Host response profiling to fungal infection: molecular cloning, characterization and expression analysis of *NPR1* gene from coffee (*Coffea arabica*)

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The *Arabidopsis thaliana* NPR1 protein (for Nonexpressor of PR genes) is a key positive regulator of the systemic acquired resistance (SAR), a general defense response in plants that is characterized by the expression of *pathogenesis-related* (*PR*) genes. Herein, we present a combination of coffee genome sequence resources, gene cloning experiments, and transient expression assays to make possible the functional characterization of the orthologous *NPR* (*CaNPR1*) gene from coffee (*Coffea arabica*). Data obtained suggest that this gene likely play a role during coffee defense responses to pathogen infection The *CaNPR1* gene may represent a promising candidate for engineering resistance in coffee to achieving broad spectrum resistance to pathogens.

Keywords plant-pathogen interaction; orange rust fungus; disease resistance.

1. General remarks

Inducible defenses against a number of pathogens are quickly expressed in the primary infection sites and are often visible as necrotic local lesions on the leaves of resistant plant varieties. This local reaction is referred to as the hypersensitive response (HR). Subsequently, the HR triggers a general resistance mechanism that renders uninfected parts of the plant tolerant to further pathogen attack, a phenomenon called systemic acquired resistance (SAR) [1]. The elicitation of the HR and SAR reactions is accompanied by the coordinated induction of a heterogeneous group of proteins which are impaired in the absence of salicylic acid (SA) [2-5]. Thus, the rise in SA levels triggers the activation of a number of defense genes. Among these genes, the best characterized code for an essential regulator of SAR, namely the *NPR1* gene (for Nonexpressor of PR genes; also known as NIM1 - non-immunity, or SAI1 - salicylic acid insensitivity) [6-8]. According to Cao *et al.* [8], the NPR1 protein is constitutively expressed and activated following pathogen recognition and SA treatment, suggesting that this protein acts as a key positive regulator of the SA-dependent resistance signaling pathway [9-10]. In healthy tissues, NPR1 is known to be an oligomeric, cytosolic protein. Following SA treatment, *Arabidopsis* NPR1 (AtNPR1) dimers become monomers and move into the nucleus to interact with TGA transcription factors [11]. It was demonstrated that NPR1 directly control the expression of *PR 1* genes which encodes a basic protein reported to be pathogen-responsive [12-14]. In Fig. 1, we summarize the data concerning the role of NPR1 proteins in plant defense responses to pathogens.

NPR1 proteins are highly conserved across many plant species and a conserved role in disease resistance has been suggested, as described by Cantu *et al.* [21]. By instance, overexpression of AtNPR1 in carrot tissues offered the ability to control a wide range of different pathogens [22]. Given the pivotal role of NPR1 gene in plant defense, we assessed the contribution and regulation of this protein during biotic stress responses in coffee (*Coffea arabica*), a key export and cash crop in tropical and subtropical developing countries. One of the major limiting factors for coffee production is coffee leaf rust (or orange rust) disease caused by the Basidiomycete fungus *Hemileia vastatrix* Berkeley and Broome [23]. This fungus infects the lower surface of the leaves where it produces large, orange colonies of urediospores, leading to premature leaf fall and yield losses in coffee (*C. arabica*) production [23].

Combining *in silico* databases searches, a genome walking strategy and phylogenetic analyses, *NPR1* orthologous sequences (termed *CaNPR1*) were isolated from the genomic DNA of *C. arabica*. Moreover, the promoter region of *CaNPR1* gene was characterized and transiently expressed by agroinfiltration in leaves of *Nicotiana benthamiana* for functional analyses. According to our results, transcript levels of *CaNPR1* were not affected following infection of coffee plants with *H. vastatrix*. Interestingly, the activity of the *CaNPR1* promoter was found to be SA-dependent. These data demonstrate that SA strongly stimulates the transcriptional activity of the *CaNPR1* promoter, and suggest that different *cis*-acting elements located in this region, such as the plant hormone- and/or infection-related motifs, might contribute to SA responsiveness.
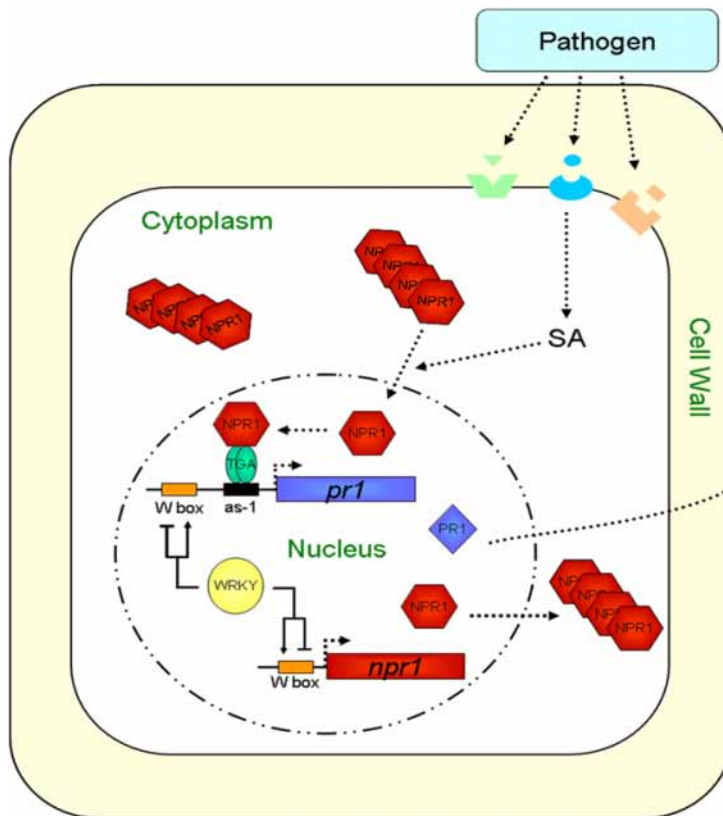
Fig. 1 Model summarizing one possible pathway of plant cell defense triggered by the recognition of pathogens. It is known that upon pathogen attack, accumulation of SA causes a change in the cytosolic cellular redox [9], leading to the conversion of the inactive NPR1, present as cytosolic disulfide-bound oligomers in the absence of pathogen attack, into active monomers [10]. NPR1 monomers are transported into the nucleus where they interact with the TGA class of basic leucine zipper transcription factors [15-17]. This interaction, in turn, stimulates the binding of TGA factors to SA responsive elements in the promoters of PR genes, launching the onset of SAR [18-19]. It is also known that the WRKY family of transcription factors can regulate the expression of PR1 and NPR1 genes, interacting with W box elements present in their promoter regions [20]. NPR1 is constitutively expressed and levels of its transcripts increased two-fold following SA treatment [8].

2. Findings

2.1. Isolation and characterization of the complete coffee *NPR1* gene sequence

To isolate the *NPR1* cDNA from *C. arabica* (*CaNPR1*), ESTs encoding a NPR1 protein were retrieved in subtractive libraries, associated with expression of early resistance mechanisms of coffee plants to the leaf rust pathogen *H. vastatrix*, constructed by Fernandez *et al.* [24] and in the Brazilian Coffee EST database [25]. These ESTs contained a 687 bp coding region that covered the C-terminal part of the encoded protein, as determined by a BLASTX search. They were used to design a pair of nested gene-specific primers to conduct the genome walking procedure for amplification of the 5' flanking region of *CaNPR1*. Hence, the 5'-flanking region of the *CaNPR1* gene was amplified using a set of nine gene-specific primers (CaNPR1-1 to CaNPR1-9). The first two (CaNPR1-1 and CaNPR1-2) were designed based on the starting EST sequence selected in the database while the others were designed based on the nucleotide sequences of the fragments amplified by genome walking.

A 4375 bp-long genomic fragment encompassing the entire *CaNPR1* coding region was obtained (Fig. 2a). The *CaNPR1* open reading frame encoded a protein of 609 amino acids (Fig. 2b). The deduced amino acid sequence of *CaNPR1* displayed highest identity with the NPR1 proteins from different Solanaceae including *Capsicum annuum* (ABG38308, 71%), *Nicotiana tabacum* (ABH04326, 71%) and *Solanum lycopersicon* (AAT57637, 71%). Detailed analysis of the deduced amino acid sequence of CaNPR1 revealed the typical highly conserved features of NPR1 proteins [7, 26-28] (Fig. 2b). Previous studies have demonstrated that residues Cys82, Cys150, Cys155, Cys160 and Cys216 present in the BTB/POZ domain are involved in oligomer-monomer transition [10, 29]. All these 5 cysteine residues are conserved in *CaNPR1* (data not shown). Curiously, an unexpected leucine zipper (LZ) motif was found in the CaNPR1 protein (Fig. 2b). This domain has never been reported in NPR1 proteins. The LZ are usually found as part of a DNA-binding domain in various transcription factors [30], and are therefore involved in many gene regulatory proteins such as octamer-binding transcription factor 2 (Oct-2/OTF-2) [31], CCATT-box and enhancer binding protein (C/EBP) [32], nuclear oncogene fos and jun [33], cAMP response element (CRE) binding proteins (CREB, CRE-BP1, and ATFs) [34], and C-myc, L-myc and N-myc oncogenes [35]. According to Viedma *et al.* [36], the LZ motif modulates the equilibrium between monomeric and dimeric forms of a protein. Interestingly, this motif was also found in Solanaceae studied herein (data not shown).

a)
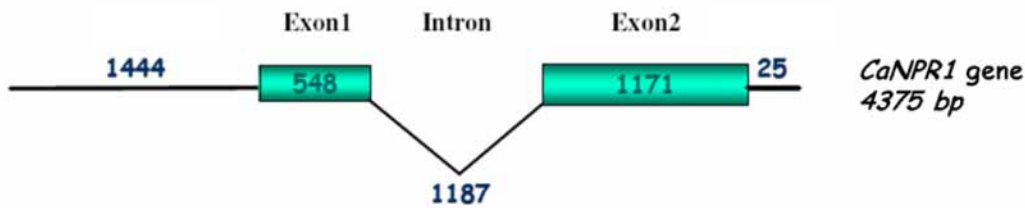
b)

Fig. 2 Genomic organization of the coffee *NPR1* gene (*CaNPR1*). a) Schematic representation of the 4375 bp-long genomic fragment amplified by genome walking of *CaNPR1*, that contains a coding region with two exons and one intron, as well as its upstream and downstream non-coding region (1444 and 25 bp respectively). b) The encoded protein (609 amino acids) harbors the three main domains of NPR1 proteins: the BTB/POZ domain, an ankyrin (ANK) repeat domain, and the nuclear localization signal (NLS). An atypical leucine zipper (LZ) domain was also observed in the CaNPR1 protein.

2.2. Phylogenetic analysis of NPR1 homologs

Phylogenetic analysis was based on the entire amino acid sequences of NPR1 proteins retrieved from public databases: *S. lycopersicum* (AAT57637.1, Le), *Capsicum annum* (ABG38308.1, Ca), *Nicotiana tabacum* (AAM62410.1, Nt), *Ricinus communis* (EEF48081.1, Rc), *Musa acuminata* (ABL63913.1, Ma), *Oryza sativa* (ABE11613.1, Os), *Hordeum vulgare* (CAJ19095.1, Hv), *Brassica napus* (AAM88865.2, Bn), *Brassica juncea* (ABC94642.2, Bj), *Arabidopsis thaliana* (NP_176610.1), *Musa* spp. ABB (ACJ04030.1, Md), *Zea mays* (NP_001147587.1, Zm), *Glycine max*: GmNPR1-1 (ACJ45013.1) and GmNPR1-2 (ACJ45015.1), *Populus trichocarpa* (XP_002322351.1, Pt), *Malus x domestica* (ACC77697.1, Mp), *Pyrus pyrifolia* (ABK62792.1, Pp), as well the sequences from *A. thaliana* NPR2 (NP_194342.1, At), NPR3 (NP_199324.2, At), NPR4 (NP_193701.2, At), NPR5 or AtBOP1 (ABH04470.1, At), and NPR6 or AtBOP2 (AAU90063.1, At).

Analysis using bootstrap consensus for neighbor joining showed that CaNPR1 grouped with members of the Solanaceae family like tobacco (*N. tabacum*), tomato (*S. lycopersicum*) and pepper (*C. annuum*) (Fig. 3). This suggests a conserved pattern in the NPR1 protein among plant families that belong to the Asterid I clade of dicots [37]. The closer taxonomic affinities of coffee and Solanaceae are also paralleled by a number of striking botanic and genetic similarities, including the production of fleshy berries [38], similar genome content [39] and similar basic number and architecture of the chromosomes [40-42]. Accordingly, plants belonging to the Brassicaceae family (*A. thaliana*, *Brassica napus* and *B. juncea*) formed a separate sub-clade. Within the monocots and dicots, a relative close grouping was obtained between NPR1 proteins from the Asterid I clade of dicots and from monocots belonging to different families (Fig. 3), which is in agreement with reports by Henanff *et al.* [27] and Zhao *et al.* [43].
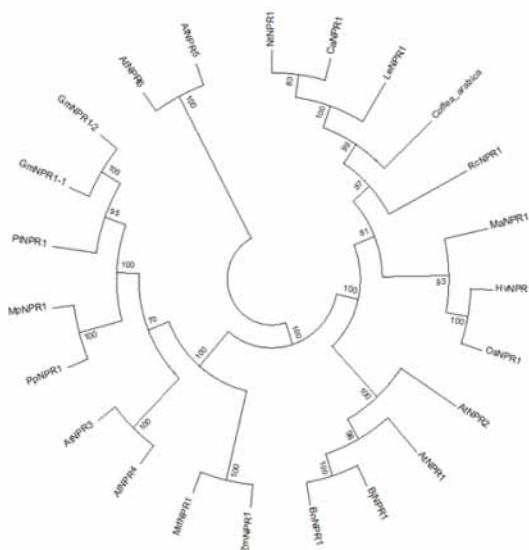

Fig. 3 Evolutionary relationship of NPR1 orthologues. The evolutionary history was inferred using the Neighbor-Joining method [44]. Bootstrap values based on 1000 replicates are indicated beside the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [45] and are in the units of the number of amino acid substitutions per site. The analysis involved 23 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 734 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [46].

2.3. Expression pattern of *CaNPR1* during compatible interaction with *Hemileia vastatrix*

Analysis of rust infection in susceptible coffee varieties showed that the fungus germinates at the leaf surface, penetrates through stomata and produces intracellular infection structure (haustoria) in leaf cells as soon as 24 hours after infection (hai) [47]. Drastic changes in defense gene expression may be observed at 6 to12 hai [47-48]. To test whether the expression of *CaNPR1* is affected by biotic stress, transcript accumulation in coffee plants exposed to *H. vastatrix* infection was monitored by quantitative real-time PCR during the first 24 hai. No significant changes in *CaNPR1* transcript levels were observed (Fig. 4), suggesting that *CaNPR1* expression is not regulated at the transcriptional level upon *H. vastatrix* infection. This is in line with previous studies showing constitutive expression of *NPR1* genes under pathogen infection [11, 27, 49].
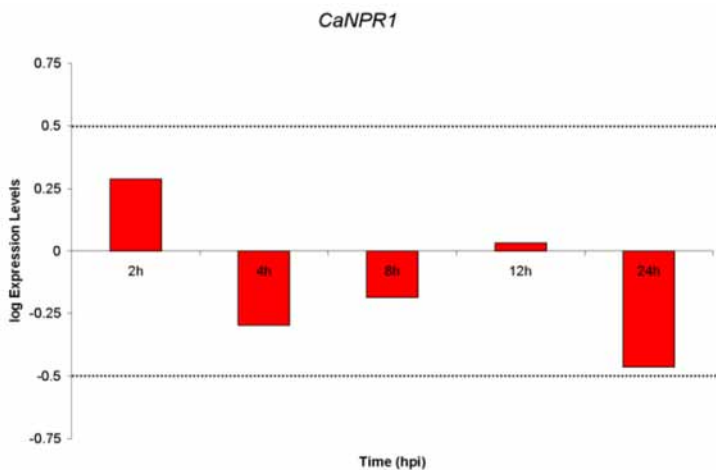

Fig. 4 Expression pattern of *CaNPR1* following infection with *H. vastatrix*. Leaves of *C. arabica* cv Mundo Novo were inoculated with *H. vastatrix* race II (10 mg spores mL^{-1}). Control leaves were sprayed with water. Leaves were collected at different time points as indicated. The log-transformed gene expression levels are presented and indicate normalized expression levels in inoculated leaves compared to normalized expression levels observed in water-treated leaves at the same time point. The *GAPDH* gene was used for normalization. The two thin dashed lines represent the minimal standard deviation, with a log expression levels difference between 0.5 and -0.5. hpi: hours post inoculation.

2.4. Sequence analysis of *CaNPR1* promoter

The nucleotide sequence of the 5' upstream region of *CaNPR1* was identical among 11 clones analyzed, suggesting either that the upstream sequences of the two *CaNPR1* copies of the tetraploid *C. arabica* genome are high similar or that our gene walking strategy impaired cloning of only one of the duplicated sequence.

To assess the set of putative *cis*-acting elements present in the *CaNPR1* promoter, named *pCaNPR1*, an *in silico* search was performed in PLACE using the 1444-bp sequence located upstream of the start codon as query. In this analysis we looked for TATA-box consensus sequences and for consensus sequences described as binding sites for defense-related transcription factors. A TATA-box was identified 496 nucleotides upstream of the start codon. The presence of CAAT motifs that are generally associated with gene regulation was also observed. The CAAT-boxes are core promoter sequences important in binding RNA Polymerase II [50]. Additional important regulatory elements detected in the *CaNPR1* promoter were those related to defense as well as ELRECOREPCRP1 [51], MYBPLANT [52], T/GBOXATPIN2 [53], GT1GMSCAM4 [54], PALBOXAPC [55] and W-box [56], suggesting a biotic regulation [57-62]. Moreover, several *cis*-elements reported to be associated with the responsiveness to plant hormones, such as salicylic acid (ASF1MOTIFCAMV), auxin (NTBBF1ARROLB), jasmonic acid (JERE), citokinin (CPBCSPOR), ethylene (ERE, WBOXNTERF3), abscisic acid (MYB1AT, MYCCONSENSUSAT, MYB2CONSENSUSAT, WRKY71OS, DPBFCOREDCDC3, LTRECOREATCOR15, ABRELATERD1), as well as gibberellin (WRKY71OS, PYRIMIDINEBOXOSRAMY1A, TATCCACHVAL21, CAREOSREP1) were observed. The presence of such large number of *cis*-elements suggests that *CaNPR1* expression might be regulated by hormonal action.

2.5. *CaNPR1* promoter activity is SA-dependent

GUS expression levels driven by the *CaNPR1* promoter was evaluated using a transient expression assay in leaves of *N. benthamiana* and compared to that obtained using the *CaMV35S* promoter. Transient GUS activity was assayed fluorometrically at 3 day post-infiltration (Fig. 5). It was observed that the level of GUS activity driven by the *CaNPR1* promoter was very weak compared to that driven by the 35S promoter. As previously mentioned, the *NPR1* gene is a key regulator of the SA-mediated SAR pathway [3, 13, 63-64] and its expression levels is slightly induced in *A. thaliana* upon foliar SA treatment [7, 65]. Given that the upstream promoter region of *CaNPR1* harbors *cis*-acting elements implicated in plant hormone responses, we examined whether this promoter showed a SA-inducible expression pattern. As a result, a significant alteration in the level of GUS activity was detected in SA-treated leaves as compared to control leaves treated with water (Fig. 5).These data demonstrate that SA strongly stimulates the transcriptional activity of the *CaNPR1* promoter.
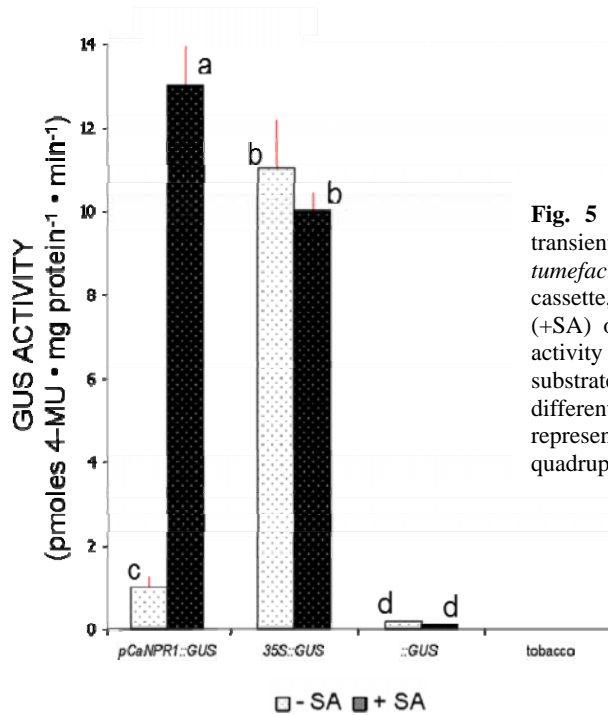
Fig. 5 Fluorimetric assay of *pCaNPR1::GUS*. After 72h of transient transformation of tobacco leaves infiltrated with *A. tumefaciens* GV3101 containing the *pCaNPR1::GUS* expression cassette, leaves were infiltrated with a 0.5 mM solution of SA (+SA) or with water (-SA), and collected after 3 hours. GUS activity was determined fluorometrically using 4-MUG as substrate. Bars without a common letter on the top are statistically different (Fisher's LSD test, P≤0.05). Standard errors are represented by *error bars*. All experiments were done in quadruplicate in three independent experiments.

3. Taken to acquire knowledge

Coffea arabica cv. Mundo Novo (IAC 388-17-1) and *C. arabica* cv. Caturra were obtained from IAC (Instituto Agronômico de Campinas - Campinas, São Paulo, Brazil). Plants were grown in potting soil for 4 months (16h/8h light/dark; 28°C; 70% RH) before use for leaf DNA extraction (*C. arabica* cv. Caturra) or pathogen inoculation (*C. arabica* cv. Mundo Novo). Plants of *Nicotiana benthamiana* were grown in potting soil under a 16/8h photoperiod for 6 weeks prior to being used for *Agrobacterium* infiltration. *Hemileia vastatrix* race II was kindly provided by Dr. Maria do Céu Silva (CIFC, Centro de Investigação das Ferrugens do Cafeeiro - Oeiras, Portugal).

For biotic stress assays, 4-month old *Coffea arabica* cv. Mundo Novo plants were kept in a growth chamber (16h/8h light/dark; 23°C; 70% RH) for at least one week, before being inoculated with the coffee leaf rust fungus *H. vastatrix* race II, that elicits a compatible reaction in coffee. The urediniospores (100 mg) were diluted in 10 mL of sterile water under dark conditions. Leaves from the second pair of plagiotropic shoots from the apex of coffee plants were inoculated with urediniospores. To allow spore germination, the inoculated leaves were covered with a wet black plastic film for 24h. The inoculated leaves were not detached from the plants. Mock-inoculated controls as well as non-inoculated controls were performed. The biological samples were obtained from three independent experiments. Leaves were randomly sampled at different time-points after inoculation: 0, 8, 12, and 24 hours, and immediately deep-frozen. To confirm the infection by the leaf rust fungus, some inoculated leaves were maintained in the plants.

RNA extraction, reverse transcription, and quantitative PCR experiments were performed as described in Barsalobres-Cavallari *et al.* [66]. The specific oligonucleotides used for gene expression studies are shown in Table 1.

Table 1 Sequence of primers used for quantitative PCR in coffee.

Primer Name	Primer Sequence (5' to 3')
CaNPR1 F	TGGACGAAGGAGCGTGATGTCAGCTT
CaNPR1 R	ACATCCTTGGGCGACGGCCTAACTCT
CaGAPDH F	TTGAAGGGCGGTGCAAA
CaGAPDH R	AACATGGGTGCATCCTTGCT

The genomic DNA from *C. arabica* cv. Caturra was isolated using the DNeasy Plant Minikit (Qiagen, France) following the manufacturer's recommendations. Gene walking by PCR was performed as described in Petitot *et al.* [67]. The promoter region of CaNPR1 was amplified using nine GSPs (termed CaNPR1-1 to CaNPR1-9), where the first two (CaNPR1-1 and CaNPR1-2) were designed based on the EST sequence retrieved in the coffee database, while the others were designed based on the nucleotide sequences of the genome walking amplified fragments (Table 2). All the amplified genomic fragments were cloned into the pGEM-T Easy vector (Promega) and sequenced (Genome Express, France).

Table 2 Primers used for genome walking.

PRIMER NAME	GENE-SPECIFIC PRIMERS
CaNPR1-1	5'CATACGCAGATCATCGCCTGCCATAGC3'
CaNPR1-2	5'CCCAGCAGTGGATCTCTTCTTTCTGCC3'
CaNPR1-3	5'GCCCTTGCAAACCAAGTTCCCTGCG3'
CaNPR1-4	5'CATCTGCAGCAGCTTTGTGAAGAACG3'
CaNPR1-5	5'GGAAACTATAGTAGGGCGTCCAGCAC3'
CaNPR1-6	5'CCAAGCAGTCAGGAGAAGACGACGCG3'
CaNPR1-7	5'GGGAGAGGCGTTGAAGTGAGACGACG3'
CaNPR1-8	5'GACGACTGAACTGGAGCCGGAGC3'
CaNPR1-9	5'CGGTCGATCGGCTAGGAGACTGACG3'

The DNA sequences were analyzed with the BLAST network services at the National Center for Biotechnology Information [68]. The software tool TSSP was used for the prediction of the transcription start site, RNA polymerase II binding site and TATA-box [69], and the PLACE database were used for the prediction of *cis*-acting elements [70]. For protein analysis, it was used the follows software: the Pfam database [71], the PSITE from Softberry [72] and the Bioinformatics Toolkit from Max-Planck Institute for Developmental Biology [73].

For the phylogenetic and molecular evolutionary analyses, the neighbor joining method with 1000 bootstraps was used. The NPR1 amino acid sequences used were: *Solanum lycopersicum* (AAT57637.1, Le), *Capsicum annum* (ABG38308.1, Ca), *Nicotiana tabacum* (AAM62410.1, Nt), *Ricinus communis* (EEF48081.1, Rc), *Musa acuminata* (ABL63913.1, Ma), *Oryza sativa* (ABE11613.1, Os), *Hordeum vulgare* (CAJ19095.1, Hv), *Brassica napus* (AAM88865.2, Bn), *Brassica juncea* (ABC94642.2, Bj), *Arabidopsis thaliana* (NP_176610.1), *Musa* spp. ABB (ACJ04030.1, Md), *Zea mays* (NP_001147587.1, Zm), *Glycine max*: GmNPR1-1 (ACJ45013.1) and GmNPR1-2 (ACJ45015.1), *Populus trichocarpa* (XP_002322351.1, Pt), *Malus x domestica* (ACC77697.1, Mp), *Pyrus pyrifolia* (ABK62792.1, Pp), as well as the sequences from *A. thaliana* NPR2 (NP_194342.1, At), NPR3 (NP_199324.2, At), NPR4 (NP_193701.2, At), NPR5 or AtBOP1 (ABH04470.1, At), and NPR6 or AtBOP2 (AAU90063.1, At).

The construction of β-glucuronidase (GUS) reporter cassettes, as well as the *Agrobacterium*-mediated transient expression assays, abiotic treatments and the GUS activity measurement were performed as described in Petitot *et al.* [67] and Barsalobres-Cavallari [74].

4. Conclusions

We have isolated the orthologous sequence of the *Arabidopsis* SAR controlling master switch *NPR1* gene in coffee, that we called *CaNPR1*. Typical features of NPR1 proteins were found in CaNPR1, including the BTB/POZ domain, an ankyrin (ANK) repeat domain, and a nuclear localization signal (NLS). As expected from *A. thaliana*, soybean and grapevine results [8, 27, 49], transcript levels of *CaNPR1* were not affected following pathogen infection. Interestingly, the activity of the *CaNPR1* promoter was found to be strongly activated by SA-treatment. The different *cis*-acting elements identified in the *pCaNPR1* sequence, such as the plant hormone- and/or infection-related motifs, might contribute to SA responsiveness. From these results, it is tempting to speculate that the mechanisms underlying *C. arabica* resistance to the rust pathogen are SA-dependent, as already suggested by other molecular studies [47, 75]. Our data also indicate that *CaNPR1* may represent a promising candidate for engineering resistance in coffee to achieving broad spectrum resistance to pathogens. Further functional studies are required for assessing the role of this gene in coffee disease resistance responses. We anticipate that transgenic plants containing *CaNPR1* promoter-reporter constructs will provide tools for the future analysis of defense signaling networks in coffee.

References

[1] Durrant WE, Dong X: Systemic acquired resistance. *Annu Rev Phytopathol* 2004, 42:185-209.
[2] Uknes S *et al.*: Regulation of pathogenesis-related protein-1a gene expression in tobacco. *Plant Cell* 1993, 5(2):159-169.
[3] Delaney TP *et al.*: *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc Natl Acad Sci U S A* 1995, 92(14):6602-6606.
[4] Lawton K *et al.*: Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol Plant Microbe Interact* 1995, 8(6):863-870.
[5] Friedrich L *et al.*: Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Mol Biol* 1995, 29(5):959-968.
[6] Ferrari S *et al.*: *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J* 2003, 35(2):193-205.
[7] Cao H *et al.*: The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 1997, 88(1):57-63.
[8] Cao H *et al.*: Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc Natl Acad Sci U S A* 1998, 95(11):6531-6536.
[9] Garreton V *et al.*: The as-1 promoter element is an oxidative stress-responsive element and salicylic acid activates it via oxidative species. *Plant Physiol* 2002, 130(3):1516-1526.
[10] Mou Z *et al.*: Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 2003, 113(7):935-944.
[11] Dong X: Genetic dissection of systemic acquired resistance. *Curr Opin Plant Biol* 2001, 4(4):309-314.
[12] van Loon LC *et al.*: Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol* 2006, 44:135-162.
[13] Cao H *et al.*: Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 1994, 6(11):1583-1592.
[14] Wang D *et al.*: Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 2005, 308(5724):1036-1040.
[15] Fan W, Dong X: *In vivo* interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in *Arabidopsis*. *Plant Cell* 2002, 14(6):1377-1389.
[16] Zhang Y *et al.*: Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc Natl Acad Sci U S A* 1999, 96(11):6523-6528.
[17] Rochon A *et al.*: The coactivation of the *Arabidopsis NPR1* requires the core of its *BTB/POZ* domain and the oxidation of C-terminal cysteines. *Plant Cell* 2006, 8:3670-3685.
[18] Dong X: NPR1, all things considered. *Curr Opin Plant Biol* 2004, 7(5):547-552.
[19] Kinkema M *et al.*: Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 2000, 12(12):2339-2350.
[20] Pandey SP, Somssich IE: The Role of WRKY Transcription Factors in Plant Immunity. *Plant Physiology* 2009, 150:1648-1655.
[21] Cantu D *et al.*: Comparative analysis of protein-protein interactions in the defense response of rice and wheat. *BMC Genomics* 2013, 14(1):166.
[22] Wally O *et al.*: Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (*Daucus carota* L.) expressing an *Arabidopsis* NPR1 gene. *Planta* 2009, 231(1):131-141.
[23] Silva M *et al.*: Coffee resistance to the main diseases: leaf rust and coffee berry disease. *Brazilian Journal of Plant Physiology* 2006, 18:119-147.
[24] Fernandez D *et al.*: Coffee (*Coffea arabica* L.) genes early expressed during infection by the rust fungus (*Hemileia vastatrix*). *Molecular Plant Pathology* 2004, 5(6):527-536.
[25] Vieira L *et al.*: Brazilian coffee genome project: an EST-based genomic resource. *Brazilian Journal of Plant Physiology* 2006, 18:95-108.
[26] Endah R *et al.*: Elicitor and *Fusarium*-induced expression of NPR1-like genes in banana. *Plant Physiol. Biochem.* 2008, 46:1007-1014.
[27] Henanff G *et al.*: Characterization of *Vitis vinifera* NPR1 homologs involved in the regulation of pathogenesis-related gene expression. *BMC Plant Biol* 2009, 9:54.
[28] Chen JW *et al.*: Molecular cloning and expression analysis of a npr1 gene from sugarcane. *Pak. J. Bot* 2012, 44(1): 193-200.
[29] Tada Y *et al.*: Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. *Science* 2008, 321(5891):952-956.
[30] Landschulz WH *et al.*: The leucine zipper: a hypothetical structure common to a new class of DNA-binding proteins. *Science* 1988, 240 (4860): 1759-1764.
[31] Clerc RG *et al.*: The B-cell-specific Oct-2 protein contains POU box- and homeo box-type domains. *Genes Dev* 1988, 2(12A):1570-1581.
[32] Vinson CR *et al.*: Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* 1989, 246(4932):911-916.
[33] O'Shea EK *et al.*: Preferential heterodimer formation by isolated leucine zippers from fos and jun. *Science* 1989, 245(4918):646-648.
[34] Maekawa T *et al.*: Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO J* 1989, 8(7):2023-2028.
[35] Collum RG, Alt FW: Are myc proteins transcription factors? *Cancer Cells* 1990, 2(3):69-75.
[36] Viedma DG *et al.*: A leucine zipper motif determines different functions in a DNA replication protein. *EMBO J.* 1996, 15:925-934.

[37] An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* 2003, 141(4):399-436.

[38] Lin C *et al.*: Coffee and tomato share common gene repertoires as revealed by deep sequencing of seed and cherry transcripts. *Theor Appl Genet* 2005, 112(1):114-130.

[39] Van der Hoeven R *et al.*: Deductions about the number, organization, and evolution of genes in the tomato genome based on analysis of a large expressed sequence tag collection and selective genomic sequencing. *Plant Cell* 2002, 14(7):1441-1456.

[40] Rick CM: Some cytogenetic features of the genome in diploid species. *Stadler Sym* 1971, 1:153-174.

[41] Pinto-Maglio CAF, Cruz ND: Pachytene chromosome morphology in *Coffea* L. II. *C. arabica* L. complement. *Caryologia* 1998, 51:19-35.

[42] Poczai P: Molecular genetic studies on complex evolutionary processes in Archaesolanum (Solanum, Solanaceae). University of Pannonia. 2011. [PhD thesis].

[43] Zhao JT *et al.*: Molecular cloning and characterization of an ortholog of NPR1 gene from Dongguan Dajiao (*Musa* spp. ABB). *Plant Molecular Biology Reporter* 2009, 27:243-249.

[44] Saitou N, Nei M: The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 1987, 4:406-425.

[45] Zuckerkandl E, Pauling L: *Evolutionary divergence and convergence in proteins*. Edited in Evolving Genes and Proteins by Bryson V, Vogel HJ. pp. 97-166. Academic Press, New York. 1965.

[46] Tamura K *et al.*: MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 2011, 28:2731-2739.

[47] Ramiro D *et al.*: Biphasic haustorial differentiation of the orange rust *Hemileia vastatrix* race II associated with differential defense responses in resistant coffee cultivar. *Plant Pathology* 2009, 58:944-955.

[48] Ganesh D *et al.*: Monitoring of the early molecular resistance responses of coffee (*Coffea arabica* L.) to the rust fungus (*Hemileia vastatrix*) using real-time quantitative RT-PCR. *Plant Science* 2006, 170:1045-1051.

[49] Sandhu D *et al.*: Systemic acquired resistance in soybean is regulated by two proteins, orthologous to *Arabidopsis* NPR1. *BMC Plant Biol* 2009, 9:105.

[50] Wenkel S *et al.*: CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*. *Plant Cell* 2006, 18(11):2971-2984.

[51] ELRECOREPCRP1. Available at: http://www.dna.affrc.go.jp/sigscan/disp.cgi?S000142. Accessed May, 13, 2013.

[52] MYBPLANT. Available at: http://www.dna.affrc.go.jp/sigscan/disp.cgi?S000167. Accessed May, 13, 2013.

[53] T/GBOXATPIN2. Available at: http://www.dna.affrc.go.jp/sigscan/disp.cgi?S000458. Accessed May, 13, 2013.

[54] GT1GMSCAM4. Available at: http://www.dna.affrc.go.jp/sigscan/disp.cgi?S000453. Accessed May, 13, 2013.

[55] PALBOXAPC. Available at: http://www.dna.affrc.go.jp/sigscan/disp.cgi?S000137. Accessed May, 13, 2013.

[56] W-box. Available at: http://www.dna.affrc.go.jp/sigscan/disp.cgi?S000390. Accessed May, 13, 2013.

[57] Logemann E *et al.*: Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. *Proc Natl Acad Sci U S A* 1995, 92(13):5905-5909.

[58] Tamagnone L *et al.*: The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell* 1998, 10(2):135-154.

[59] Park HC *et al.*: Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol* 2004, 135(4):2150-2161.

[60] Boter M *et al.*: Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. *Genes Dev* 2004, 18(13):1577-1591.

[61] Rushton PJ *et al.*: Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. *Plant Cell* 2002, 14(4):749-762.

[62] Laloi C *et al.*: The *Arabidopsis* cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. *Plant Physiol* 2004, 134(3):1006-1016.

[63] Shah J *et al.*: Characterization of a salicylic acid-insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. *Mol Plant Microbe Interact* 1997, 10(1):69-78.

[64] Glazebrook J *et al.*: Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 1996, 143(2):973-982.

[65] Ryals J *et al.*: The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell* 1997, 9(3):425-439.

[66] Barsalobres-Cavallari CF *et al.*: Identification of suitable internal control genes for expression studies in *Coffea arabica* under different experimental conditions. *BMC Molecular Biology* 2009, 10(1):1.

[67] Petitot AS *et al.*: Promoter analysis of the WRKY transcription factors CaWRKY1a and CaWRKY1b homoeologous genes in coffee (*Coffea arabica*). *Plant cell reports* 2013: 1-14.

[68] Altschul SF *et al.*: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997, 25(17):3389-3402.

[69] Shahmuradov IA *et al.*: PlantProm: a database of plant promoter sequences. *Nucleic Acids Res* 2003, 31(1):114-117.

[70] Higo K *et al.*: Plant cis-acting regulatory DNA elements (PLACE) database. *Nucleic Acids Res* 1999, 27(1):297-300.

[71] Pfam 27.0 database. Available at: http://pfam.sanger.ac.uk/search. Accessed May, 13, 2013.

[72] Softberry. Available at: http://linux1.softberry.com/berry.phtml. Accessed May, 13, 2013.

[73] Bioinformatics Toolkit. Available at: http://toolkit.tuebingen.mpg.de/sections/search. Accessed May, 13, 2013.

[74] Barsalobres-Cavallari CF: Identificação e caracterização de promotores de genes de café (*Coffea arabica*). UNESP/UM2. 331p. 2009. [Tese de Doutorado].

[75] Ramiro D *et al.*: Identification of coffee WRKY transcription factor genes and expression profiling in resistance responses to pathogens. *Tree Genetics and Genomes* 2010, 6:767-781.