

Short communication

Molecular characterization of a cytokinin-inducible periwinkle protein showing sequence homology with pathogenesis-related proteins and the Bet v 1 allergen family

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Abstract

Cytokinin treatment of periwinkle callus cultures increased the accumulation of a protein, designated T1, in two-dimensional separated protein extracts. The first 30 NH₂-terminal amino acids were determined by Edman degradation and showed significant sequence homology with intracellular pathogenesis-related (IPR) plant proteins and the Bet v 1 allergen family. The deduced amino acid sequence of cDNAs coding for T1, isolated by RT-PCR and 5' RACE-PCR, exhibited an average sequence identity of 40% with both IPR and Bet v 1-related allergens. T1 and all related proteins contained a p-loop motif typically found in nucleotide-binding proteins as the most conserved sequence feature. Northern blot analysis showed that cytokinin treatment of periwinkle callus induced T1 transcripts, whereas addition of 2,4-dichlorophenoxyacetic acid inhibited this accumulation. Hybridization of genomic periwinkle DNA with the T1 cDNA suggested that the protein is encoded by a single-copy gene. Immunoblot studies with a panel of Bet v 1-specific antibodies and sera from Bet v 1 allergic individuals identified T1 as a protein that is immunologically distinct from the Bet v 1 allergen family and has no allergenic properties.

Cytokinins (CKs) regulate several aspects of plant development, growth and metabolism [1]. These phytohormones have been shown to modulate the accumulation of various mRNAs and proteins in plant cells, however most responses to CKs appear as very complex: first, the reported effects of CK treatment include up- or down-regulation in cellular enzyme activities and mRNA amounts; second, the regulation of gene expression occurs at the level of transcription and/or post-transcription (for example, through stabilization of gene transcripts); third, CK-modulated genes are often also regulated by other chemical and/or phys-

al environmental factors such as auxin, abscisic acid, nitrate or light [7, 8]. The isolation of CK-modulated genes should allow a detailed study of their expression in relation to several aspects of the physiology and biochemistry of plant cells.

We previously found that adding a CK to the culture medium of a suspension-cultured cell line of periwinkle (*Catharanthus roseus* [L.] G. Don, Apocynaceae) enhanced the indole alkaloid accumulation in the cells [10] and induced the accumulation of a few polypeptides. In particular, a polypeptide of 17 kDa (referred to as T1) was detected as a well-separated spot by analytical two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) when the cells were treated by CK for 48 h [26]. While we were previously unable

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y10612.

to obtain sufficient amounts of T1 from the cell suspension, the availability of a new periwinkle line growing as callus tissues allowed the isolation of T1 and the proper *in vitro* study of this polypeptide in the tissues under different hormonal conditions. We describe here the microsequencing of T1 and the cloning and sequence characterization of the corresponding cDNA. We show that T1 is closely related to the so-called intracellular pathogenesis-related proteins (IPR proteins).

The periwinkle (cv. Little Pinkie) callus culture (NT-CRLp line) has been initiated from cotyledons [15] and maintained in darkness on a culture medium containing 58 mM sucrose, 4.5 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and the mineral salts and vitamins of Gamborg *et al.* [14]. To induce the accumulation of T1, the tissues were subcultured for one passage on a 2,4-D-free culture medium containing 5 μ M zeatin (Figure 1). Total proteins were extracted from two-week-old tissues, then separated by preparative 2D-PAGE as previously described [26] except that 200 μ g of proteins were loaded per gel. The spot corresponding to protein T1 was excised from 4 preparative gels and the protein was eluted from the gel fragments by incubation overnight at 37 °C in 200 μ l 0.1 M sodium acetate (pH 8.5) containing 0.1% (w/v) sodium dodecyl sulfate (SDS) [18]. The eluate was adsorbed onto a polyvinylidene difluoride membrane using ProSorb cartridges (Applied), then submitted to automatic Edman degradation in an Applied Biosystems Procise 492 protein sequencer. The N-terminal amino acid sequence of T1 (30 residues) was identical at 53% with the N-terminus of the major allergen of birch pollen Bet v 1 [2], i.e. the main cause of allergic rhinoconjunctivitis and allergic bronchial asthma in Europe and North America [19].

We search a cDNA for T1 protein by the following RT-PCR procedure. Total RNA was extracted by an usual phenol-SDS procedure [9] from NT-CRLp callus tissues grown for 10 days on a 2,4-D-free medium supplemented with 5 μ M zeatin. Complementary DNA was prepared from 9 μ g total RNA in a reaction volume of 40 μ l RT buffer [23] containing 0.5 mM each of dATP, dCTP, dGTP and dTTP, 40 units of RNasin, and 2.2 mM of each of 5'-GCTCGAGC T11 VN-3' oligonucleotide (V = A/C/G; N = A/C/G/T). These oligonucleotides (primer 1) were designed for priming the cDNA in a location corresponding to the beginning of the poly(A) tail of the mRNA [23]. Each oligonucleotide was composed of 11 dT residues, extended with a *Xho*I restriction site (underlined) in the 5' end (in order to direct cloning into an appro-

priate expression vector at a later step). The first-strand cDNA was synthesized by reverse transcription at 37 °C for 90 min. For PCR, two degenerate oligonucleotide primers were synthesized (Oligo-Express, Paris, France). Their nucleotide sequences were derived from two stretches of 6 amino acids each (namely YDMEIK and KLFKAF) present in the N-terminal sequence of T1. The sequence of primer 2 (5'-TAYGAYATGGARATHAAR-3'; R = A/G; H = A/C/T; Y = C/T) corresponded to the sense orientation of YDMEIK; the sequence of primer 3 (5'-AARITITTYAARGCI TTYGT-3'; R, H and Y as above; I = inosine) corresponded to the sense orientation of KLFKAF (Figure 2) completed by the first two bases of the codon of valine, i.e. the following amino acid. PCR-mediated amplification of the first-strand cDNA synthesis products was performed with 0.02 μ M of each 5'-GCTCGAGC T11 VN-3' oligonucleotides (primer 1), 0.2 μ M of primer 2 and 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase (Amersham). Thirty cycles of 30 s-denaturation at 94 °C, 1 min annealing at 45 °C and 1 min extension at 72 °C were performed. Amplified cDNA products were submitted to a second PCR with primers 1 and 3, using the conditions of the first PCR except higher annealing temperature (50 °C). The reaction was completed with a 10 min-extension at 72 °C. This procedure yielded a single PCR product of about 600 bp which was cloned into pTag vector (R&D Systems, Abingdon, UK) after adding A overhangs to the 3' blunt-ended PCR fragments, according to the manufacturer's instructions. The ligations were transformed into *Escherichia coli* JM 109 (Promega) and recombinant bacteria chosen for analysis.

After digestion of recombinant pTag vector with *Bam*HI and *Xho*I, 8 positive cDNA clones were selected and partially sequenced. One of them (*pckr 3a*) was completely sequenced on both strands by the dideoxy chain-termination method. Its nucleotide sequence, together with the deduced amino acid sequence, is displayed in Figure 2. The clone comprised 604 bp and had an open reading frame of 140 amino acids terminated by two TAA stop codons. To know the nucleotide sequence corresponding to the N-terminal amino acid sequence of T1, we adopted a 5' RACE-PCR procedure (Rapid Amplification of cDNA Ends, version 2.0 from Gibco-BRL, Cergy-Pontoise, France). Two specific primers were used: for reverse transcription, primer 4: 5'-AGTGTGTGCTTGCA-3'; for PCR, primer 5: 5'-AGTGTGTGCTTGCAAGACGGA-3' (Figure 2) and a specific primer provided in the RACE-PCR kit. The amplification of the 5' cDNA ends from a total RNA

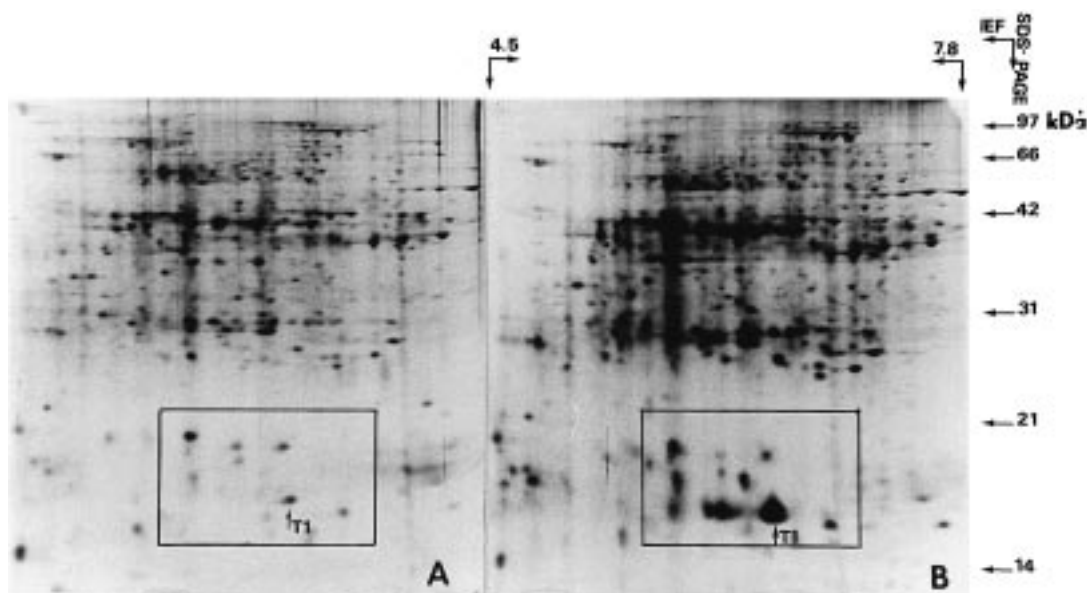


Figure 1. The effect of cytokinin treatment on the 2D-PAGE pattern of polypeptides from the callus line (NT-CRlp) of periwinkle. Callus tissues were cultured for two weeks in absence (A) or presence (B) of 5 μ M zeatin. Fifteen μ g of total proteins were loaded on IEF gel rod, then resolved on 12.5% (w/v) polyacrylamide gel and silver stained. The boxed area displayed the region where the 17 kDa polypeptide (T1) was observed. Note that a few other polypeptides are also up-regulated by CK.

template was achieved according to the manufacturer's instructions. The obtained PCR fragment was cloned in pTag vector and the corresponding clone (*pckr 3b*) sequenced on both strands. The clone comprised 453 bp. The 303 bp of overlap between the sequences of *pckr 3a* and *pckr 3b* clones revealed no sequence discrepancies. Moreover, the deduced first 30 amino acid sequence was identical with the N-terminus of T1 previously determined by microsequencing.

Results from RT-PCR and RACE-PCR are displayed in Figure 2. The polypeptide T1 contains 155 amino acids. The predicted molecular mass (16805 Da) and pI (6.55) correlate well with the values determined by SDS-PAGE. The coding sequence shows two consensus sites (NXS/T) for glycosylation. A search in the nucleotide and protein databases confirmed significant similarities between the sequences of T1 and those of Bet v 1 (40.4% identity; 69.8% relatedness), and more generally with those of IPR proteins (Figure 3).

Northern blot analysis was carried out as previously described [15], using the *pckr3a* cDNA as a probe in order to determine if the transcript accumulation was regulated in a similar manner than protein T1. Periwinkle callus cultures were grown on media with different hormonal composition and harvested after ten

days (northern analysis) or two weeks (2D-PAGE analysis). Figure 4 shows that both T1 protein and T1 transcripts accumulated in very low amounts in callus grown on hormone-free medium. Treatment with 5 μ M zeatin increased the amount of T1 transcripts as well as the content of T1 protein. Addition of 4.5 μ M 2,4-D together with 5 μ M zeatin decreased the enhancing effect of the CK on both accumulation of transcripts and protein T1.

The gene encoding T1 was cut by *Xba*I but not by *Eco*RI. Digestion of periwinkle genomic DNA with these enzymes produced single bands and these data suggest that T1 is encoded by a single-copy gene in the genome of periwinkle (not shown). Our results differ from those obtained with the IPR proteins SAM 22 and Bet v 1 which are encoded by multiple copies [6, 28], but resemble those obtained with ABR 17, a T1 homologous IPR from pea, which is also encoded by a single-copy gene [17].

In order to investigate whether T1 shares immunological properties with the Bet v 1 allergen family, immunoblot experiments with a panel of Bet v 1-specific mouse monoclonal antibodies and serum IgE Bet v 1-allergic individuals were performed. In a first set of experiments, membranes containing 2D-separated CK-induced periwinkle callus extracts were

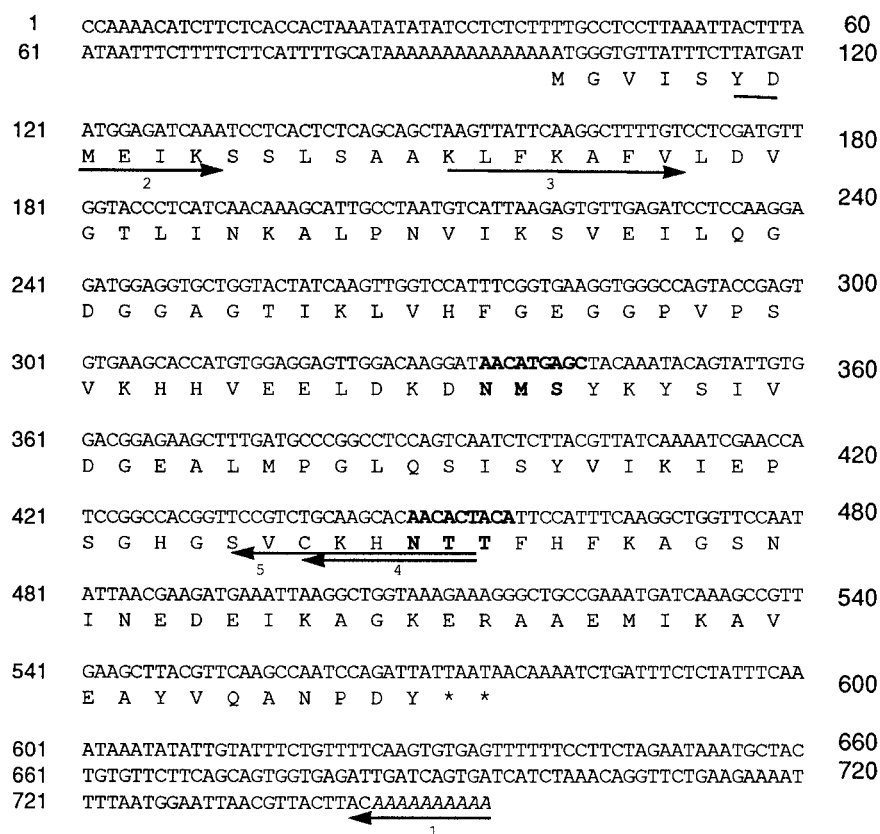


Figure 2. The nucleotide sequence and the corresponding amino acid sequence of T1, as deduced from RT-PCR and 5' RACE-PCR procedures. Sequencing of clones *pckr 3a* (obtained by RT-PCR) and *pckr 3b* (obtained by RACE-PCR) has given the nucleotide sequences 151 to 754 and 1 to 453, respectively. Primers 1, 2 and 3 (for RT-PCR), 4 and 5 (for 5' RACE-PCR) are indicated by arrows. Stop codons are denoted by asterisks. The two putative sites of glycosylation are in bold.

probed with Bip 1, a mouse monoclonal anti-Bet v 1 antibody and a mixture of four other mouse monoclonal anti-Bet v1 antibodies (Nos. 14–18), all of them showing extensive cross-reactivity with Bet v 1-related plant allergens, as described [20, 22]. Whereas in the control experiment these antibodies strongly reacted with nitrocellulose-blotted Bet v 1, T1 was not recognized (not shown). In a second set of experiments, blotted periwinkle extracts were exposed to sera from Bet v 1-allergic individuals who displayed IgE-cross-reactivity to all of the Bet v 1-related tree pollen (Bet v 1, Aln g 1, Cor a 1, Car b 1) and plant-food (Mal d 1, Api g 1, Dau c 1, Pru a 1) allergens. The results of these experiments indicated that T1 did not bind Bet v 1-specific IgE antibodies. The lack of IgE-binding capacity of T1 was further confirmed by an IgE-inhibition experiment (Figure 5). Membranes containing 2D-separated periwinkle extracts were probed with serum IgE from two patients (serum pool) who reacted with Bet v 1,

profilin (= Bet v 2), a conserved actin-binding protein that was identified as a highly cross-reactive allergen [10, 30, 31] and a '60 kDa' cross-reactive plant allergen [16]. The presence of Bet v 1 and Bet v 2-specific IgE in the serum pool was confirmed by reactivity with nitrocellulose-blotted recombinant Bet v 1 and Bet v 2 (Figure 5A). In one experiment, serum was preadsorbed with recombinant Bet v 1 (Figure 5B) and in the parallel experiment with recombinant Bet v 2 (birch profilin) (Figure 5C). Serum IgE reacted with periwinkle profilin, which was confirmed by the fact that recombinant birch profilin inhibited IgE-binding to the 14 kDa protein and by the finding that two different rabbit anti-profilin antisera detected the same spot in the 2D-separated and blotted extract. In addition, reactivity with several spots migrating at ca. 40 kDa was observed. These spots are likely immunologically-related with the '60 kDa' conserved plant allergen [16] but it is not yet resolved whether the difference in Mr is

	GXGGXGXXK	
T1	GVISYDMEIKSSLSAAKLFKAFVLDVGLINKALPNVIKSVEIL QDGGAGTIX L VHFGE GGPVPSVKHHVEELDKDNMSYKYSIVDGE	90
Betv 1a	M--FN-ET-TT-VIP--R-----I--GDN-FP-VA-QA-S---NIE- N--P ----KIS-P--F-FKY--DR-D-V-HT-FK-N--VIE-G	
Betv 1d	M--FN-EI-TT-VIP--R-----I--GDN-VP-VA-QA-S---NIE- N--P ----KIN-P--F-FKY--DR-D-V-HT-FK-N--VIE-G	
Betv 1l	M--FN-ET-AT -VIP--RM-----I--GDK-VP-VA-QA-S---NIE- N--P ----KIN-P--F-FKY--DR-D-V-HT-FK-N--VIE-G	
Aln g 1	--FN-EA-TP-VIP--R-----I--GDK-LP-VA-EAVS---NIE- N--P ----KIT-P--S-FKY--ER-D-V-RV-FK-SF-VIE-G	
Cor a 1	--FN-EV-TP-VIP--R---SY---GDK--P-VA-QA-T---NVE- N--P -----NIT----SRYKY--ER-D-V-NT-FT-S-TVIE-D	
Car b 1	--FN-EA-TP-VIP--R---SY---GDK--P-VA-Q--S---NVG- N--P -----NIT-A--I-FKF--ER-D-V-NA-FK-N-TVIE-D	
Mal d 1	--YTFEN-FT-EIPPSR-----ADN--P-IA-QA--QA---E- N--P ----KIT----SQYGY--RIDSIEASY--S-TLIE-D	
Api g 1	--QTHVL-LT--V--E-I-QG--I--D-VLP--A-GAY-----K---P--L-IITLFD---ITMTLRIDGVN-EALTFD--VI--D	
Phavu	--FTFEDQTT-PVAP-T-Y--VAK-AD-IFP-----DSF-----VE- N--P ----KIS-V-D-ETKF-L-KI-SI EA-LG-S-----G-V	
SAM 22	M--FTFED--N-PVAP-T-Y--L-T-ADNV-P---DSF-----NVE- N--P ----KIT-L-D-ETKF-L-KI-SI-EA-LG-S--V-G-A	
AB 18	M--FT-ENDTT-TVPP-----V-H-ADLIVP-VV.DS--T---E- N--P--V -KLT-V---QTLY-L-K--AI-DAKFE-N-----G-V	
T1	ALMPGLQSIISYVIKIEPSGHGSVCKHNTTFHFKAGSNINEDEIKAGKERAEMIKAVEAYVQANPD.Y	160
Betv 1a	PIGDT-EK--NE---VATPDG--IL-ISNKY-T-GDHEVKAEQV--S--MGETLLR---S-LL-HS-A-N	
Betv 1d	PVGDT-EK--NE---VATPDG-C-L-ISNKY-T-NDHEVKAEQV--S--MGETLLR---S-LL-HS-A-N	
Betv 1l	PVGDT-EK--NE---VATPDG-C-L-ISNKY-T-NDHEVKAEQV--S--MGETLLR---S-LL-HS-A-N	
Aln g 1	-VGDA-EKVCNE---VAAPDG--IL-ISNK--T-GDHE--AEQ--IE--K-VGLL---S-LL-HS-A-N	
Cor a 1	V-GDK-EKVCHL--VAAPGG--IL-ISSK--A-GDHE--AE-M-GA--M-EKLLR---T-LL-HSAE-N	
Car b 1	V-GDK-EKV-HEL--VAAP-G--IV-ISSK--A-GYHEV-AEKM-GA--M-EKLLR---S-LL-HTAE-N	
Mal d 1	--TDIEK---ET-LVAC-S.--TI-SISHY-T-GNIE-K-EHV-V---K-HGLF-LI-S-LKDH--A-N	
Api g 1	I-LGFIE--ENHVIV-TADG--I--TTAI--T-GDAVVP-EN--YAN-QNTALF--L---LI--	
Phavu	--PETAEK-TFDS-LSDPNG--LI-LSI-Y-S-GDAPP---L---AKSDSLF-----LL---	
SAM 22	--PDTAEK-TFDS-LVAGPNG--AG-LTVKYET-GDAEP-Q--L-T--AK-DALF--I---LL-H--.-N	
AB 18	GISDIVEK--FEA-LF-GPNG--G-MIVKY-T-GDAKPI-K-VEE--AKGDALF--I---L---N.-N	

Figure 3. Comparison of the deduced amino acid sequence of T1 with amino acid sequences of proteins of the Bet v 1 allergen family as well as with some other intracellular-pathogenesis-related proteins. T1 homologous allergens which were shown to bind IgE-antibodies from allergic patients comprise: the T1 homologous tree pollen allergens, Bet v 1, the major birch pollen allergen [2], Aln g 1, the major alder pollen allergen [4], Car b 1, the major hornbeam pollen allergen [21] and Cor a 1, the major hazel allergen [4]; the T1 homologous plant food allergens: Mal d 1, the apple allergen [32], and Api g 1, the celery allergen [5]. The T1 homologous pathogenesis-related proteins comprise Phavu from kidney bean [34], SAM22 from soybean [6], and AB18 from pea [13, 17]. Besides Bet v 1 are included two isoforms (Bet v 1 d and Bet v 1 l) [28] which have a strongly reduced IgE-binding capacity [12] despite their closely related sequence. The p-loop motif which is the most striking feature of the sequence alignment is in bold.

due to glycosylation, other posttranslational modification, or degradation. As in the previous experiments, T1 was not recognized by serum IgE and preincubation with recombinant Bet v 1 did not affect IgE binding to any of the detected proteins. These results demonstrate that despite significant sequence similarity of T1 with the Bet v 1 allergen family, T1 represents a protein that is immunologically distinct from Bet v 1 and lacks allergenic property. Whether the lack of antibody binding is due to its reduced immunogenicity cannot be answered at that point. It is however well known that antibody recognition of Bet v 1 is dependent on the presence of conformational epitopes [20, 33] and may be dramatically altered by the exchange of only a few amino acid residues [12]. Bet v 1 isoforms with reduced anaphylactic activity have therefore been proposed as candidates for specific immunotherapy of Bet v 1-induced allergy in order to increase the dose of allergens that can be injected without anaphylactic

activity. The use of Bet v 1 homologous proteins with a similar lack of IgE-binding capacity, such as T1, for immunotherapy of Bet v 1 related allergies may therefore be considered as well. Note on the other hand that in contrast to the considerable variability of structures among Bet v 1-related proteins (as is evidenced by their immunological reactivities), a phosphate-binding loop motif (GXGGXGXXK) [27] shows a remarkable conservation among T1 homologous proteins pointing to a common biological function of these proteins, probably as nucleotide-binding proteins since this motif is engaged in ATP or GTP binding in other proteins.

The function of T1 remains unknown in periwinkle tissues. Indeed, the protein is not implicated as a regulatory enzyme of the indole alkaloid pathway, as we expected at the beginning of our work, however, its accumulation is associated with the CK-induced alkaloid accumulation in periwinkle cells [26]. The high similarities between the amino acid sequence of T1

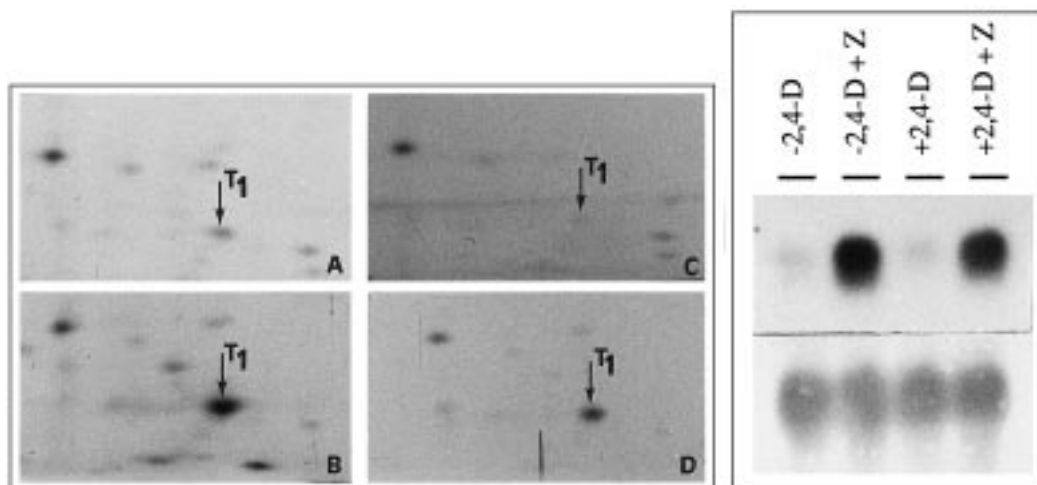


Figure 4. The effect of hormonal treatments on the accumulation of T1 (on the left) and T1 transcripts (on the right). Callus tissues were harvested after ten days (northern) or fifteen days (2D-PAGE). Polypeptide profiles were studied as described in Figure 1 (each panel is an enlargement of the boxed area in Figure 1). For northern analysis, total RNA was electrophoresed in a formaldehyde-agarose gel and transferred onto a nylon membrane. Each lane contained 20 μ g of total RNA. Hybridization was carried out with [^{32} P]-labelled insert from the *pckr 3a* cDNA clone. Control hybridizations to standardize the amounts of RNA loaded were performed with a 18S rDNA fragment. Treatments were as follows: A, tissues grown on hormone-free medium; B, tissues treated with 5 μ M zeatin; C, tissues treated with 4.5 μ M 2,4-D; D, tissues treated with 4.5 μ M 2,4-D + 5 μ M zeatin.

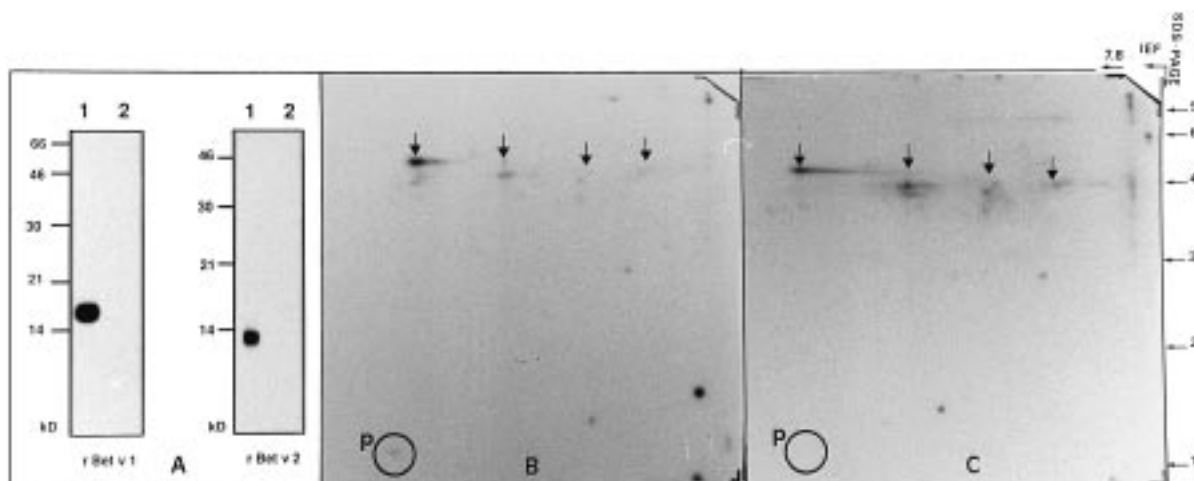


Figure 5. IgE-immunoblot inhibition studies. Proteins from periwinkle callus tissues grown for two weeks on 2,4-D-free medium containing 5 μ M zeatin were separated by 2D-PAGE and transferred to nitrocellulose membranes by semi-dry blotting. A. Reactivity of the serum pool and a control serum with recombinant Bet v 1 and Bet v 2. Ca. 5 μ g/ml purified recombinant Bet v 1 and Bet v 2 were separated by 12.5% preparative SDS-PAGE and blotted onto nitrocellulose. Nitrocellulose-blotted r Bet v 1 and r Bet v 2 were exposed to the same serum pool used in B and C (lanes 1), and for control purposes to serum from a non-allergic individual (lanes 2). B. Preadsorption with r Bet v 1. C. Preadsorption with r Bet v 2 (birch profilin). Note that the reaction of 14 kDa spot (P) with anti-profilin IgE antibodies was inhibited by recombinant birch profilin. Arrows designate several spots reacting with serum IgE.

and those of IPR proteins (which are known to be strongly up-regulated in plants by pathogen attacks or wounding) might indicate that T1 play a role against environmental stresses. Moreover, many active defense reactions in plants combined an increase in some sec-

ondary metabolites and activation of genes encoding stress compounds [24, 25], and there are now some evidences that stress responses are features of normal development [6].

This paper shows that CKs up-regulate the expression of a gene encoding an IPR protein in periwinkle callus cultures. Previously, a gene encoding the IPR protein SAM 22 in soybean suspension cells was also reported to be modulated by CK but, on the contrary of what is found in periwinkle cultures, CK treatment of soybean cells led to a severe decrease in the accumulation of transcripts [6]. Therefore, it should be of interest to compare the promoters of both genes. Further studies will have to clarify the function of T1 in the physiology of the cells, as well as to specify which hormonal and environmental factors regulate the accumulation of the protein. Moreover, recombinant 'hypo-allergenic' T1 protein might be useful for therapy of Bet v 1-related allergies.

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