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-expressor 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 (Li 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/plantcare/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 analysis 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, 1998).

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...
In *s*ilico 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 PRI, 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 mediate 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 assessed 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, CUL5A 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 CUL3A 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. 3. Chromosomal distribution of NPR1 gene in *A. thaliana*, constructed by Chromosome Map Tools available at TAIR.

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

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

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. 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. 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.

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.

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defense response to bacterium</td>
<td>TGA3,NPR3,NPR4,TGA1,</td>
</tr>
<tr>
<td>Response to salicylic acid</td>
<td>TGA3,NPR1,NPR4</td>
</tr>
<tr>
<td>Regulation of responses to stresses (biotic and abiotic)</td>
<td>TRX5,NPR1,NPR3,NPR4</td>
</tr>
<tr>
<td>Response to fungi</td>
<td>NPR1,NPR3,NPR4</td>
</tr>
<tr>
<td>Immune responses</td>
<td>TGA3,NPR1,NPR3,NPR4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Factor</th>
<th>Location</th>
<th>Signal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYB1ST1</td>
<td>295 (-)</td>
<td>GGATA</td>
</tr>
<tr>
<td>MYBPLANT</td>
<td>1,152 (-)</td>
<td>MACCWAMC</td>
</tr>
<tr>
<td>MYBCORE</td>
<td>1,266 (+)</td>
<td>CNGTTR</td>
</tr>
<tr>
<td>MYB1AT</td>
<td>1,431 (+)</td>
<td>WAACCA</td>
</tr>
<tr>
<td>MYB2CONSENSUSAT</td>
<td>1,441 (-)</td>
<td>YAACKG</td>
</tr>
<tr>
<td>MYBCORE</td>
<td>1,441 (+)</td>
<td>CNGTTR</td>
</tr>
<tr>
<td>MYBCORE</td>
<td>1,509 (+)</td>
<td>CNGTTR</td>
</tr>
<tr>
<td>WRKY71OS</td>
<td>766 (+)</td>
<td>TGAC</td>
</tr>
<tr>
<td>WRKY71OS</td>
<td>1,293 (+)</td>
<td>TGAC</td>
</tr>
<tr>
<td>WRKY71OS</td>
<td>1,387 (+)</td>
<td>TGAC</td>
</tr>
<tr>
<td>WRKY71OS</td>
<td>1,392 (+)</td>
<td>TGAC</td>
</tr>
<tr>
<td>WRKY71OS</td>
<td>1,408 (-)</td>
<td>TGAC</td>
</tr>
</tbody>
</table>

Table 3. Expression analysis of NPR1 co-expressed genes under salicylic acid treatment. The relative expression ratios are shown as log2 (treated/control) of three biological replicates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPR1</td>
<td>1.6</td>
</tr>
<tr>
<td>NPR2</td>
<td>0.11</td>
</tr>
<tr>
<td>NPR3</td>
<td>3.2</td>
</tr>
<tr>
<td>NPR4</td>
<td>2.1</td>
</tr>
<tr>
<td>TGA3</td>
<td>1.6</td>
</tr>
<tr>
<td>Cal3A</td>
<td>-0.7</td>
</tr>
<tr>
<td>WRKY1</td>
<td>0.14</td>
</tr>
</tbody>
</table>
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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https://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp
http://bar.utoronto.ca database
http://www.genemania.org
http://plantpan2.itps.ncku.edu.tw/promoter.php
http://www.phytozome.net/
http://string-db.org