greener than any remaining leaves on control plants (Fig. 4). The chlorophyll *a*/*b* content in these leaves was more than two times that of control tissues. The PI-II-*ipt* leaf chlorophyll levels were ca. 2300 μg/g fresh weight. Levels of zeatin had zeatin riboside cytokinins, which are major cytokinins produced in tissues transformed with the *ipt* gene, were greatly elevated [1, 29, 30, 31]. In partially consumed leaves, zeatin and zeatin riboside concentrations averaged 500 to 550 pmol/g fresh tissue, a greater than 70-fold increase over endogenous cytokinin levels in control tissues (7 pmol/g). Cytokinin levels in detached leaves at the end of a 72 h infestation were slightly lower (ca. 400 pmol/g). Just prior to larval infestation and wounding, leaf cytokinin levels were 35 to 70 pmol/g.

Transcription of one of the coordinately regulated defense related genes previously reported to be modulated by cytokinins was also examined in

Table 2. Results of green peach aphid feedings on flowering homozygous R2 PI-II-*ipt* plants (102, 108) without and with exogenously supplied zeatin (10 μg/ml and 20 μg/ml). Presented are the percent of nymphs still alive and the percent of adult females (if any) that had reproduced at the end of the 8 day test. Each number represents an average of four independent tests without added zeatin done in replicates of 10 disks per treatment. The zeatin results are averages of two independent tests with 5 disks per treatment.

Plants	Percent alive			Percent adult females with nymphs		
	0	10 μg/ml zeatin	20	0	10 μg/ml zeatin	20
102	40	10	10	53	0	0
108	32	50	10	83	50	0
Control	67	20	20	89	0	0
Normal	80	20	60	97	50	40

PI-II-*ipt* plants [22]. Acidic chitinase gene (PR-Q) transcripts were analyzed in the upper and lower leaves, stems, and roots of preflowering and flowering plants. In unwounded flowering PI-II-*ipt* plants 102 and 108, chitinase message levels in leaves and stems were generally low as in untransformed plants (Fig. 5). However, in the roots transcript levels were higher in PI-II-*ipt* plants than in the controls. Within 3 to 6 h after wounding of the lower leaves, there was a slight induction of the message in leaves and stems and a 3- to 5-fold increase in roots (Fig. 5A). In preflowering and control plants, chitinase message levels were low in all tissues analyzed and did not increase significantly after wounding (data not shown).

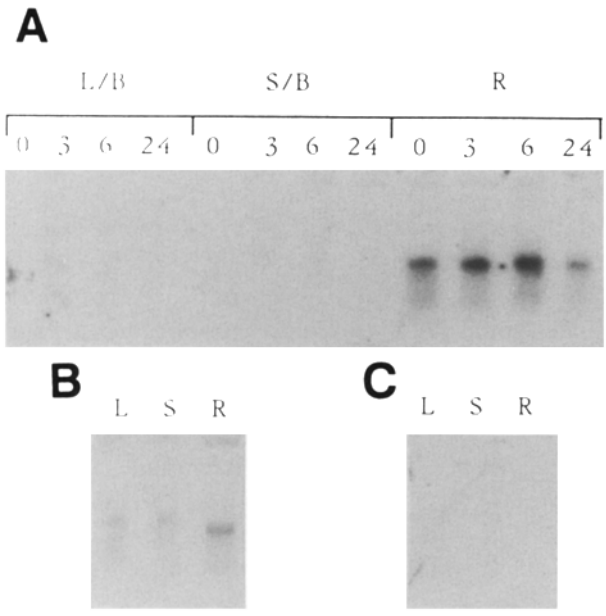

Fig. 5. Analysis of chitinase gene (PR-Q) transcription in PI-II-*ipt* plants. A. Transcript levels in lower leaves (L/B), lower stems (S/B), and roots (R) of a homozygous 108 plant at 0, 3, 6, and 24 h after wounding of the lower leaves. Transcript levels in leaves (L), stems (S) and roots (R) of unwounded 102 (B) and untransformed plant (C).

Discussion

Cytokinins comprise a major group of plant hormones that regulate normal growth and development and influence secondary metabolic pathways that have been shown to produce compounds with insecticidal properties. In order to assess the potential role of cytokinins for increasing natural defenses of plants by genetic engineering, a chimeric cytokinin gene was constructed that is expressed in plant tissues preferentially consumed by insects [35]. The potato PI-IIK gene promoter, which was fused to the cytokinin biosynthesis gene, has been shown to be regulated by developmental and environmental stimuli when fused with numerous reporter genes [20, 35, 36]. Wound-induced expression of the reporter genes appeared not to affect insect feeding [35]. By introducing the chimeric cytokinin gene into tobacco, we were able to demonstrate an increase in *ipt* gene transcription upon

mechanical wounding of the leaves and that the *ipt* transcript levels were 2- to 5-fold higher in fully expanded leaves from flowering plants as compared to preflowering plants (Fig. 1A and 1B). This is not unexpected since it is well established that the promoter is not only induced in plant foliage by mechanical wounding but also becomes constitutively expressed in tubers and flowers during the reproductive stage of plant growth [20].

The relatively high levels of transcript of the *ipt* gene in leaves of flowering PI-II-*ipt* plants provided material for testing the defensive properties of cytokinins in *N. plumbaginifolia* against *M. sexta* and *M. persicae*. When the *M. sexta* larvae were fed leaf disks or whole leaves from flowering PI-II-*ipt* plants, they consumed significantly less of the plant material than larvae feeding on leaves from control plants (Figs. 2 and 3); Table 1). A corresponding decrease in larval weight gain was also observed. At the whole plant level, less PI-II-*ipt* leaves were consumed but no significant differences in larval weights were recorded (Fig. 4). It appears that sufficient feeding material is provided by younger leaves and the abundance of lateral buds released during reproductive stage of growth of the PI-II-*ipt* plants (A. Smigocki, unpublished). On normal plants, newly emerging and younger leaves have been reported to be preferred by these insects [35]. We find lower *ipt* gene transcript levels and cytokinin concentrations in younger leaves of PI-II-*ipt* plants (Fig. 1B, A. Smigocki, unpublished). Green peach aphid feedings on leaf material from flowering PI-II-*ipt* plants delayed normal development of newly hatched nymphs into adult females by about 50% (Table 2). In addition, of the nymphs that reached maturity, fewer were able to reproduce as compared to controls.

Enhanced resistance to the tobacco hornworm and green peach aphid is observed when PI-II-*ipt* plants are in the mid to late flowering stage of growth (Table 1). Since the PI-IIK gene promoter is constitutively expressed during tuber and flower development, we speculate that it is the combination of induction of the cytokinin gene by wounding and normal development that raises the cytokinin concentrations to levels which may be necessary for the increased resistance. Highest concentrations of zeatin and zeatin riboside cytokinins, known to be overproduced in *ipt* transformed tissues, were found in leaves remaining on PI-II-*ipt* plants after tobacco hornworm feeding [1, 31]. Cytokinin levels were elevated by ca. 70-fold in comparison to controls. Enhanced resistance to the tobacco hornworm was induced in leaves from preflowering plants by boosting the endogenous cytokinin levels with exogenous applications of zeatin (Table 1). A higher degree of resistance was also observed when leaves from flowering PI-II-*ipt* plants were supplied with zeatin. This response to zeatin was not observed with leaves from normal, untransformated plants and may reflect problems associated with sufficient uptake, metabolism, or compartmentalization of exogenously supplied cytokinins necessary to retard hornworm feeding. We speculate that in preflowering PI-II-*ipt* plants even low levels of zeatin uptake are sufficient to further increase the endogenous cytokinin concentrations up to levels which are effective in retarding larval feedings. Similar uptakes in normal plants, however, would still not raise the endogenous concentrations to effective levels. The effects of zeatin applications on delaying the green peach aphid development were more dramatic in that most of the nymphs did not reach maturity. The green peach aphid tolerance to cytokinin effects appears to be lower than that of the tobacco hornworm and may be directly related to their much reduced overall body mass. Zeatin application results suggest that use of a stronger constitutive promoter to express the cytokinin gene would increase endogenous cytokinin concentrations to even higher levels than those in PI-II-*ipt* plants and result in better insect control. We have previously reported that overexpression of the *ipt* gene with the 35S promoter from cauliflower mosaic virus increases zeatin levels up to several hundred fold in *N. plumbaginifolia* [31]. However, the constitutive overproduction of cytokinin in plant cells inhibits regeneration of whole plants. Temporal and tissue specific expression allows for regeneration of plants and is preferred for expression of a for-

eign gene as for example in leaves upon insect feeding.

Presently, the mode of action of the cytokinin gene product on enhanced resistance is not clear. Cytokinin has been reported to modulate the expression of coordinately regulated defense-related genes such as chitinase [22]. But, chitinase gene transcription in leaves from flowering or preflowering PI-II-*ipt* plants was low and minimally inducible (Fig. 5). Higher levels of the transcript were detected only in roots of flowering plants before and after leaf wounding. Therefore, direct involvement of these defense genes in the observed resistance is doubtful. Transformation of tobacco plants with the proteinase inhibitor gene has been shown to be inhibitory to insect growth, but we did not specifically look for expression of this gene in our PI-II-*ipt* plants because to our knowledge no homologous sequences have been detected in tobacco [19, 27]. We speculate that products of secondary metabolic pathways affected by elevated cytokinin levels are the likely candidates for the observed enhanced resistance.

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