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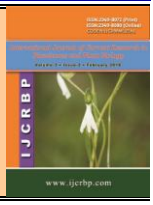

Original Research Article

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Cloning and Characterization of *NPR1* Gene from *Arabidopsis thaliana* ecotype Col-0

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Abstract

In the world of plants the non-expresser of pathogenesis-related gene 1 (*NPR1*) is a key regulator of salicylic acid (SA) mediated systemic acquired resistance (SAR). It also plays an important role in the Jasmonic acid (JA) induced systemic resistance (ISR) signaling pathway and also arbitrates the crosstalk between SA-JA defense pathways to fine-tune defense responses of whole plant system. *NPR1* is one of the most agronomically important genes which are under extensive research for development of transgenic with broad spectrum disease resistance. In this study, we have isolated *NPR1* gene from genomic DNA of *Arabidopsis thaliana* ecotype Col-0. 2277 bp *AtNPR1* PCR product with single complete open reading frame has been cloned in pJET1.2 cloning vector and sequence characterized. *AtNPR1* encode a putative functional protein of 593 amino acids long. Sequence analysis in NCBI CDD domain reveals presence of important Ankyrin repeats domain and a BTB/POZ domain, found in some regulatory proteins and both of which mediate protein-protein interactions. Among the SAR-related gene with vast potential in disease resistance, *AtNPR1* is a leading candidate represents promising results in engineering resistance to broad-spectrum of pathogens.

Article Info

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Keywords

Ankyrin repeats domain
Arabidopsis thaliana ecotype Col-0
AtNPR1
BTB/POZ domain
Induced Systemic Resistance (ISR)
Jasmonic acid (JA)
Salicylic acid (SA)
Systemic Acquired Resistance (SAR)

Introduction

With continuously changing climatic condition of the earth number of plant diseases are emerging stronger than ever and are tangible threat to worldwide agricultural production. Fungal, bacterial and viral diseases cause significant yield losses in most of the agricultural and horticultural species. Fungi cause more than 70% of all major crop diseases (Agrios, 2005; Sarah and Paul, 2005). Fungal diseases are rated either the most important or second most significant factor contributing to yield losses in crops like tomato (Lee and Raikel, 1995), wheat (Huang and Gill, 2001), barley (Smith, 2002), cotton (Cui et al., 2000), groundnut (Mace et al., 2006), grapevine (Dhekney et al., 2007) and others. In India, losses due to diseases have been estimated to be more than 10%, accounting for an annual loss to the tune of Rs. 1200 million (Rangaswami and Mahadevan, 2005). In India, on an average 18% crop loss due to pests with a monetary loss of 90,000 Cr annually (CARE, 2009).

All high yielding verities are not mostly resistance enough to plant pathogens, as they should be. We have to use chemical pesticides and fungicides to control these diseases. Chemical control might be very effective for some diseases, but the effects are often non-specific. It kills beneficial organisms in the environment along with plant pathogens, and definitely they have undesirable health effects, safety and environmental risks (Manczinger et al., 2002). Pest and pathogens developing resistance to chemical pesticides is again a matter of great concern. Apart from chemical control, biological control measures have been used but with limited

success. Deployments of resistant cultivars, selected or bred through conventional breeding are considered the most common and dependable method of controlling plant diseases. However, biotic loss to the tune of 42% occurs despite the use of all these conventional management practices. Alternative strategies including search for novel genes and their effective deployment through biotechnological approach would be a rapid and eco-friendly approach for controlling the crop diseases.

For biotechnologists, diseases control is a subject of great curiosity. Gene isolation and genetic transformation to develop transgenic resistance to diseases was the most noteworthy development in the area of varietal development for disease resistance. Improvements in genetic transformation technology allowed the genetic modification of almost all important food crops like rice, wheat, maize, mustard, pulses and fruits. Genetic engineering technology has proved to be beneficial in managing fungal (Lin et al., 2004; Wani, 2010, Parkhi et. al., 2010a and 2010b; Kumar et al., 2012), viral (Wani and Sanghera, 2010b) and bacterial (Jube and Borthakur, 2007; Sanghera et al., 2009) diseases in plants. Additionally, genetic engineering has the potential to increase disease tolerance to a range of pathogens, with no side effect on beneficial soil microbes (Liu et al., 2005).Genetic engineering of disease-resistance through transfer of plant defense-related genes or pathogen-originated genes into crops is valuable in terms of cost, efficacy and reduction of pesticide usage (Shah et al., 1997; Salmeron and Vernooij, 1998; Rommens and Kishore, 2000; Stuiver and Custers, 2001).

A composite grid of defense responses have been evolved in plants associated with the response to local site of infection. Interaction between avirulence (*avr*) gene from pathogen and cognate resistance (R) gene from plants are the key players in these responses (Heath, 2000; Durrant and Dong, 2004; Attaran et al., 2009; Mur et al., 2008). Further, in the process called as systemic acquired resistance (SAR), these defense responses are systemically induced in remote parts of the plants (Durrant and Dong, 2004; Dong, 2004; Mukhtar et al., 2009). Induction of the SAR pathway confers an increased, long-lasting, and broad-spectrum resistance to subsequent pathogen attacks for the whole plant (Ryals et al., 1996; Durrant and Dong, 2004; Mukhtar et al., 2009). Many research labs working on monocots and dicots established salicylic acid (SA) as a key player as a signaling molecule in SAR (Cao et al., 1994; Liu et al., 2002; Fitzgerald et al., 2004; Vlot et al., 2008; Yuan et al., 2007; Chen et al., 2009; Makandar et al., 2006; Le et al., 2009). There is considerable amount of increase in SA levels both locally and systemically in plants, after the attack by pathogens (Malamy et al., 1990; Metraux et al., 1990; Rasmussen et al., 1991). In addition, SA is required for the induced expression of a set of pathogenesis-related (PR) genes (Cao et al., 1994; Gaffney et al., 1993; Rochon et al., 2006; Loake and Grant, 2007; Ward et al., 1991; Ryals et al., 1996).

NPR1 is a key regulator of SAR (Conrath et al., 2002; Kohler et al., 2002). Initial screening of *Arabidopsis* mutants that were insensitive to SA (or its chemical analogs, 2,6-dichloroisonicotic acid (INA) or benzothiadiazole (BTH)) reveled presence of *NPR1* gene(Cao et al., 1994; Shah et al., 1997; Delaney et al., 1995; Glazebrook et al., 1996; Ryals et al., 1997; Clarke et al., 1998). These screens identified a mutation designated as Non-Expressor of PR1 (*NPR1*). Further studies reveled that *npr1* mutants displayed reduced expression of PR genes upon SA treatment and were more susceptible to pathogens (Cao et al., 1994; Shah et al., 1997; Glazebrook et al., 1996; Ryals et al., 1997). Contrariwise, after over expression of *NPR1* gene, the transgenic plants exhibited increased resistance to pathogens, and were able to induce improved levels of PR genes in a dose-dependent fashion (Cao et al., 1998).

NPR1 expression is very low under normal circumstances. But, *NPR1* gene expression level elevated by treatment of plants with SA, 2,6-dichloroisonicotinic acid (INA) or benzo thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Heath MC, 2000; Durrant and Dong, 2004; Attaran et al., 2009; Cao et al.,1997; Ryals et al., 1997), and regulated through SA-induced WRKY DNA binding proteins (Yuet al., 2001) and redox changes (Mou et al.,2003). *NPR1* also plays an important role in the Jasmonic acid (JA)/Ethylene dependent Induced Systemic Resistance (ISR) signaling pathway and also mediates the crosstalk between SA-JA defense pathways to fine-tune defense responses (Feys and Parker 2000; Ndamukong et al., 2007; Spoel et al., 2003; Leon-Reyes et al., 2009; Koornneef and Pieterse, 2008). Biotrophic pathogens are controlled by SA-mediated defense pathway, while necrotrophic pathogens and herbivorous insects are countered by JA-mediated defense mechanism. *NPR1* mediates the antagonistic effect of SA on JA signaling by suppressing the expression of JA-responsive genes upon combined treatment of SA and Methyl Jasmonate (MeJA) (Spoel et al., 2003).

Evidenced by several studies, it is well established fact that *NPR1* belongs to a multigene family in genome of plant kingdom, 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). Similar pathways like that of the *Arabidopsis NPR1* (*AtNPR1*) mediated signaling pathway were also confirmed in rice (Chern et al., 2001). Additionally, homologs of *AtNPR1* have been cloned and characterized in numerous crop plants including cotton (Zhang et al., 2008), banana (Endah et al., 2008; Zhao et al., 2009), grapevine (Henanff et al., 2009), rice (Chern et al., 2005; Yuan et al., 2007), apple (Malnoy et al., 2007) and rosaceous tree (Pilotti 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 boost fungal and bacterial resistance.

All these outcomes indicated that *NPR1* represents a creed of desirable candidate genes for genetic modifications in crop plants to improve disease resistance. There are reports of use of this *AtNPR1* gene from Col-0 ecotype in transgenic tomato which showed improved resistance to bacterial wilt (BW) and Fusarium wilt (FW), and modest degree of enhanced resistance to gray leaf spot (GLS) and bacterial spot (BS) (Lin et al., 2004). Keeping these results in mind we have also isolated this *AtNPR1*gene from *Arabidopsis thaliana* ecotype Col-0 for its further application in development of cotton transgenic for improved disease resistance.

Materials and methods

Genomic DNA isolation

Arabidopsis genomic DNA was extracted by CTAB buffer miniprep protocol method. 0.5 gm of tissue sample (including leaf, stem and roots) was crushed and extracted in 800 µl of Extraction Buffer (100mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 2% PVP and 0.5 M glucose) in a two ml centrifuge tube. The mixture was vortexed for 45 secs and incubated at 65 °C for 1 hr with occasional shaking. Equal volume of chloroform : isoamyl alcohol (24:1) was added after incubation, mixed and centrifuged at 4 °C for 10 min at 12,000 rpm. The supernatant was removed carefully and put into the fresh two ml centrifuge tube. To this 0.8 volume of ice-cold isopropanol was added and mixed gently by inverting the tube for 5-6 times. This mixture was incubated at -20°C for half an hour. Centrifuge the mixture at 12,000 rpm at 4°C for 15 min. Discard supernatant, let the DNA dry for two min and dissolve the DNA pellet in 500 µl of sterile double distilled water.

RNaseA treatment was given by adding RNaseA (20 µg/ml final concentration) and incubating 37°C for half an hour. This treated DNA was re-precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and double volumes of ice cold ethanol. Mixture was incubated at -20°C for an hour. Precipitated DNA was collected by centrifugation at 12,000 rpm at 4°C for 10 min. Re-precipitated DNA was rinsed with 70% ethanol. Dry this DNA at room temperature for two minutes and re-dissolved in 200 µl of sterile double distilled water. Check DNA concentration and purity by measuring the absorbance ratio A260/280. The quality of the DNA was further established by resolving 1 µl DNA on 0.8% agarose gel.

Primer designing for *AtNPR1* gene amplification

Primers for amplification of complete functional gene were designed based on the *Arabidopsis NPR1* gene sequence available at National Centre for Biotechnology Information (NCBI) online. Forward primer (NPR1-F: 5'-CTTGGCTCTGCTCGTCAATGG-3') was designed 98 bp upstream from the start codon and reverse primer (NPR1-R: 5'-GGATGCAAAACGAAGAGCGA-3') was designed 95 bp downstream from stop codon so as to cover complete sequence.

Amplification of full length AtNPR1 gene by touchdown PCR

These primers were used to standardized PCR protocol for amplification of *NPR1* gene from *Arabidopsis* genomic DNA. Platinum *Pfx* DNA Polymerase (11708-013, Invitrogen, ThermoFisher Scientific, USA) was used for amplification. This is high fidelity and high endpoint Taq, with the proofreading (3′→5′ exonuclease) activity with automatic hot-start effect. PCRx Enhancer Solution is included for higher primer specificity, broader magnesium concentration allowances, and broader annealing temperature parameters.

PCR cocktail for amplification of full length *AtNPR1* gene was made as detailed in Table1a. PCR protocol was standardized as mentioned in Table 2. Touchdown PCR technique was applied to increase specificity of the primers. In 45 cycle's repeats, primer annealing was started at 68°C in the first cycle and then one degree temperature was reduced per cycle, up to seven cycles to reach 60°C and from that point remaining 38 cycles were

continued at 60°C primer annealing temperature. Negative controls were maintained to check non-specific amplification, if any. The amplified product was checked by resolving on 1.0% agarose gel and it was documented on gel documentation system (AlphaimagerTM 2200, USA).

Table 1a. PCR cocktail for Platinum *Pfx* DNA Polymerase.

Sr. No.	Components	Volume (µl)
1	10x PCR amplification buffer	10.0
2	10 mM dNTPs	1.0
3	10 mM $MgSO_4$	1.0
4	NPR1-F (10 mM)	1.0
5	NPR1-R (10 mM)	1.0
6	DNA template (50 ng/µl)	2.0
7	Platinum Pfx DNA Polymerase (5 U/µl)	0.3
8	10x PCR Enhancer solution	5.0
9	Sterile double distilled water	25.4
	Total volume	50.0

* Whole reaction was set up in ice bath below 4°C.

Table 1b. PCR cocktail for Genaxy Taq DNA Polymerase.

Sr. No.	Components	Volume (µl)
1	10x PCR amplification buffer (+$MgCl_2$)	2.5
2	10x BSA	0.5
3	25 mM dNTPs	0.5
4	NPR1-F (10 mM)	0.5
5	NPR1-R (10 mM)	0.5
6	DNA template (50 ng/µl)	2.0
7	Taq DNA Polymerase (5 U/µl)	0.5
8	Sterile double distilled water	18.0
	Total volume	25.0

* Whole reaction was set up in ice bath below 4°C.

Table 2. Details of touchdown PCR protocol optimized for transgenic event characterization.

Primer combination	Initial denaturation		Denaturation		Annealing		Extension		Final extension		Product size (bp)
	Temp (°C)	Time (min)	Temp (°C)	Time (Sec)	Temp (°C)	Time (Sec)	Temp (°C)	Time (Sec)	Temp (°C)	Time (min)	
NPR1-F	94	5	94	40	***68-60**	30	72	180	72	5	2300
NPR1-R	***01 Cycle***		***45 Cycles***						***01 Cycle***		

* Touchdown PCR temperature, annealing temperature decrease from 68°C to 60°C.

Gene cloning in PCR cloning vector

Before proceeding for cloning, PCR product was purified with the help of PCR purification kit (12500-50, UltraClean® PCR Clean-Up Kit, Genaxy, USA). This purified *AtNPR1* amplicon was cloned in pJET1.2 cloning vector (CloneJET PCR Cloning Kit, #K1231, Thermo Scientific, USA). The kit features the novel positive selection cloning vector pJET1.2/blunt. This vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate, eliminating the need for expensive blue/white screening. Our PCR product was blunt end product and can be cloned directly into pJET1.2 vector. The ligation reaction was prepared as per Table 3 and the ligation mix was incubated at 22°C for 20 min for optimum performance. This ligation mix after incubation was directly used for transformation procedure.

For transformation *E. coli* XL1-Blue strain was used and the transformation was done by chemical transformation method. For this TransformAid Bacterial Transformation Kit (#K2711, Thermo, USA) was used. From a fresh culture plate of XL1-Blue, overnight culture of single colony of *E. coli* in 2 ml C-medium was start at 37°C in incubator shaker. Further treatment was done as per the manufacturer's protocol. At the end, 5µl fresh ligation

mix was added in 200 µl PCR tube and chilled on ice. To this chilled 5µl ligation mix, 50 µl freshly prepared competent cells were added. Mixed by tapping and centrifuged shortly to collect mixture at bottom. The mixture was incubated on ice for 5 min and immediately plated on pre-warmed LB plate with ampicillin (final concentration of 75 µg/ml). This seeded plate was incubated at 37°C overnight in incubator.

Table 3. DNA ligation reaction on ice.

Sr. No.	Components	Volume (µl)	Mixing	Incubation
1	2x Reaction buffer	10.0	Vortex briefly and centrifuge for 3-5s	Incubate the ligation mixture at 22°C for 20 min.
2	Purified PCR product	01.0		
3	pJET1.2/blunt cloning vector (50 ng/µl)	01.0		
4	T4 DNA ligase	01.0		
5	Nuclease free sterile water	07.0		
	Total volume	20.0		

*Use this cocktail directly for transformation.

Analysis of recombinant clones

Colonies appeared on plate the next day can be considered positive because of special selection strategy of vector. But we also confirmed the recombinant clones by single and double restriction digestion and PCR with gene specific primers. First grid plate was prepared on LB + ampicillin plate. While streaking the grid with sterilized tooth pick, start overnight culture in 2ml LB + ampicillin broth with the same tooth pick. Make sure to annotate the grid plate and broth culture tubes properly. These tubes were incubated in incubator shaker at 37°C and 90rpm overnight. Next day, this fresh bacterial culture was used to isolate plasmid DNA. Plasmid isolation was done by the miniprep protocol described by Chakrabarty et al. (2010). Only first five colonies (pJET-AtNPR1-1 to pJET-AtNPR1-5) were processed for analysis. Plasmid DNA was checked by resolving on 0.8% agarose gel and documented on gel documentation system (AlphaimagerTM 2200, USA).

*BamH*I *single* digestion cocktail consist of 3µl plasmid DNA, 2 µl 10x *BamH*I buffer, 0.25 µl of 10 U/µl *BamH*I (Fermentas, USA) restriction enzyme and sterile double distilled water added up to 20 µl (Table 4a). *Sal*I s*ingle* digestion cocktail consist of 3µl plasmid DNA, 2 µl 10x buffer-O, 0.25 µl of 10 U/µl *Sal*I (Fermentas, USA) restriction enzyme and sterile double distilled water added up to 20 µl (Table 4a). Double digestion cocktail consist of 3µl plasmid DNA, 2 µl 10x *BamH*I buffer, 0.25 µl of 10 U/µl *BamH*I (Fermentas, USA) restriction enzyme, 0.25 µl of 10 U/µl *Sal*I (Fermentas, USA) restriction enzyme and sterile double distilled water added up to 20 µl (Table 4b). Digestion cocktail were incubated at 37°C for two and a half hour. Digested plasmid DNA was checked by resolving on 1.2% agarose gel and documented on gel documentation system (AlphaimagerTM 2200, USA).

Positive clones were also confirmed by PCR amplification of the *At-NPR1* gene using gene specific primers, NPR1-F and NPR1-R. PCR cocktail was prepared as per Table 1b. Here, Taq DNA polymerase from Genaxy (USA) was used for amplification. The PCR protocol was followed as explained in Table2. PCR products were checked by resolving on 1.0% agarose gel and documented on gel documentation system (AlphaimagerTM 2200, USA).

Gene Sequencing and characterization

The positive recombinant clones *pJET-At-NPR1*in *E. coli* XL1-Blue were further cultured in 5 ml LB + Ampicillin broth at 37°C overnight in incubator shaker. Negative control without any inoculum was kept to check any contamination in liquid broth itself. Next day, this overnight grown culture was used to isolate plasmid DNA with the help of AxyPrep Plasmid Miniprep Kit (AP-MN-P-50, Genaxy, USA). This plasmid DNA was then sent for sequencing to Xcelris Labs Ltd., Ahmedabad, India. The sequences obtained were put together using software DNA BASER Sequence Assembler4.10. This sequence was submitted in GenBank (KF564649). Alignment and base composition analysis of the nucleotide sequence of the *AtNPR1* gene was performed with the BLASTn (Gotea et al., 2003). Also amino acid sequence of *AtNPR1* protein was compared online using BLASTp software and conserved domains in the *AtNPR1* protein were identified using the National Center for Biotechnology Information (NCBI) Conserved Domains software (Marchler-Bauer et al., 2005) online. This *AtNPR1* sequence was compared with *ArabidopsisNPR1* mRNA sequence (NM_105102) to get an idea about *AtNPR1*mRNA sequence and amino acid sequence.

Table 4a. Single digestion cocktail.

Sr. No.	Components	Volume (µl)	Incubation
1	10x Buffer *BamH*I / Buffer O	2.00	Incubate the mixture at 37°C for two and a half hour
2	*BamH*I or *Sal*I enzyme(10 U/µl)	0.25	
3	Plasmid DNA	2.00	
4	Sterile double distilled water	15.75	
	Total volume	20.00	

* Whole reaction was set up in ice bath below 4 °C.

Table 4b. Double digestion cocktail.

Sr. No.	Components	Volume (µl)	Incubation
1	10x Buffer *BamH*I	2.00	Incubate the mixture at 37°C for two and a half hour
2	*BamH*I enzyme (10 U/µl)	0.25	
3	*Sal*I enzyme (10 U/µl)	0.25	
4	Plasmid DNA	2.00	
5	Sterile double distilled water	15.60	
	Total volume	20.00	

* Whole reaction was set up in ice bath below 4 °C.

Results and discussion

Quality genomic DNA of *Arabidopsis thaliana* was extracted in lab using CTAB buffer miniprep protocol (Fig. 1). Gene specific primers NPR1-F and NPR1-R were used in touchdown PCR with Platinum Taq have given expected amplicon of 2277 bp (Fig. 2). This blunt ended PCR product after purification cloned in pJET1.2 vector and the positive recombinant clones were confirmed by single digestion, double digestion and gene specific PCR. First five recombinant clones (pJET-AtNPR1-1 to pJET-AtNPR1-5) were processed for plasmid DNA isolation by lab miniprep protocol (Chakrabarty et al., 2010) (Fig. 3a). Single digestion of these plasmids with enzyme *BamH*I and *Sal*I simultaneously produced liner plasmid band of nearly 5.3 kb (Fig. 3b, 3c), which was expected, as the size of vector was 3 kb and PCR product was of 2277 bp. And hence, double digestion with enzyme *BamH*I and *Sal*I have produced two bands, first was of 3 kb (cloning vector) and second was of approximately 2.3 kb (PCR product) (Fig. 3d). All these five plasmids were also tested with gene specific primers NPR1-F and NPR1-R, and produced PCR product of approximately 2.3 kb as expected (Fig. 3e). These tested positive clone plasmids were sequenced for gene of interest cloned in them.

The *NPR1* gene was isolated and cloned from *Arabidopsis thaliana* ecotype Col-0 that has one uninterrupted ORF. Assembled sequence is of 2277 bp and from start codon to stop codon it is 2079 bp in length. Gene contains four coding regions i.e. 4 exons which are interrupted by 3 noncoding regions i.e. introns. First exon is of 561 bp, second exon is of 736 bp, third exon is of 204 bp and fourth exon is of 281 bp. Combining all complete coding sequence i.e., mRNA sequence of *AtNPR1* is of 1782 bp (Fig. 4) which encode a putative protein of 593 amino acids (aa). The nucleotide sequence was submitted in GenBank (Accession no. KF564649). Protein information of *AtNPR1* coding region is available in GenPet at NCBI (Accession no. AIK27558).

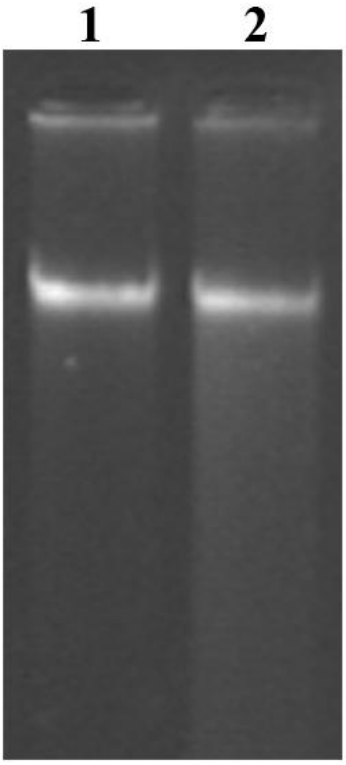

Fig. 1: *Arabidopsis* Genomic DNA (2 µl) separated on 0.8 % agarose gel. Lane 1 and 2, genomic DNA (2 µl) samples.

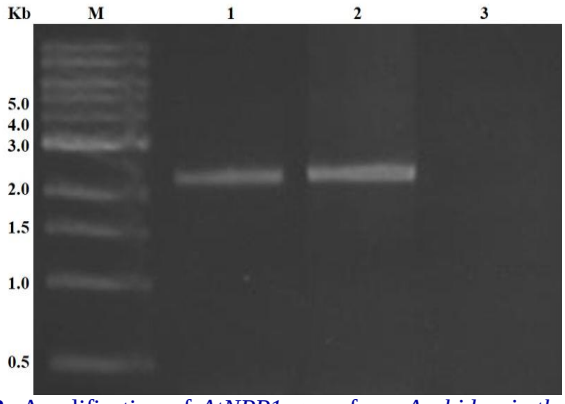

Fig. 2: Amplification of *AtNPR1* gene from *Arabidopsis thaliana* ecotype Col-0 using gene specific primers NPR1-F and NPR1-R. Lane M, 1 Kb DNA ladder, Lane 1 and 2, *Arabidopsis* samples, Lane 3, PCR negative.

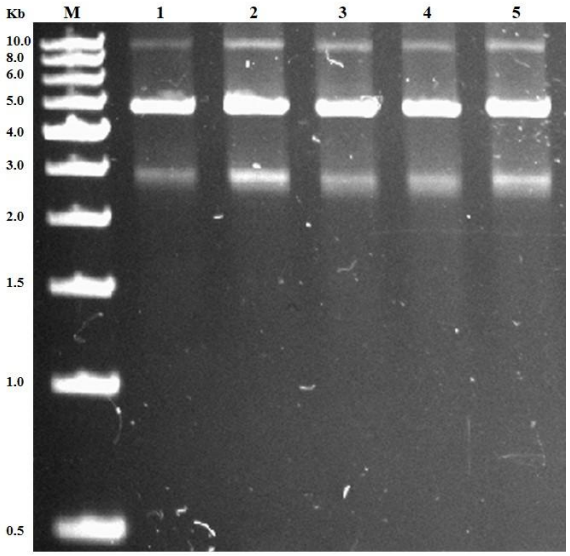

Fig. 3a: First five recombinant clones (pJET-AtNPR1-1 to pJET-AtNPR1-5) plasmid (3 µl) separated on 0.8 % agarose gel. Lane 1, pJET-AtNPR1-1; Lane 2, pJET-AtNPR1-2; Lane 3, pJET-AtNPR1-3; Lane 4, pJET-AtNPR1-4; Lane 5, pJET-AtNPR1-5.

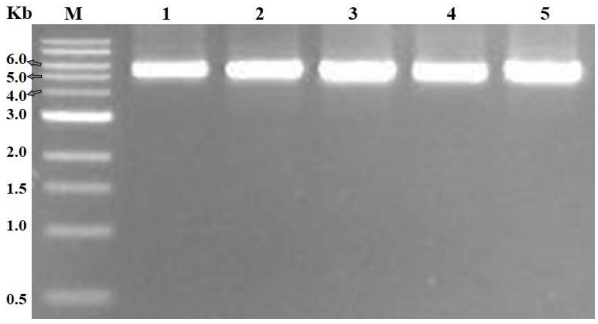

Fig. 3b: Single digestion with *BamH*I restriction enzyme. 3 µl plasmid DNA from first five recombinant clones (pJET-AtNPR1-1 to pJET-AtNPR1-5) were subjected to single digestion to confirm *AtNPR1*gene integration. Digested product (5.3 Kb linear plasmid) was separated on 1.2% agarose gel. Lane M, 1 Kb DNA ladder. Lane 1, pJET-AtNPR1-1; Lane 2, pJET-AtNPR1-2; Lane 3, pJET-AtNPR1-3; Lane 4, pJET-AtNPR1-4; Lane 5, pJET-AtNPR1-5.

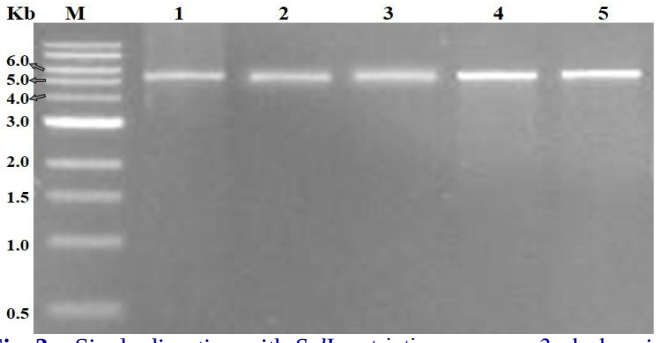

Fig. 3c: Single digestion with *Sal*I restriction enzyme. 3 µl plasmid DNA from first five recombinant clones (pJET-AtNPR1-1 to pJET-AtNPR1-5) were subjected to single digestion to confirm *AtNPR1*gene integration. Digested product (5.3 Kb linear plasmid) was separated on 1.2 % agarose gel. Lane M, 1 Kb DNA ladder. Lane 1, pJET-AtNPR1-1; Lane 2, pJET-AtNPR1-2; Lane 3, pJET-AtNPR1-3; Lane 4, pJET-AtNPR1-4; Lane 5, pJET-AtNPR1-5.

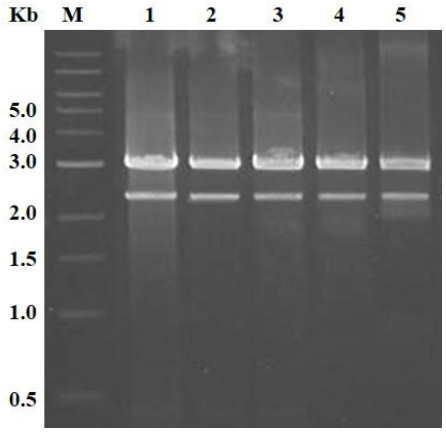

Fig. 3d: Double digestion with *BamH*I + *Sal*I restriction enzyme. 3 µl plasmid DNA from first five recombinant clones (pJET-AtNPR1-1 to pJET-AtNPR1-5) were subjected to double digestion to confirm *AtNPR1*gene integration. Digested product (3.0 Kb vector and 2.3 Kb PCR product) was separated on 1.2 % agarose gel. Lane M, 1 Kb DNA ladder. Lane 1, pJET-AtNPR1-1; Lane 2, pJET-AtNPR1-2; Lane 3, pJET-AtNPR1-3; Lane 4, pJET-AtNPR1-4; Lane 5, pJET-AtNPR1-5.

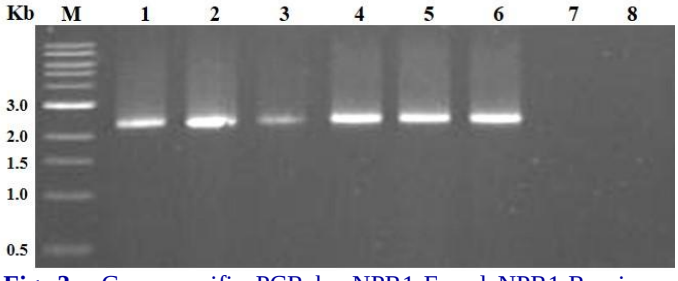

Fig. 3e: Gene specific PCR by NPR1-F and NPR1-R primers. Plasmid DNA from first five recombinant clones (pJET-AtNPR1-1 to pJET-AtNPR1-5) were subjected PCR detection to confirm *AtNPR1* gene integration. PCR product was separated on 1.0 % agarose gel. Lane M, 1 Kb DNA ladder. Lane 1, pJET-AtNPR1-1; Lane 2, pJET-AtNPR1-2; Lane 3, pJET-AtNPR1-3; Lane 4, pJET-AtNPR1-4; Lane 5, pJET-AtNPR1-5.

Nucleotide BLAST search of GenBank revealed that *AtNPR1* shared the identity of 78% to 99% with *Arabidopsis NPR1*sequences available. Protein BLAST results showed highest homology with 100% amino acid identity to non-expressor of PR1 protein of *Arabidopsis lyrata* subsp. Lyrata (XP_002886354). The concise results of the conserved domains analysis of the deduced *AtNPR1* amino acid sequence using the NCBI CDD program indicated that it contains Ankyrin repeats (ANK) domains(ARD) (aa 271-369), NPR1/NIM1 like defense protein C terminal (aa 370-565), BTB zinc finger domain (for BR-C, ttk and bab) or POZ (for Pox virus and Zinc finger) domain (aa 66-192) and a domain of unknown function (aa 231-271) (Fig. 5). These are typical features of *NPR1* genes that are highly conserved across many species (Cao et al., 1997; Aravind and Koonin, 1999; Chern et al., 2005; Endah et al., 2008). Amino acids critical for the *NPR1* function as well-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 the sequence there are conserved eight Cys residues which are possibly engaged in the oligomerization and the nuclear localization of *NPR1* or NPR1-like proteins (Mou et al., 2003; Yuan et al., 2007).

An evolutionary relationship between *AtNPR1* proteins with other *NPR1* proteins from other plants species, a phylogenetic tree was produced. This was based on the alignment of presumed amino acid sequences of *AtNPR1* and amino acid sequences involved in BLASTn results, using bootstrap consensus for neighbor joining, maximum parsimony and maximum likelihood. There are two main clusters in phylogenetic tree (Fig. 6) which was in agreement with reports by Zhao et al., (2009) and Henanff et al., (2009). *AtNPR1* was closely related non-expressor of PR1 from *Arabidopsis lyrata* subsp. Lyrata (XP_002886354) and regulatory protein NPR1 of *Arabidopsis thaliana* (NP_176610).

Nuclear localization of *NPR1* is mediated by a bipartite nuclear localization sequence at the carboxyl end of *NPR1*, and is required for PR genes induction (Kinkema et al., 2000).There are two protein-protein interaction domains present, first a BTB/POZ domain (Aravind and Koonin, 1999) at the N-terminal end and second an Ankyrin-repeat domain (ARD) inthe center of the *NPR1* protein (Cao et al., 1997). Though, most of the proteins possess either ARD or BTB/POZ domains, *NPR1* has its place in an exclusive group of proteins that possess both domains. Ankyrin repeats domain and a BTB/POZ domain, found in some regulatory proteins and both of which mediate protein-protein interactions (Stogios et al., 2005; Cao et al., 1997; Ryals et al., 1997). Detailed studies on various *npr1* mutants known with amino acid changes in the consensus of these domains, have emphasized the functional importance of these protein–protein interaction domains (Cao et al., 1997; Ryals et al., 1997).

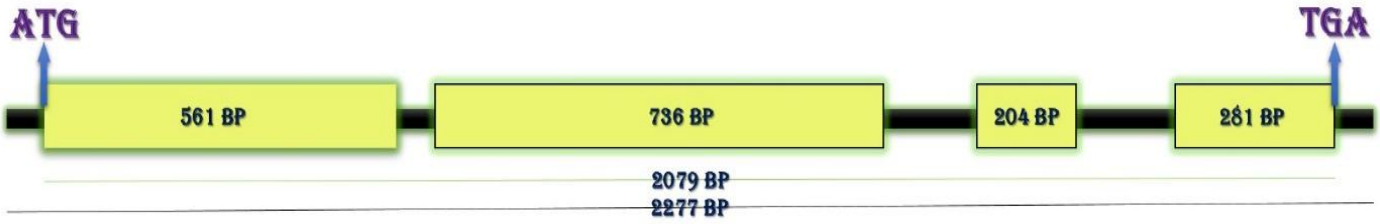

Fig. 4: *AtNPR1*gene assembly. Big rectangular boxes represent exons and thin black intervals represent introns. Size of the gene and size of exons are indicated.

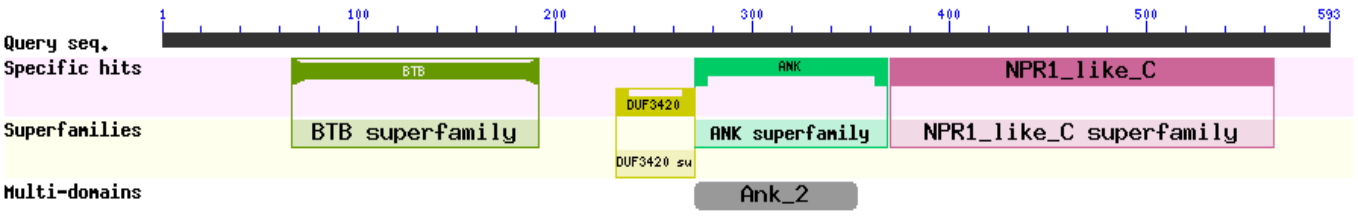

Fig. 5: The conserved domains analysis of the deduced AtNPR1 amino acid sequence using the NCBI CDD program.

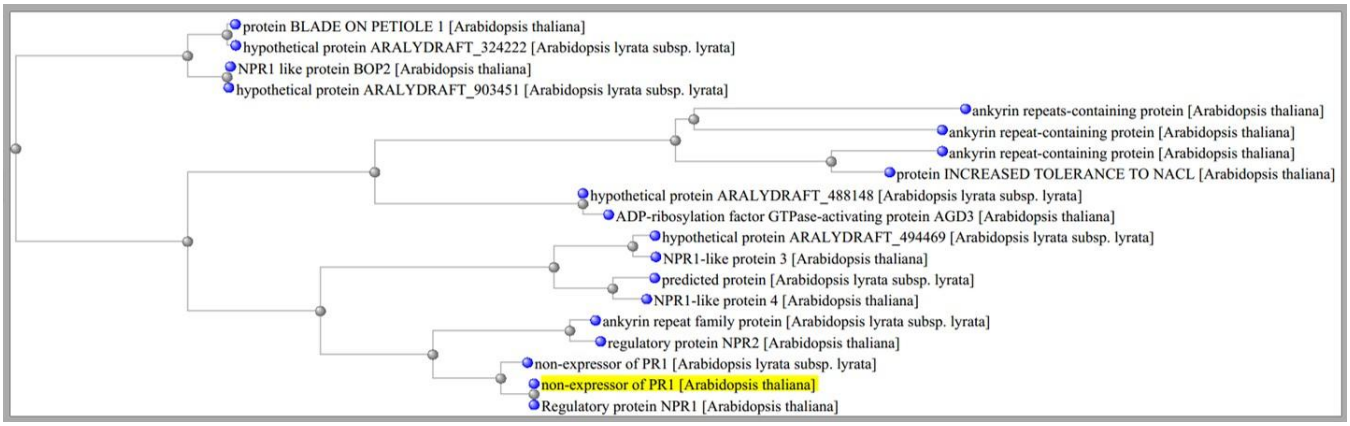

Fig. 6: A phylogenetic tree of *AtNPR1* with 18 other known *Arabidopsis* NPR1 proteins, made using online NCBI distance tree result domain in BLASTp.

In genetic engineering of economically important crops for improving resistance to spectrum of diseases, there is a vast potential use of SAR-related genes. We have chosen to clone this *Arabidopsis NPR1*gene, as its effectiveness in conferring an enhanced degree of non-specific disease resistance has been previously demonstrated in transgenic studies. In earlier studies, over expression of *NPR1* in plants have confer enhanced non-specific resistance plant pathogens, such as, enhanced resistance to an oomycete pathogen and a bacterial pathogen in *Arabidopsis* (Cao et al., 1998), resistance to bacterial blight in rice plants (Chern et al., 2001; Fitzgerald, 2004), enhanced resistance to Bacterial wilt (BW), Fusarium wilt (FW), Gray Leaf Spot (GLS) and Bacterial Spot (BS) in Tomato (Lin et al., 2004), resistance to Citrus canker, caused by *Xanthomonas citri* in Citrus plant (Zhang et al., 2010), enhanced resistance to *Verticillium dahlia* isolate TS2, *Fusarium oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani*, *Alternaria alternata and* Reniform Nematodes in cotton plant (Parkhi et al., 2010b) and resistance to root-knot nematode infection caused by *Meloidogyne incognita* in Tobacco (Priya et al., 2011).

For transgenic studies it is very much important to isolate and clone a full length functional gene. In our study, mRNA and amino acid sequence predicted, showed presence of full length functional protein which showed high homology towards the similar functional genes and proteins. Since, our ultimate goal is to explore the potential use of this *AtNPR1*gene for the genetic engineering of long lasting and broad-range disease-resistance, we have cloned and sequence characterized this gene in our lab for further studies. This *AtNPR1* gene is ready to use for genetic transformation of agricultural important crops.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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