Two distinct isopentenyl diphosphate isomerases in cytosol and plastid are differentially induced by environmental stresses in tobacco[1]

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Received 26 June 2001; revised 14 August 2001; accepted 26 August 2001

First published online 14 September 2001

Edited by Marc Van Montagu

Abstract Two distinct cDNA clones (*IPI1* and *IPI2*) encoding *IPI* were isolated from *Nicotiana tabacum*. In situ expression of isopentenyl diphosphate isomerase-1 (IPI1)– and IPI2–green fluorescent protein fusion constructs revealed that IPI1 and IPI2 were localized in chloroplast and cytosol, respectively. The level of *IPI1* mRNA was increased under high-salt and high-light stress conditions, while that of *IPI2* mRNA was increased under high-salt and cold stress conditions. Both *IPI* transcripts were increased in an abscisic acid-independent manner. This is the first report of a cytosolic IPI. The results indicated that two distinct IPIs were differentially induced in response to stress.

Key words: Isopentenyl diphosphate isomerase; Isoprenoid; Stress; Tobacco; Chloroplast; Cytosol

1. Introduction

Isoprenoids constitute a large class of natural products playing important roles in plants. Most isoprenoids have been assumed to be synthesized in the classical cytosolic mevalonate pathway from acetyl-CoA via isopentenyl diphosphate (IPP; C5). Recently, however, a novel isoprenoid biosynthetic pathway (non-mevalonate pathway), where IPP is formed from pyruvate via 1-deoxy-D-xylulose-5-phosphate, was discovered in bacteria [1]. In the cytosolic mevalonate pathway, steroids, sesquiterpenes, ubiquinones and dolicohols are synthesized. In contrast, phytohormones (e.g. abscisic acid, gibberellin and cytokinin), phytols, carotenoids and monoterpenes are derived via a plastidic non-mevalonate pathway [2]. IPP is a common precursor of all isoprenoids in both pathways. IPP isomerase (IPI; EC 5.3.3.2) catalyzes the conversion of IPP to dimethylallyl diphosphate to form the basic five-carbon isoprene unit for IPP condensation. IPI was suggested to be localized in cytosol, plastid, mitochondria and endoplasmic reticulum, by the location of isoprenoids and geranylgeranyl diphosphate (GGPP) synthase, which condenses IPP to GGPP. However, although many IPI cDNAs have been isolated from plants, the presence of cytosolic IPI has not been demonstrated [3,4].

In plants, isoprenoids function in the protection from and adaptation to stresses. For example, terpenoids as phytoalexin have been investigated in detail. It was reported that 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme of the mevalonate pathway, was induced by elicitor treatment [5,6]. Some isoprenoids play roles in protection against environmental stresses. For protection of the photosynthetic apparatus, the quantity and composition of carotenoid compounds, particularly xanthophylls, are increased to detoxificate free radical and active oxygen caused by high-light [7,8]. Like carotenoids, tocopherols are also effective antioxidants. Isoprene has been suggested to enhance the thermo-tolerance of leaves in some plant species [9]. ABA, which is synthesized from carotenoid, is accumulated in response to salt and cold stress, and induces many stress-responsive genes [10]. However, the regulation of isoprenoid synthesis under these environmental stresses has yet to be clarified.

In this study, two types of *IPI* cDNAs were isolated, and the localizations of these IPIs were determined. We also investigated the mRNA expression of the two IPIs under salt, high-light and cold stresses, and discussed the difference of the expression between the IPIs in view of their localization.

2. Materials and methods

2.1. Cloning of the IPP isomerase gene

Based on the highly conserved region of IPP isomerases in higher plants, two primers, 5′-TGAVAATYTRYTDCAYAGAGCTTT-CAGTGT-3′ and 5′-RGWBCCYTTYTCRACRTGRTCCCAC-CACTT-3′, were designed. The PCR products (94°C, 30 s; 60°C, 30 s; 72°C, 60 s; 30 cycles), obtained using the SR1 cDNA library as a template, were used for screening as probe. Candidate clones of *IPI* cDNA, were subcloned into the *Eco*RI site of pBluescript SK(−) (Stratagene) and sequenced.

2.2. Northern blot analysis

Total RNAs (10 μg) from each sample were separated in denaturing 1.2% (w/v) agarose gel and transferred onto a nylon membrane filter. The filters were hybridized with a ^{32}P-labeled gene-specific (3′-non-encoding region) probe of *IPI1* or *IPI2* in Church buffer containing 7% (w/v) SDS, 1 mM EDTA and 0.5 M sodium phosphate (pH 7.2) [11] at 65°C. The filters were washed with 2×SSC and 0.1% SDS at 65°C.

2.3. Transient expression analysis of green fluorescent protein (GFP) fusion protein

A plasmid vector having GFP CaMV-sGFP(S65T)-nos3′ was obtained from Dr. Niwa of University of Shizuoka, Japan [12]. Full-

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[1] The nucleotide sequence data in this paper have been submitted to the DDBJ data base (IPI1, accession number AB049815; IPI2, AB049816).

Abbreviations: GFP, green fluorescent protein; IPI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate

0014-5793/01/$20.00
PII: S0014-5793(01)02870-8

length *IPI1* and *IPI2* open reading frames were subcloned into the *Nco*I site of the vector just upstream of the GFP gene. Gold particles (1 mg) coated with the each vector (4 μg) were shot into 3-week-old tobacco (SR1) leaves by a particle delivery system (Model PDS-1000/He Biolistic Particle Delivery System, Bio-Rad). The leaves were incubated under white light at 27°C, and the glow of GFP fusion protein in cells was detected by a fluorescence microscopy (Axioplan2 and Axiophoto2, Zeiss) after 24 h incubation.

2.4. Growth conditions, stress and ABA treatment

Nicotiana tabacum SR1 plants were grown under continuous white light (3 W/m^2) at room temperature (27 ± 2°C) for 40 days. High-salt stress treatment was performed with 300 mM NaCl (final concentration). For high-light stress, plants were illuminated at 90 W/m^2. The low-temperature treatment was performed by exposing plants at 8 ± 2°C under continuous white light. ABA treatment was performed with 100 μM ABA (final concentration). ABA was dissolved in dimethylsulfoxide (DMSO; final concentration was 0.01%). Harvested leaves were stored at −80°C until used.

3. Results and discussion

3.1. Isolation of cDNAs encoding the IPP isomerase gene from tobacco

Two cDNA clones for the *IPI* gene (*IPI1* and *IPI2*) were obtained from the *N. tabacum* cDNA library. *IPI1* consisted of 1107 bp, and encoded an open reading frame of 290 amino acids with a molecular mass of 33.2 kDa. IPI1 possessed an N-terminal amino acid extension (1–62) showing characteristics of a chloroplast targeting signal. The nucleotide sequence of *IPI2* was 993 bp, and encoded an open reading frame of 236 amino acids with a molecular mass of 27.3 kDa. To exclude the possibility that this cDNA contains only part of the open reading frame of *IPI2*, 5′-RACE and primer-extension assays were performed to determine the 5′-transcription starting point of *IPI2* mRNA (data not shown). The results suggested that the *IPI2* cDNA contained the translation initiation site of *IPI2* mRNA. Therefore, this cDNA contains a full-length open reading frame of *IPI2*, and IPI2 had no N-terminal signal peptide. The amino acid sequences of the mature polypeptides of tobacco IPI1 and IPI2 were very similar to each other (92% identity). These IPIs were also similar to the IPIs of other plants in the mature region, for example, 86–89% identical to *Arabidopsis thaliana* IPIs and *Oryza sativa* IPI. IPI1 and IPI2 in tobacco, as well as other plants, contained conserved cysteine and glutamic acid residues at the catalytic active site reported in *Saccharomyces cerevisiae* IPP isomerase [13]. IPI activity was measured in transformed *Escherichia coli* harboring the *IPI1* and *IPI2* genes of tobacco [14]. *E. coli* strains containing IPI1 and IPI2 showed 1.6-fold and 4.2-fold more activity than the control transformant harboring TA cloning vector without insert DNA, respectively. This result confirmed that both clones encoded the functional IPI proteins in tobacco.

3.2. Subcellular localization of two types of IPP isomerases in tobacco

To investigate the localization of IPI1 and IPI2 in tobacco cells, we constructed two plasmids (IPI1–GFP and IPI2–GFP) to express IPI1 and IPI2 fused with GFP, and introduced them into tobacco leaves by particle bombardment (Fig. 1). The fluorescence of IPI1–GFP fusion protein was detected in chloroplasts in leaves, showing that IPI1 was localized in chloroplast. On the other hand, the fluorescence of IPI2–GFP was detected in the cytosol, showing that IPI2 is a cytosolic pro-

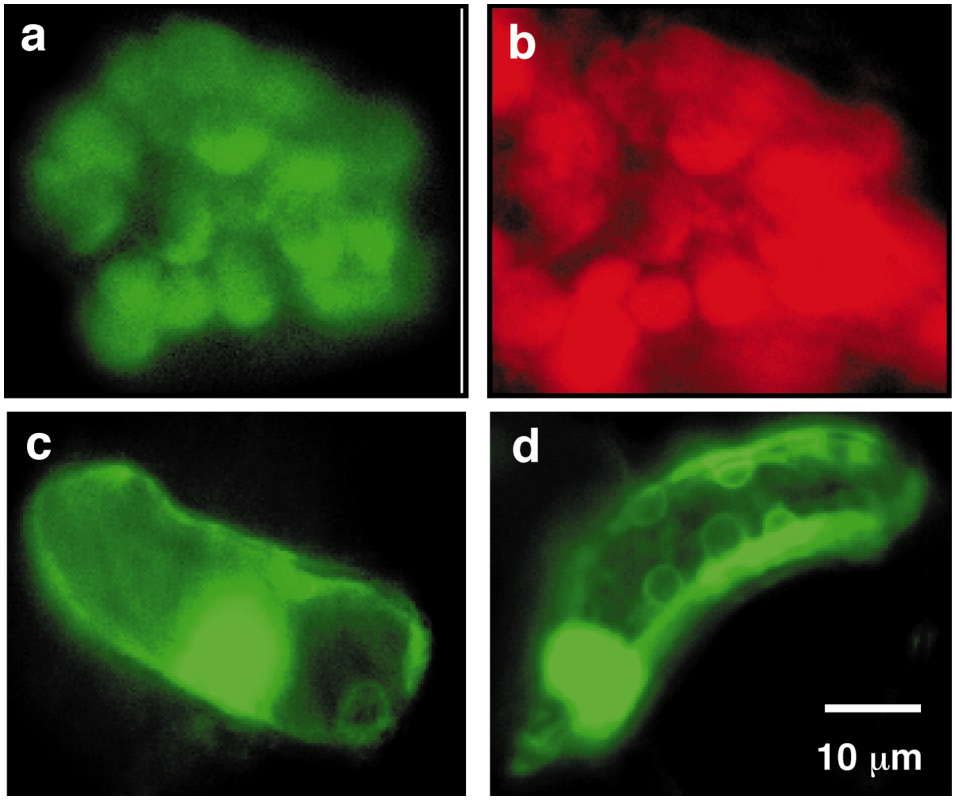

Fig. 1. Subcellular localization of the GFP fusion proteins. a: Fluorescence of IPI1–GFP; b: autofluorescence of chloroplast at the same position as (a); c: fluorescence of IPI2–GFP; d: fluorescence of cytosolic control GFP. The fluorescence was observed after 24 h of incubation.

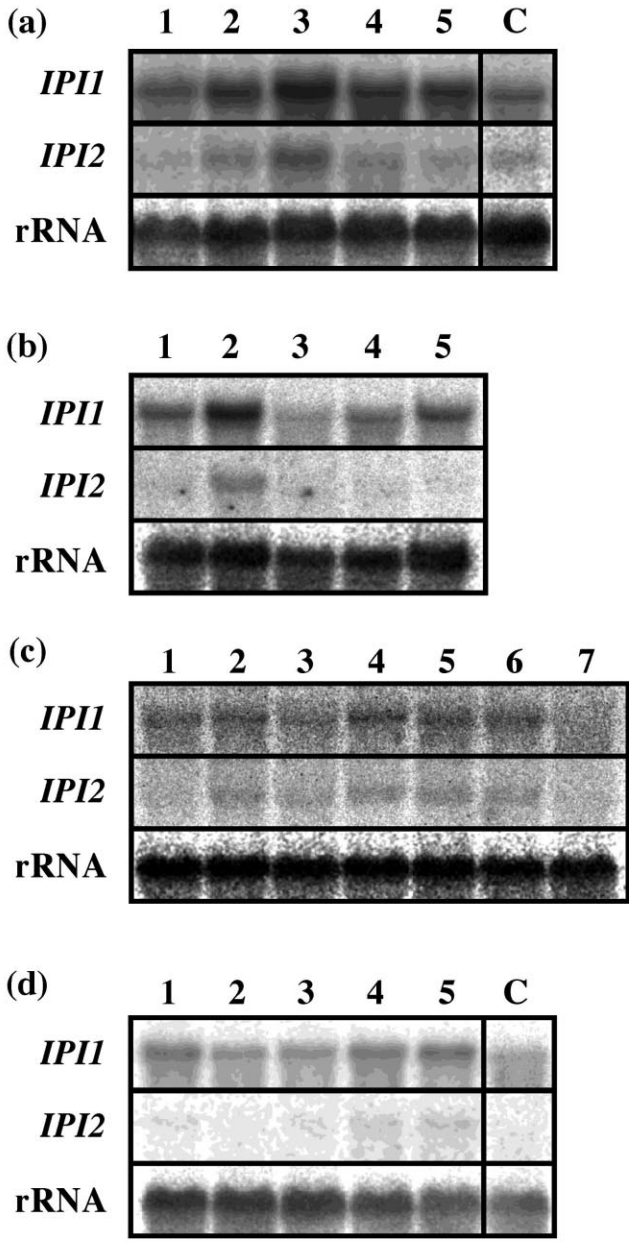

Fig. 2. Time courses of *IPI1* and *IPI2* mRNA levels with various treatments. a: Effect of NaCl treatment. NaCl treatment was performed with MS medium containing 300 mM NaCl. Lanes 1–5 indicate 0, 12, 24, 48 and 72 h treatment, respectively. Lane C indicates control treatment with pure MS solution for 24 h. b: Effect of high-light. Leaves of *N. tabacum* SR1 were illuminated by 30-fold stronger light than normal conditions. Lanes 1–5 indicate 0, 1, 3, 6 and 12 h treatment, respectively. c: Effect of cold stress. Leaves were exposed at 8 ± 2°C under continuous white light. Lanes 1–7 are 0, 3, 6, 9, 12, 24 and 48 h treatment, respectively. d: Effect of ABA treatment. Treatment was performed with 50 ml of MS medium containing 100 μM ABA. Lanes 1–5 are 0, 1, 3, 6 and 12 h treatment with ABA, respectively. Control treatment (lane C) was performed for 12 h with MS solution containing 0.01% DMSO.

tein. We emphasize that this is the first direct evidence of localization of IPI protein in cytosol, although many IPI cDNAs have been isolated from higher plants so far. Genome sequence analysis revealed that *A. thaliana* has only two types of *IPI*. The amino acid sequences of *A. thaliana* IPIs, IPP1 and IPP2, suggested that both isozymes are localized in plastid [15]. However, *Camptotheca acuminata* IPI2 and *O. sativa* IPI seem to have no signal peptide, and thus are cytosolic type. *Ricinus communis* IPI was suggested to be localized in plastid and mitochondria [3]. These finding confirmed that IPI was localized in various cellular compartments, which probably differed among plant species.

3.3. Response of IPP isomerase genes to stresses and ABA in tobacco

Northern hybridization analyses were performed with the gene-specific 3′-non-encoding region of each clone. Under normal growth conditions, the level of *IPI1* mRNA was about three-fold higher than that of *IPI2*. The abundance of *IPI1* mRNA may be explained by the variety and abundance of isoprenoids in chloroplast, such as chlorophylls, carotenoids, quinones, tocopherols and some of the phytohormones. Thus, a quantity of IPI1 was probably required for the biosynthesis of these isoprenoids. Cytosolic isoprenoids, such as steroids, sesquiterpenes and polyterpenes, however, are relatively less abundant and metabolically stable under normal conditions, and hence, the requirement for IPI2 would not be great.

To address which environmental stresses are responsible for the individual expression of IPI genes, we investigated the effect of three representative stresses (high-salt, high-light and cold) on the expression of *IPI* genes. The level of *IPI1* mRNA reached almost 4.5-fold that at 0 h after 24 h high-salt treatment (Fig. 2a). The high accumulation continued for 72 h after the treatment. *IPI2* transcripts were also increased by NaCl treatment, but showed a different induction profile. The increase in *IPI2* mRNA was transient; after 24 h of treatment, *IPI2* mRNA had accumulated to 7.5-fold the level of the untreated control.

The high-light treatment caused a clearer induction of both types of *IPI* mRNA than the NaCl treatment. The level of *IPI1* mRNA increased within 1 h and decreased rapidly after 3 h treatment, and then gradually recovered to the steady-state level (Fig. 2b). A slight induction of *IPI2* mRNA was also observed after 1 h treatment, and then the level gradually decreased.

Among our stress conditions, low-temperature treatment with illumination caused severe stress in tobacco plants. Tobacco plant could not tolerate these conditions and died within 4 days. During the low-temperature treatment, changes in the level of transcripts were observed more clearly for *IPI2* than *IPI1* (Fig. 2c). The highest level of mRNA of *IPI2* was 1.8-fold that at 0 h after 12 h treatment. The decrease in mRNA of both *IPI1* and *IPI2* 48 h after the cold stress, was probably attributable to the decline in the viability of tobacco.

ABA is known as a central mediator in the regulation of responses to salt and cold stresses. To examine the involvement of ABA in the up-regulation of *IPI* transcription caused by these stresses, we investigated the effects of exogenous ABA without stress. The levels of both types of *IPI* transcripts were not changed by exogenous ABA (Fig. 2d), indicating that transcription of *IPI* under salt and cold stresses was enhanced in an ABA-independent manner.

As shown by the data, NaCl and high-light stresses increased the amount of *IPI1* mRNA, and NaCl and cold stresses induced expression of *IPI2* mRNA. Because IPI is a rate-limiting enzyme of both the isoprenoid biosynthetic path-

ways, the increases of *IPI* mRNA in each compartment should increase the flux of the biosynthesis of each isoprenoid for protection against stress in plants. Under NaCl treatment, the accumulation of plastidic *IPI1* mRNA may enhance synthesis of ABA from carotenoid in chloroplast and induce mRNAs of many stress-response genes. High-salt stress also probably increased the level of *IPI2* mRNA to accelerate the synthesis of cytosolic isoprenoid compounds, which may increase the protection against the stress. Under high-light and cold stresses, harmful reactive oxygen is produced by absorbed excess light energy. For protection of the photosynthetic apparatus from damage caused by reactive oxygen, the amount of carotenoids, particularly zeaxanthin, was increased by these stresses (data not shown). The transient accumulation of *IPI1* mRNA under high-light may be required for synthesis of new carotenoids. However, under cold stress, the mRNA of *IPI1* was not increased. Under the conditions, the amount of chlorophyll was decreased and that of carotenoid was increased (data not shown), so the reaction product of IPI may be mainly used for carotenoid biosynthesis without an increase in *IPI1* mRNA. In the case of cold stress, the amount of membrane components is known to be changed to maintain the membrane fluidity. It was reported that a kind of plant sterol, stigmasterol, increased the membrane fluidity [16]. Thus, we suggest that cold stress increased the level of *IPI2* mRNA for the synthesis of the sterols, which increases membrane fluidity.

Acknowledgements: We thank Professor Y. Niwa of University of Shizuoka for kindly providing the plasmid vector of GFP.

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