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Cloning of a gene encoding a putative ethylene receptor in *Catharanthus roseus* and its expression in plant and cell cultures

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Abstract

A full length cDNA (designated *CrETR1*) was isolated by polymerase chain reaction amplification of a cDNA library from periwinkle (*Catharanthus roseus*) cell cultures. *CrETR1* cDNA encodes a polypeptide of 740 amino acids with a predicted molecular mass of 82 kDa. The deduced protein contains a hydrophobic ethylene-binding transmembrane region, a GAF domain, a third domain homologous to the histidine protein kinase domain of the prokaryotic two-component systems, and a fourth carboxyl-terminal domain homologous to the receiver domain of the response regulators, as found in the *A. thaliana* ethylene receptor ETR1. *CrETR1* transcripts are strongly accumulated in petals and ovaries of *C. roseus* young plants whereas no significant changes are detected in cell cultures submitted to various stress or hormonal (including ethylene) treatments. The amount of the monoterpene indole alkaloid ajmalicine in the cells treated by ethylene is reduced after addition of inhibitors of histidine kinases showing a possible involvement of CrETR1 protein in the ethylene-related signalling pathway leading to alkaloid biosynthesis enhancement in *C. roseus* cell cultures.

Additional key words: cell culture, histidine kinase, Madagascar periwinkle, salicylanilides.

Introduction

Ethylene is a gas that regulates many aspects of developmental programs in plants, including germination of seeds, expansion and senescence of leaves, ripening and abscission of fruits, and nodulation of roots (Abeles *et al*. 1992, Chen and Chang 2003). It also modifies the plant responses to chilling, pathogen attack and wounding through positive or negative regulation of gene expression. For instance, it enhances the expression of genes encoding several plant defence proteins (Ecker and Davis 1987) and induces the accumulation of 6-methoxymellein in carrot suspension cells (Guo and Ohta 1994). On the contrary, it decreases the expression of *GS-II*, a gene that encodes leaf lectin in *Griffonia simplicifolia* (Zhu-Salzman *et al.* 1998), and it suppresses the production of nicotine in tobacco plants (Kahl *et al*. 2000).

Two subfamilies of ethylene receptors have been recognized in the last decade. Members of subfamily 1 (represented by AtETR1, AtETR2 and AtEIN4 in *Arabidopsis thaliana*) are composed of a sensory histidine kinase coupled to a response regulator. Members of subfamily 2 (AtERS1 and AtERS2 in *A. thaliana*) have the sensory histidine kinase only (Bleecker *et al.* 1998). There has been significant progress in elucidating the ethylene signal transduction pathway from AtETR1. The receptor is localized at the level of the endoplasmic reticulum (Chen *et al.* 2002). In the absence of ethylene, it activates CTR1, a Raf-like

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Abbreviations: ABA - abscisic acid; *CrETR1* - *Catharanthus roseus* ethylene receptor 1 mRNA; CrETR1 - putative protein; 2,4-D - 2,4 dichlorophenoxyacetic acid; DMF - *N,N'*-dimethylformamide; JA - jasmonic acid; MIAs - monoterpene indole alkaloids; RACE - rapid amplification of cDNA ends; TCSA - 3,3',4',5-tetrachlorosalicylanide; t-Z - *trans*-zeatin.

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The nucleotide sequence reported in this paper has been submitted to the GenBank, EMBL and DDBJ nucleotide sequence data bases with the accession number AF 534892.

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MAP kinase kinase kinase (Clark *et al*. 1998). In turn, CTR1 initiates a MAP kinase cascade (Ouaked *et al.* 2003) that negatively regulates the activity of EIN2, a protein with high similarity to the mammalian metal-ion transporters (Alonso *et al.* 1999). In the presence of ethylene, AtETR1 (and also AtERS1) forms homodimers that binds the gaseous molecules; binding is mediated by a copper cofactor (Rodriguez *et al.* 1999). This leads to the inactivation of CTR1 and therefore to the activation of EIN2. The downstream transcription factor EIN3 binds to the EBS element of the *ERF1* gene promoter (Chao *et al*.1997), then the ERF1 protein reacts in turn with the GCC-box of various ethylene target genes (Stepanova and Ecker 2000).

We are currently studying how the biosynthesis of the therapeutically valuable monoterpenoid indole alkaloids (MIAs) is regulated in the Madagascar periwinkle. In the course of these studies, we previously observed that treating periwinkle suspension cells with ethefon led to an increase of MIAs production (Yahia *et al.*1998). In order to study more in details the role of ethylene on MIAs biosynthesis, we attempted to isolate elements possibly involved in ethylene signalling. Here, we report on the cloning and characterization of *CrETR1*, a cDNA encoding a putative histidine kinase ethylene receptor in *C. roseus* and we describe the accumulation of the corresponding mRNAs in plants and cell cultures. We also show that two drugs known to be inhibitors of bacterial histidine kinases can decrease the ethylene-enhanced accumulation of MIAs in periwinkle cell cultures.

Materials and methods

Chemicals: 3,3',4',5-tetrachlorosalicylanide (TCSA) was purchased from *Acros Organics* (Geel, Belgium). N-[5-chloro-4-[(*R*,*S*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodo-benzamide (*Closantel*®) was provided and authorized by Janssen Pharmaceutica Research Foundation (Beerse, Belgium). Other chemicals were from *Sigma* (L'Isle d'Abeau, France).

Plants and cell cultures: Two-month-old plants of Madagascar periwinkle (*Catharanthus roseus* [L.] G. Don, *Apocynaceae*) were grown in a greenhouse under natural light at 25 °C. Suspension cells (line C20D) were maintained on a 7-d-growth cycle in the B5 medium of Gamborg *et al*. (1968) supplemented with 58 mM sucrose and 4.5 µM 2,4 dichlorophenoxyacetic acid (2,4-D). They were cultured in 250 cm^3 Erlenmeyer flasks (containing 50 cm^3 culture medium) on a rotary shaker (100 rpm), at 25 °C in the dark.

***CrETR1* cDNA isolation and characterization**: Comparison of the ETR1-like gene sequences from *Arabidopsis thaliana* (L24119), *Vitis vinifera* (AF243474), *Mangifera indica* (AF227742) and *Brassica oleracea* (AF047476) allowed us to locate an N-terminal conserved motif that was used to design a degenerated primer designated *ETL3*, the structure of which was the following:
5'-gC(A,g)CC(A,g)AACTg(g,A,C)AC(A,g)Ag(A,T,g,C) ACCCA-3'. Asymmetric PCR were performed on a periwinkle cDNA library using this primer and the T3 universal primer. DNA amplification was carried out through 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 30 s extension at 72 °C. This was completed by a final extension of 10 min at 72 °C. A 480 bp PCR product (designated ET10) was cloned into pGEM-T Easy vector (*Promega*, Charbonnières les Bains, France) and sequenced. In order to obtain the 3' end, RACE-PCR (*Gibco-BRL*, Paisley, Scotland, UK) was performed with mRNA from C20D cells, using a specific primer designed from the 5' non-coding end of ET10. A final PCR was used to verify the entire sequence of the full-length cDNA, which was designated *CrETR1*.

Cell treatments: All treatments were achieved on the third day after subculturing the C20D cells in growth regulator-free B5 medium. Mannitol (final concentration 200 mM), NaCl (35 mM), ABA (10 µM), 2,4-D (4.5 µM), t-Z (5 µM), JA (400 (µM) or ethefon (10, 100, 500, 1000 µM) were dissolved in water and filter-sterilised before addition to the culture medium. *Closantel*® (1µM) and TCSA (0.4 µM) were dissolved in DMF (the final concentration of DMF does not exceed 0.03 % v/v in the culture medium). For studying the *CrETR1* transcript amounts, the cells were harvested after one hour of treatment, then quickly frozen in liquid nitrogen. For studying ajmalicine concentration, the cells were harvested after 4 d of treatment**,** frozen in liquid nitrogen and freeze-dried. Quantitation of ajmalicine was as previously described (Oudin *et al.*1999).

Northern blot analysis: 3 g (fresh mass) frozen cells were ground to a fine powder in liquid nitrogen. Total RNAs were extracted with RNAeasy Plant Mini Kit (*Qiagen*, Courtaboeuf, France) as described by the manufacturer. 15 µg of total RNA were fractionated on a 2.2 formaldehyde + 1.5 % (m/v) agarose gel, capillary transferred with 20X SSC to a nylon membrane Hybond-N+ (*Amersham Bioscience*, Saclay, France), and subsequently baked for 2 h at 80 °C. The membrane was pre-hybridized (42 °C, 3 h) in UltraHYB solution (*Ambion*, Cambridgeshire, United Kingdom). The ET10 insert was labelled with *Prime-a-gene* labelling system (*Amersham Bioscience*), then used as a probe. The

[α-dCT32P]-labelled probe was added to hybridize the membranes (12 h, 42 °C) with the same UltraHYB solution. The membrane was washed (50 min, 42 °C) with 2X SSPE / 0.5 % SDS and auto-radiographed.

Results and discussion

Cloning of a cDNA encoding a putative periwinkle ethylene receptor: We characterised a 2674 bp cDNA (designated *CrETR1*) that encodes a putative protein of 740 amino acids (predicted molecular mass: 82 kDa) showing high similarities with AtETR1 from *A. thaliana*. The protein is composed of the following domains (Fig.1): *1*) At the N-terminus, three putative membrane segments similar to those essential for ethylene binding in

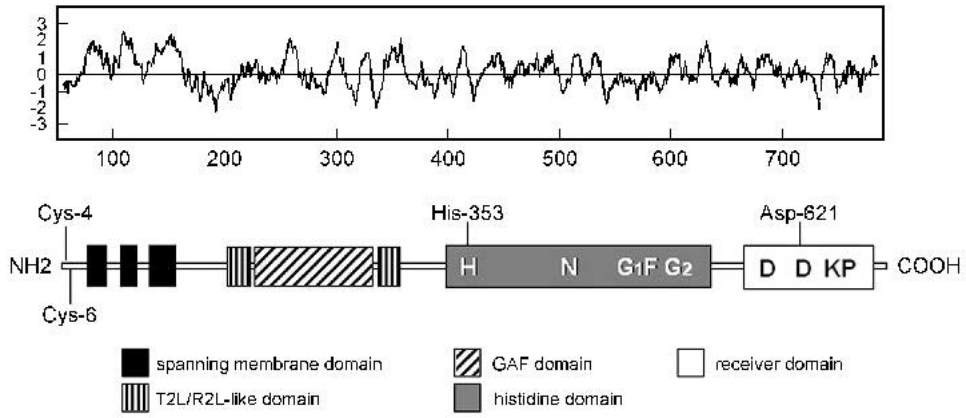

Fig. 1. Schematic representation of the domain organisation of the CrETR1 ethylene receptor.

AtETR1 (Schaller and Bleecker 1995). Residues found to be important in various ETR1 homologues are also present in this hydrophobic domain, *i.e.* the pair of cysteine residues (Cys-4 and Cys-6) required for dimerisation of AtETR1 through disulfide cross-linkage (Schaller *et al.* 1995); the Cys-65 and His-69 residues considered to be ligands for the Cu(I) cofactor of ethylene binding (Bleecker *et al.* 1998, Rodriguez *et al.* 1999); the Ala-31, Ile-62 and Ala-102 residues, the mutation of which caused insensitivity of AtETR1 to ethylene (Chang *et al.* 1993). *2*) a GAF domain usually conserved within the plant and cyanobacteria ethylene receptors (Mount and Chang 2002) and thought to be associated with cyclic-GMP binding proteins (Aravind and Ponting 1997). The GAF domain is flanked by the T2L and R2L regions found in the phytochromes from higher plants and cyanobacteria (Kehoe and Grossman 1996). *3*) An histidine kinase domain with five typical boxes that define the catalytic core of histidine kinases, *i.e.* H-box (amino acids 351-358) containing a presumptive autophosphorylated histidine at position 353, N-box (amino acids 463-471), G1 and G2 glycine-rich boxes (amino acids 515-523 and 543-551) which are both needed for histidine kinase activity in AtETR1 (Gamble *et al.* 1998, 2002), and F-box (aa 530-534) indispensable for the ATP-binding pocket activity (West and Stock 2001). *4*) At the C-terminal region, a typical response regulator domain very similar to the canonical prokaryotic receiver domain. This domain exhibits five highly invariant residues, *i.e.* Asp-620, Asp-621, Asp-663

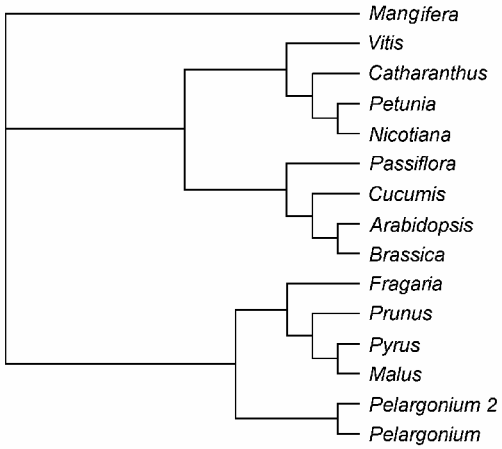

Fig. 2. Phylogenetic tree of plant histidine kinases identified as putative ethylene receptors. The tree was constructed with the amino acid sequences by the UPGMA method. *Catharanthus roseus* (AF534892) *Petunia* × *hybrida* (AF145972), *Pyrus communis* (AF386509), *Prunus persica* (AF124527), *Nicotiana tabacum* (AF022727), *Fragaria* × *ananassa* (AJ297511), *Malus* × *domestica* (AF032448), *Passiflora edulis* (AB015496), *Pelargonium* × *hortorum* (AF141929), *Vitis vinifera* (AF243474), *Pelargonium* × *hortorum* (AF141928), *Mangifera indica* (AF227742), *Cucumis melo* var. *reticulatus* (AF054806), *Arabidopsis thaliana* (L24119), *Brassica oleracea* (AF047476).

(all three form an acidic pocket), Lys717 (which protrudes into the latter) and Pro-718.

We compared the amino acid sequence of CrETR1 with sequences from other ethylene receptors. The highest percentage of homology (84 %) was found with the ETR1 homologue receptors identified from *Petunia* × *hybrida*, *Nicotiana tabacum* and *Vitis vinifera* (Fig. 2). CrETR1 has 81 % homology with the well-known ethylene receptor AtETR1.

Expression pattern of *CrETR1*: The expression patterns of *CrETR1* was investigated in various organs of periwinkle plants, as well as in periwinkle suspension cells submitted to several treatments. In plants, the highest transcript levels were found in petals and ovaries; apex, leaves, stems and roots accumulated lesser amounts (Fig. 3*A*). In C20D cells, kinetic and dose-response experiments indicated that the accumulation of *CrETR1* transcripts was not affected by ethefon (Fig. *3B*). Moreover, other hormonal (abscisic acid, auxin, jasmonic acid, cytokinin) or stress (low or high temperature, high osmolarity) treatments did not significantly modulate *CrETR1* transcription (Fig. 3*C*).

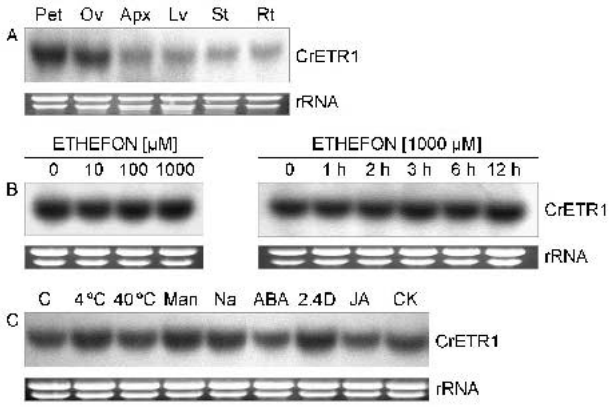

Fig. 3. Northern blot analysis of *CrETR1* mRNA levels. *A* - periwinkle organs: petals (Pet), ovaries (Ov), shoot apex (APx), expanded leaves (Lv), stem (St), roots (Rt). *B* - C20D cells cultivated in hormone-free medium for 3 d, then treated for 1 h with increasing amounts (10, 100, 1000 µM) of ethefon (*left panel*) or with 1 mM ethefon for increasing times (1, 2, 3, 6, 12 h) (*right panel*). *C* - C20D cells cultivated in hormone-free medium for 3 d then cold-shocked (4 °C), heat-shocked (40 °C), or treated with 200 mM mannitol (Man), 35 mM NaCl (Na), 10 µM ABA, 4.5 µM 2.4-D, 400 µM JA or 5 µM t-Z (CK) for 1 h prior RNA extraction; control (C). 20 µg of total RNA were loaded in each lane. Photographs of rRNA stained with ethidium bromide accounts for equivalent loading.

Effect of salicylanilide compounds on MIA accumulation in C20D cells: Salicylanilides such as *Closantel*® and TCSA have been reported as inhibitors of bacterial histidine (Stephenson *et al.* 2000). We recently showed that these drugs are active on the cytokinin transduction pathway acting through histidine kinase modules in *C. roseus* since they can inhibit the cytokinin-induced accumulation of type-A response regulators transcripts in periwinkle cells within 1 h (Papon *et al.* 2003). Ethefon increases the accumulation of MIAs in the cells, whereas *Closantel*® and TCSA used at concentrations unaffecting cell growth (expressed in dry mass or fresh mass) inhibit the MIA production in the presence or absence of ethefon (Fig. 4)

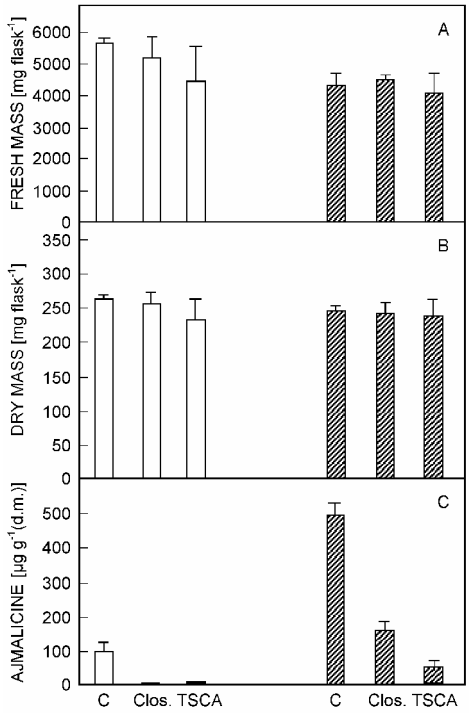

Fig. 4. Effects of inhibitors of bacterial histidine kinases on periwinkle cell growth and on ajmalicine accumulation. The cells were grown in hormone-free medium for three days. Two series of flasks (5 flasks each condition) were treated with either 1 µM *Closantel*® (Clos.) or 0.4 µM TCSA (TCSA) or untreated (C - control cells), then one serie was treated with 500 µM ethefon (hatched columns). The cells were harvested at day 7. *A* - fresh mass per flask; *B* - dry mass per flask; *C* - ajmalicine accumulation.

General discussion: *Catharanthus roseus* C20D suspension cells grown in their maintenance (2,4-D-containing) medium do not accumulate MIAs. Omitting 2,4-D from the medium induced MIA production, which was further enhanced by adding the ethylene-releasing agent ethefon to the medium (Yahia *et al.* 1998). In a first step to investigate the molecular control of MIAs production by ethylene, we characterized a periwinkle cDNA homologous to the ethylene-response sensor *AtETR1* from *A. thaliana*. The deduced protein shared 81 % aminoacid homology with AtETR1 and exhibits all the domains characteristic of the ethylene receptors belonging to the subfamily I (*i.e.* hybrid histidine kinase type).

Several data have proven that ethylene receptors act

as negative regulators of the response (Hua and Meyerowitz 1998) and that ethylene treatments could affect the amount of receptors at the plasma membrane level, providing the cells with a fine tuning mechanism to adapt their response to the hormone (Klee 2002). We have investigated the expression of the gene *CrETR1* in periwinkle cells submitted to ethefon and we have found that the gene appeared constitutively expressed in the cells, indicating that the enhancing effect of ethylene on MIAs production is probably not correlated with changes of the steady state level of *CrETR1*. Moreover, the expression pattern of *CrETR1* was not significantly affected by diverse stress and hormonal treatments that were previously reported to modulate MIAs production in periwinkle (Moreno *et al.* 1995). This suggests that, although the participation of endogenous ethylene in the response of the cells to these treatments cannot be ruled out, its effects on MIAs accumulation can hardly be explained by a modification of the amount of receptors at the cell surface. Concerning expression of *CrETR1* in plant organs, the gene appeared also to be expressed constitutively, but its transcript steady state level was markedly high in flowers, organs which are well known to be engaged in senescent processes by ethylene treatments (Abeles *et al*. 1992).

Since ethylene enhances MIAs production in periwinkle cells, it was expected that inhibitors of histidine kinase receptors may decrease the response to ethylene. We recently reported that the bacterial histidine kinase inhibitors *Closantel*® and TCSA are also active on the cytokinin transduction pathway in C20D cells since they can inhibit the cytokinin-induced accumulation of the response regulator *CrRR1* gene transcripts (Papon *et al.* 2003). In the present work, we used these drugs at concentrations much lower than those utilized in bacterial assays (Stephenson *et al.* 2000) or in studies with cytokinin (Papon *et al.* 2003). Indeed, the drugs exhibited no effect on C20D cell growth but severely inhibited the MIAs production. The effect observed on MIAs production even in the absence of added ethefon is probably due to the inhibition of the cell response to endogenous production ethylene since the gas is synthesised in C20D cells (Yahia *et al.* 1998).

In addition to ethylene, previous works have shown that jasmonic acid and cytokinin can also enhance the production of MIAs in C20D cells (Gantet *et al.* 1998, Oudin *et al.* 1999). There are also indications for complex two-component histidine kinase signalling network in plant cells. In *Arabidopsis*, ETR1 (ethylene pathway) seems to interact with AHP1 and AHP2 (cytokinin pathway) (Urao *et al.* 2000); EIN2, which acts downstream CTR1 in the ethylene pathway is probably also acting in the jasmonate pathway (Lorenzo *et al*. 2003). Therefore, in our opinion, C20D cells represent a convenient system to study the molecular biochemistry of ethylene response and to investigate the crosstalks putatively existing between hormone signalling pathways.

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