

Inhibition of the plant cytokinin transduction pathway by bacterial histidine kinase inhibitors in *Catharanthus roseus* cell cultures

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Abstract We describe the isolation of two *Catharanthus roseus* cDNAs encoding proteins putatively involved in the final steps of a 'histidine-to-aspartate' phosphorelay in cytokinin (CK) signaling. The expression of one of these genes, *CrRRI*, was specifically up-regulated by CKs in *C. roseus* cell suspensions. We used this system as a biological model to test the activity of bacterial histidine kinase inhibitors. Our data demonstrate that these inhibitors are active on the CK transduction pathway and represent powerful chemical tools to study hormone signal transduction in plants. Moreover, these data suggest a strong conservation of functional features between prokaryotic and plant signaling pathways utilizing histidine kinases.

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Key words: Anilide derivative; Cytokinin; Histidine kinase; Response regulator; *Catharanthus roseus*

1. Introduction

Bacteria sense and transduce many extracellular signals through histidine-to-aspartate phosphotransfers. Typically, the simplest 'two-component system' is composed of: (i) a trans-membrane histidine kinase receptor (HK) with a conserved autophosphorylatable histidine residue; (ii) a cognate cytosolic response regulator (RR) exhibiting a phospho-accepting aspartate and activating gene transcription through its output domain (Fig. 1). In some cases, more complex multi-step phosphorelays include an additional signaling module, namely a histidine-containing phosphotransfer protein (HPT). Moreover, sophisticated systems implicating the presence of both conserved histidine and aspartate residues within hybrid signaling proteins have emerged likely from gene duplication and fusion [1,2]. Because of their promising applications in antimicrobial research, compounds targeting 'His-to-Asp' phosphotransfer in bacteria have been studied through extensive screening strategies (see [3] for a review).

Recently, 'His-to-Asp' phosphorelays utilizing elements homolog to bacterial modules have been identified in plants, more precisely in hormone signalization, a four-step phosphorelay being crucial for cytokinin (CK) signaling in *Arabi-*

dopsis thaliana [4–6] (Fig. 1). Upon CK perception, the receptor (HK), built of an histidine kinase domain and an aspartate phospho-accepting domain, fuses together autophosphorylates and activates an additive histidine-containing phosphotransfer domain (HPT). Activated HPT shuttles to the nucleus and likely releases type-B RR from a repressor (R). Although it is still debated whether this release requires the phosphorylation of the conserved aspartate within the receiver domain of the RR [5,7], it actually allows type-B RR to bind promoters of target genes and to activate their transcription. Among these target genes, type-A RRs are CK primary response genes that negatively regulate the signaling pathway. CKs are plant growth regulators with pleiotropic functions, including regulation of cell division, control of morphogenesis/embryogenesis and inhibition of senescence [8]. They can also control some secondary metabolite biosynthetic pathways like anthocyanin production in maize and *Arabidopsis* [9,10] and terpenoid indole alkaloids (TIAs) in periwinkle cells [11]. However, it remains unclear whether wholly or partly these effects implicate the activation of 'His-to-Asp' phosphorelays. We previously isolated from periwinkle two cDNAs encoding a HK receptor (*CrCKRI*, accession number AY092025) and a HPT (*CrHPT1*, accession number AF346308). In the present work, we reported the isolation and the characterization of two cDNAs corresponding to type-A-like and type-B-like RRs. This allowed us to design a biological plant cell system to test the activity of salicylanilide compounds, recently reported as inhibitors of bacterial HK receptors, on the CK signaling pathway.

2. Materials and methods

2.1. Chemicals

3,3',4',5-tetrachlorosalicylanilide (TCSA) was purchased from Acros Organics (Geel, Belgium). Closantel[®], (*N*-[5-chloro-4-[(*R,S*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide) was provided and authorized by Janssen Pharmaceutica Research Foundation (Beerse, Belgium). *trans*-Zeatin, 2,4 dichlorophenoxyacetic acid (2,4-D), NaCl, jasmonic acid (JA), ethefon, were from Sigma (L'Isle d'Abeau, France). TCSA, Closantel[®], *trans*-zeatin, jasmonic acid, were dissolved in dimethylformamide. Ethefon, 2,4-D and NaCl were dissolved in distilled water.

2.2. Plant materials and growth conditions

Two month old periwinkle (*Catharanthus roseus* [L.] G. Don, Apocynaceae) plants were grown at 25°C in a greenhouse. Periwinkle suspension cells (line C20D) were maintained on a 7 day growth cycle in 250 ml Erlenmeyer flasks each containing 50 ml B5 medium [12]

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supplemented with 58 mM sucrose and 4.5 μ M 2,4-D. The cultures were shaken (100 rpm) at 25°C in the dark. For experimental use, the cells were subcultured in the same maintenance medium but 2,4-D was omitted. On the third day of culture, one of the following substances was added to the medium: 2,4-D (4.5 μ M), NaCl (50 mM), JA (400 μ M), *trans*-zeatin (5 μ M), ethefon (500 μ M). TCSA and Closantel® were added 1 h before CK treatment. For heat-shock experiments, C20D cells were placed at 37°C for 1 h prior to harvest.

2.3. cDNA cloning and analysis

cDNA encoding type-A-like RRs were isolated by polymerase chain reaction (PCR) using an oriented periwinkle cDNA library and the following two degenerated primers: 5'-CA(C/T)gT(A/T/g/C)(C/T)T(A/T/g/C)gC(A/T/g/C)gT(A/T/g/C)gA(C/T)gA(C/T)-3' and 5'-(C/T)T(g/C)(A/T/g/C)gC(A/T/g/C)CC(A/T/g/C)TC(C/T)TC(A/T/g/C)A(A/g)(A/g)CA-3'. Each of these primers encodes the characteristic HVLAVDD and CLEEGAE/D/Q motifs conserved in the *Arabidopsis* response regulator (ARR) family. The amplified PCR products were cloned into pGEM-T easy vector system II (Promega). Four clones were sequenced, which allowed us to obtain a truncated open reading frame, the 3' end of which was further characterized by RACE-PCR (3' RACE System, Gibco-BRL) performed on *C. roseus* mRNA with specific sense primer designed from the sequence. The 5' end was amplified by asymmetric PCR on the *C. roseus* cDNA library using antisense-specific primers and T3 universal primer. The full-length cDNA sequence was designated as *CrRR1* (GenBank accession number AF534888).

cDNA encoding type-B-like RRs were isolated following the same strategy with the degenerated primers 5'-ATgCC(A/T/g/C)gA(C/T)-ATggA(C/T)ggTTT-3' and 5'-gC(A/T/g/C)CTCAT(A/T/g/C)Ag(A/g)-TC(C/T)TC(C/T)Tg-3' (corresponding to MPDMDGFK and NQED-LMSA conserved motifs from ARR1–2). The full-length cDNA sequence was designated as *CrRR5* (GenBank accession number AF534891).

The cDNA (GenBank accession number AF329435) encoding the HSP60 (chaperonin β -subunit) utilized in heat-shock experiments was isolated in [13].

2.4. RNA gel blot analysis

Harvested cells or plant organs were frozen, then 3 g fresh mass was ground to a fine powder in liquid nitrogen. Total RNAs were extracted with RNeasy Plant Mini Kit (Qiagen). 15 μ g of total RNA was fractionated on a 2.2 M formaldehyde/1.5% (w/v) agarose gel, capillary transferred onto a nylon membrane (Hybond-N+, Amersham-Pharmacia-Biotech), and subsequently baked for 2 h at 80°C. The membrane was prehybridized (42°C, 1 h) in UltraHYB solution (Ambion). cDNAs were labeled with Prime-a-gene Labelling system (Amersham-Pharmacia-Biotech). 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 \times SSPE/0.5% SDS and autoradiographed. The signals were quantified with Quantity One software (Bio-Rad) considering the highest expression level of each experiment as a 100% reference.

3. Results

3.1. Isolation of RRs in periwinkle

PCR strategies based on protein-conserved domains were used to screen a *C. roseus* cDNA library and to isolate two full-length cDNAs encoding putative RRs. The deduced amino acid sequence of *CrRR1* (254 amino acids; calculated mass: 27.5 kDa) showed 40–70% identity with several members of the ARR family described in [14]. It contains a typical 126 aa plant DDK receiver domain with the three invariant residues conserved among all plant RRs (aspartic acids 21 and 75, lysine 127). *CrRR1* is closely related to ARR4 and ARR7 because it includes an acid/S-rich charged C-terminal end (Fig. 2A). Thus, according to [14], it could be assigned to the type-A RR subfamily. The amino acid sequence of *CrRR5* (643 amino acids, calculated mass 70 kDa) also contains a typical DDK receiver domain (aspartic acids 43 and

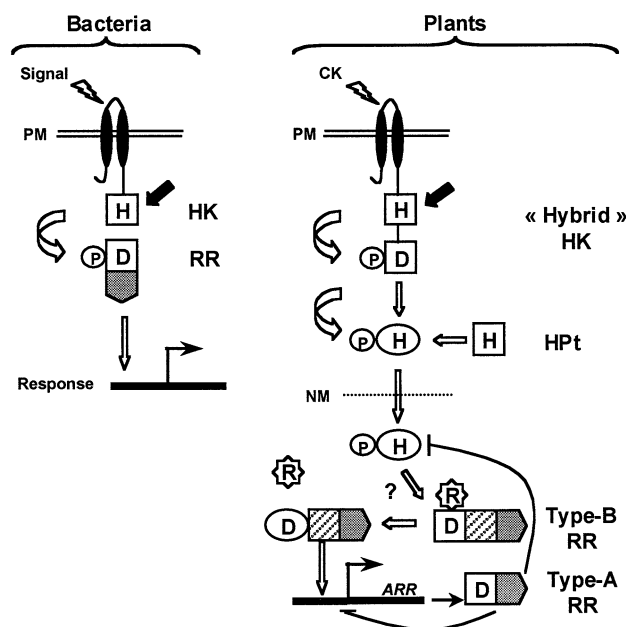


Fig. 1. Comparison of the schematic organization in the prototype bacterial 'two-component' system or in the plant 'multi-step' phosphorelay involved in CK signaling. H and D symbolize phosphorylatable histidine and aspartate residues putatively engaged in the phosphorelay. R represents a yet unknown repressor. Grey boxes show the output domain of RRs and hatched boxes correspond to the GARP domain in type-B RRs. A black arrow indicates the probable site of action of the inhibitors tested in our work. For more clarity, a single monomer of the HK dimer is represented.

88, lysine 137) but it exhibits the large C-terminal extension characteristic of the type-B RRs of *A. thaliana*. The organization of this C-terminal extension is extremely similar to that of ARR1–2 described in [15]: an acidic domain (ca. 70 residues) and a glutamine-rich domain (ca. 370 residues) flanking a highly conserved stretch of 60 amino acids referred to as the GARP motif [16] (Fig. 2B). This motif, folding into an α -helix structure, is related to basic helix–loop–helix DNA-binding motifs and to MYB-like transcription factors [17,18]. Moreover, as described for ARR1, ARR2 and ARR10, putative nuclear localization signals (amino acids 151–156 and 217–221) could be detected in *CrRR5* immediately after the DDK receiver domain and at the beginning of the GARP motif, respectively. Considering these important similarities with ARR1–2 both in sequence (ca. 50% overall identity, up to 93% for the GARP motif) and in organization, we can conclude that *CrRR5* is a type-B-like RR possibly acting as a transcription factor in CK signaling in *C. roseus*.

3.2. Expression analyses

Using standard Northern blot analysis, we investigated the expression of *CrRR1* and *CrRR5* in flowers, stem apex, young leaves, stems and roots of 2 month old periwinkle plants, as well as in periwinkle suspension cells submitted to various hormonal or stress treatments. *CrRR1* and *CrRR5* showed a similar pattern of expression in whole plants with transcripts preferentially accumulated in roots and flowers (Fig. 3A). In cell suspensions, *CrRR1* transcripts accumulated dramatically upon treatment with a CK (*trans*-zeatine) while neither other hormonal (auxin, jasmonic acid, ethylene) nor stress (NaCl) treatments affected both *CrRR1* and *CrRR5* transcription (Fig. 3B). The response of *CrRR1* to exogenous CKs was

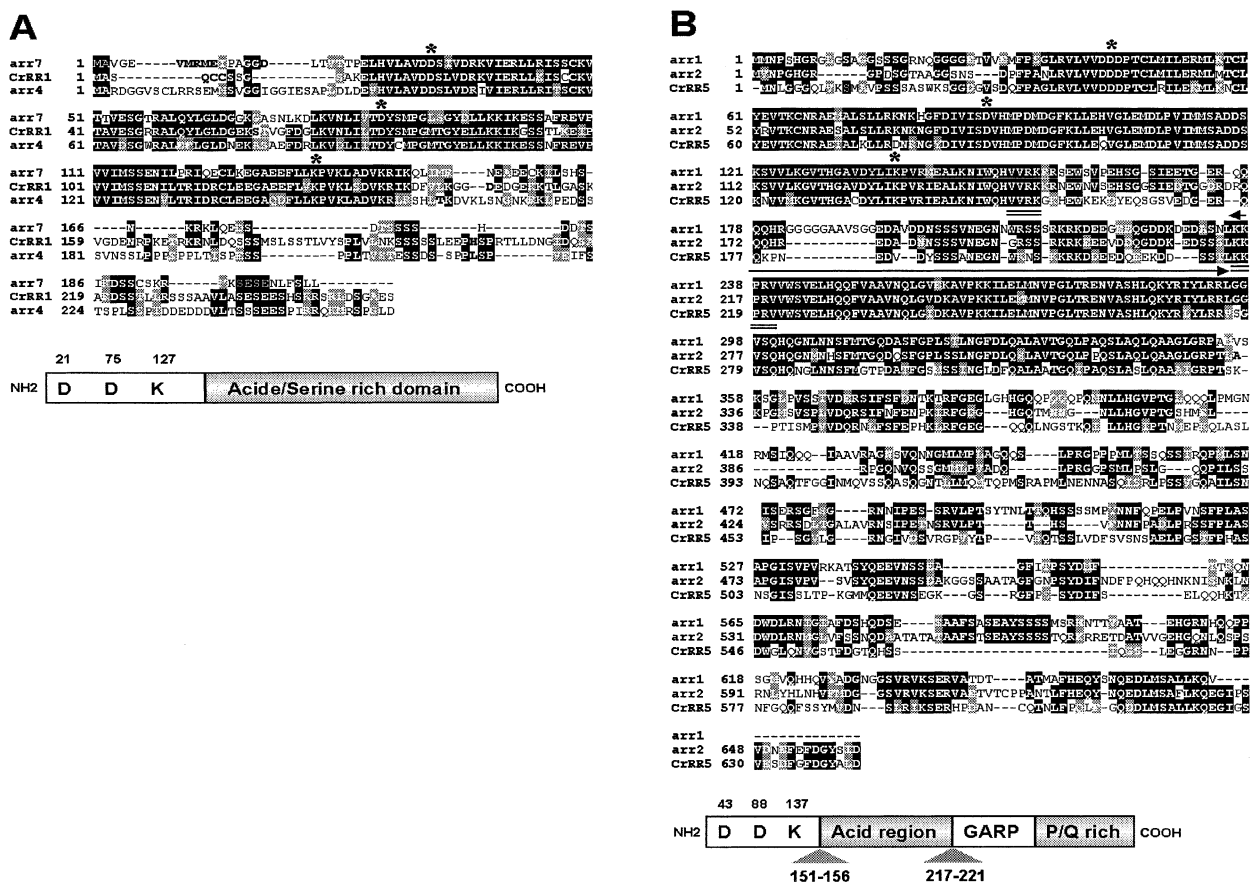


Fig. 2. Alignment of deduced amino acid sequences of *C. roseus* RRs with their homologs in *A. thaliana* and schematic domain organization (A: CrRR1, B: CrRR5). Homolog residues are boxed (similar in gray and identical in black boxes). Asterisks indicate the position of the three invariant DDK residues. Putative nuclear localization signals (NLS) are doubly underlined. GARP motif is represented with a black double-head arrow. In diagrams of domain organization, the numbers give the position of the residues relative to the position 1 of the methionine. In panel B, gray arrowheads indicate the position of NLS.

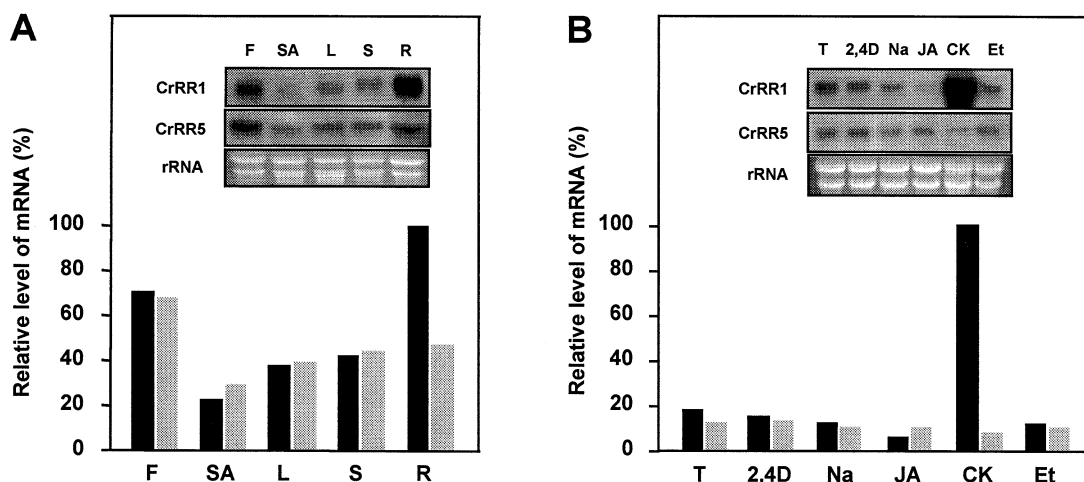


Fig. 3. Expression analyses of the *CrRR1* and *CrRR5* genes in periwinkle. A: Organ-specific expression in plant. Total RNA was prepared from flowers (F), shoot apex (SA), expanded leaves (L), stems (S) and roots (R) of 2 month old plants. B: Expression in C20D cell cultures under various hormonal or stress treatments. Cells were treated for 1 h with 5 μ M 2,4-D, 50 mM NaCl (Na), 400 μ M jasmonic acid (JA), 5 μ M *trans*-zeatin (CK) and 500 μ M ethephon (Et). Each lane was loaded with 10 μ g of total RNA. The signals were quantified with Quantity One software, with the highest signal as 100% reference (*CrRR1*: black bars; *CrRR5*: gray bars). Photographs of rRNA stained with ethidium bromide account for equivalent loading.

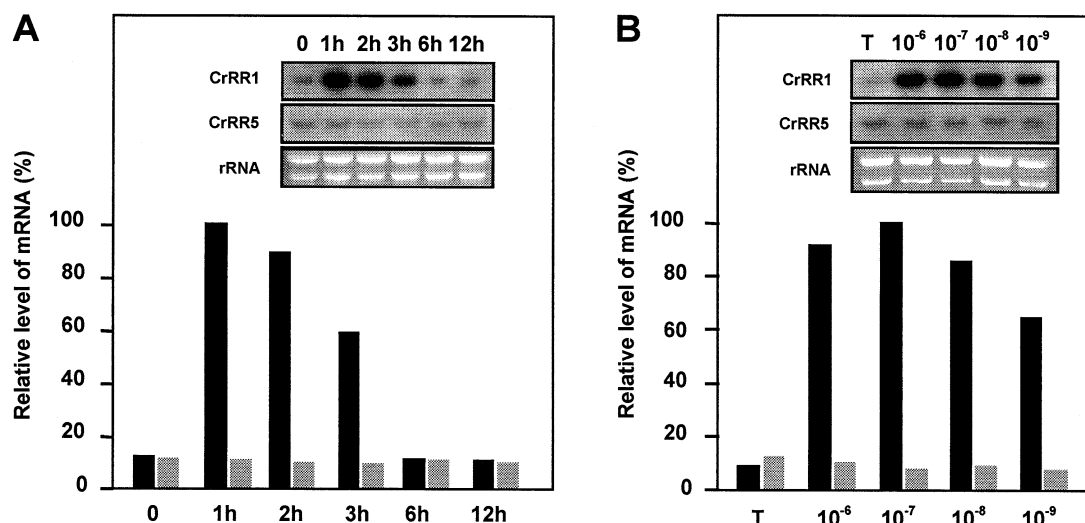


Fig. 4. Kinetic and dose-response analysis of *CrRR1* and *CrRR5* expression in C20D cells. A: Cultures were treated for 1, 2, 3, 6, 12 h with 5 μ M *trans*-zeatin; RNA was extracted immediately after each treatment. B: Cultures were treated with increasing amounts (from 10^{-6} to 10^{-9} M) of *trans*-zeatin for 1 h prior to RNA extraction. Loading and signal quantification as in Fig. 3.

further characterized through kinetic and dose-response analysis. Fig. 4A shows that transcript accumulation in the cells was maximum after 1 h of treatment with 5 μ M *trans*-zeatin and returned to the steady-state control levels within 6 h. These observations are consistent with previous results showing that among the 10 *Arabidopsis* type-A RRs tested for their response to exogenous CKs, maximum induction occurred for the *ARR4-ARR7* sub-group during a 10–100 min period [14]. The effect of *trans*-zeatin on *CrRR1* transcription was efficient for concentrations ranking from 10^{-6} to 10^{-9} M (Fig. 4B), which is compatible with most of the physiological responses to CKs in plant cells. Moreover, adenine, dimethyladenine and *cis*-zeatin, all structurally related to CKs but biologically inactive, failed to enhance *CrRR1* transcription, while the biologically active ‘cytokinin-like’ thidiazuron did (data not shown). The accumulation of *CrRR5* transcripts was not af-

fected in any of these experiments, confirming that it probably represents a CK non-inducible type-B-like RR.

3.3. Inhibition of the CK transduction pathway by salicylanilide derivatives

To further characterize the possible involvement of a phosphorelay in CK signaling in periwinkle, we checked whether the inhibition of CK sensing at the receptor level could affect the rate of transcription of *CrRR1* in the cells. For this purpose, we investigated the effects of two salicylanilide derivatives, TCSA and Closantel®, recently reported as potent inhibitors of bacterial HKs [19,20]. Cells of the C20D line were pre-treated for 1 h with increasing amounts of either TCSA (0–2 μ M) or Closantel® (0–8 μ M), then treated for 1 h with 5 μ M *trans*-zeatin and immediately frozen. Fig. 5 shows that both compounds strongly reduced, in a dose-dependent man-

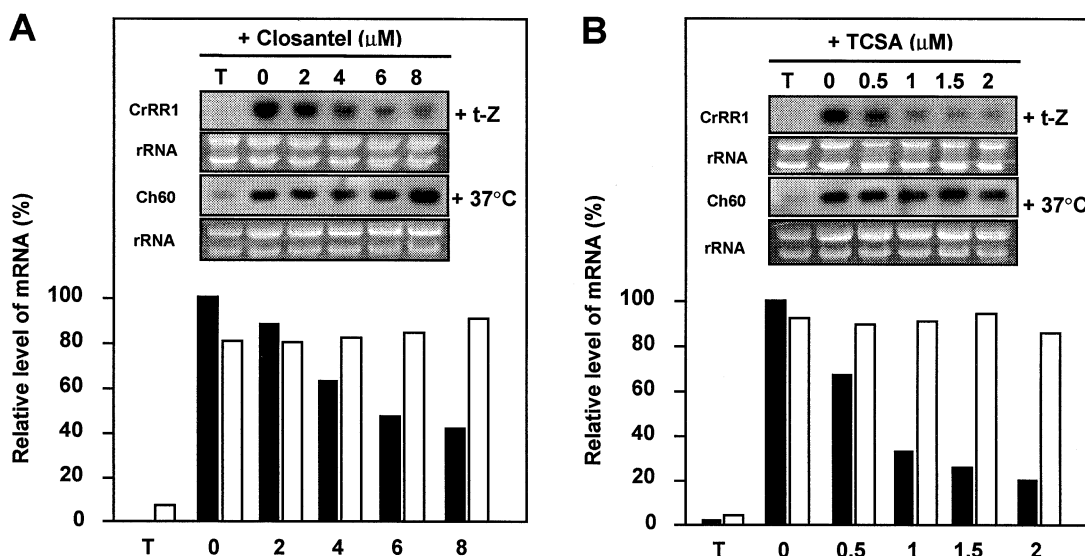


Fig. 5. Dose-response effects of salicylanilide derivatives on the CK-induced *CrRR1* expression. A: Closantel®. B: TCSA. Each compound was added at the different concentrations indicated 1 h before the cells were treated with CK (5 μ M of *trans*-zeatin for 1 h). Accumulation of *HSP60* transcripts after heat shock (37°C for 1 h) in the cells treated with equivalent amounts of inhibitors represent a control of their global transcription capacities. Loading and signal quantification (black bars: *CrRR1*; white bars: *HSP60*) as in Fig. 3.

ner, the transcription of *CrRRI* in cells treated by CKs, the percentage of inhibition at the highest concentration reaching 60% and 80% for Closantel® and TCSA, respectively. As these products were reported to exhibit possible non-specific inhibitory effects on growth in bacterial assays, probably due to their detergent action on the plasma membrane [21], we checked that the inhibition of *CrRRI* transcripts was not imputable to alterations of the whole transcription process in the cells. Fig. 5 indicates that the accumulation of HSP60 transcripts induced by a 37°C heat shock was not affected in these conditions. Moreover, the cell viability was confirmed (by blue Evans and/or triphenyltetrazolium chloride assays) after 1 h of treatment with each inhibitor (data not shown).

3.4. Effects of salicylanilides on CK-enhanced alkaloid production

In the C20D cells, the accumulation of TIAs is dramatically enhanced by CKs [11], thus we tested whether Closantel® and TCSA could affect the stimulation of TIA biosynthesis by CKs when applied at concentrations that inhibit *CrRRI* transcription. For this purpose, 3 day old cells were treated for 1 h with 0.5–2 µM TCSA or 2–8 µM Closantel® prior to the addition of 5 µM *trans*-zeatin to the culture medium, and harvested 4 days later. Alkaloid measurements indicated that TIA biosynthesis was completely abolished in the cells but the effects on the cell growth were too deleterious to conclude whether or not inhibition of TIA biosynthesis and inhibition of *CrRRI* transcription could be correlated (data not shown).

4. Discussion

The results presented here show that the elements of a functional circuitry utilizing ‘His-to-Asp’ phosphotransfer to mediate CK signal transduction are active in periwinkle. We used the fast and specific expression of *CrRRI*, a gene encoding a type-A-like RR, to monitor the early steps of CK signalization. More precisely, we were able to investigate the effects of salicylanilide derivatives, namely Closantel® and TCSA, on CK perception in plants. Our data confirmed that a transduction pathway involving HK receptors operates in plants in response to CKs as was previously reported by genetic approaches in *A. thaliana*. In bacteria, salicylanilides were proven to strongly reduce the autokinase activity of HK receptors, probably through structural alteration of their catalytic domain [21]. These compounds have never been tested on plant HKs before. Our results show that they efficiently interrupt the circuitry between CK perception and subsequent activation of the primary response gene *CrRRI*. This suggests that, despite structural divergences, functional features are conserved between prokaryotic and plant HKs. Moreover, as both compounds are active in plant cells at lower concentrations than in bacterial assays (0–10 µM vs. 100–200 µM), their effects on the membrane integrity should be notably

reduced. These inhibitors could be advantageously used as HK inhibitors in plants, in short-term experiments. However, after long-term exposure to these products, toxic effects on cell growth prevented us from drawing any correlation between the inhibition of the CK signaling pathway and the subsequent inhibition of alkaloid accumulation. We are currently testing this hypothesis by an alternative transgenic approach utilizing RNA interference to specifically and independently inhibit each element of the CK transduction pathway in periwinkle cells.

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