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Light-induced expression of *ipt* from *Agrobacterium tumefaciens* results in cytokinin accumulation and osmotic stress symptoms in transgenic tobacco

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Abstract

Cytokinins are plant growth regulators that induce shoot formation, inhibit senescence and root growth. Experiments with hydroponically grown tobacco plants, however, indicated that exogenously applied cytokinin led to the accumulation of proline and osmotin. These responses were also associated with environmental stress reactions, such as salt stress, in many plant species. To test whether increased endogenous cytokinin accumulation led to NaCl stress symptoms, the gene *ipt* from *Agrobacterium tumefaciens*, encoding isopentenyl transferase, was transformed into *Nicotiana tabacum* cv. SR-1 under the control of the light-inducible *rbcS-3A* promoter from pea. In high light (300 μmol PPFD m^{-2} s^{-1}), *ipt* mRNA was detected and zeatin/zeatin glucoside levels were 10-fold higher than in control plants or when transformants were grown in low light (30 μmol PPFD m^{-2} s^{-1}). High light treatment was accompanied by increased levels of proline and osmotin when compared to low light grown transformed and untransformed control plants. Elevated *in planta* cytokinin levels induced responses also stimulated by salt stress, suggesting either common or overlapping signaling pathways are initiated independently by cytokinin and NaCl, setting in motion gene expression normally elicited by developmental processes such as flowering or environmental stress.

Abbreviations: IPT, isopentenyl transferase; *rbcS-3A*, gene encoding a small subunit protein (SSU) of Rubisco from *Pisum sativum*; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase

Introduction

Cytokinins are generally considered adenine derived plant growth regulators with several important biological properties. It has been widely accepted that one function attributable to cytokinin is the inhibition of plant senescence [29, 20]. However, some studies argue that natural and synthetic cytokinins can also act as effective defoliants [44, 34, 36]. Such conflicting responses to cytokinin treatment indicate that the linkage between cytokinin and senescence is not suffi-

ciently understood. Likewise in a related process, increased exogenous cytokinin can mimic salt-induced responses [39, 40], yet cytokinin levels tend to decrease under salt-stress conditions [18, 39].

The paradox that cytokinin concurrently promotes and discourages senescence and other developmental programs, such as vegetative growth, is also observed during flower initiation. Cytokinin levels decreased in roots with advanced age, but accumulated in phloem and adjacent leaves just prior to floral initiation [29, 35]. Furthermore, cytokinins are known to participate in the complex combinatorial processes of flower initiation [4, 22]. Increased cytokinin concentration in floral buds has been shown to stimulate early flower-responsive genes, while negatively regulating the accumulation of three later-stage floral homeotic genes [14]. Thus, conclusions based on the effects of a plant growth regulator such as cytokinin on a given plant tissue or organ must take into account the developmental state of the plant material being treated.

Regarding the normal course of plant development and flowering, the halophyte *Mesembryanthemum crystallinum* L. (the common ice plant) initiates a series of metabolic changes gradually leading to the metabolic switch from C3 to crassulacean acid metabolism (CAM) as the plant ages [5]. NaCl stress within a temporal window, determined largely by growth conditions, accentuates a developmental program leading to rapid changes in osmoprotecting metabolites (proline and D-pinitol, a cyclic polyol), in accumulation of the pathogenesis-related protein, osmotin, and in the synthesis of CAM enzymes (e.g. phosphoenolpyruvate carboxylase) [1, 5, 40, 43]. These responses may be viewed as the induction of defense mechanisms against abiotic stress. All responses are also elicited by cytokinin treatment, albeit only in whole plants [38, 39, 40]. It appears that in the ice plant multiple stimuli trigger several stress-related responses, perhaps a general observation as similar results have been reported in tobacco for other inducible genes [13, 25].

A comparison of plant gene expression, senescence and morphology during uninduced and induced cytokinin synthesis and salt stress may provide insights into the role of cytokinin *in planta* during various environmental and developmental processes. One tool to examine this question would be to engineer cytokinin synthesis under the control of an inducible promoter. The *Agrobacterium tumefaciens* gene, *ipt,* encodes isopentenyl transferase, IPT, the enzyme which catalyzes the rate-limiting step in cytokinin biosynthesis, condensation of AMP and isopentenyl pyrophosphate to form isopentenyl-AMP [20]. Previous studies have used the overexpression of the *ipt* gene to demonstrate that resulting plant phenotypes are similar to those obtained when cytokinin is applied exogenously. For example, expression of *ipt* under the control of the constitutive CaMV-35S promoter resulted in a ca. 100-fold increase in cytokinin levels, which subsequently increased the rate of organogenesis and adventitious shoots on tobacco leaves [31]. Heat shock (HS)-inducible promoters have also been used for similar purposes [24, 2].

Questions arising from our previous work are focused here on whether cytokinin could stimulate salt stress-associated responses, proline and osmotin accumulation, in glycophytes as in the halophytic ice plant, given that glycophytes and halophytes differ greatly in survival under salt stress. Secondly, would induction of endogenous cytokinin accumulation, using a transgenic approach, produce analogous responses to those seen with exogenous application? To examine these questions, we treated *Nicotiana tabacum* L. cv. SR-1 with cytokinin and measured changes in proline and osmotin levels. In addition, we constructed, transformed and monitored the expression of the *ipt* gene of *A. tumefaciens* under control of a light-inducible promoter in transgenic plants. Under high or low light levels, cytokinin concentration, plant morphology, proline and osmotin were recorded. Our results indicated that the two salt-stress-linked responses, proline and osmotin accumulation, were induced by exogenous cytokinin application and by induced expression of the transferred *A. tumefaciens ipt* gene. In addition, plants overproducing cytokinin were characterized by wilting as observed during NaCl

stress. These results suggest that the similar responses upon NaCl stress and exposure to augmented cytokinin levels may be due to either a similarity between the respective signaling mechanisms, or due to overlapping elements, such as induction or synergism with other plant growth regulators, possibly reflecting the evolution of abiotic stress response pathways.

Materials and methods

Whole plant studies and suspension cells

Whole *N. tabacum* L. cv. SR-1 plants were grown hydroponically in 5 l of Hoagland's solution according to Thomas [39]. After five weeks, the plants were treated either with 250 mM NaCl or 10 μM 6-benzylaminopurine (6-BAP) in Hoagland's solution. Leaf samples were harvested after 1, 3 and 7 days and the tissue frozen in liquid nitrogen.

Suspension cells of tobacco (SR-1) were obtained by initiating callus on MS medium plus 30 g/l sucrose, 11 μM naphthalenacetic acid (NAA), 0.5 μM 6-benzylaminopurine (6-BAP) and 1% Bacto agar. Friable callus was placed in identical medium and grown at 25 °C, 30 μmol PPFD m^{-2} s^{-1} with 2 μM 2,4-dichlorophenoxyacetic acid (2,4-D). A rapidly growing cell line was diluted 1 to 10 in fresh medium (30 ml in a 125 ml Erlenmeyer flask) in MS medium containing 30 g/l sucrose with 2 μM 2,4-D and either without or with 250 mM NaCl, or with 10 μM 6-BAP. Cultures were harvested after 1, 3 and 7 days and analyzed for proline.

DNA construction

The *ipt*-coding region of pHSCkn312 [32] was excised with *Eco* RI and *Hin*d III, and both the 1.3 kb (heat shock 70 promoter of *Drosophila* + *ipt* gene) fragment and a larger fragment (including the SSU 3′ + vector) were isolated with GeneClean (Bio 101). The larger fragment (SSU 3′ fragment) was digested with *Cla* I and the 600 bp fragment containing the SSU 3′ polyadenylation site gel-purified. The SSU 3′ fragment was ligated into *Hin*d III/*Cla* I digested pBS + vector to yield pJTSSU 3′. The 1.3 kb *Eco* RI/*Hin*d III fragment from pHSCkn312 was digested with *Rsa* I and the 990 bp *ipt* gene fragment inserted into the *Sma* I and *Hin*d III sites of pJTSSU 3′. The resultant plasmid, pJT *ipt* SSU3′, was restricted with *Xba* I and *Sal* I, overlaps were filled in with Klenow fragment of DNA pol I, and the insert gel-purified. The isolated fragment was ligated into an *Eco*-RV digested pJTSSU-B3, a clone of a pea *rbcS-3A* small subunit gene of Rubisco previously inserted into the *Hin*d III site of pBS+ (Fig. 3). The resulting plasmid, pJTSSU-*ipt*, was digested with *Kpn* I and *Sst* I and the SSU-*ipt*-*RbcS-3A* fragment inserted into the identical site in pBin 19. Mating into *A. tumefaciens* LBA 4404 and generation of transgenic tobacco plants were according to Thomas [37].

pJTSSU-GUS was constructed by first subcloning pRJ275 (Clonetech) into pBS BS+ as a *Pst* I, *Eco* RI fragment. Next, the nopaline synthase polyadenylation site from pJTNos A (unpublished) was added as an *Eco* RI and *Xba* I fragment and inserted into identical sites 3′ of the GUS gene. The SSU promoter of Rubisco *rbcs-3A* from pea was isolated from pJTSSU-B3 as a *Hin*d III fragment and inserted 5′ upstream of the GUS-Nos A construction. The entire SSU-GUS-Nos A insert was restricted with *Kpn* I and *Sst* I and ligated into identical sites in pBIN 19. The resultant pJTSSU-GUS plasmid was transferred into LBA4404 and used to transform tobacco leaf disks as described above.

RNA analysis

RNA was extracted from tobacco leaves in 2 volumes extraction buffer [21] with the addition of 1% (w/v) lithium dodecyl sulfate (LDS), 1.5% (w/v) sarcosyl and 1% (w/v) NP-40. Twenty μg of RNA was electrophoresed in a formaldehyde 1% agarose gel, RNA transferred to GeneScreen Plus (NEM) and UV cross-linked according to

the manufacturer's instructions. The 990 bp *ipt* gene was isolated from pJTSSU-*ipt*, labeled with αdATP^{32} (random primer method), denatured and added to 10 ml of hybridization buffer (50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate with 200 μg/ml salmon sperm DNA). Blots were hybridized for 16 h at 42 °C, washed in 2–0.1 × SET (1 × SET = 100 mM NaCl, 1 mM EDTA, 50 mM Tris pH 6.8) with 1% SDS at 60 °C, and exposed on Kodak X-OMAT/AR film with intensifying screens at −70 °C.

Proline analysis and protein blotting

Proline and immunological detection of osmotin with a chicken anti-osmotin antibody (a gift of Dr R.A. Bressan, Purdue University), was as described in Thomas and Bohnert [40].

Zeatin + and zeatin-related compounds

Zeatins were extracted from 20 mg of lyophilized leaf material in 5 ml of 80% (v/v) methanol at 4 °C for 16 h, filtered, applied to a Sep-Pack C_{18} column (Alltech Associates, Milwaukee, WI) and eluted with 80% methanol. Samples were evaporated, dissolved in water and analyzed by enzyme-linked immunosorbent assay (ELISA). Zeatin standards were used for calibration (PhytoScience Biotechnologie Végétale, Angers, France), and the analysis error was calculated as ±4.3% per sample.

Results

Either 250 mM NaCl or 10 μM 6-BAP was added to 5-week old hydroponically grown tobacco plants. After 1, 3 and 7 days randomly selected leaves were removed, pooled and analyzed for proline. Both NaCl and cytokinin induced the accumulation of proline in whole tobacco plants (Fig. 1). In contrast, non-salt adapted suspension grown cells responded to NaCl stress, but not cytokinin, by accumulating proline (Fig. 1). No

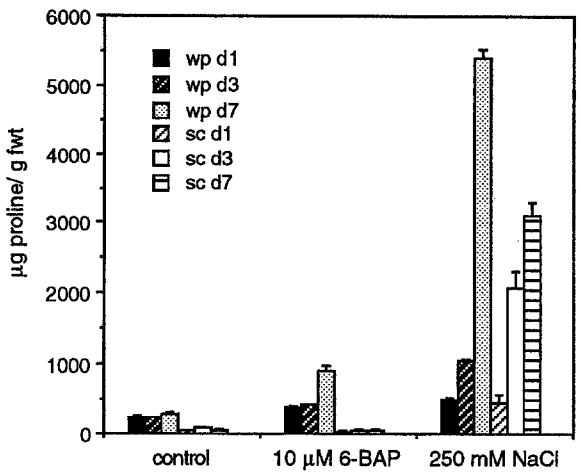


Fig. 1. Proline accumulation in whole plants and suspension cells in response to NaCl and cytokinin. Five-week old tobacco (cv. SR-1) were grown at 24 °C for 16 h in high light (250 μmol PPFD m^{-2} s^{-1}) in 5 l/plant of Hoagland's solution and the roots aerated. At the initiation of the experiment, fresh nutrient solution was added either with 10 μM 6-BAP, 250 mM NaCl or no additive control. Random samples of 3 leaves (pooled) were taken 1, 3 and 7 days after experiment initiation. Samples were ground in liquid N_2 and analyzed for proline. Data represent the mean and SE of three experiments. Wp, whole plants; Sc, suspension cells.

further experiments were conducted with suspension cells.

Protein extracts from the hydroponically grown plants were prepared and subjected to SDS-PAGE, blotted to nitrocellulose and reacted with an anti-osmotin antibody. Three osmotin isoforms of different mobility were detected. In NaCl-stressed plants a protein of 24 kDa was prevalent after 7 days (Fig. 2). A second, less abundant, protein was also observed, migrating at approximately 29 kDa. In whole plants treated for 7 days with cytokinin two isoforms of osmotin, 24 and 26 kDa accumulated.

The *ipt* coding sequence originally from *A. tumefaciens* was assembled under the control of the *rbcS-3A* promoter and 3′ polyadenylation site (Fig. 3). Following introduction into tobacco, several plant phenotypes were observed. In low light, ca. 10% of the transformants appeared normal and produced roots. This phenotype was considered wild type-like. In low light the remaining 90% of the transformants formed a shooty

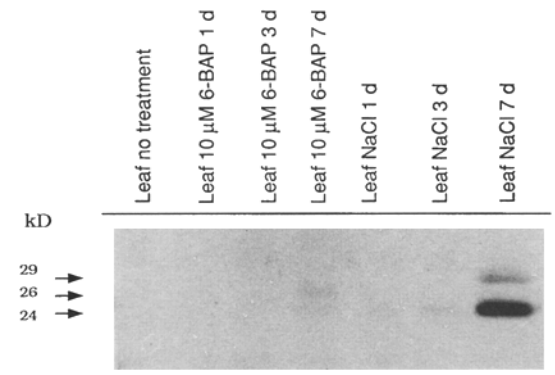


Fig. 2. Osmotin induction by NaCl and 6-BAP. Samples were from the same experiment as in Fig. 1. Each lane contained 40 μg total protein. Arrows indicate the 24, 26 and 29 kDa isoforms detected with the chicken anti-osmotin antibody.

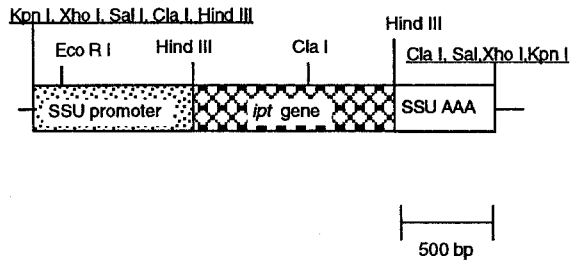


Fig. 3. pJTSSU-*ipt* DNA construction. SSUp, promoter of small subunit of RUBISCO from pea; SSU 3′, polyadenylation site of the SSU gene of small subunit of Rubisco from pea. Restriction sites: E, *Eco* RI; H, *Hind* III, Xh, *Xho* I; Xb, *Xba* I, S, *Sst* I.

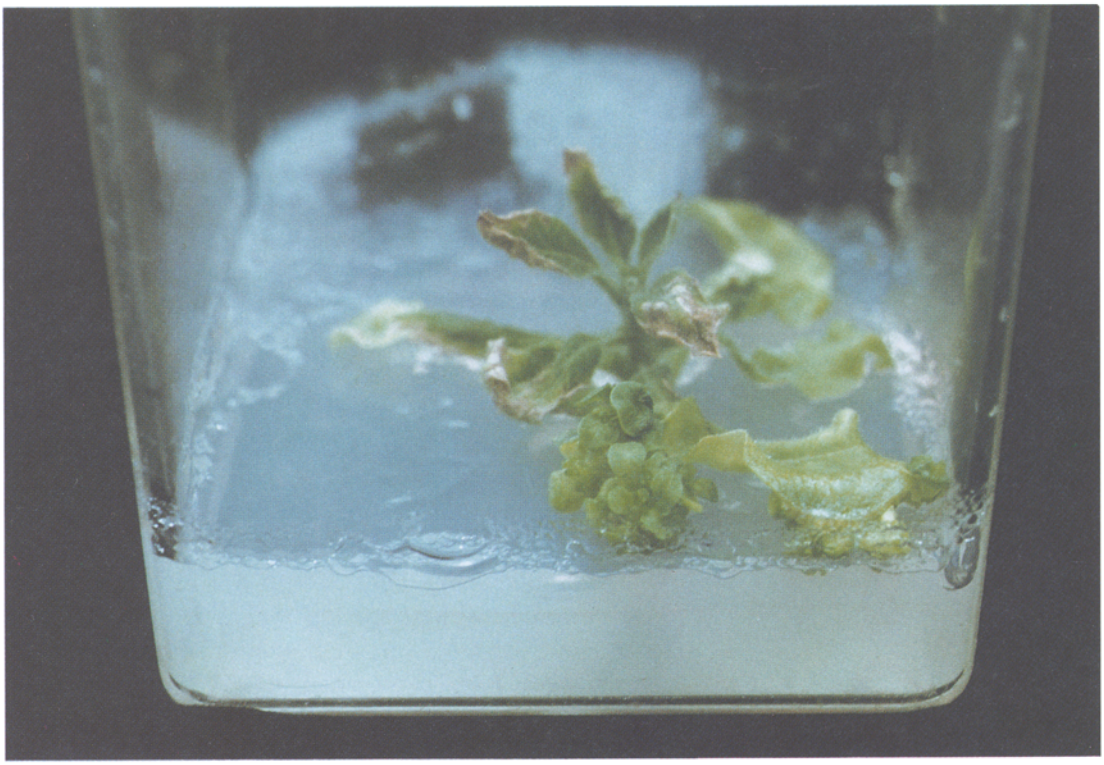
and leafy mass with little apical dominance, structures characteristic of increased cytokinin to auxin ratios in tobacco plants (Fig. 4a). In this group, plants developed with little apical dominance, very reduced internodal distances and eventually rooted when grown for extended periods in low light (30 μmol PPFD m^{-2} s^{-1}).

Under high light, wilting and browning of leaves were also observed in SSU-*ipt* plants prior to the appearance of multiple green regions on the leaves where new shoots formed (Fig. 4b). In some of the transformed plants where rooting was observed, chlorophyll levels were reduced in leaves (Fig. 4c). Seeds (T-1 generation) from the SSU-*ipt* 1 transformant were selected for kanamycin resistance. These progeny plants displayed a loss of apical dominance and multiple side-shoot development when seedlings were grown for 3 weeks under high light (12 of 19 plants), as compared to 26 of 26 transformant seedlings displaying apical dominance when grown under low light. Apical dominance and the number of side shoots was unaffected in high or low light grown SSU-GUS control progeny, all displaying apical dominance. Furthermore, when leaves of the progeny of the SSU-*ipt* 1 transformant were wounded and placed on growth regulator free medium, a shooty phenotype was again observed.

Cytokinin levels were determined for each light treatment in control and SSU-*ipt* transformed plants. In high light (300 μmoles PPFD m^{-2} s^{-1}), zeatin/zeatin glucoside levels were 10-fold higher in SSU-*ipt* plants than in control or when transformants were grown in low light. After prolonged periods of high light the three shooty phenotypes (SSU-*ipt* 1, 2 and 3) contained 6.60, 1.21 and 1.24 pmol/g fresh weight of cytokinin compared with 0.30, 0.27 and 0.29 pmol/g fresh weight found in transformed plants after incubation in low light. Control SSU-GUS plants also contained 0.47 and 0.29 pmol/g fresh weight in low and high light respectively, similar to amounts reported by Smigocki and Owens in tobacco [31, 32].

Northern analysis of control (SSU-GUS) and three independent SSU-*ipt* transformants indicated that after a 14-day culture period under high light a 990 bp band, corresponding to the size of the expected *ipt* transcript, hybridized to the *ipt* gene probe (Fig. 5). No hybridization to the *ipt* probe was observed in low light-grown SSU-*ipt* and in control plants.

When incubated *in vitro* in high light (300 μmol PPFD m^{-2} s^{-1}), proline accumulation was nearly 100-fold higher than in the low light (30 μmol PPFD m^{-2} s^{-1}) grown plants in three SSU-*ipt* transformants analyzed (Fig. 6). This was also true for the kanamycin-resistant segregants of the progeny line of SSU-*ipt* 1, which behaved as the original transformant (Fig. 6). Not all transformants, however, contained elevated proline levels when grown in high light. A kanamycin-resistant line from one of the (10%) wild-type like regenerants (SSU-*ipt* 4) did not ac-



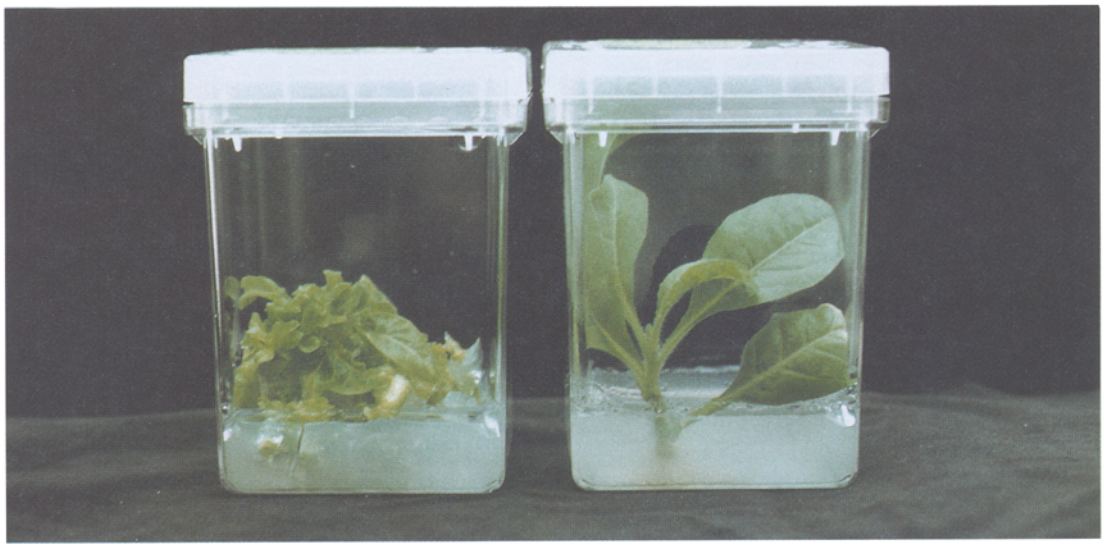


Fig. 4. Phenotype of transformed tobacco expressing *Ipt*. A (top). Shooty phenotype of low light grown SSU-*ipt* transformants (left) and a SSU-GUS transformant (right). B (middle). Senescence induced in high light grown SSU-*ipt* transformed plants (left) and SSU-GUS on right. Note browning of leaves and diminutive size of the SSU-*ipt* plant. Photographed after 2 weeks of light treatment after subculture. C (bottom). Comparison of the growth habit and greening of SSU-GUS control plant (center), and SSU-*ipt* transformants, one with a shooty morphology (right) and another with altered chlorophyll distribution (left).

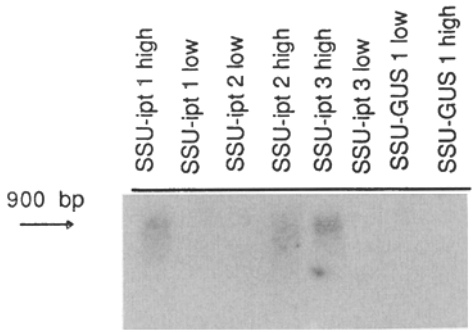


Fig. 5. Northern analysis of high light (H) or low light (L) grown SSU-*ipt* transformants. SSU-*ipt* 1 indicates transformant 1, SSU-*ipt* 2 etc. Samples are for identical experiment as shown in Fig. 4. Control RNA was extracted from SSU-GUS plants. A total of 25 μg/lane of total RNA was used.

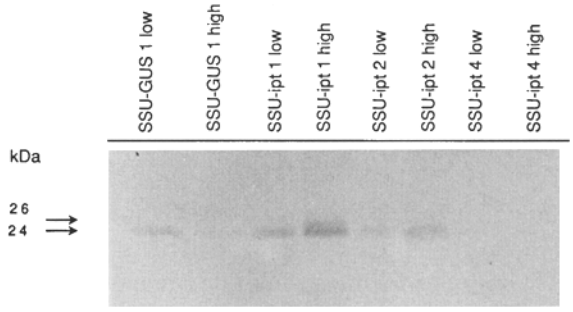


Fig. 7. The effects of high or low light on osmotin isoform prevalence in SSU-GUS and SSU-*ipt* plants. Samples are identical to those in Figs. 4, 5 and 6. Arrows indicate the 24, 26 and 29 kDa isoforms detected with the chicken anti-osmotin antibody.

cumulate proline when grown in high light (Fig. 6). This transformant also did not contain detectable *ipt* transcript (data not shown). The behavior of this transformant supported the interpretation that the biochemical stress symptoms observed were correlated with decreased apical dominance and a shooty phenotype, indicative of cytokinin over-expression. Control plants (SSU-GUS) did not respond to high light by accumulating proline (Fig. 6).

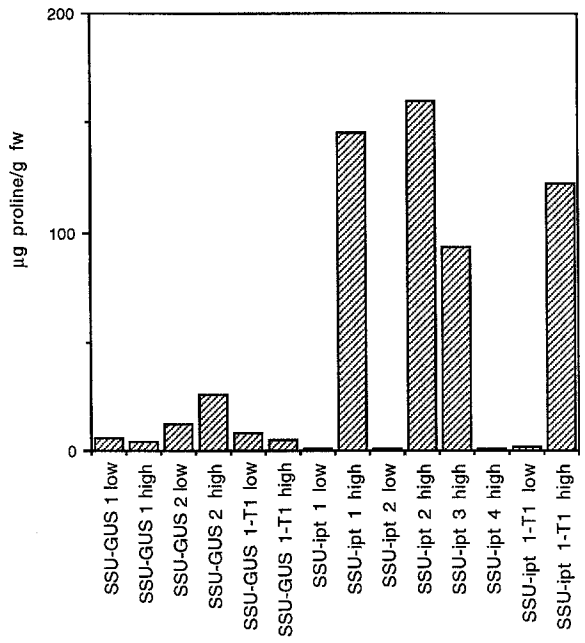


Fig. 6. Proline accumulation in high light or low light grown SSU-*ipt* transformants 1, 2, 3 etc. and SSU-*ipt* 1 progeny seedlings (T1-P). Nomenclature and samples as in Figs. 4 and 5.

Using an antibody to osmotin, the SSU-GUS control transgenic plants were shown to contain higher levels of osmotin (24 kDa isoform) under low light compared to the amount found after high light treatment. In contrast, the 24 kDa isoform and 26 kDa isoform increased in high light as compared to low light in the SSU-*ipt* plants (Fig. 7). The wild-type like SSU-*ipt* 4 transformant did not accumulate appreciable levels of osmotin (24 kDa) either after high or low light treatment (Fig. 7).

Discussion

Are responses to cytokinins related to osmotic stress?

To protect plants against osmotic stress, several strategies or combinations of mechanisms seem to be important. These mechanisms include: (1) augmented changes in development, such as the timing of flowering, (2) altered structural traits, such as leaf form or trichomes and changes in the cuticle, (3) compartmentalization and excretion of salt (to the vacuole on a cellular level), such as glands or by assigning specific storage tissues, or exclusion systems through alterations in uptake, (4) changes in photosynthesis and carbon meta-

bolism, such as the switch from C3 to CAM in the ice plant, and (5) osmotic adjustments, exemplified by the accumulation of proline or other metabolites [23, 5]. Proline accumulation following stress occurs in isolated cells as well as in whole plants [38, 39]. However, it remains to be seen whether this response is an adaptive mechanism to combat stress or whether proline accumulation reflects other changes in metabolism, as not all plants can resist osmotic stress even when proline is accumulated [12]. It has been proposed that proline accumulation may limit localized cell damage in salt-stressed plants. Supporting this idea, salt-induced proline accumulation is only observed upon cell injury [15]. Alternatively or additionally, transient protection by increases in proline may also provide time for signal molecules to be generated and transmitted to other, as yet unstressed tissues.

Osmotin gene expression is highly regulated in tobacco in a tissue-specific way [16]. Accumulation of osmotin is localized mainly to roots, flowers and to the epidermis of stems in tobacco, and its expression is regulated by both transcriptional and post-transcriptional mechanisms [19, 26]. Cytokinin could act on either of these control processes. Induction of osmotin synthesis by a variety of environmental factors lead to its classification as a member of the PR-5 subgroup of pathogenesis related proteins [25]. In SR-1 tobacco, 24 and 29 kDa osmotin proteins were induced following salt stress, while 24 and 26 kDa protein isoforms were detected with a 7 day cytokinin treatment (Fig. 3). Similarly, SSU-*ipt* containing transformants produced a 24 kDa and a 26 kDa protein. The amount of both isoforms increased when the plants were grown in high light, and they were correlated with increased accumulation of cytokinin in these same tissues.

From the results reported here and from our previous investigations [38, 39, 40] it is clear that cytokinins elicit responses that are similar to those generally associated with responses to environmental stresses. Many of the changes in gene expression and related stress reactions are also induced by the growth regulator abscisic acid (ABA) [46]. Thus it appears that developmental and environmental sensor and/or response pathways are in some way inter-connected.

Cytokinin, senescence and environmental stress

Other work has indicated a role for cytokinins in establishing juvenile plant characteristics [29, 20]. Both exogenous cytokinin application [29] and genetic modification leading to endogenous accumulation of cytokinins and increased cytokinin/auxin ratios inhibit plant senescence and stimulate shoot regeneration in tobacco [32, 30]. Cytokinin estimates from SSU-*ipt* and SSU-GUS plants are consistent with the idea that the shooty phenotype and loss of apical dominance may result from the accumulation of cytokinin and the greater cytokinin to auxin and or ethylene ratios. Similar results have been obtained by *Ipt* expression under control of a heat shock promoter (HSP70) [24]. Mimicking exogenous application, Smart [31] demonstrated that expression of *Ipt* effectively delayed senescence in transformed tobacco plants. Contrarily, cytokinins have been linked to stimulation of the plant growth regulator ethylene [44, 34], whose action is generally viewed as opposing the action of cytokinins. Application of cytokinin can induce senescence in *Arabidopsis thaliana* [45], and excessive endogenous cytokinin overproduction can also cause a senescence-like response in transgenic tobacco [2].

From the above discussion, the existence of overlapping and/or opposing response networks to multiple stimuli in higher plants is likely. A number of different environmental stimuli have been shown to affect NaCl stress and ABA stimulated responses in tomato and the ice plant [7, 43]. At the physiological level, salt stress, ABA and cytokinin can affect the stomatal response [6]. Correlations between osmotic stress, cytokinins, and flowering have also been established before [25, 13]. Thus, senescence and anti-senescence activities exerted by cytokinins are not clearly separated and likely depend not only on the absolute cytokinin amount, but also the cytokinin ratio with respect to other growth regula-

tors (e.g. auxin, ethylene). One report suggested that cytokinin treatment in the presence of Ca^{2+} stimulated ethylene formation 15-fold compared to controls [44]. In *Arabidopsis*, cytokinin inhibited ethylene production in detached leaves cultured in the dark, but in high light cytokinin concentrations stimulated ethylene formation 4-fold and hastened senescence [45]. Gibberellins have also been implicated in interacting with cytokinin during plant senescence [28]. Thus, by having multiple positive and presumably negative signaling steps, greater coordination of the complex responses to growth, development and stress may be highly and tightly regulated.

Gene expression patterns under stress in the ice plant suggest a mechanism for how different stimuli may act to induce the expression of genes that respond to several stimuli. A key regulatory enzyme in the CAM pathway, phospho*enol*-pyruvate carboxylase (PEPCase), is induced in response to salt stress, drought, ABA, and cytokinin treatment [9, 10, 11, 42, 39]. While experimentally inducible by the above-mentioned stimuli, in the absence of stress PEPCase accumulates gradually during development leading to flowering. Recently, a cytokinin response element has been reported in *Agrobacterium tumefaciens* (ATGCCCCACA) [27], which is 70% similar to a sequence 560 bp upstream of the start of PEPCase transcription in the ice plant C [11]. Response elements for ABA and a salt-responsive *cis*-acting element appear to be present and can be identified in the ice plant PEPCase promoter (J.C. Cushman, personal communication). It is conceivable that the cytokinin responsive *cis*-acting element in this promoter may respond to transacting factors activated by increased cytokinin levels accompanying the gradual increase of PEPCase as this halophyte approaches flowering [see 10, 11].

Independent signal pathways to flower induction have been proposed, dependent on both cytokinin and gibberelic acid modulations [3]. This view is consistent with a role for cytokinin in late-flowering *Arabidopsis* mutants [17] and mutants that accumulate large amounts of cytokinin, resulting in plants with small hypocotyls, etiolated leaves and which flower precociously [8]. A phloem-localized increase of cytokinin may initiate flower development [35, 41, 4, 22] and may also, *via* common or overlapping pathways, initiate salt stress-associated responses. While zeatin and zeatin riboside levels do not increase dramatically during salt stress [18, 39] we cannot exclude possible localized changes in growth regulator concentration during salt stress or changes in titer of distinct cytokinin derivatives, some of which specifically promote flowering responses [41].

Cytokinins, which stimulate pathways that lead to completion of development, flowering in particular, may act on NaCl-related signal transduction pathways and *vice versa* using common *cis*- and *trans*-acting components and modifiers in the promoters of the affected genes. Because the glycophyte tobacco and the halophytic ice plant both respond to cytokinins and salt stress by inducing the same pathways [39, 40], perhaps some of the salt stimulated signal transduction pathways used in halophytes have been adapted or recruited from normal developmental pathways in glycophytes to cope with environmental stress. The data presented here, using a transgenic tobacco model which can be induced to express Ipt, points towards an evolutionarily conserved mechanism in sensing and responses to salt stress and cytokinin, perhaps utilizing several divergent secondary messengers (e.g. ethylene, Ca^{2+}). Future investigations aimed at understanding environmental stress tolerance must consider interrelationships between the particular stress and the developmental state of the stressed plants.

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