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 MIM1 (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 (Henanf 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 & Glazmann, 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 polypeptides (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; Schögl 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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[References omitted for brevity.]

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 immersing 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 U. scitaminea Syd collected from the fields. 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 RNase Plant Mini Kit according to the manufacturer’s instruction (QIAGEN, Germany). Total RNA content was quantified by spectrophotometry (BioPhotometer plus, Eppendorf, 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 superscript III reverse transcriptase (Invitrogen) and Oligo (dT)$_12$-20 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'-GGTGGTGGCGAAGGCGGTCTTCT-3' and antisense: 5'-CCTCTTGTGGTAATCTGTTG-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: ccactccgagctgactctt. 3′RACE1: caagtgtagatgcgtgag and 3′RACE2: cttgtgtgcgaaggaggag. 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 bootstrapping iterations. The 16 NPR1 homologues sequences registered in GenBank are AtNPR1 (At1g64280), AtNPR2 (At4g26120), AtNPR3 (AtSG45110), and AtNPR4 (At4G19660) from Arabidopsis thaliana; NINPR1 (AAC62410.1) from tobacco; OsNPR1 (AAV18700.1) from rice; LeNPR1 (AAT57637.1) from tomato; ZmNPR1 (NP_001147587.1) from maize; GmNPR1-1 (ACJ450313.1) and GmNPR1-2 (ACJ45015.1) from soybean; PpNPR1 (ABK62792.1) from pear; MpNPR1 (AC778976.1) from apple; MdNPR1 (ACJ04030.1) from banana; CaNPR1 (ABG38308.1) from pepper; RcnPR1 (EEF44801.1) from castor bean, and PtNPR1 (XP_002522351.1) from poplar.
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'-TGTCTTCTCATGTCGTGGCGT-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 constitutively expressed and conserved regions of the sugarcane leaves that had one uninterrupted ORF. It was 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 among 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 AtNPR1 were 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 ScNPR1 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 “#”.
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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$_2$O$_2$ 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 defense 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.
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. Further studies are needed to fully understand the protective role of ScNPR1 gene in disease defense responses.

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