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Original Research Article

A simple method for screening of plant NBS-LRR genes that confer a hypersensitive response to plant viruses and its application for screening candidate pepper genes against *Pepper mottle virus*

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Plant NBS-LRR genes are abundant and have been increasingly cloned from plant genomes. In this study, a method based on agroinfiltration and virus inoculation was developed for the simple and inexpensive screening of candidate *R* genes that confer a hypersensitive response to plant viruses. The well-characterized resistance genes *Rx* and *N*, which confer resistance to *Potato virus X* (PVX) and tobamovirus, respectively, were used to optimize a transient expression assay for detection of hypersensitive response in *Nicotiana benthamiana*. Infectious sap of PVX and *Tobacco mosaic virus* were used to induce hypersensitive response in *Rx*- and *N*-infiltrated leaves, respectively. The transient expression of the *N* gene induced local hypersensitive response upon infection of another tobamovirus, *Pepper mild mottle virus*, through both sap and transcript inoculation. When this method was used to screen 99 candidate *R* genes from pepper, an *R* gene that confers hypersensitive response to the potyvirus *Pepper mottle virus* was identified. The method will be useful for the identification of plant *R* genes that confer resistance to viruses.

1. Introduction

Most plant viruses have the ability to systemically infect their hosts. This process involves a series of interactions between the genome and proteins of the virus and proteins of the host (Schoelz, 2006). To systemically infect the host after initial invading a small number of its cells, viruses express their proteins, replicate their genomes, and move from invaded cells to other cells (Pallas and García, 2011). In turn, plants have developed a variety of defense mechanisms to interrupt viral infection and prevent the disease development. RNA silencing, for example, degrades virus RNA and helps plants recover after virus infection (Ratcliff et al., 1997). Some viruses, however, have adapted to RNA silencing and are able to counter this defense by deploying suppressors of RNA silencing (Kasschau and Carrington, 1998).

To prevent such adapted viral pathogens, plants have evolved additional defense mechanisms that are mediated by resistance genes (Kang et al., 2005). Such genes are classified into two groups: (i) recessive resistance genes provide a passive resistance in which the pathogen is unable to utilize the host cell machinery (Diaz-Pendon et al., 2004), and (ii) dominant resistance genes (*R* genes) confer an active resistance by encoding resistance proteins that recognize pathogen effectors or so-called avirulence (*Avr*) factors (Chisholm et al., 2006). Most *R* genes encode proteins with a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) region (Ellis et al., 2000). *R* genes can generate extreme resistance, in which the pathogen is localized to a single cell, or a hypersensitive response, in which the pathogen is localized to a few cells (Carr et al., 2010). Hypersensitive response is easier to recognize and quantify than extreme resistance because it produces visible symptoms and distinctive histological and biochemical changes (Heath, 2000; Richael and Gilchrist, 1999).

Agrobacterium-mediated transient expression enables the rapid accumulation of a large amount of recombinant proteins in plants (Horsch and Klee, 1986). Using this approach and hypersensitive response as an indicator, researchers have screened for plant *R*

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genes. The *Potato virus X* (PVX) resistance gene *Rx2* from *Solanum acaule*, for example, was isolated from among 200 *Rx1* homologues by agroinfiltration in transgenic *Nicotiana tabacum* leaves expressing PVX coat protein (Bendahmane et al., 2000). In another example, candidate resistance genes to *Phytophthora infectans* were screened by transient expression in *Nicotiana benthamiana* (Li et al., 2011). Transient expression is widely used to screen candidate genes because it is fast, inexpensive, and reproducible.

In this study, we developed a method for the screening of genes that confer resistance to viruses based on virus inoculation and transient over-expression of candidate *R* genes by agroinfiltration in *N. benthamiana*. The advantages of this method are: (i) it does not require a transgenic host plant that expresses an elicitor, and (ii) it can be carried out for viruses with unknown elicitors. With this method, an *R* gene that induces hypersensitive response to *Pepper mottle virus* (PepMoV) in pepper was isolated.

2. Materials and methods

2.1. Plant virus isolates and generation of PMMoV transcripts

The viruses used in this study include PVX USA strain (Park and Kim, 2006), *Tobacco mosaic virus* (TMV) U1 strain (V01408), *Pepper mild mottle virus* (PMMoV) Kr strain (AB216003),and PepMoV Kr strain isolate 134 (EU586123). Each virus isolate was obtained from a local lesion host at 7–14 days post-inoculation and was maintained in *N. benthamiana*.

To make PMMoV transcripts, total RNAs were extracted from PMMoV-infected *N. benthamiana* leaves using Isol-RNA lysis reagent (5Prime, Hamburg, Germany) according to the manufacturer's instructions. PMMoV cDNA was amplified by RT-PCR using GoScript Reverse Transcriptase (Promega, WI, USA) and LA *Tag* DNA polymerase (Takara, Tokyo, Japan). Sequences of the primers were designed to produce a T7 promoter and *Sma*I restriction enzyme (RE) site on the corresponding 5′ and 3′ end of the PCR products, respectively (Table 1). Amplified PMMoV dsDNA was then cloned into the pGEM®-T Easy Vector (Promega) and linearized by *Sma*I (Takara) for *in vitro* transcription. PMMoV transcripts were synthesized using T7 RNA polymerase (Takara) and G(5′)ppp(5′)G RNA cap structure analog (NEB, Hertfordshire, England) as described previously (Ryu, 2005).

2.2. Construction of vectors for transient expression of the resistance genes Rx and N

For amplification of the *Rx* gene, chromosomal DNAs were extracted from leaves of the transgenic *N. benthamiana*, which contained the cDNA sequence of *Rx*, using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The *Rx* gene was amplified by PCR using EX*Tag* DNA polymerase (Takara). Sequences of the primers were designed so that the *Mlu*I RE site was incorporated into the two ends of the *Rx* gene (Table 1). The amplified *Rx* gene was digested by *Mlu*I and then cloned into the modified pPZP212 binary vector (Fig. 1A) (Park et al., 2009) using *T4* DNA ligase (Promega).

For amplification of the *N* gene and its regulatory sequences, total RNAs and chromosomal DNAs were extracted from *Nicotiana glutinosa* leaves using Isol-RNA Lysis Reagent and the DNeasy Plant Mini Kit, respectively. From the total RNA, full-length cDNA

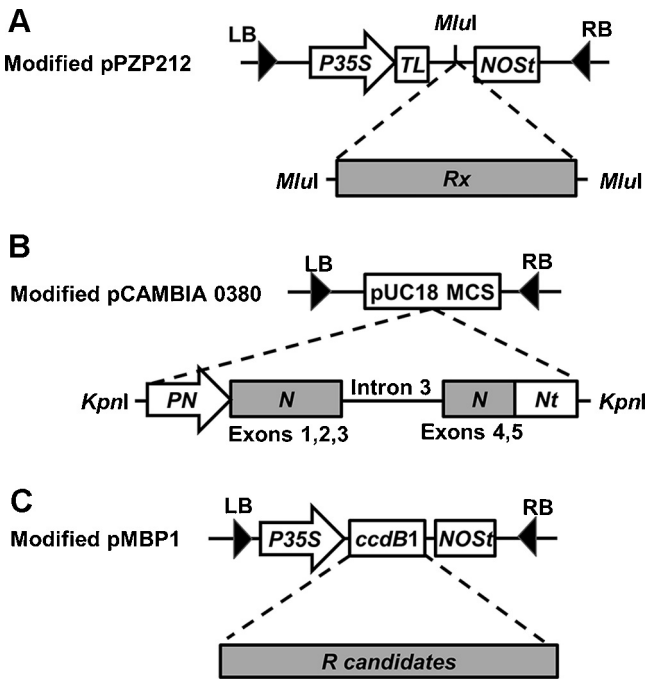

Fig. 1. Schematic representation of constructs used for agrobacterium-based transient expression of *R* genes and candidates. All constructs, which were cloned in binary Ti-vectors, were flanked by a left border (LB) and a right border (RB) of T-DNA. (A) *Rx* gene was cloned into modified pPZP212 vector under control of CaMV 35S promoter (*P35S*), *Tobacco etch virus* leader sequence (*TL*) and NOS terminator (*NOSt*). (B) The cassette of *N* gene and its regulatory sequences, which were cloned in modified pCAMBIA 0380 binary vector, included *N* natural promoter (*PN*), *N* coding sequences (exons 1–5), *N* intron 3, and *N* natural terminator (*Nt*). The restriction enzyme cleavage sites used to make the constructs are shown. (C) By ligation-independent cloning, *R* candidates were individually cloned into modified pMBP1 in which the cytotoxic *ccdB1* gene was used for selection.

Table 1
Primers used for cloning of candidate R genes, PMMoV cDNA, the *Rx* gene and the *N* gene.

Primer name	Primer sequence (5′ → 3′)	Amplified sequence
Rpi-blb2 ortholog Fw	CCAATCCCTCTACGGATGGAAAAACGAAAAGATAWTG	*Rpi-blb2* orthologs
Rpi-blb2 ortholog Rv	TATCCTCCTACGGTCCTACTTAAATAAGGGGATATG	*Rpi-blb2* orthologs
T7 promoter-PMMoV Fw[a]	TAATACGACTCACTATAGTAAATTTTTCACAATTTAACA	PMMoV
PMMoV SmaI Rv[b]	CCCGGGTGGGCCGCTACCCGCGGTTC	PMMoV
Rx *Mlu*I Fw	CGACGCGTATGGCTTATGCTGCTGTTAC	*Rx*
Rx *Mlu*I Rv	CGACGCGTCTACTCGCTGCAGAAAAATA	*Rx*
N Fw	ATGGCATCTTCTTCTTCTTCTTCTAG	*N*
N Rv	TCACCCATTGATGAGCTCATAAAAGG	*N*
N Promoter *Sac*II-*Kpn*I Fw	GGCCGCGGTACCCAAGTTGACAGTTACCTCTTTCTCCTCC	*N* promoter
N 492 Rv[c]	GAGCCTTTGAGATTGGCCGCT	*N* promoter
N 6039 Fw	TGTCGCGCATGACCCAGAAACT	*N* terminator
N Terminator *Mlu*I-*Kpn*I Rv	CGACGCGTGGTACCTAGAATTTGTCAACAATGTTAGCTCA	*N* terminator
N 2512 Fw	ACGACATAAGGGAAGGGGGCA	*N* Intron 3
N 5634 Rv	GGTAGCTGTGTAAGCCTCTGGC	*N* Intron 3

[a] Fw stands for forward primer, and Rv stands for reverse primer.
[b] The fused enzyme sites are included in primer names, with the underlined sequences.
[c] The number indicates the start position of the primer on the genomic sequence of the *N* gene.