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MOLECULAR CLONING AND EXPRESSION ANALYSIS OF A *NPR1* GENE FROM SUGARCANE

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Abstract

The *NPR1* genes play a pivotal role in systemic acquired resistance in plants. In the present work, a full-length sugarcane *NPR1* gene, designated as *ScNPR1*, was isolated and identified. The full-length cDNA was 2184 bp in length with a 1758 bp open reading frame (ORF), which encoded a 586 amino acids protein with an estimated molecular mass of 65.17 kDa and a calculated isoelectric point (pI) of 5.88. Homology analysis suggested that the ScNPR1 protein was significantly similar to maize ZmNPR1, and shared common features with *NPR1* from other plants. Real-time quantitative PCR (RT-qPCR) results indicated that the expression of *ScNPR1* was obviously up-regulated after treatment with salicylic acid (SA) and inoculation with the smut disease fungus *Ustilago scitaminea*. Its expression level was reduced after methyl jasmonate (MeJA) or ethylene treatment. In addition, higher levels of *ScNPR1* transcripts were observed in the leaf and sheath tissues of sugarcane cultivars resistant to the smut disease. These results clearly demonstrated that the *ScNPR1* gene was likely to be involved in SA-mediated signaling pathway and might play a role in the defense response to sugarcane smut disease.

Introduction

Plants have evolved a variety of mechanisms to protect themselves against attack by pathogens. Among them, it has been well documented that systemic acquired resistance (SAR) confers an increased, long-lasting, and broad-spectrum resistance to subsequent pathogen attacks for the whole plant (Durrant & Dong, 2004; Mukhtar *et al*., 2009). *NPR1* (nonexpressor of pathogenesis-related genes 1), also known as *NIM1* (noninducible immunity) and *SAI1*, has been identified as a key positive regulator of the salicylic acid (SA)-dependent signalling pathway and is required for the transduction of the SA signal to activate *PR* (pathogenesis-related) gene expression and SAR in *Arabidopsis* (Loake & Grant, 2007; Bari & Jones, 2009). NPR1 also mediates the cross-talk between the SA signaling pathway and the jasmonic acid (JA) signaling pathway, and the antagonistic effect of SA on JA signaling requires NPR1 (Liu *et al*., 2005; Spoel *et al*., 2003, 2007). A disease resistance pathway similar to that of the *Arabidopsis NPR1* (*AtNPR1*)-mediated signaling pathway was also demonstrated in rice (Chern *et al*., 2001). Moreover, homologs of *AtNPR1* have been cloned and characterized in several crop plants including rice (Chern *et al*., 2005; Yuan *et al*., 2007), apple (Malnoy *et al*., 2007), banana (Endah *et al*., 2008; Zhao *et al*., 2009), grapevine (Henanff *et al*., 2009), rosaceous tree (Pilotti *et al*., 2008) and cotton (Zhang *et al*., 2008). Over-expression of *AtNPR1* in *Arabidopsis* (Cao *et al*., 1998), rice (Chern *et al*., 2001), tomato (Lin *et al*., 2004), wheat (Makandar *et al*., 2006) and apple (Malnoy *et al*., 2007) has been shown to enhance bacterial and fungal resistance. These results indicated that *NPR1* represented a desirable candidate gene for transgenic manipulation in crops for enhanced disease resistance. Moreover, functional analysis of rice *NPR1* (*OsNPR1*) has revealed that although rice and *Arabidopsis* share conserved defense pathways, the regulation of these pathways and the links to other plant pathways may be quite divergent (Chern *et al*., 2005). Thus, there is a demand for the identification, isolation, and characterization of *NPR1* homologues from more plant species.

Sugarcane (*Saccharum* spp. hybrids), a major industrial crop, is widely cultivated in tropical and subtropical regions for sugar production. It is also a vital component in the economies of many tropical regions and a prime candidate as a future fuel crop because of its efficient biomass production (Zhang *et al*., 2006). Several agents of crop loss affect sugarcane productivity, including viruses, bacteria, fungi and invertebrates (Rasool *et al*., 2010). Smut disease of sugarcane, one of the most severe diseases, caused by the fungus *Ustilago scitaminea* Sydow, can cause considerable yield losses and reductions in cane quality (Olweny *et al*., 2008). The only viable form of protection against many sugarcane diseases is through varietal resistance. As a result, the greatest losses due to disease occur when resistance breaks down owing to more virulent forms of the pathogen (Glynn *et al*., 2008). However, the development of pathogen-resistant sugarcane varieties is limited by the complexity of the sugarcane genome (D'Hont & Glaszmann, 2001) and the poor availability of genetics tools for *Saccharum spp*. hybrids (Grivet & Arruda, 2001). On the other hand, resistance to smut has been associated with the accumulation of free or conjugated polyamines (Legaz *et al*., 1998; Piñón *et al*., 1999), phenolic compounds (de Armas *et al*., 2007; Santiago *et al*., 2009) in sugarcane tissues and with the production of several glycoproteins in juice (Martínez *et al*., 2000) which affect germination of fungal spores (Fontaniella *et al*., 2002; Millanes *et al*., 2005). Although a large amount of DNA sequence information for sugarcane was released into the public domain as expressed sequence tags (ESTs) derived from cDNA libraries (Casu *et al*., 2004; Camargo *et al*., 2007; Rocha *et al*., 2007; Papini-Terzi *et al*., 2009), few genes that govern biotic or abiotic stress responses have been molecularly characterized (Glynn *et al*., 2008; Schlögl *et al*., 2008; Trujillo *et al*., 2008) and no full-length *NPR1* gene has been isolated and characterized from this crop to date.

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In the present work, a full-length *NPR1* gene, termed *ScNPR1*, was isolated and characterized from sugarcane. Furthermore, its expression profiles under treatments with *U. scitaminea* inoculation, SA, methyl jasmonate (MeJA) and ethylene were investigated by RT-qPCR. Finally, its tissue expression patterns in three varieties of sugarcane, defined by their relative resistance to smut, including ROC22 (susceptible), Q190 (moderate resistance) and Badila (extremely resistant), were also analyzed.

Materials and Methods

Plant materials and treatments: Three sugarcane (*Saccharum spp*. hybrids) varieties, ROC22, Q190 and Badila were maintained in the greenhouse of Guangzhou Sugarcane Industry Research Institute, Guangzhou, China. All plants were grown under controlled greenhouse conditions (28°C, 70% relative humidity, approximately 2,000 Lux with a 12 h natural light/12 h darkness photoperiod).

Six-week-old plantlets were used for RT-qPCR analysis. To determine the expression pattern of *ScNPR1* under different stress stimuli, plantlets of ROC22 were uprooted from the soil, rinsed with water, and placed in a beaker that contained either 40 ml (just immerging the roots) of water (control), or 5 mM SA, or 0.1 mM MeJA, or 2 mM ethephon (a liquid ethylene) for 72 h. For inoculation treatment, plantlets of ROC22, Q190 and Badila were rinsed with water and were inoculated with the teliospore suspension of *U. scitaminea* Syd collected from the fields. (viz. : the plantlet's basal outside sheaths were removed and exposed 1-2 axillary buds, then covered with the agglomerate cotton which sucking in the teliospore suspension of 5×10^6 spores/ml on the bud. The cottons were removed after 72 hours.) At 0, 1, 6, 12, 24, 48 and 72 h after each treatment, leaf tissues were collected randomly from six plantlets, then frozen in liquid nitrogen and then stored at -80°C until use. To evaluate the tissue expression pattern, the leaf, sheath and root tissues from six-week-old plantlets of ROC22, Q190 and Badila, that are susceptible, moderate resistance, and extremely resistant to the smut disease, respectively, were also randomly sampled, frozen in liquid nitrogen, and stored at -80°C until use.

RNA extraction, cDNA synthesis and Cloning of the full-length *ScNPR1* Cdna: Total RNA was isolated from sugarcane tissues using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Each RNA sample was subjected to DNase digestion (TaKaRa, Shiga, Japan) to remove any remaining DNA and purified through an RNeasy Plant Mini Kit according to the manufacturer's instruction (QIAGEN, Germany). Total RNA content was quantified by spectrophotometry (BioPhotometer plus, Eppendoff, Germany). In addition, each sample was assessed by gel electrophoresis for integrity and contaminating genomic DNA. The presence of known transcripts was confirmed by reverse transcription polymerase chain reaction (RT-PCR) and RT-qPCR with diagnostic primer sets, respectively (data not shown). All RNA samples were stored at -80°C until required.

First-strand cDNA was synthesized using 1 µg of treated total RNA with superscrip III reverse transcriptase (Invotrogen) and Oligo $d(T)_{18}$ to a total volume of 20 µl, according to the manufacturer's instructions. Synthesized cDNA was used as templates for PCR amplification. A pair of degenerate primers were designed with reference to the conserved amino acids sequences of NPR1 (i.e., sense: 5'-GGTGRTGGCNAYGCGTTCATNT-3' and antisense: 5'-CCTCTTSGTGGATYTCTGRGGT-3', where S = C or G, N = A, T, C, G, Y = C or T, R = A or G). RT-PCR reactions were subjected to one cycle of 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min, followed by one cycle of 72°C for 10 min. RT-PCR products of the predicted size (about 950 bp in length) were purified and cloned into the pMD18-T vector (Takara, Shiga, Japan). The nucleotide sequences of the cDNA inserts were determined using the Thermo Sequenase dye terminator cycle sequencing kit and a 377 DNA sequencer (PerkinElmer Applied Biosystems, Waltham, MA, USA).

Consequently, 3′- or 5′ rapid amplification of cDNA ends (3′- or 5′-RACE-PCR) were performed using cDNA amplification kits (Takara, Shiga, Japan) according to the manufacturer's protocol. In order to amplify 3′-end and 5′-end fragments, the specific primers for *ScNPR1* were designed based on the nucleotide sequences of the cDNA fragments already cloned by RT-PCR, viz. 5'RACE-INNER: agggagctccttatccaaagat and 5'RACE-OUTER: catcagcagttggtgacttctt, 3'RACE1: caagggtagatgcactgatgaa and 3'RACE2: cttctgtgagctgaaggaggac. The 5'- and 3'-RACE-PCR products were cloned and sequenced by using the same kit and sequencer as described above.

cDNA sequence analysis, alignment, and comparisons: Identification of nucleotide sequences from the RT-PCR clones was established using the NCBI Blast program [http://www.ncbi.nlm.nih.gov/BLAST]. Sequence alignment and comparison of sequences were made using the ClustalW program (http://www.ebi.ac.uk/clustalw). Open reading frame (ORF) and protein were predicted using NCBI ORF Finder [http://www.ncbi.nlm.nih.gov/gorf/gorf.html]. The theoretical isoelectric point (pI) and mass values were calculated using the PeptideMass program [http://us.expasy.org/tools/peptide-mass.html]. The phylogenetic tree was generated from the deduced amino acid sequences for *ScNPR1* and 16 *NPR1* homologues from other species using the MEGA 4.0 software with 1,000 bootstraping iterations. The 16 *NPR1* homologues sequences registered in GenBank are *AtNPR1* (At1g64280), *AtNPR2* (At4g26120), *AtNPR3* (AT5G45110), and *AtNPR4* (AT4G19660) from *Arabidosis thaliana*; *NtNPR1* (AAM62410.1) from tobacco; *OsNPR1* (AAX18700.1) from rice; *LeNPR1* (AAT57637.1) from tomato; *ZmNPR1* (NP_001147587.1) from maize; *GmNPR1-1* (ACJ45013.1) and *GmNPR1-2* (ACJ45015.1) from soybean; *PpNPR1* (ABK62792.1) from pear; *MpNPR1*(ACC77697.1) from apple; *MdNPR1* (ACJ04030.1) from banana; *CaNPR1* (ABG38308.1) from pepper; *RcNPR1* (EEF48081.1) from castor bean, and *PtNPR1* (XP_002322351.1) from poplar, respectively.

Gene expression analysis by RT-qPCR: The synthesized cDNA was diluted 1:40 with water, and 4 µl of the diluted cDNA was used as a template for RT-qPCR analysis. PCR reactions were performed in a total volume of 20 µl, 2 µl for each primer, 10 µl for Platinum®SYBR®Green qPCR SuperMix-UDG (Invitrogen) and 0.1 µl ROX Reference Dye (Invitrogen) on an Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. The RT-qPCR programme included a preliminary step of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95 °C for 15 s, 55°C for 30 s and 72°C for 30 s. No-template controls for each primer pair were included in each run. Gene-specific primers were designed for non conserved areas using the Primer Expression 3.0 software (Applied Biosystems, Foster City, CA) and the length of all PCR products ranged from 100 to 200 bp. The primer sequences used for *ScNPR1* were 5'-TGTCTTCATCGTCGTCGTGCGT-3' (forward) and 5'-TCCCAGGTCTCCAAAACCGTGAT-3' (reverse). The sugarcane actin gene was used as an internal control to normalize small differences in template amounts with the forward primer 5'-GCCAACAGGGAGAAGATGACC-3' and the reverse primer 5'-AGCAAGATCCAAACGAAGAATG-3'. At least three different RNA isolations and cDNA syntheses were used as replicates for the RT-qPCR analysis. Expression levels at each time point were expressed as a ratio relative to the control at hour 0 time point, which was set to 1, using the $\Delta\Delta C_T$ method described in ''Getting Started Guide: Relative Standard Curve and Comparative C_T Experiments" (Applied Biosystems) and by Kenneth & Schmittgen (2001). Dissociation curves were run to determine the specificity of the amplification reactions. In addition, the amplified products were sequenced as described above.

Standard curves for all RT-qPCR by all primers have been established using twofold dilutions of cDNA in concentrations between 0.5 ng and 8 ng. The curves obtained allowed the determination of PCR efficiency according to Pfaffl *et al*., (2002).

Results and Discussion

Isolation of the full-length *ScNPR1* cDNA: Previous studies have documented that *NPR1* belongs to a multi-gene families in the genome of many plant species, comprising up to six members in *Arabidopsis*, 5 in rice and 3 in banana (Chern *et al*., 2005; Yuan *et al*., 2007; Endah *et al*., 2008; Zhao *et al*., 2009). In this study, one *NPR1* homolog of approximately 950 bp was cloned from sugarcane leaves that had one uninterrupted ORF. It was also observed that both the nucleotide and amino acid sequences shared high similarity to the corresponding region of the *NPR1* gene of maize and banana. The full-length *ScNPR1* cDNA contained an ORF of 1758 with a 5'-untranslated region of 112 bp and a 3'-untranslated region of 314 bp terminated by a string of A residues. It encoded a putative protein of 586 amino acids with a predicted molecular weight of 65.17 kDa and an isoelectric point of 5.88.

Sequence and phylogenetic analysis of ScNPR1: A BLAST search of GenBank revealed that ScNPR1 shared the highest identity of 83% with maize ZmNPR1 (NP_001147587.1). The ScNPR1 contained a predicted BTB zinc finger domain (amino acids 65-140) and an ankyrin repeats (ANK) (amino acids 268-396), as also found in AtNPR1 and OsNPR1 (Fig. 1). These are typical features of *NPR1* genes that are highly conserved across many species (Cao *et al*., 1997; Chern *et al*., 2005; Endah *et al*., 2008). Amino acids critical for the NPR1 function as defined by genetic mutants, such as npr1-1 (H) and npr1-2 (C) (Cao *et al*., 1997), and nim1-4 (R) (Ryals *et al*., 1997), were also conserved in ScNPR1 (Fig. 1B). Expectedly, eight Cys residues were completely conserved among all the sequences and these residues were probably involved in the oligomerization and the nuclear localization of NPR1 or NPR1-like proteins (Mou *et al*., 2003; Yuan *et al*., 2007). In addition, the carboxy terminal domain of ScNPR1 is rich in basic amino acids typical of the nuclear localization signals (NLS, Fig. 1B). Kinkema *et al*., (2000) showed that the 5 residues in the C-terminus of AtNPR1, i.e., KK-R-KK, were essential for its nuclear translocation and constituted the NLS1, which was essential for SA-mediated *PR* gene expression. Four of the 5 amino acids were conserved in ScNPR1. However, basic amino acids of the second NLS2 in AtNPR1were found to be unnecessary for nuclear targeting, and were less conserved among five different NPR1 homologs (Fig. 1B) (Kinkema *et al*., 2000; Henanff *et al*., 2009). These results indicated that S*cNPR1* shared some common features with *NPR1* genes from other plants.

To reveal the evolutionary relationship between ScNPR1 and NPR1 proteins from other plant species, a phylogenetic tree was generated based on the alignment of deduced amino acid sequences of ScNPR1 and 16 other NPR1 homologues using bootstrap consensus for neighbour joining, maximum parsimony and maximum likelihood. As shown in Fig. 2, the tree was branched into two main clusters, which was in agreement with reports by Henanff *et al*., (2009) and Zhao *et al*., (2009). ScNPR1 had a closer relationship to maize ZmNPR1 and banana MdNPR1 within the same cluster (Fig. 2), suggesting that it might have a similar function to that of ZmNPR1 and MdNPR1.

Expression analyses of *ScNPR1* in response to signal molecules and *Ustilago scitaminea* inoculation: It has been shown that the *NPR1* genes can be induced by exogenous defense molecules, such as SA, MeJA, ethylene and so on, to activate plant disease resistance response (Mou *et al*., 2003; Yuan *et al*., 2007; Endah *et al*., 2008; Henanff *et al*., 2009; Zhang *et al*., 2009; Zhao *et al*., 2009). In this study, the expression levels of *ScNPR1* in sugarcane leaves following treatments with SA, MeJA, or ethylene were analyzed by RT-qPCR, respectively, as shown in Fig. 3. When plants were treated with 5mM SA, *ScNPR1* was up-regulated 1.53-fold, which was significant compared to the untreated control at 1 h, but declined back to the level of control (Fig. 3A). On the contrary, the *ScNPR1* transcription level in MeJA or ethylene- treated plants were obviously reduced to a lower level during the whole experiment period (Fig. 3B and C).

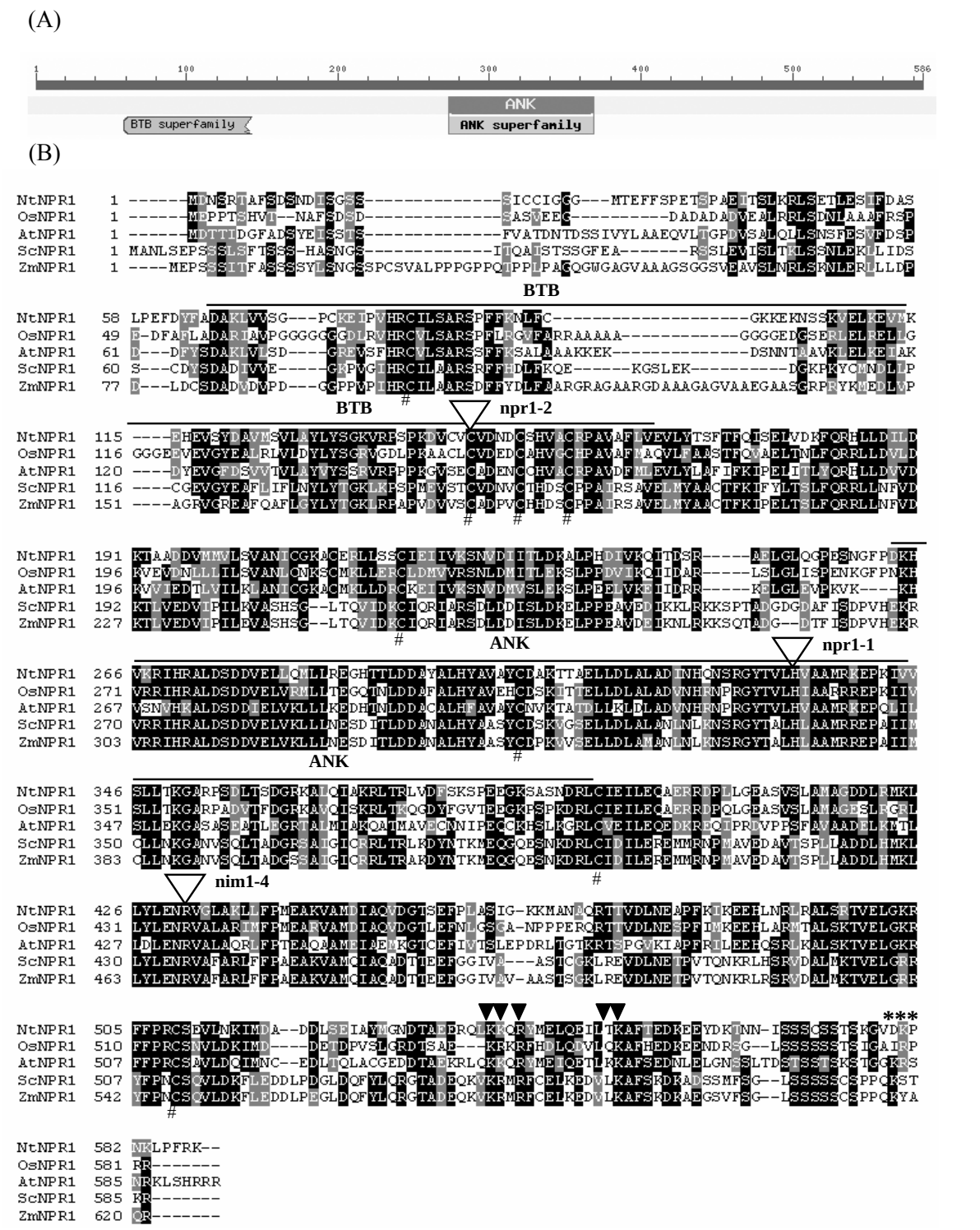

Fig. 1. Comparison of ScNPR1 with other four plant NPR1 homologues. (A) Domain structure of the ScNPR1 protein. A BTB domain and an ANK domain are indicated. (B) Alignment of the ScNPR1 predicted proteins with tobacco NtNPR1, rice OsNPR1, *Arabidopsis* AtNPR1 and maize ZmNPR1. Black shading identifies the residues shared by at least three NPR1 proteins. Conservative amino acid substitutions are represented by gray shading. Gaps are introduced to optimize alignment. The protein domains are indicated above the sequences. The amino acid changes in npr1-1 (H), npr1-2 (C), and nim1-4 (R) mutants are marked by open triangles. Amino acids required for nuclear localization of NPR1 (NLS1) in *Arabidopsis* and the second NLS2 are marked with filled triangles and asterisks, respectively. Eight Cys residues are labeled by"#".

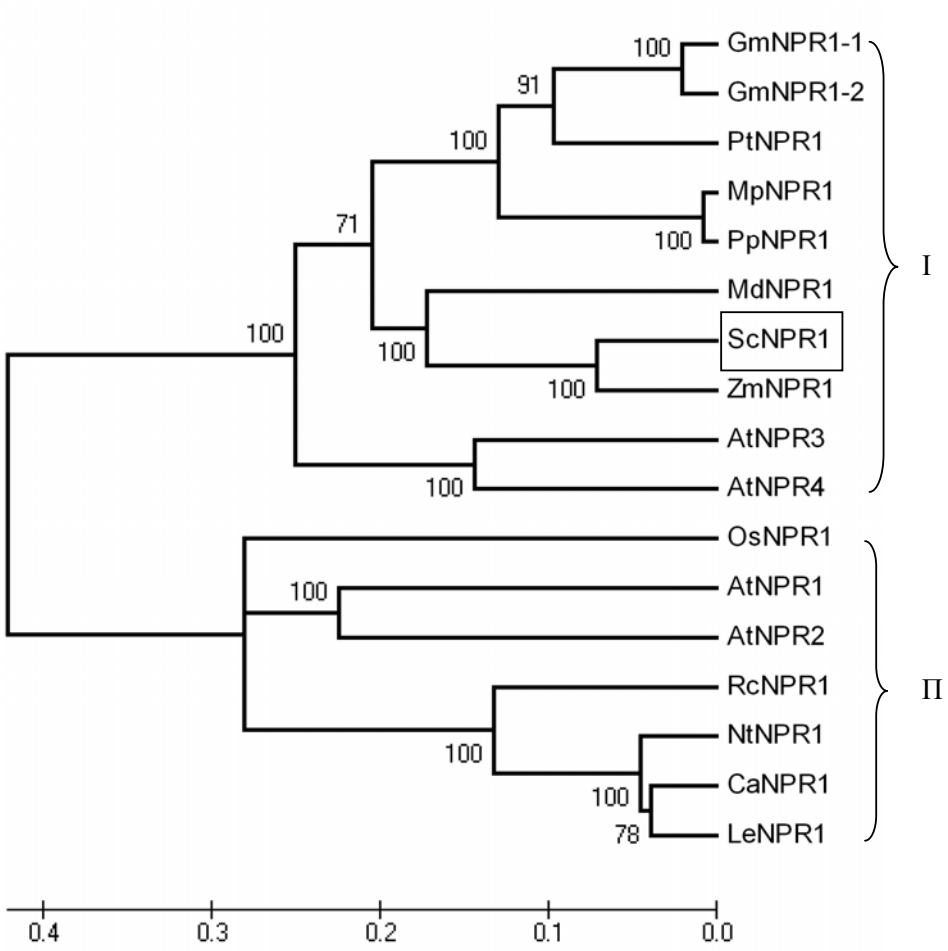

Fig. 2. A phylogenetic tree of ScNPR1 and 16 other known NPR1 proteins was made using the ClustalW program. ScNPR1 is indicted by a rectangle. Numbers at the branch points indicate bootstrapping values. A scale of distance is shown at the bottom.

The RT-qPCR analysis also showed that the *ScNPR1* expression levels were apparently up-regulated by *U. scitaminea* infection, reaching a peak at 24 h after inoculation (Fig. 3D), basically, is consistent with this fungus' invading time of 6 to 36 hours (Alexander & Ramakrishnan, 1980), suggesting a potential role of *ScNPR1* gene in sugarcane resistance to the smut disease. Similar results were also obtained in rice, banana, tobacco and *Arabidopsis* that *NPR1* expression was enhanced when plants sensed pathogen attack (Mou *et al*., 2003; Yuan *et al*., 2007; Endah *et al*., 2008; Henanff *et al*., 2009; Zhao *et al*., 2009; Zhang *et al*., 2010).

Plant signal molecules, including SA, MeJA, ethylene, H_2O_2 and abscisic acid (ABA), which may accumulate upon pathogen infection, are involved in the signaling network that mediate defense responses (Leon-Reyes *et al*., 2009). However, each signal molecule or pathogen has its specific mechanism (Kunkel & Brooks, 2002). It has been well accepted that two major pathways are involved in the induction of systemic plant resistance: SAR and induced systemic resistance (ISR). SAR leads to the expression of PR proteins, is SA-dependent and was initially described as being pathogen inducible. ISR was initially described in plants colonized by nonpathogenic rhizobacteria, is JA/ethylene-dependent, and induces a primed state, which enhances defence gene expression in plants following pathogen attack (Segarra *et al*., 2007). The crosstalk between SA and JA signaling pathways exists to adapt to infection by different pathogens and to fine-tune defense responses. In general, this crosstalk is considered to be antagonistic (Pozo *et al*., 2004). Therefore, the expression patterns of *ScNPR1* induced by disease pathogen, SA, MeJA, or ethylene were different (Fig. 3). In this study, *ScNPR1* expression was induced to higher levels in pathogen infected and SA-treated plants (Fig. 3A and D), but was weakened in MeJA or ethylene-treatment plants (Fig. 3B and C), which was different from previous reports on tobacco, banana and grapevine plants that NPR1 gene was enhanced upon MeJA treatment (Endah *et al*., 2008; Henanff *et al*., 2009; Zhang *et al*., 2010). These results indicate that *ScNPR1* was likely to be involved in SA-mediated signaling defended pathway, which was consistent with the sequence analysis data that *ScNPR1* contained a nuclear localization signal (NLS1) that was essential for SA-mediated *PR* gene expression (Fig. 1B). In addition, *ScNPR1* was rapidly induced upon SA treatment and pathogen inoculation within 24 h (Fig. 3A and D), suggesting that this gene may be involved in the early stages of defense response to pathogen infection and exogenous treatment with plant signal molecules.

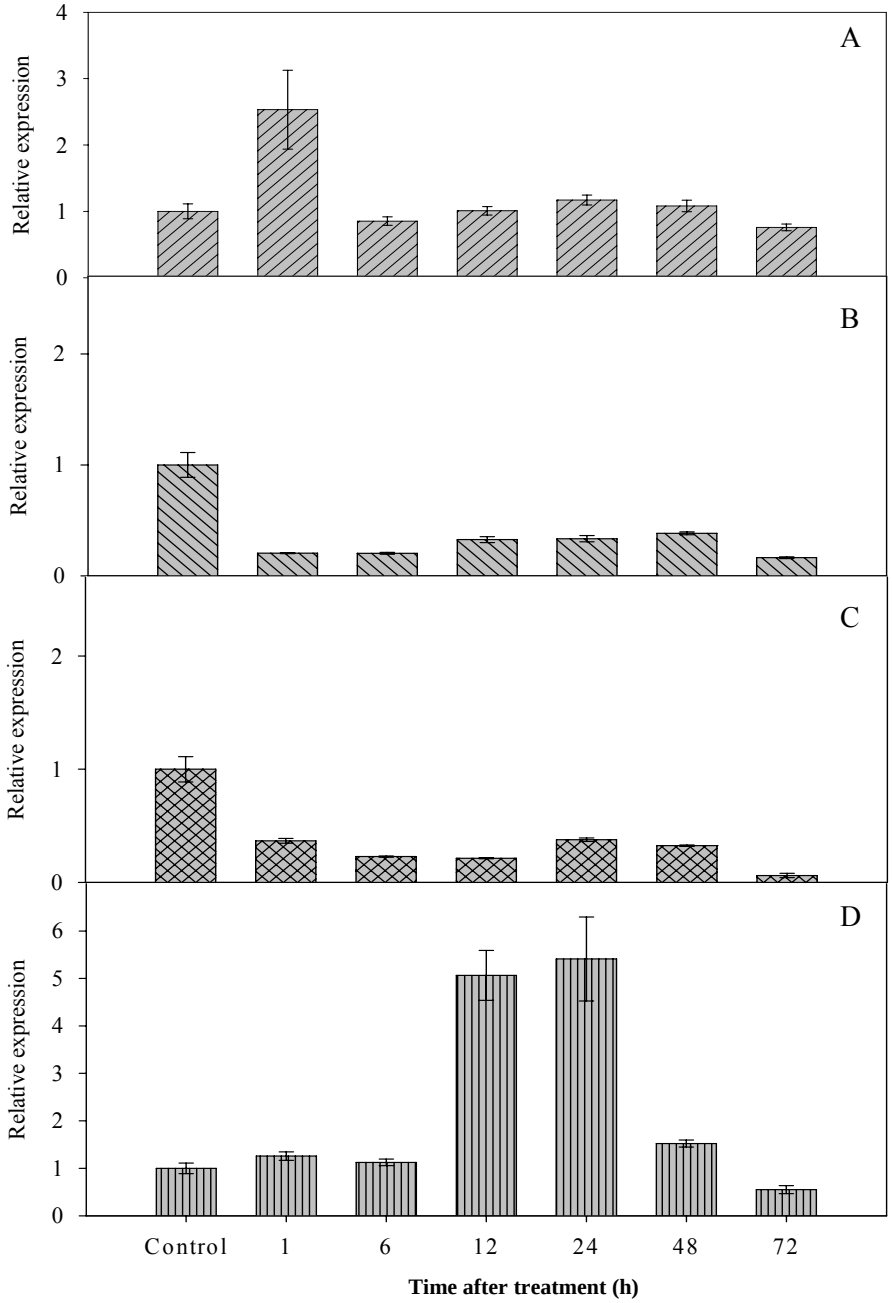

Fig. 3 Expression patterns of *ScNPR1* gene in sugarcane leaves of ROC22 upon treatments with SA (A), MeJA (B), ethylene (C), and inoculation with *U. scitaminea* (D), respectively. The expression level at each time point was expressed as a ratio relative to the control sugarcane plant at hour 0 time point, which was set to 1.

Tissue-expression patterns of *ScNPR1* in three varieties of sugarcane: The expression patterns of *ScNPR1* in leaf, sheath and root tissues of 3 varieties of sugarcane are presented in Fig. 4. Cultivar ROC22 is susceptible to smut, Q190 is moderately resistant to smut, and Badila is extremely resistant to smut. Accumulation of *ScNPR1* transcripts was remarkably higher in the leaf and sheath tissues of Q190 and Badila than in ROC22. Similar results were also reported in rice and banana that expression of *OsNPR1* or *MdNPR1* was stronger in resistant cultivars than in susceptible ones (Yuan *et al*., 2007; Zhao *et al*., 2009). Interestingly, a higher accumulation of *ScNPR1* transcripts in the root tissue of Badila was also observed (Fig. 4). Badila belongs to *S. officinarum*. According to the previous reports, most of *S. officinarum* is immune or highly resistance to sugarcane smut, and is deemed to the resistance source for this disease (Martin *et al*., 1962). Here, our results are consistence with the previous research. To sum up, the above results further indicated that *ScNPR1* might play a role in the defense response to the sugarcane smut disease.

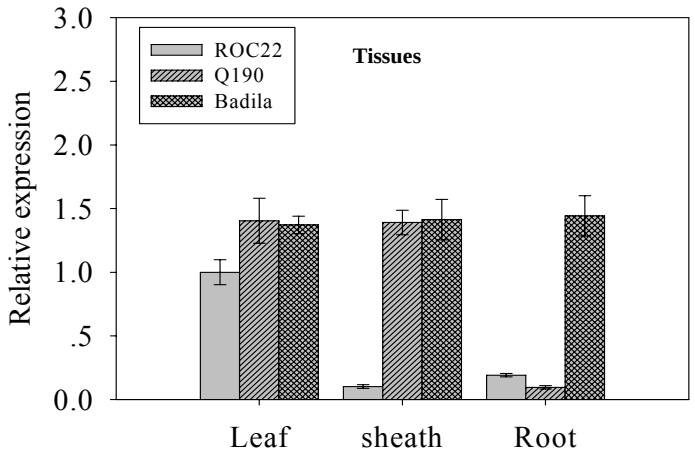

Fig. 4 *ScNPR1* expression in different tissues of three sugarcane cultivars, namely, ROC22, Q190, and Badila. The expression level at each tissue was expressed as a ratio relative to that in the leaf of ROC22, which was set to 1.

In conclusion, the results of this study clearly demonstrated that the *ScNPR1* was likely to be involved in SA-mediated signaling pathway and might play a role in the defense response to sugarcane smut disease. This study has provided some new information on understanding the mechanism of disease-resistance in relation to the *NPR1* gene in sugarcane. Further studies are needed to fully understand the protective role of *ScNPR1* gene in disease defense responses.

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