Histidine-containing phosphotransfer domain extinction by RNA interference turns off a cytokinin signalling circuitry in *Catharanthus roseus* suspension cells

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Received 19 November 2003; revised 19 December 2003; accepted 22 December 2003

First published online 7 January 2004

Edited by Ulf-Ingo Flügge

Abstract **We previously reported that cytokinins (CK) induce the fast and specific transcription of *CrRR1*, a gene encoding a type A response regulator in *Catharanthus roseus* cell cultures. Here, we characterized the *CrHPt1* gene that encodes a histidine-containing phosphotransfer domain. *CrHPt1* was silenced through RNA interference (RNAi) to test its possible implication in the CK signalling pathway. In transgenic lines stably transformed with an intron-spliced construct, the degradation of *CrHPt1* transcripts abolishes the CK inductive effect on *CrRR1* transcription. These result give a new in vivo functional argument for the crucial role of HPt proteins in the CK signalling pathway leading to the expression of the genes encoding type A response regulators. They also show that RNAi is a powerful strategy to turn off the CK signalling circuitry.**

Key words: Histidine-containing phosphotransfer; Cytokinin; *Catharanthus roseus*

1. Introduction

Cytokinins (CKs) are implicated in the regulation of several physiological processes in plant growth and development [1]. Many studies have been dedicated to analyze in detail their signalling pathways. It is thought that upon CK perception, membrane-localized protein kinases acting as receptors dimerize and autophosphorylate on a conserved histidine residue. The phosphoryl group is transferred successively to an aspartate residue within a receiver domain fused to the receptor, then to a mobile histidine-containing phosphotransfer (HPt) protein that shuttles from the cytosol to the nucleus. Finally, the phosphate is transferred to an aspartate residue in the receiver domain of type B response regulators (RRs). These activate the transcription of type A RR genes, the products of which feed back to inhibit their own expression and probably also interact with various effectors [2–4].

Five genes (*AHP1–5*) encode HPt proteins in *Arabidopsis thaliana* and several experiments have suggested their function and their role in CK signalling: (i) AHP1–3 proteins can complement the deletion of *YPD1*, a gene encoding an endogenous HPt in a budding yeast mutant [5]; (ii) in yeast two-hybrid assays AHP1–3 interact physically with the histidine-aspartate kinase AHK4/CRE1/WOL or CKI1, as well as with several type B RRs; (iii) in vitro biochemical assays prove the transfer of a phosphoryl group during these interactions [6,7]; (iv) in vivo localization of the fusion proteins GFP/AHP1–4 shows the transient transfer of these HPts from the cytoplasm to the nucleus in a CK-dependent manner [8,9]; (v) *AHP2* ectopic expression in transgenic plants led to CK hypersensitivity [10].

As two-hybrid and complementation assays as well as in vitro transient test systems could lead to non-physiological interactions [11] and considering possible redundant functions between the different members of the multigene family, definitive arguments about the functional role of HPts in CK signalling are still missing. In the present work, we used RNA interference (RNAi) to stably silence a gene encoding a HPt protein in a *Catharanthus roseus* cell culture system [12] to investigate the role played by HPt proteins in the physiological processes of CK signalling.

2. Materials and methods

2.1. Plant material and treatments

Leaves, stems and flowers of *C. roseus* [L.] G. Don (cv. Pink Pacifica, Ducrettet Nursery, Thonon, France) were harvested from 2-month-old plants grown in a greenhouse at 25°C, then rapidly frozen in liquid nitrogen. Cell suspension cultures (line C20D) were maintained on a 7-day growth cycle in B5 medium [13] supplemented with 58 mM sucrose and 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4D). The cells were agitated in 250-ml Erlenmeyer flasks containing 50 ml medium on a rotary shaker (100 rpm) at 25°C, in the dark [12].

For experimental use, the cells were subcultured in 2,4D-free B5 medium, grown for 3 days, then subjected for 1 h to one of the following treatments: *trans*-zeatin (5 μM), abscisic acid (ABA, 10 μM), 2,4D (4.5 μM), jasmonic acid (JA, 400 μM), ethephon (1 mM), mannitol (200 mM), NaCl (35 mM). For cold or heat shock, the cells were shaken (100 rpm) during 1 h in an ice bath or an oven (40°C), respectively. The cells were filtrated from the culture medium and rapidly frozen in liquid nitrogen.

2.2. Plasmid constructions

A cDNA encoding the C-terminus of a *C. roseus* HPt protein was amplified by asymmetric polymerase chain reaction (PCR) performed on a λ*Zap*II-oriented *C. roseus* library (obtained from Dr. J. Memelink, Leiden University, The Netherlands) using the degenerate primer CHP3, 5′-TA(C,T)GT(A,T,G,C)CA(C,T)CA(A,G)(C,T)T(A,T,G,C,)AA(A,G)GG-3′ (sense orientation), and the T7 universal primer (antisense orientation). A complete cDNA was then obtained by an asymmetric PCR with a sense primer designed from the 3′ non-coding

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0014-5793/04/$30.00
doi:10.1016/S0014-5793(03)01522-9

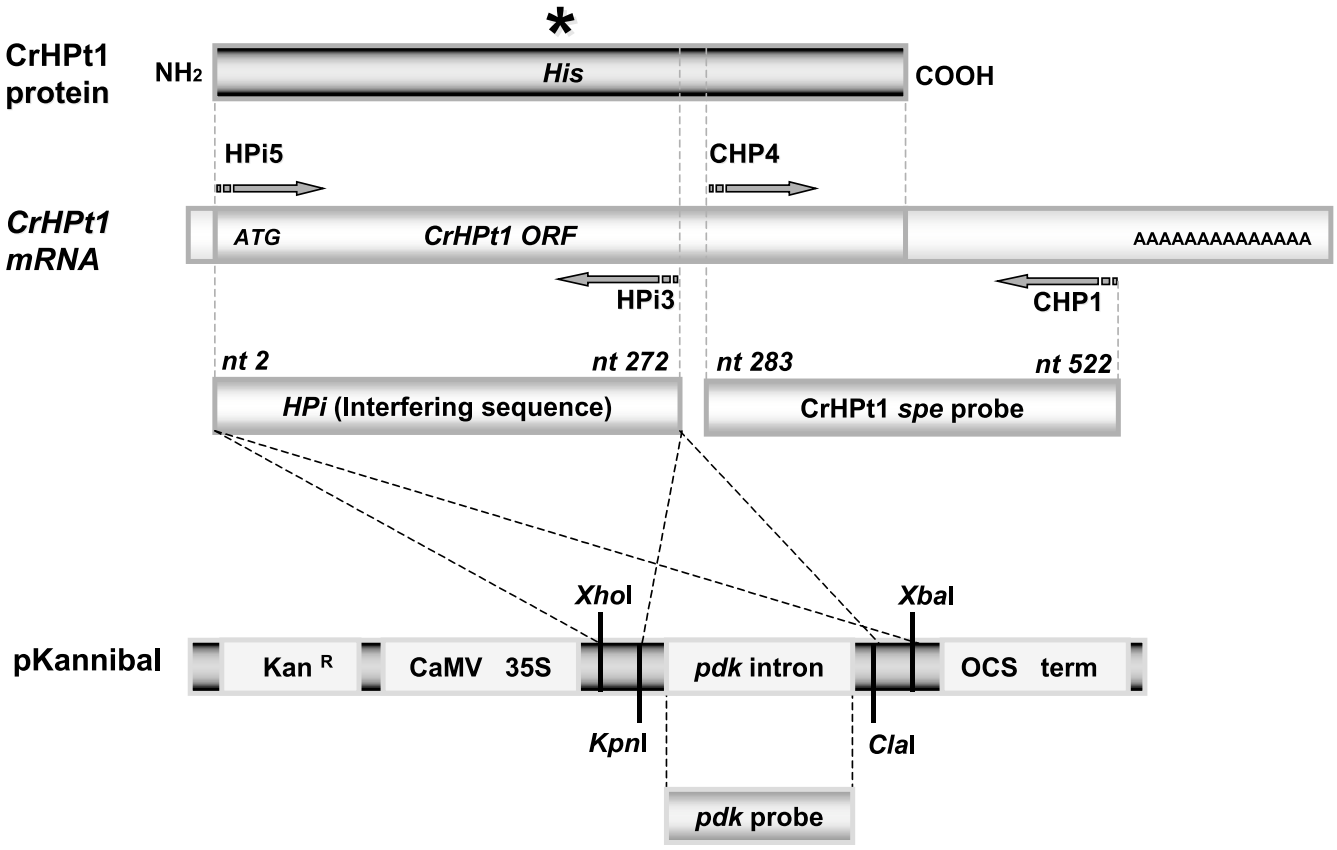

Fig. 1. PiH2 intron-spliced construction in the pKANNIBAL vector. The *HPi* sequence encodes the region of CrHPt1-deduced protein which contains the putative phosphorylatable histidine residue indicated by an asterisk. The positions of the probes utilized for Northern blot analysis are indicated.

end of the partial cDNA and the M13 reverse universal primer. The resulting cDNA was cloned into pGEM-T Easy vector and sequenced (GenBank accession number AY346308).

For RNAi experiments, a 270-bp *CrHPt1* partial sequence (nt 2–272) was amplified using HPi5 (sense) primer, 5′-TTCTAGA CTCGAGTGGAGGTCGGTCAATTGCAG-3′ (which contains *Xba*I and *Xho*I restriction sites), and HPi3 (antisense) primer, 5′-AATCGAT GGTACCAGCACCTATGCTGGAGCTGC-3′ (which contains *Cla*I and *Kpn*I restriction sites). The amplified sequence (named *HPi*) was cloned in both sense and antisense orientations between the *Xho*I/*Kpn*I and the *Xba*I/*Cla*I sites respectively of the pKANNIBAL vector [14,15]. The construct will be referred to as PiH2 (Fig. 1).

2.3. Biolistic experiments

For transformation, 4 ml of 4-day-old suspension cells was collected on a 1MM Whatman paper disk, placed onto semi-solid culture medium (0.7% agar) and bombarded with a mixture of either empty pKANNIBAL vector and PGL2 plasmids, or PiH2 and PGL2 plasmids in a ratio of 4:1. PGL2 plasmids contain a hygromycin selection gene [16] using a PDS-1000 Bio-Rad system. Twenty-four hours after bombardment, filters were transferred onto a culture medium containing 50 μg/ml hygromycin B. Independent transformed calluses that appeared on the filters after 3–4 weeks of selection were individually harvested and grown, first on solid, then in liquid maintenance culture medium.

2.4. RNA gel blot analysis

Total RNAs were extracted with RNAeasy Plant Mini Kit (Qiagen). Ten μg of total RNA was fractionated on a 2.2 M formaldehyde/1.5% (w/v) agarose gel, capillary-transferred onto nylon membranes (Hybond-N^+, Amersham Bioscience), and subsequently baked for 2 h at 80°C. The membranes were prehybridized (42°C, 3 h) in UltraHYB solution (Ambion). cDNAs were labelled with the Prime-a-gene Labelling system (Amersham Bioscience). Hybridization was carried out for 12 h at 42°C in UltraHYB solution. The membrane was washed for 30 min at 42°C in 0.1×SSPE/0.5% sodium dodecyl sulfate and autoradiographed.

The following labelled probes were used (see also Fig. 1): (i) the *pdk* probe was obtained by a PCR on the pKANNIBAL vector, using a sense primer designed from the end of the CaMV 35S promoter and the beginning of the *pdk* intron, and an antisense primer designed from the end of the *pdk* intron and the beginning of the *ocs* terminator. It was designed to detect *C. roseus* cell lines stably transformed with an empty pKANNIBAL vector; (ii) the *HPi* sequence was used as a probe to detect expression of the transgenic construction in the cells transformed with the pKANNIBAL vector harboring the PiH2 construct; (iii) the *spe* probe was amplified by PCR using the following two primers: CHP4 (5′-AATGCCTGCATTGCATTCCGA-3′) and CHP1 (5′-GAGGCATCGGCATAGACAA-3′). The *spe* probe, which contains a *CrHPt1* fragment (nt 283–522) not overlapping the *HPi* sequence (Fig. 1), was utilized to detect the endogenous *CrHPt1* transcripts; (iv) the *CrRR1* and *CrRR5* probes are described in [12].

3. Results

3.1. Expression of CrHPt1 in whole plant and cell cultures of C. roseus

A full-length cDNA (designated *CrHPt1*) was isolated by PCR amplification of a cDNA *C. roseus* library. It encodes a 151-aa protein, CrHPt1, which contains the 12-aa hallmark motif of the HPt proteins [17] and the histidine-79 found to be crucial for the phosphorelay [18]. The protein is about 60–70% identical to the HPt protein AHP1 from *A. thaliana*. Despite the sequence similarity between CrHPt1 and AHP1, the corresponding genes have different expression patterns in

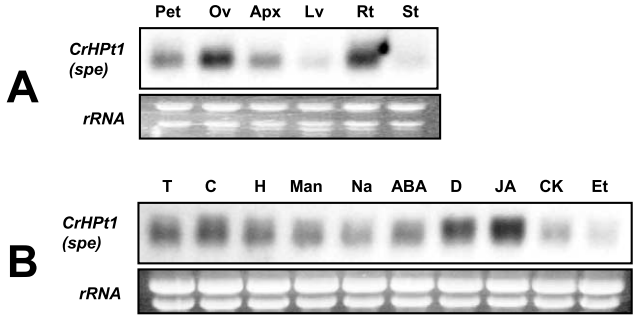

Fig. 2. Expression analyses of the *CrHPt1* gene in *C. roseus*. A: Organ-specific expression in plant. Total RNA were prepared from petals (Pet), ovaries (Ov), shoot apex (Apx), expanded leaves (Lv), roots (Rt) and stems (St) of 2-month-old plants. B: Three-day-old cell cultures were treated for 1 h as described in Section 2, then harvested and frozen prior to RNA extraction. C: cold shock (4°C), H: heat shock (40°C), Man: mannitol (200 mM), Na: NaCl (35 mM), ABA: abscisic acid (10 μM), D: 2,4D (4.5 μM), JA: jasmonic acid (400 μM), CK: *trans*-zeatin (5 μM), Et: ethephon (1 mM). *CrHPt1* transcripts were detected using the *spe* probe. Each lane was loaded with 10 μg of total RNA. Photographs of rRNA stained with ethidium bromide show equivalent loading.

plants: *AHP1* is exclusively expressed in roots [5] whereas *CrHPt1* is expressed at high level in roots, petals, shoot apex and ovaries, and at low level in leaves and young stems (Fig. 2A). The transcripts of *CrHPt1* accumulated also in *C. roseus* suspension cells (Fig. 2B). After subjecting the cells to various hormonal or stress treatments for 1 h, which is a sufficient time to detect CK-enhanced transcription of type A RR genes [12], the amount of *CrHPt1* transcripts was unchanged in most cases, except for JA and ethephon treatments.

3.2. Generation and selection of transgenic C. roseus cell suspensions

Previous observations have shown that supplying the C20D cells with *trans*-zeatin for 1 h enhanced the expression of *CrRR1*, which encodes a type A RR [12]; (see also Fig. 3B, untransformed line UL). To test whether the stable transformation and subsequent cell selection processes may induce change in the response of *CrRR1* to CK, we bombarded the cells with particles coated with PGL2 and the empty pKANNIBAL vector. Upon selecting several lines that were resistant to hygromycin, the presence of the pdk intron was tested by Northern blotting. About 70% of these lines were found to have integrated the pKANNIBAL vector. Five of them (Fig. 3A) were further studied and treated on the third day of culture with 5 μM *trans*-zeatin. *CrRR1* expression was induced in the transformed lines (Fig. 3B, C1–C5) to the same extent as in the untransformed C20D line (Fig. 3B, UL). These five C lines were then added to the untransformed C20D (UL line) as negative control for further experiments.

In addition, several hygromycin-resistant lines were obtained after bombarding C20D cells with particles coated with a mixture of PGL2 and PiH2 vectors. Northern hybridization with the *HPi* probe showed that about 50% of these lines expressed the PiH2 construct and thus had been transformed by the intron-spliced hairpin RNA. Five of them were selected for further experiments (Fig. 3C, lines iS1–iS5). The hybridization profile revealed by the *HPi* probe also shows the severe degradation of the corresponding RNAs in these transgenic lines.

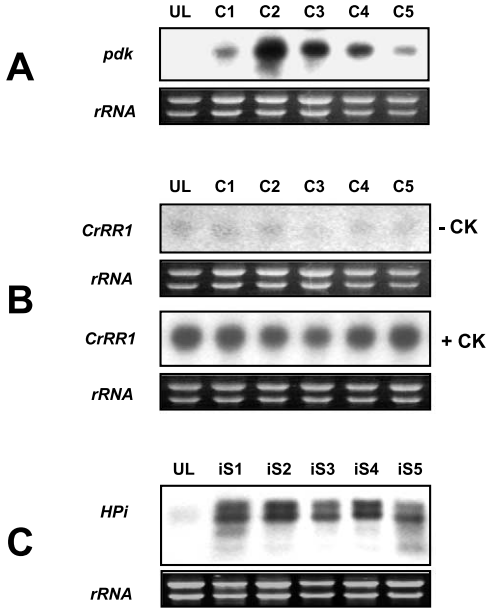

Fig. 3. Screening of the transgenic cell lines by Northern blot analysis. A: Transcription levels of the *pdk* intron in five control cell lines transformed with an empty pKANNIBAL vector. C1–C5: control lines; UL: untransformed C20D line. B: The corresponding cell lines were treated (+CK) or not (−CK) during 1 h with 5 μM *trans*-zeatin before RNA extraction. Membranes were hybridized with labelled *CrRR1* full-length probe. C: Selection of transformed cells. iS1–iS5: PiH2-transformed lines. Hybridization was performed with the *HPi* probe. Each lane was loaded with 10 μg of total RNA. Photographs of rRNA stained with ethidium bromide show equivalent loading.

3.3. Inhibition of the CK signalling pathway in HPt-interfered cell lines

The untransformed UL line, the C lines transformed with the empty pKANNIBAL and the iS lines harboring the PiH2 construct were treated for 1 h with 5 μM *trans*-zeatin. Then, the expression of *CrHPt1* and *CrRR1* was investigated by Northern blotting (Fig. 4). *CrHPt1* transcripts were present to the same extent in the control UL and in the C lines but were rather undetectable in the iS lines. *CrRR1* was highly induced by CK in UL and C lines, but the expression in iS lines remained very weak. *CrRR5* (which encodes a type B RR

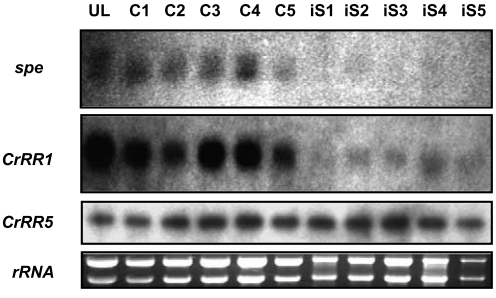

Fig. 4. Effect of CrHPt1 mRNA interference on CK signalling in PiH2-transformed cells. Cells were treated during 1 h with *trans*-zeatin 5 μM. Endogenous CrHPt1 mRNAs were detected after hybridization of the membrane with the *spe* probe. The same membrane was subsequently rehybridized with the *CrRR1* and *CrRR5* probes. UL: untransformed C20D line, C: transgenic control lines, iS: PiH2-transformed lines. Each lane was loaded with 10 μg of total RNA. Photographs of rRNA stained with ethidium bromide show equivalent loading.

not regulated by CK) was expressed at similar levels in all lines.

4. Discussion

We reported previously that CK trigger the fast and specific transcription of *CrRR1*, a type A RR considered a primary CK response gene, in the *C. roseus* cell line C20D [12]. In the past 5 years, the role of HPt proteins acting upstream of type A RRs in the CK signalling pathway has been investigated by various approaches in *A. thaliana* and *Zea mays* (see Section 1 and [19]) but no data concerning the physiological effect of mutating the corresponding genes have yet been reported. To examine the role of HPt proteins in *C. roseus*, the endogenous *C. roseus CrHPt1* gene was targeted for silencing through the technique of intron-spliced hairpin constructs [15]. *CrHPt1* encodes a HPt protein with high similarities to AHP1-AHP2 from *A. thaliana*.

In iS transgenic lines, the dramatic degradation of endogenous *CrHPt1* transcripts clearly correlates with the suppression of the CK-inducible *CrRR1* transcription. These results are unlikely to be imputable to non-specific effects on the CK signalling due to the transformation by the pKANNIBAL vector and/or to the biolistic method by itself. Indeed, the CK-induced expression of *CrRR1* in UL or C cells transformed with an empty vector is not affected while the expression of *CrRR5* (a type B RR gene unregulated by CK) remains unchanged in all the lines. This indicates that the whole transcriptional machinery is operative and that cell viability is not affected by the transformation procedure. Therefore, our results give in vivo functional evidence that the CrHPt1 protein is crucial in the CK signalling pathway leading to the induction of *CrRR1* expression in *C. roseus*. Five and three *HPt* genes have been identified in *A. thaliana* and *Z. mays*, respectively [11,19]. The failure in the identification of mutants affecting one of these gene based on a CK sensitivity phenotype screening could in part be explained by a functional redundancy between the different HPt isoforms present in plants. This also suggests that *CrHpt1* is likely a member of a multigene family in *C. roseus* and asks if all *HPt* genes were silenced in the iS transgenic cell lines that we have used. It was reported that efficient RNA silencing does not require a complete identity between the double-stranded RNA and the target RNA sequences [15,20]. Since high similarity exists within the HPt sequences of a given plant species, and since we have used a conserved part of these proteins to generate RNAi, we think that all *HPt* genes may be silenced in the iS transgenic lines.

Classically, sustained growth of plant cells in vitro requires the presence of both CK and auxin. CK has been proposed to regulate the cell cycle by inducing cyclin D3 transcription [21], possibly via the mediation of a RR protein [22]. The C20D line has become CK-habituated during subcultures, and C20D cells do not now require exogenous CK to divide: this may explain why the growth of the iS transgenic cell lines was not initially altered in their division process by *CrHPt1* silencing. Nevertheless, a progressive decrease in the growth rate of the transgenic lines was observed along subcultures leading to some lethality in the iS lines. Molecular mechanisms leading to CK habituation remain poorly understood but the existence of specific positive feedback gene regulation for the synthesis of CK has been proposed [23], and *CrHPt1* silencing could have progressively interrupted this regulation. Taken with other data previously published [5–10], our study give a new experimental argument in favor of a specific implication of HPts in the CK signalling transduction pathway in plants.

In other works, we have reported that CK enhanced the production of monoterpene alkaloids in C20D cells [24] and it will be interesting to know whether the effect of CK on alkaloid accumulation is correlated with the activation of the CK signalling described above. The present study shows the efficiency of the RNAi method to turn off the CK circuitry in *C. roseus*. To avoid lethal effects occurring in the transgenic cell lines along cell subcultures, the intron-spliced constructs will be improved by substituting an inducible promoter to the 35S promoter in the pKANNIBAL vector.

Acknowledgements: This research was supported by the Ministère de l'Education Nationale et de la Technologie (MENRT, France). A doctoral grant was awarded by the MENRT to N.P. We are grateful to Dr. P. Waterhouse (CSIRO Plant Industry, Canberra, Australia) who kindly provided the pKANNIBAL vector.

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