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INDUCING GENE SILENCING USING A CONSTRUCT CAPABLE OF TRANSCRIPTION FROM BOTH DIRECTIONS*

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Abstract

A DNA fragment of a *Potato leaf roll virus* full-length cDNA (part of the virus replicase gene) was amplified by PCR. The resulting fragment was inserted as a sense fragment into a vector flanked by a promoter at both sides. Tobacco and potato plants were transformed with this construct. The resulting transgenic plants were tested for resistance to PLRV. Fifty-nine percent of the transgenic tobacco lines showed lower titer of PLRV. It was also shown that the resistant transgenic tobacco lines express both sense and antisense mRNAs of the transgene. Using graft-inoculation method, no PLRV resistant transgenic potato line was identified. This is the first report of testing a single construct capable of expressing mRNA from both directions. It seems that the simultaneous expression of sense and antisense mRNA followed by hybridization and dsRNA formation have triggered post transcriptional gene silencing in transgenic tobacco lines.

Keywords: Simultaneous expression, Sense, Antisense, Gene silencing, Transcription.

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Introduction

A highly efficient way of conferring a form of gene silencing-mediated resistance against plant viruses is to transform plants with transgenes that simultaneously express sense and antisense mRNA or hairpin loop structures capable of dsRNA formation (Waterhouse *et al*. 1998, Wang *et al*. 2000). It has been suggested that dsRNA induces a mechanism that will degrade single stranded RNAs containing the same sequence in the genomic RNA of the invading virus and it acts as a key initiator molecule (Wang *et al*. 2000; Wesley *et al*. 2001). The dsRNA molecules necessary for activation of the degradation system are provided in various ways including exogenous delivery, *in vivo* production by host- or virus-encoded RNA-dependent RNA polymerase (RdRp), and by transcription either through inverted repeats or from converging promoters (Moissiard & Voinnet, 2004).

Waterhouse and colleagues (1998) reported that transformation of plants with the virus-derived sequences or reporter genes that produced RNAs capable of duplex formation conferred virus immunity or gene silencing to the transformed plants. They showed that transgenic tobacco plants co-expressing sense and antisense transcripts of the PVY protease gene were much more resistant to PVY than those that expressed either sense or antisense mRNA of the transgene alone. Nikan & Barker (2007) by crossing the transgenic tobacco plants expressing sense mRNA of part of the PLRV replicase gene with those expressing antisense mRNA of the same transgene produced transgenic plants capable of simultaneously expressing both sense and antisense mRNAs of the transgene. They reported that 50% of the transgenic lines co-expressing sense and antisense orientations of the transgene were resistant to PLRV, whereas only 25% of those expressing either sense or antisense mRNA alone were resistant. Waterhouse and colleagues (1998) suggested that in some cases, dsRNA could be produced by a single sense transgene being integrated into the plant genome such that its 3´ end is adjacent to an endogenous promoter, thus producing an antisense mRNA that could hybridize with the sense transgene mRNA to form a duplex. To produce a transgenic plant that can simultaneously express both sense and antisense mRNAs of the transgene, it was proposed to insert a gene (part of the PLRV ORF2 sequence) into a transformation vector flanked by a promoter at either side and to transform plants with that vector.

Materials and Methods

The cloning vector construct pJB1 (Nikan & Barker, 2007) contains a DNA fragment (697bp in size) that comprises part of the PLRV OFR2 sequence. The fragment has an *Xba*I site at one end and a *Kpn*I site at the other end. Using the *Xba*I and the *Kpn*I restriction enzymes, the interested fragment was cut from pJB1 and ligated into the pKDW20 vector (H. Barker, unpublished results) which had also been cut by the same enzymes. The pKDW20 vector has two promoters, the *Cauliflower mosaic virus* (CaMV) 35S promoter in sense orientation and the *Strawberry vein banding virus* (SVBV) 35S promoter in antisense orientation, flanking either side of its polylinker site (Fig.1). The constructed vector was named pPLRV-JB/S+AS and cloned in *Escherichia coli*. Size and direction of the inserted fragment into the vector pPLRV-JB/S+AS was checked by PCR using different sets of primers and by digestion with appropriate restriction enzymes (H Barker, unpublished results). Transformation of *A. tumefaciens* with pPLRV-JB/S+AS plasmid was carried out using the tri-parental method (Armitage *et al*. 1988). Presence of the fragment of interest in selected colonies was tested by PCR (Fig.2).

Transformation of *Nicotiana tabacum* var. Samsun NN and potato (cultivar Maris Piper) with the plasmid pPLRV-JB/S+AS was carried out as described by Horsch *et al*. (1985) and Rooke & Lindsey (1998), respectively. Screening of the transgenic tobacco and potato plants that expressed mRNAs of the transgene, determining the reaction of selected transgenic tobacco lines to PLRV inoculation, estimating the reduction of PLRV titers in resistant transgenic tobacco lines, finding the expression status of the transgene in transgenic tobacco lines and statistical analysis of the data obtained from resistance tests on transgenic tobacco lines were carried out as described by Nikan & Barker (2007).

The reaction of transgenic potato plants to PLRV inoculation was performed by graft-inoculation of four plantlets of each independent transgenic potato line and of the wild type plants propagated by stem cutting. Three weeks later, leaf

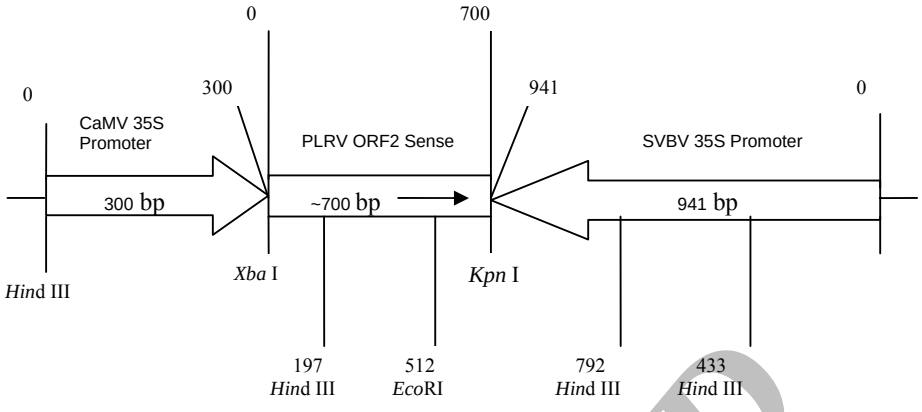

Fig. 1 Location of 697bp fragment of PLRV cDNA between two promoters in pPLRVJB/S+AS vector

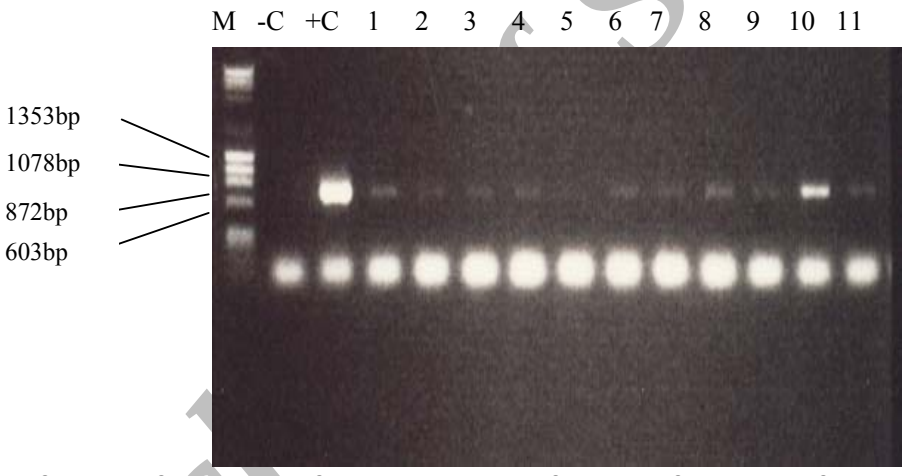

Fig. 2 Amplification of the target fragment by PCR from purified DNA of *A. tumefaciens* cells transformed with pPLRV-JB/S+AS, M: Marker DNA; -C: blank control; +C: positive control; 1 - 11: selected colonies.

samples taken from side shoots developed on the inoculated plants were tested by ELISA.

Results and Discussion

Comparisons of the means of ELISA absorbance values (an indication of resistance to virus accumulation) for PLRV-JB/S+AS transgenic tobacco lines showed that, 16 of 27 (59%) of the lines had significantly lower ELISA absorbance values than inoculated wild type controls and therefore were considered to be resistant to PLRV accumulation (Table 1). However, comparisons of the proportions of infected plants (an indication of resistance to virus infection) showed that among those lines of transgenic tobacco plants that were tested more than once, only line K27 was resistant to PLRV infection (Fig.3).

The results of quantitative ELISA tests indicated that the infected plants of the resistant transgenic tobacco lines K27, K35 and K23 had 25%, 33% and 50%, respectively, of the PLRV titer estimated in infected plants of their corresponding wild type controls. Results of RT-PCR for determining the expression status of transgene mRNAs in PLRV-JB/S+AS transgenic tobacco lines revealed that the examined PLRV-JB/S+AS transgenic lines (K27 and K29)

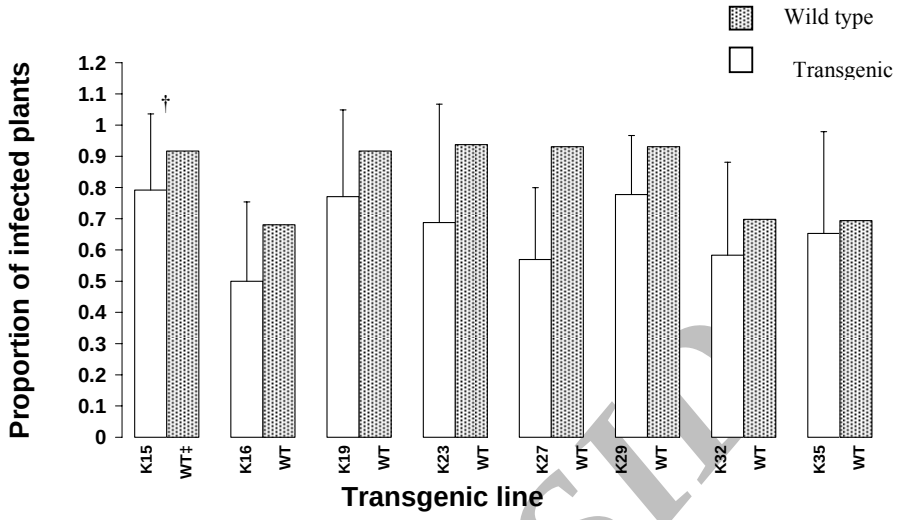

Fig. 3 Proportion of infected plants in selected PLRV-JB/S+AS transgenic lines compared to wild type control

†Error bars represent the Least Significant Difference (LSD, *P* = 0.05)

‡ Wild type control inoculated at the same time as the transgenic line

expressed both sense and antisense mRNAs of the transgene. One transgenic line with resistance to PLRV infection was found in the S_1 generation of the PLRV-JB/S+AS tobacco transformants (line K27). Likewise, Nikan & Barker (2007) found three transgenic resistant lines to PLRV infection in the F_1 generation of PLRV-JB/S x AS.

Nikan & Barker (2007) also reported that nearly 25% of the transgenic lines expressing either sense or antisense mRNA of the transgene alone and 50% of those co-expressing sense and antisense orientations of the transgene (progenies of the sense x antisense crosses) were resistant to PLRV accumulation. We found a higher percentage of PLRV accumulation-resistant lines (59%) among the S_1 generation of PLRV-JB/S+AS transgenic tobacco lines. The results of our experiments are also in agreement with those obtained by Waterhouse *et al.* (1998).

No resistant line was identified among our PLRV-JB/S+AS ^{potato} transgenic lines transformed with the same vector (pPLRV-JB/S+AS). Transgenic potato lines resistant to PLRV accumulation due to sense and antisense RNA-mediated resistance was found by Kawchuk *et al*. (1991) who inoculated their transgenic potato plants by using viruliferous aphids. We inoculated our transgenic potato plants by graft inoculation which, compared to aphid inoculation, introduces relatively more virus particles directly into the phloem tissues where the replication of PLRV takes place. This could have overcome any resistance that might have been induced due to transformation with the transgene.

The transgene used in our transgenic plants neither has a start codon nor is a complete gene. Thus, it is very unlikely that transcription of the transgene results in translation of a functional protein. By means of strand specific RT-PCR tests in which either the forward or reverse primer alone was used at the cDNA synthesis stage (reverse transcriptase) we showed that in the PLRV resistant transgenic tobacco lines (e.g. line K27) both sense and antisense mRNAs of the transgenes were expressed. Therefore, the transgenic resistance observed in our transgenic plants is probably RNA-based, a phenomenon that has been reported in a variety of transgenic plants (Kawchuk *et al*., 1991; Barker *et al*., 1993; Presting *et al*., 1995; Tacke *et al*., 1996; Waterhouse *et al*., 1998; Nikan & Barker, 2007).

The effectiveness of RNA-mediated resistance has been shown to correlate with high transgene copy number in the plant genome (Lindbo &

Table 1. Means of ELISA absorbance values (A_{405}) of inoculated PLRV-JB/S+AS transgenic tobacco lines

Line	Mean[a] of ELISA absorbance values		S.E.D[c]
	Transgenic	WT[b]	
K2	0.683 *	0.905	0.0958
K3	0.210 ns	0.560	0.2460
K4	0.720 ns	1.130	0.2230
K5	1.130 ns	0.930	0.3560
K7	0.997 ns	0.905	0.278
K8	0.756 ns	0.905	0.734
K9	0.620 ns	1.100	0.2580
K10	0.702 *	0.905	0.1010
K13	0.711 ns	0.923	0.1133
K15	0.785 ***	1.480	0.1434
K16	0.160**	0.520	0.0268
K19	0.790 ***	1.496	0.1328
K20	0.741*	0.905	0.0617
K23	0.448 ***	0.923	0.0879
K24	0.120 ns	0.560	0.2370
K25	0.610 ***	0.923	0.0746
K26	1.140 ns	1.160	0.1750
K27	0.578 ***	1.490	0.1294
K28	0.500 ns	1.110	0.3420
K29	0.558 ***	1.480	0.1141
K30	0.100 ns	0.550	0.2490
K31	0.040*	0.540	0.210
K32	0.170 *	0.300	0.0140
K33	0.698 *	0.923	0.0924
K34	0.440 ***	1.140	0.2040
K35	0.160 ***	1.210	0.0632
K36	0.576 ***	0.923	0.0797

[a] The means have been calculated following statistical analysis of the raw data

Dougherty, 1992). Nikan & Barker (2007) argued that in their transgenic plants, all S_1 generation plants (progenies of the self-fertilized PLRV-JB/S or PLRV-JB/AS) and F_1 generation of the PLRV-JB/S x AS transgenic lines would have the same chance of receiving one or more copies of the transgenes. This has also been reported by Waterhouse *et al*. (1998) who did not find any convincing correlation between high transgene copy number and immunity to PVY in the tobacco lines containing two transgenes, sense and antisense, brought together by crossing. Indeed, they found 4 of 11 PVY immune plants each contained only a single copy of the transgene. The hypothesis can also be extended to the progenies of PLRV-JB/S+AS self-fertilized lines we tested.

Waterhouse *et al.* (1998) compared PVY resistance in progenies obtained from the PVY susceptible Pro-s (sense) x PVY susceptible Pro-a/s (antisense) crosses with resistance in progenies of the self-fertilized parents, Pro-s x Pro-s and Pro-a/s x Pro-a/s. They concluded that it was the expression of the sense and antisense mRNA of the transgene together in the same plant, and not the genomic arrangement of the transgenes that induced the PVY immunity. This is because a proportion of the progenies of each Pro-s x Pro-a/s cross was immune to PVY whereas all of other progenies were susceptible. Waterhouse *et al*. (1998) concluded that their results were due to the fact that some progenies of each Pro-s x Pro-a/s cross should have both Pro-s and Pro-a/s genes but at different locations in the genome. Likewise, the increased frequency of resistant lines among the progenies of the sense x antisense crosses obtained by Nikan & Barker (2007) and those of our PLRV-

JB/S+AS self-fertilized lines can be ascribed to the expression of the sense and antisense mRNA of the transgene together in the same plant that induced the PLRV resistance. It was shown that some of our PLRV resistant transgenic tobacco plants expressed both sense and antisense mRNAs of the transgene.

The transgene PLRV-JB/S+AS was designed to express both sense and antisense mRNAs of the virus sequence it carries. The basis of the design relates to the proposal of Waterhouse *et al*. (1998) who suggested that in some cases, although rarely, dsRNA could be produced by a single sense transgene being integrated into the plant genome such that its 3´ end is adjacent to an endogenous promoter. The simultaneous expression of both sense and antisense mRNAs from the transgene by some of our transgenic lines (e.g. line K27) transformed with the vector construct pPLRV-JB/S+AS indicated that this construct was functional. Some lines (e.g. line K27) were resistant to PLRV accumulation on all the occasions they were tested but some showed no resistance in some replicate tests. These variations which could have been caused by inevitable differences in environmental conditions under which each test was conducted (i.e. the effects of Gene x Environment, G x E) show the importance of considering many factors when developing transgenes for use in crop plants where stability of phenotypic expression will be important.

The development of transgenic resistance due to the transformation of tobacco plants with the transgene PLRV-JB/S+AS is the first example of resistance conferred by this type of transgene. Recent preliminary evidence has shown that resistance to PVY can also be developed based on the same approach (H. Barker unpublished results). Simultaneous expression of sense and antisense mRNAs from such a transgene is a novel method for developing PTGS-based resistance which compared to other methods could save time and reduce the costs of cloning and transformation.

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