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Original Article

Regulatory Network Identification, Promoter and Expression Analysis of *Arabidopsis thaliana* NPR1 in Defense Responses against Stresses

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Abstract

Salicylic acid (SA) and jasmonic acid (JA) phytohormones have been known for their roles in plant defense behaviour against biotic and abiotic stresses. They regulate defense pathways by antagonistic interaction. NPR1 as a key regulatory factor in the cross-talk between SA and JA, signaling is essential for the inhibition of JA-responsive gene expression by SA. *In silico* promoter analysis of 1.5 kb promoter regions of *NPR1* gene revealed that NPR1 contains 23 MYB and 20 WRKY transcription factor binding sites. Different *cis*-elements associated with various stress responses were identified in *Arabidopsis thaliana NPR1*. The most common element was allocated to the defense responses against biotic stresses. Based on gene network analysis, NPR1, TGA2 and TGA3 were predicted to have functional cooperation with each other. Affymetrix microarray data analysis of *A. thaliana* under SA treatment demonstrated that most genes involved in NPR1 network are up-regulated under SA treatment. Therefore, interaction and cooperation between these factors might serve to fine-tune regulation of defense and immune responses against biotic and abiotic stresses.

Keywords: abiotic and biotic stresses; *Arabidopsis thaliana*; gene network; *in silico* analysis; NPR1; regulatory elements

Introduction

Plants are exposed to various biotic and abiotic stresses. Perception of stress signals often results in the biosynthesis of signaling molecules like jasmonic acid (JA) and salicylic acid (SA) phytohormones (Turner *et al*., 2002; Shah, 2003). Production of these hormones mediates signal transduction cascades that lead to physiological adaptation of the plant to stresses (Kunkel and Brooks, 2002).

SA plays a significant role in plant resistance to biotic and abiotic stress factors (Loake and Grant, 2007; Vlot *et al*., 2009; Miura and Tada, 2014). JA pathway is also involved in different processes including responses to biotic and abiotic stresses (Howe and Jander, 2008; Browse, 2009).

SA and JA exhibit antagonistic interactions in response to plant pathogens. The activation of SA biosynthesis or signaling in many plant-pathogen interactions has been shown to result in the inhibition of JA biosynthesis or signaling through non-expresser of pathogenesis-related genes 1) (NPR) and WRKY70 proteins (Koornneef and Pieterse, 2008; Yasuda *et al*., 2008; Bari and Jones, 2009; Antico *et al*., 2012; Thaler *et al*., 2012). Conversely, induction of the JA pathway represses the SA signaling through MAPK4 and JIN2 factors (Kachroo and Kachroo, 2007; Koornneef and Pieterse, 2008).

Transcription factors (TFs) bind to *cis*-acting regulatory elements (CAREs) with short conserved nucleotide motifs at the 5' end of the genes and upstream of the gene transcription start site known as the promoter (de Boer *et al*., 1999). Plant promoter identification may increase fundamental information in the understanding of the regulation of gene expression (Shahmuradov *et al*., 2003).

TFs regulate gene expression via interaction with CAREs. The interaction between TFs and *cis*-acting DNA elements might activate or repress genes (Moore *et al*., 2011). TFs can interact with jasmonate signaling pathway and mediate response to biotic and abiotic stresses (Century *et al*., 2008). The role of TFs in hormone signaling can also activate defence genes (Lu *et al*., 2011).

The NPR1, as a positive regulator of SA, is essential for the inhibition of JA-responsive gene expression by SA (Zhang *et al*., 1999; Després *et al*., 2000; Zhou *et al*., 2000). In the SA pathway, the activity of NPR1 is regulated by several SA-dependent modifications (reviewed in Fu and Dong, 2013). By interacting with TGACGTCA cis-

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element-binding protein (TGA) transcription factors, NPR1 acts as a co-activator of SA-induced gene transcription and activates SA marker genes such as pathogenesis related protein (PR1). It has been shown that expression of NPR1 is regulated by WRKY TFs (Yu *et al*., 2001). WRKY TFs fine-tune and amplify downstream transcriptional responses (Wang *et al*., 2006; Eulgem and Somssich, 2007). WRKY factors act as positive regulators of the SA-mediated defenses while these factors repress the JA response functioning (Li *et al*., 2004). Moreover, several WRKY and TGA TFs have been shown to be important for SA/JA crosstalk and contribute to the complexity of the SA- and NPR1-dependent signaling network (Wang *et al*., 2006; Pieterse *et al*., 2012; Gimenez-Ibanez and Solano, 2013). V-myb myeloblastosis viral oncogene homolog (MYB) family has been found to be involved in a variety of biological functions such as biotic and abiotic stresses (Lippold *et al*., 2009; Segarra *et al*., 2009), hormone responses (Urao *et al*., 1993) and plant defense responses (Liu *et al*., 2008).

The identification of CAREs and their interaction with TFs could give some insight into the plant defense against stresses and presents a comprehensive understanding of the regulation of NPR1 gene expression.

Therefore, in the present study, *in silico* promoter analysis and affymetrix microarray data analysis of *A. thaliana* under salicylic acid treatment was followed to study NPR1 CAREs, TFs and expression analysis of genes involved in *Arabidopsis thaliana* NPR1 signaling network which are involved in defense responses against biotic and abiotic stresses.

Due to the pivotal role of transcription factors, such studies will advance the knowledge of host-stress interactions and can further provide the development of new strategies for the plant genetic engineering for management of biotic and abiotic stresses.

Materials and Methods

Promoter analysis of NPR1 gene

The genomic sequence of *NPR1* gene from *A. thaliana* (AT1G64280) was applied as a platform in order to recognize the promoter region of the *NPR1* gene by using the Phytozome database (http://www.phytozome.net/). After identifying the *Arabidopsis NPR1* on the chromosome using the BLAST-n algorithm, the 1,500 bp upstream of the start codon (ATG) was taken as a promoter. The upstream region sequences of the *NPR1* in *Arabidopsis* was analyzed via the PLANT CARE (http: // bioinformatics. psb.ugent. be /webtools/ plantcar e/html/) and Plant Pan (http:/ / plantpan2 .itps.ncku.edu .tw / promoter. php) databases to predict their key CAREs and the precise location of these elements.

NPR1 regulatory network analysis

In order to study the regulatory network of NPR1, GeneMANIA (http://www.genemania.org) database was used to identify different categories of co-expressed genes, physically interacted genes and functionally related genes with NPR1 regulatory factor. Also, STRING 9.0 (http://string-db.org) was used to predict all the proteins that interact with the NPR1 protein in *A. thaliana*.

NPR1 chromosome map

Chromosome map of *A. thaliana NPR1* was constructed by Chromosome Map Tools available at TAIR (https://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp).

Expression analysiss of NPR1 gene based on microarray data

To analyze the expression pattern of *NPR1* and co-regulated genes, affymetrix microarray data (http://bar.utoronto.ca database) was used. The relative expression ratio of genes under 10 mM SA treatment was obtained after 3 hours post treatment with SA based on Log2 (treated/control) ratio of three biological replicates.

Results and Discussion

Figs. 1 and 2 show the exon-intron arrangement and promoter map of *NPR1* in *A. thaliana*. *NPR1* contains 4 exons with transcript length of 2,522 bps, translation length (peptide sequence) of 593 residues and genomic sequence of 2,819 bps. *In silico* chromosome mapping of *NPR1* gene of *A. thaliana* is presented in Fig. 3. *NPR1* gene of *A. thaliana* was shown to be distributed on only 1 out of 5 chromosomes. In *A. thaliana*, chromosome 1 (23,852,748-23,855,566 position) harbored *NPR1* gene.

Different *cis*-elements associated with various stress responses such as cell death, response to heat, response to bacterium, response to insect, response to wounding, systemic acquired resistance (SAR), defense response to fungi and negative regulation of defense response were shown in *A. thaliana NPR1* (Fig. 4). These results were in accordance with NPR1 regulatory network, which showed that different functional groups predicted based on NPR1 co-regulated gene by Genemania database, play a role in defense response to bacterium, response to salicylic acid, regulation of responses to stress, response to fungus and immune responses (Table 1).

The frequency of various regulatory elements with stress-(abiotic or biotic)-responsive function on *NPR1* promoter suggests that this gene recruits several TFs to mediate gene expression which leads to enhanced resistance to biotic and abiotic stresses.

In another study, a relationship between architecture of promoter sequence and tobacco PR-1a expression has been reported (Lodhi *et al*., 2008). Therefore, gene expression regulation studies can increase the current knowledge to understand the molecular mechanisms of plant defense response.

On the other hand, it has been shown that TFs play a key role in the response to different stresses with their roles in the regulation of genes expression (Meshi and Iwabuchi, 1995).

According to Fig. 4, the most common element was allocated to SAR function with 52.17%. SAR as a systemic defense response is developed to enhance disease resistance

against viral, bacterial and fungal pathogens in plants through up-regulation of pathogenesis-related (PR) genes expression (Metraux *et al*., 1990).

In silico promoter analysis of 1.5 kb promoter regions of *NPR1* gene revealed that this gene is significantly enriched in the WRKY and MYB transcription factor binding sites (TFBSs) (Fig. 5, Table 2). Promoter analysis showed that the NPR1 contains 23 MYB and 20 WRKY TFBS (Fig. 5).

WRKY elements with common TGAC signal sequence were observed in *NPR1* promoter (Table 2).

Analysis of motifs in the promoter of *NPR1* gene suggests that the TGAC signal sequence may be a target for crosstalk regulation. TGA and WRKY TFs further contribute to the complexity of the SA- and NPR1-dependent signaling network involved in SA-dependent SAR (Wang *et al*., 2006) and some of the WRKY TFs have been implicated as a positive regulator of the SA-mediated defenses (Li *et al*., 2004). Therefore, WRKYs seem to play important roles in the suppression of JA responses. Analysis of protein-protein interaction network is presented in Fig. 6. Based on STRING web server's results, NPR1 mostly interacts with the different types of TGA TFs (Fig. 6).

In addition to promoter analysis of *NPR1* gene and STRING results, the NPR1 regulatory network was analyzed to identify other genes that participate in this regulatory network. Construction of gene networks can provide a powerful tool for identification of genes involved in biotic and abiotic stress responses (van Verk *et al*., 2011).

According to the results, NPR1, 2, 3 and 4, TGA2 and 3, NIMIN-2 and 3, and CUL3A, showed physical interaction with each other while only PR1, 2, 3 and 4, TGA3 and CUL3A are expressed together (Fig. 7 and 8). Also, NPR genes including NPR1, 2, 3, 4, 5 and 6 showed common protein domains (Fig. 9). Analysis of NPR protein structure in NCBI-Conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed that NPR1, 2, 3 and 4 have three common domains including NPR1, Ankrin and BTB. NPR1 acts as a defense-related domain in *Arabidopsis*. Ankrin domain mediates the protein-protein interaction which is necessary for NPR protein family by their regulatory role through linking with other proteins (Alvo *et al*., 2010). Based on the hereby findings, NPR1, TGA2 and TGA3 are suggested to cooperate with each other in a common network (Fig. 10).

NPR1 interacts with TGA TFs, while they can target WRKY TFs. Moreover, WRKY can regulate the expression of NPR1 (Yu *et al*., 2001). NPR1-dependent crosstalk and TGA TFs can play a role in SA/JA crosstalk. Therefore, it can be hypothesized that NPR1-TFs interactions might play a role in the SA-mediated suppression of JA-responsive gene expression. Similarly, other studies showed that plant hormones biosynthesis and signaling pathways are controlled by different TFs (Jensen *et al*., 2013; Sakamoto *et al*., 2013).

MYB and WRKY families can likely be critical players in responses to biotic and abiotic stresses. The antagonistic effects of SA on the JA signaling pathways are partly dependent on the opposite effects of the transcription factors (Zander *et al*., 2010).

To study the expression pattern of *NPR1* and other related genes, expression analysis of co-expressed network genes was assesed based on *Arabidopsis* microarray data under salicylic acid treatment. These data were extracted from bar.utoronto.ca database. All genes involved in NPR1 regulatory network were up-regulated under SA treatment except for *Cul3A*. *Cul3A* encodes ligase enzymes which participate in degradation of regulatory proteins such as NPR1 (Thomman *et al*., 2005).

According to the results, the expression ratio of *CulA3* is down-regulated under salicylic acid treatment (Table 3). The highest up-regulation ratio belongs to NPR3 (Table 3). The NPR1 and TGA3 relative expression ratios showed the same value. The current findings are in agreement with the results of another study (Yu *et al*., 2001). Therefore, it seems that NPR1, 3, 4 and TGA3 genes are probably co-regulated. Thus, interaction and cooperation between these factors might fine-tune regulation of defense and immune responses through SA signaling pathway. In another study, PR proteins were up-regulated after wounding and drought stress; this can be due to the presence of stress-related elements in their promoters (Kaur *et al*., 2017).

Thus, the key role of promoters in controlling gene expression has been confirmed (Marino-Ramirez *et al*., 2009).

Fig. 1. Exon-intron arrangement of *NPR1* gene using the Phytozome database. Exons are represented by red boxes and connecting lines represent introns in *A. thaliana*

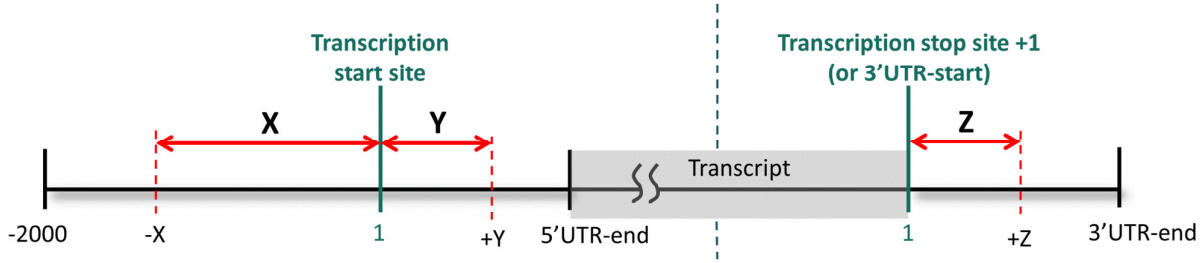

Fig. 2. Upstream and downstream coordinates of *A. thaliana NPR1* promoter obtained using Plant PAN

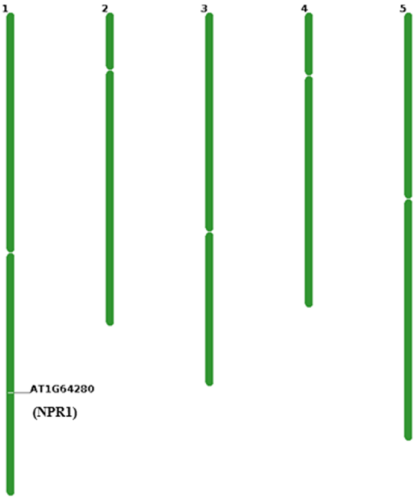

Fig. 3. Chromosomal distribution of *NPR1* gene in *A. thaliana*, constructed by Chromosome Map Tools available at TAIR

Fig. 5. The TFBS frequencies of MYB and WRKY in *NPR1* promoter of *A. thaliana*

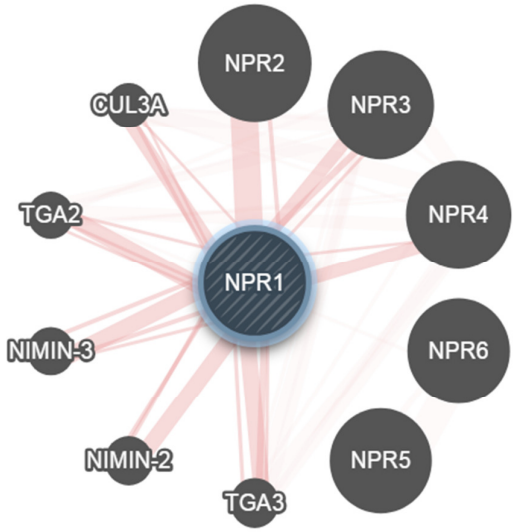

Fig. 7. Physically interacted proteins with *NPR1* identified using Genemania database

According to the database predictions, gene products are considered as physical interacted if they were found to interact in a protein-protein interaction according to any previous study or report specifically based on proteome-wide binary protein-protein interaction map

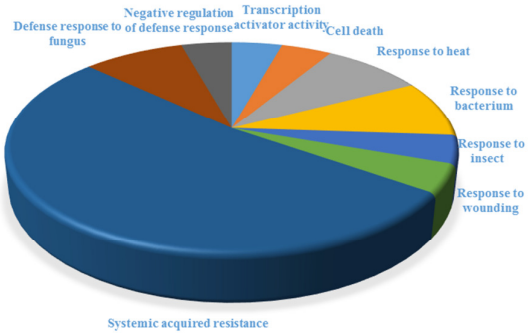

Fig. 4. Pie distribution of identified motifs of *A. thaliana* NPR1 obtained using AtPAN, based on their biological functions

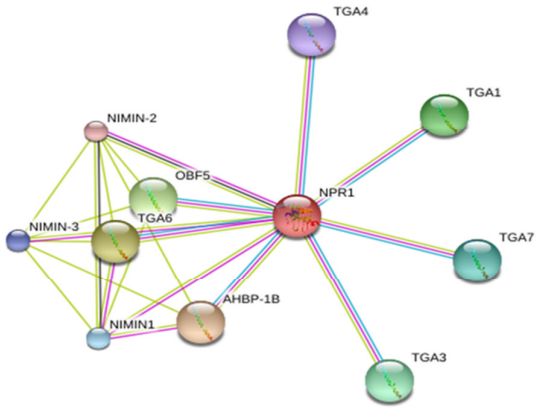

Fig. 6. Protein-protein interaction network analysis of *A. thaliana NPR1* obtained using STRING database

Protein nodes in the network are automatically highlighted in color. Colored lines between the proteins indicate the various types of interaction evidence. an edge may be drawn with up to 7 differently colored lines - these lines represent the existence of the seven types of evidence used in predicting the associations. Red line - indicates the presence of fusion evidence, Green line - neighborhood evidence, Blue line - co-occurrence evidence, Purple line - experimental evidence, Yellow line - text mining evidence, Light blue line - database evidence, Black line – co-expression evidence

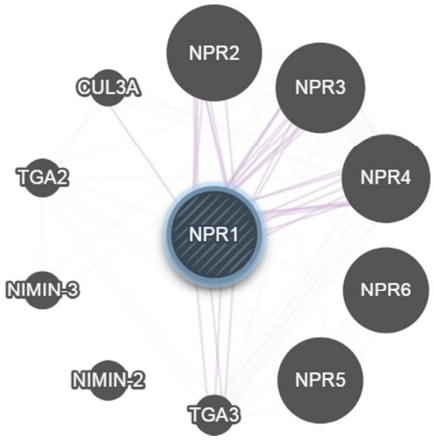

Fig. 8. Regulatory network of *NPR1* co-expressed genes using Genemania database

The co-expressed genes are linked together if their expression levels are similar across conditions in any reported gene expression study. The data are collected from the Gene Expression Omnibus (GEO)

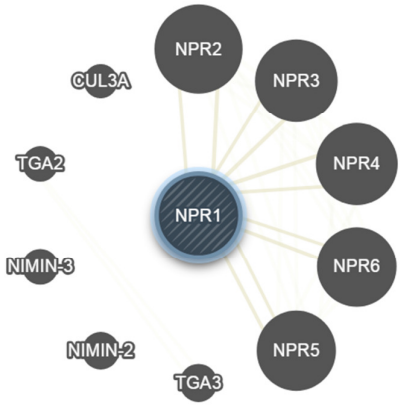

Fig. 9. Shared protein domains in *NPR1* related genes depicted using GeneMania based on PFAM and INTERPRO domain databases

According to the Genemania analysis, two gene products are linked as shared protein domains if they showed same protein domains

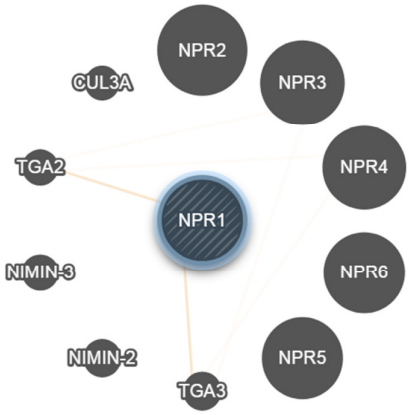

Fig. 10. The genes predicted to have functional relationships with *NPR1* gene using Genemania

Two proteins are predicted to interact if their orthologs are known to interact in another organism

Table 1. Different functional groups in *NPR1* regulatory network depicted using Genemania database. The identified genes in NPR1 gene regulatory network were categorized as different functional groups based on their functions in *Arabidopsis*

Functional group	Genes
Defense response to bacterium	TGA3,NPR3,NPR1,NPR4,TGA1,
Response to salicylic acid	TGA3,NPR1,NPR4,
Regulation of responses to stresses (biotic and abiotic)	TRX5,NPR1,NPR3,NPR4
Response to fungi	NPR1,NPR3,NPR4
Immune responses	TGA3,NPR1,NPR3, NPR4

Table 2. TFBS of MYB and WRKY in *NPR1* promoter of *A. thaliana*

Factor	Location	Signal sequence
MYBST1	295 (-)	GGATA
MYBPLANT	1,152 (-)	MACCWAMC
MYBCORE	1,266 (+)	CNGTTR
MYB1AT	1,431 (+)	WAACCA
MYB2CONSENSUSAT	1,441 (-)	YAACKG
MYBCORE	1,441 (+)	CNGTTR
MYBCORE	1,509 (+)	CNGTTR
WRKY71OS	766 (+)	TGAC
WRKY71OS	1,293 (+)	TGAC
WRKY71OS	1,387 (+)	TGAC
WRKY71OS	1,392 (+)	TGAC
WRKY71OS	1,408 (-)	TGAC

Table 3. Expression analysis of *NPR1* co-expressed genes under salicylic acid treatment. The relative expression ratios are shown as $\log_2$ (treated/control) of three biological replicates

Gene	Relative expression ratio
NPR1	1.6
NPR2	0.11
NPR3	3.2
NPR4	2.1
TGA3	1.6
Cul3A	-0.7
WRKY1	0.14

Conclusions

Taken together, identification of interacting proteins constitutes an important step in understanding the mechanism of their activation in defense signaling pathways and their roles in regulating the expression of related genes. Therefore, such analysis can provide new tools for the plant genetic engineering to protect crops against biotic and abiotic stresses.

Acknowledgements

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http://bar.utoronto.ca database

http://bioinformatics.psb.ugent.be/webtools/plantcare/html/bar.utoronta database

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https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

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