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Short communication

Systemic induction of a *Capsicum chinense* nitrate reductase by the infection with *Phytophthora capsici* and defence phytohormones

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ABSTRACT

The mRNA differential display technique was used to identify genes from Habanero pepper (*Capsicum chinense* Jacq.) seedlings whose expression is modified systemically by infection with the oomycete *Phytophthora capsici* L. Experiments with different oligonucleotide primer combinations revealed that no single gene was synthesised *de novo*. Instead, the quantitative accumulation of multiple transcripts was found. From these transcripts, levels of a nitrate reductase (*Capsicum chinense* nitrate reductase, CcNR), which has a high percentage of identity with other Solanaceae NRs, showed a consistent increase a few hours after inoculation (hai) with *P. capsici*. Reverse northern blotting revealed the existence of basal levels of CcNR transcripts in different adult tissues; however, systemic levels rose dramatically after spraying seedlings with salicylic acid (SA) and ethephon (ET) but not with methyl jasmonate (MeJa). Both *P. capsici* and defence phytohormones (DP) also modified NR enzymatic activity (nitrite:NAD^+ oxidoreductase; EC 1.7.1.1) with similar kinetics. Because the application of DP induced and activated the CcNR differentially, it is possible that the activity of CcNR is related to a specific host defence response.

1. Introduction

Phtytopththora blight (PB) is a lethal disease in peppers caused by the oomycete *Phytophthora capsici* L [1]. In fact, there is a small number of pepper species with consistent levels of resistance to PB [2,3]. In contrast to what has been classically defined as compatible and incompatible interactions, which supposedly involve one-for-one gene interactions [4], the compatibility between *P. capsici* and susceptible pepper species appears to be a complex phenomenon not exclusively due to a specific single factor [5]; thus, this interaction could be defined as a non-host compatible interaction [3,6,7]. Blocking of the defence response and the manipulation of the host's metabolism by oomycete pathogen effectors have been proposed as key events for the success of an infection [8]. However, regardless of the efforts made to elucidate the mechanisms of oomycete pathogenicity, few advancements have been made at this time [9].

Searching for differentially expressed genes has been used as a general strategy to elucidate the molecular relationships between plants and their pathogens [10–13]. In peppers, this approach has led to the identification of several genes induced by infections with compatible and incompatible pathogens [14–16]. By means of mRNA differential display, it was shown that pepper cDNAs encoding aldehyde dehydrogenase, P23 protein, NP24 protein, cytochrome P450 protein, esterase, and MADS-box proteins are expressed differentially in resistant fruits 24 and 48 h after infection with the fungus *Colletotrichum gloeosporioides*, which coincided with the period between fungal invasion and colonisation [17]. Kim and Hwang [18] demonstrated that genes encoding pathogenesis-related (PR) proteins, such as β-1,3-glucanase and chitinase, are induced in the leaves and stems of peppers (*Capsicum annuum*) by *Xanthomonas campestri* pv vesicatoria or *P. capsici* infections. Because the expression of PR genes was higher and faster in incompatible interactions than in the compatible interactions, they suggested that the time and intensity of gene expression could lead to differences between susceptibility and resistance.

To obtain further evidence of the cellular modifications produced in the host during the early stages of PB, we attempted to identify *Capsicum chinense* genes whose expression is modified systemically by infection with *P. capsici*. For that purpose, we applied the mRNA differential display technique to identify genes expressed in leaves after *in vitro* inoculation with mycelia of

Abbreviations: SA, salicylic acid; ET, ethephon; MeJa, methyl jasmonate; CcNR, nitrate reductase from *Capsicum chinense* Jacq.; PB, Phytophthora blight disease; DP, defence phytohormones; NR, nitrate reductase; hai, hours after the inoculation; haa, hours after the application; NO, nitric oxide; MS, Murashige and Skoog.

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P. capsici in the seedling roots. From the few genes identified, a nitrate reductase transcript (CcNR) demonstrated a minor, but consistent, accumulation soon after infection. Interestingly, the inoculation also induced NR activity with similar kinetics. In addition, both gene expression and protein activity were quickly enhanced by the external addition of defence-related phytohormones, which suggests CcNR could be part of a pepper incipient strategy to fight against *P. capsici* infection.

2. Results

2.1. Isolation and analysis of the CcNR cDNA sequence

By using the mRNA differential display technique, no gene transcripts synthesised *de novo* were found in leaves of *C. chinense* seedlings after they were inoculated in the roots with a virulent strain of *P. capsici in vitro*. Instead, few transcripts that displayed only differential expression were detected. A BLAST analysis showed that nucleotide sequences from clones CCA-1, CCB-2, and CCC-3 matched reported sequences of putative proteins with unknown functions (data not shown). Conversely, clones CCE-5, CCF-6, and CCG-7 share high similarity levels with zinc finger protein (*Arabidopis thaliana*), asparaginase (*O. sativa*), and vacuolar protein sorting-associated protein 26-like protein (*Solanum tuberosum*) genes, respectively (data not shown). Particularly, the cDNA sequence of clone CCD-4, with a size of 395 pb, had high similarity with nitrate reductase genes from other species of the Solanaceae family (data not shown). The deduced amino acid sequence of clone CCD-4 had 95% identity with NR from *S. tuberosum* and 89% with NR from *Nicotiana benthamiana* (Fig. 1A), and it also has the NR conserved domains HAEM and FAD (Fig. 1B). HAEM domains of NR from *S. tuberosum* (StNR2 and StNR3) possess a subdomain of 11 amino acid residues conserved in all members of the cytochrome *b*5 superfamily, including two invariable histidine residues (His-571 and His-594) involved in Fe binding [24]. The sequence track of clone CCD-4 contains His 594. Both StNR2 and StNR3 also possess a motif characteristic of NADH-specific NRs, which includes two conserved arginine residues (Arg-706 and Arg-868) that may be involved in the catalytic activity of the FAD domain [24]. Clone CCD-4 possesses the Arg-706. Based on its sequence similarity and the presence of conserved domains in its deduced amino acid sequence, clone CCD-4 most likely encodes a fragment of a *C. chinense* NADH-dependent nitrate reductase, which was named CcNR (*C. chinense* nitrate reductase gene homologue).

2.2. CcNR is expressed in adult tissues and is induced systemically by inoculation with P. capsici

Quantification of CcNR transcripts showed that, under normal conditions, there is a basal steady state level in leaves, stems and roots of Habanero pepper seedlings cultivated *in vitro* under hydroponic conditions (Fig. 2A). When the mycelium of *P. capsici* was deposited in the liquid medium, without touching the root epidermis directly, there was a noticeable increase in CcNR transcripts in the seedling leaves from at least six hai (Fig. 2B). This increase reached a maximum by 12 hai and remained high for at least 72 hai. Although the application of a PDA plug (Mock-treatment) also produced a slight increment of CcNR transcripts, the accumulation was faster and much higher in seedlings inoculated with the mycelium of *P. capsici* (Fig. 2B).

These results corroborated that CcNR was induced in systemic tissues early after Habanero pepper roots were infected with *P. capsici*. In some plant species the induction of NR is related to defence against pathogens [25–28]; however, in our system all the infected seedlings died, suggesting that the induction of CcNR is

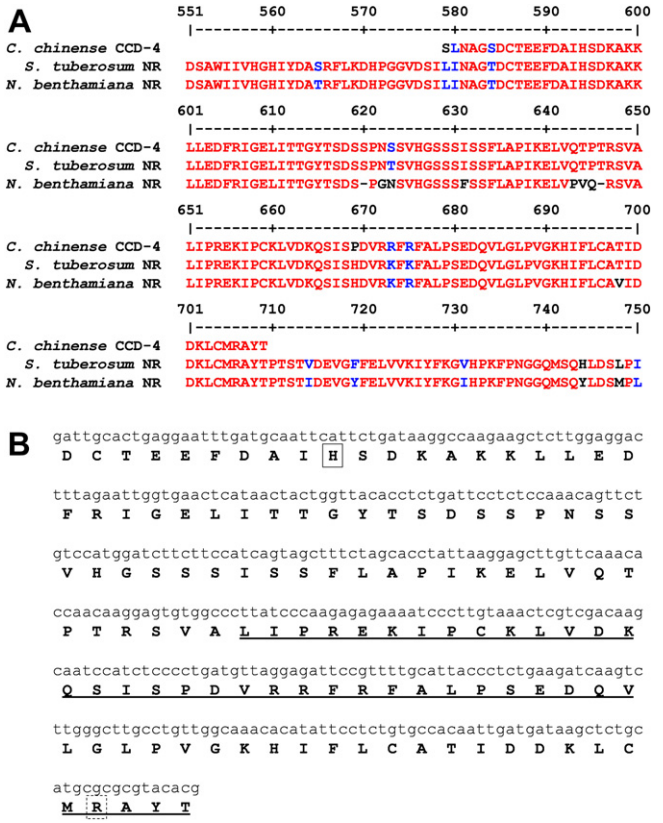

Fig. 1. Analysis of the nucleotide and deduced amino acid sequences of the *C. chinense* clone CCD-4. A, The deduced amino acid sequence of the *C. chinense* clone CCD-4 was aligned with the amino acid sequences of NR from *S. tuberosum* (GenBank accession code BAB935349) and *N. benthamiana* (GenBank accession code AB12345) [21]. Perfect matches are colored in red. Conserved amino acid changes are in blue. Non-conserved amino acid changes are in black. B, The protein functional domains of the deduced amino acid sequence of the clone CCD-4 were obtained using the NCBI Conserved Domain Search service (CD search). Amino acids from the HAEM and FAD domains of plant NRs are underlined. A conserved Hys-594 residue from the Fe binding domain is boxed. A conserved Arg-706 residue from the NADH-binding domain is boxed with dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ineffective or is not related to defence. To further investigate whether the cloned NR was also activated by pathogen-activated systemic signals, we sprayed the pepper seedlings with defence phytohormones and analyzed its effect on the expression of CcNR.

2.3. CcNR is induced by the exogenous application of defence phytohormones

Spraying with 5 mM SA or 5 mM ET solutions on the leaves of seedlings cultivated in hydroponics induced rapid expression of CcNR (Fig. 2C). The accumulation of CcNR transcripts induced by SA and ET reached a maximum between 0 and 6 h after application (haa) and remained high until at least 72 haa. However, the application of 100 μM MeJa exerted only a modest influence on the basal levels of CcNR transcripts, especially during the first haa (Fig. 2C). The rapid and robust induction suggested that CcNR could play a role in the systemic response mediated by SA and ET but not by MeJa.

2.4. Nitrate reductase activity is modified in pepper leaves by the addition of defence phytohormones and inoculation with P. capsici

The existence of a peptide domain characteristic of the NADH-dependent plant nitrate reductases in the deduced amino acid