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BRIEF COMMUNICATION

# Resistance to *Grapevine leafroll associated virus-2* is conferred by post-transcriptional gene silencing in transgenic *Nicotiana benthamiana*

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**Abstract** *Grapevine leafroll-associated virus-2* (GLRaV-2) is an important component of the leafroll disease complex in grapevine. We have previously sequenced the GLRaV-2 genome and identified the coat protein (CP) gene. The objective of this study is to test the concept of pathogen-derived resistance against a closterovirus associated with grapevine leafroll disease. Because GLRaV-2 is capable of infecting *Nicotiana benthamiana*, we decided to test the concept on this herbaceous host. Thirty-seven $T_0$ transgenic *N. benthamiana* plants expressing the GLRaV-2 CP gene were regenerated following *Agrobacterium*-mediated transformation. Disease resistance was evaluated in greenhouse-grown $T_1$ and $T_2$ plants by mechanical inoculation with GLRaV-2. Although all the inoculated non-transgenic plants showed symptoms 2–4 weeks post inoculation, various numbers of transgenic plants (16–100%) in 14 of 20 $T_1$ lines tested were not infected. In these resistant plants, GLRaV-2 was not detectable by enzyme linked immunosorbent assay. Although virus resistance was confirmed in $T_2$ progenies, the percentage of resistant plants was generally lower (0–63%) than that of the corresponding $T_1$ lines (0–100%). Northern blot and nuclear run-off results showed that virus resistance in the transgenic plants was consistently associated with the low level of transgene RNA transcript suggesting a post-transcriptional gene silencing. The success of pathogen-derived resistance to GLRaV-2 in transgenic *N. benthamiana* plants represents the first step towards eventual control of the leafroll disease in grapevines using this strategy.

**Keywords** *Closteroviridae* · Coat protein · Pathogen derived resistance · Grapevine · *Vitis vinifera*



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## Introduction

Leafroll is an important viral disease of grapevines worldwide. The disease affects both grape yield and its sugar content, thereby altering wine quality. Etiological studies on the leafroll disease complex

are complicated by the fact that a total of nine serologically distinct viruses in the family *Closteroviridae* are associated with the disease (Alkowni et al. 2004; Fauquet et al. 2005). One major virus component in this disease complex is *Grapevine leafroll associated virus-2* (GLRaV-2), a species of genus *Closterovirus*, family *Closteroviridae*. With 16,493 nt in its genome, GLRaV-2 is composed of nine open reading frames (ORF) organized in a typical closterovirus arrangement (Dolja et al. 1994; Zhu et al. 1998; Meng et al. 2005). The coat protein (CP) gene is encoded at the 3′ terminal portion of the genome and is likely expressed via a subgenomic RNA (Zhu et al. 1998).

Since the first report that transgenic tobacco plants expressing the CP gene of *Tobacco mosaic virus* (TMV) confer resistance to TMV infection (Powell-Abel et al. 1986), there have been numerous examples on the successful application of the concept of pathogen-derived resistance (PDR) against different types of plant viruses (Prins 2003; Tenllado et al. 2004). However, success in using the PDR strategy to confer resistance to closteroviruses has been limited. Much of its progress is concentrated with *Citrus tristeza virus* (CTV) (Batuman et al. 2006; Fagoaga et al. 2006).

Although grapevine transformation with virus-derived sequences has been successful (Krastanova et al. 1995; Mauro et al. 1995; Xue et al. 1999; Golles et al. 2000; Radian-Sale et al. 2000; Spielmann et al. 2000; Gambino et al. 2005; Maghuly et al. 2006), testing virus resistance in grapevine is complicated by the lack of efficient mechanical or vector transmission. Evaluation of virus resistance by symptom expression in grapevine would take years to complete. GLRaV-2 is the only one of nine grapevine leafroll associated viruses that is capable of infecting an herbaceous host, *Nicotiana benthamiana* (Goszczynski et al. 1996). The objective of this study is to test the concept of pathogen-derived resistance against a closterovirus associated with grapevine leafroll disease in *N. benthamiana*. Here we describe the development of transgenic *N. benthamiana* plants expressing the GLRaV-2 CP gene and evaluation of transgenic plants against GLRaV-2 infection. We report that resistance to GLRaV-2 in transgenic *N. benthamiana* is via the mechanism of post-transcriptional gene silencing (PTGS).

## Methods, results and discussion

Double-stranded RNA (dsRNA) purified from GLRaV-2 (isolate PN) infected grapevine tissue (Zhu et al. 1998) was used as template for CP gene amplification. The complete CP gene plus 56 nt downstream region was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with primers CP-96F (cggaattccaATGGAGTTGATGTCCGACAG, nt positions 13,084–13,103), and CP-96R (agcggatccatggCAGATTCGTGCGTAGCAGTA at nt 13,714–13,733). An *Nco* I restriction site was introduced at the beginning of each primer (underlined) to facilitate the cloning process. The RT-PCR amplified product was purified from a low melting temperature agarose gel, digested with *Nco* I, and cloned into plant expression vector pEPT8 (Ling et al. 1997). The sequence and orientation of the recombinant CP gene were then confirmed by enzyme restriction analysis and sequencing (data not shown). The resulting expression-cassette, which consisted of a double enhanced (Enh) *Cauliflower mosaic virus* (CaMV) 35S-promoter (35S-P), 5′ untranslated leader of *Alfalfa mosaic virus* RNA4 (AMV 5′UT), CP gene of GLRaV-2 (GLRaV-2 CP) and 3′ terminal untranslated sequence of CaMV 35S (35S-T), was digested with *Hind* III, isolated from a low melting point temperature agarose gel and cloned into *Hind* III restricted binary vector pGA482GG (Quemada et al. 1991). The resulting binary vector, pGA482GG/EPT8CP-GLRaV2, was transformed by electroporation into *Agrobacterium tumefaciens* strain LBA4404. Agrobacterium-mediated transformation and regeneration of transgenic *N. benthamiana* plants were performed essentially as described by Horsch et al. (1985).

Upon *Agrobacterium*-mediated transformation, 37 kanamycin resistant *N. benthamiana* plants with positive reaction for neomycin phosphotransferase II (NPTII) protein (5′ Prime to 3′ Prime, Inc. Boulder, CO) were considered transgenics. Five other plants with negative NPTII reaction were regarded as non-transgenics. Presence of CP gene of GLRaV-2 (663 bp) in transgenic plants was confirmed by PCR with GLRaV-2 specific primers (CP-96F and CP-96R) (data not shown). CP expression in transgenic plants was analyzed by indirect ELISA with the antibody prepared against a recombinant CP of GLRaV-2 (Ling et al. 2007). Results showed that

transgenic CP expression in $T_0$ transgenic plants was extremely low to non-detectable (OD405 nm: 0.12–0.13), similar to that of a non-transgenic healthy control (OD405 nm: ~0.12). Although Western blot detected the expected 22 kDa CP in GLRaV-2 infected samples, such product was not detectable in transgenic plants (data not shown). Nevertheless, $T_0$ plants were self-pollinated in greenhouse. Resistance screening experiments were conducted with $T_1$ and $T_2$ seedlings, respectively.

Prior to being tested for resistance, $T_1$ seedlings from 20 transgenic lines were screened for NPT II protein to identify transgenic from non-transgenic plants. The isolate 94/970 of GLRaV-2, which was originally identified and transmitted from grapevine to *N. benthamiana* in South Africa (Goszczynski et al. 1996), was used as inoculum. CP gene sequence of the isolate 94/970 was identical to that of the PN isolate used in vector construction (Meng et al. 2005). At the six to seven leaf stage, two developed lower leaves were collected and saved at –80°C for further laboratory analyses. Two youngest leaves were challenge inoculated with GLRaV-2 isolate 94/970. Inoculum was prepared by grinding 1 g of GLRaV-2 infected *N. benthamiana* leaf tissue in 5 ml of 0.01 M phosphate buffer (pH 7.0). Test plants were lightly dusted with Carborundum and rubbed gently with the prepared virus inoculum. Non-transformed *N. benthamiana* plants were included as controls in all screening experiments. The inoculated plants were maintained under greenhouse conditions and observed for symptom development every other day for at least 60 days. Upon infection with GLRaV-2, all nontransgenic control plants produced chlorotic, and occasionally necrotic lesions followed by systemic vein clearing and necrosis. Eventually, GLRaV-2 infected susceptible plants died.

Responses of the transgenic plants to GLRaV-2 infection could be grouped into three types: (1) resistant, the tested plants remained asymptomatic throughout the test; (2) tolerant, delay and attenuation in disease symptom expression; (3) susceptible, typical symptoms observed 2–4 weeks post inoculation (wpi). In the first screening experiment, resistant plants were obtained in $T_1$ progenies from 14 of 20 lines tested (Fig. 1a, Table 1). The percentage of resistant plants in these 14 putative resistant lines varied widely, ranging from 100% in line 19 to 16% in line 8. When tested by indirect ELISA, the resistant plants were shown to have no apparent virus accumulation. Six other lines did not produce any resistant plants, and 93–100% of the progeny was susceptible (Table 1). Plants rated as susceptible showed severe symptoms (Fig. 1a) and eventually died within 3–8 wpi.

In a separate experiment, additional $T_1$ progenies were tested for four transgenic lines that produced variable number of resistant plants (lines 1, 4, 5, and 19), and two lines that produced only susceptible plants (lines 12 and 13) (Table 1). $T_1$ progenies from three of the lines (1, 4, and 19) produced again resistant plants although at a lower percent (range 23–42%) than that in the first screening (range 23–100%). Although 43% of $T_1$ plants in line 5 were considered resistance in the first screening, none of the tested plants were resistant in the second test. As expected, progenies from the two susceptible lines (12 and 13) remained susceptible.

To validate these results, additional screening was done with $T_2$ plants derived from selfing of three $T_1$ lines (1, 4 and 19). In the first screening experiment, we tested plants generated from five $T_2$ progenies for line 1 (designated as 1–22, 1–30, 1–31, 1–35, and 1–41), four $T_2$ progenies for line 4 (4–139, 4–149, 4–152, and 4–174), and four $T_2$ progenies for line 19 (19–650, 19–657, 19–659, and 19–660). In the second test with $T_2$ progenies, additional plants derived from these same resistant lines were used. Interestingly, greater variability in plant response to virus infection was observed in the $T_2$ progenies (Table 2). In general, the percentage of resistant plants in the $T_2$ progenies was lower than that of the corresponding $T_1$ plants (Table 1): for line 1, 0–57% in the $T_2$ vs. 42–56% in the $T_1$; for line 4, 0–50% in the $T_2$ vs. 33–71% in the $T_1$; and for line 19, 0–63% in the $T_2$ vs. 23–100% in the $T_1$. Resistant plants remained asymptomatic throughout the test and were able to develop to normal maturity (Fig. 1).

To evaluate whether the mechanism of PTGS was involved in conferring transgenic *N. benthamiana* plant resistance to GLRaV-2, Northern blot and nuclear run-off transcription experiments were performed on the selected resistant and susceptible plants. Total RNA was extracted from collected leaf tissues following the method described by Napoli et al. (1990). About 10 μg of total RNA per well was electrophoresed on a denatured agarose gel. After electrophoresis, the gel was stained with ethidium

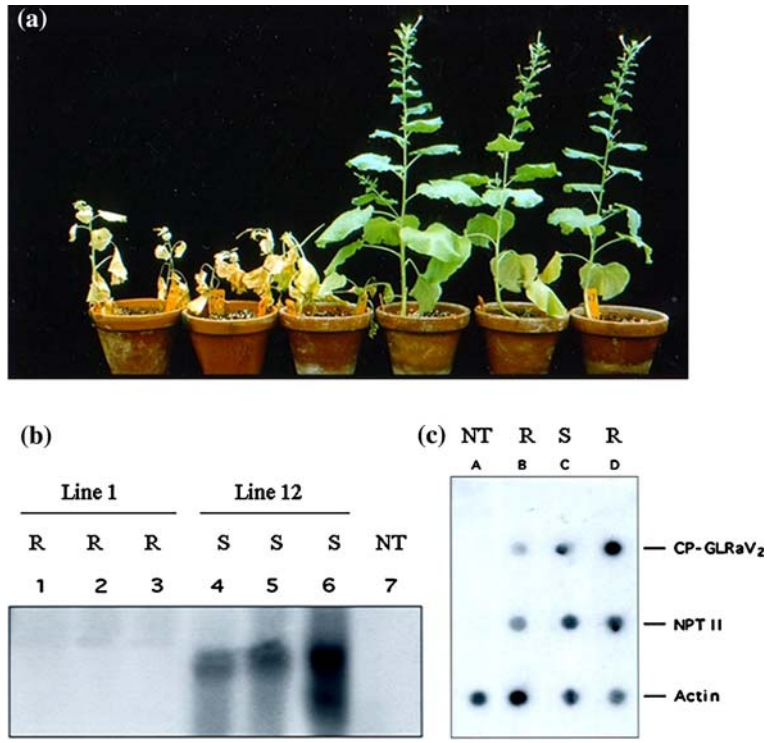


**Fig. 1** Responses of transgenic *Nicotiana benthamiana* plants to GLRaV-2 infection, and analyses of transgenic plants by Northern blot and nuclear run-off transcription. (**a**) Severe disease symptoms (dying plants) developed on non-transgenic plants (the left 3 potted plants). The resistant response in $T_2$ transgenic plants (line 1–22) was asymptomatic (the right 3 potted plants). (**b**) Northern blot analysis, lanes 1–3: three resistant $T_1$ plants from line 1; lanes 4–6: three susceptible $T_1$ plants from line 12; and lane 7: non-transgenic plant. (**c**) Nuclear run-off transcription experiments, lane A: non-transgenic plant (NT); lane B: a resistant $T_1$ plant in line 1; lane C: a susceptible $T_1$ plant in line 12; lane D: a resistant $T_1$ plant in line 19

bromide to reveal a relative similar amount of ribosomal RNA for each sample (data not shown). The conditions for Northern blot hybridization were those recommended by the manufacturer (DuPont NEN, Boston, MA). The probe used was a PCR amplified GLRaV-2 CP gene product, randomly labeled with $^{32}P$ (α-dATP) using the Klenow fragment of DNA polymerase I (Feinberg and Vogelstein 1983). Results in Northern blot analyses showed that while resistant $T_1$ plants (line 1) had low to non-detectable level of steady state transgene RNA transcript, higher accumulation was observed in the susceptible plants (line 12) (Fig. 1b). Isolation of nuclei and nuclear run-off transcription assays were performed essentially as described (Pang et al. 1996). The same amount of labeled nascent RNA was hybridized to dot blot membranes that contained 0.2 μg of CP-GLRaV-2, Actin, or NPT II genes respectively. Results in nuclear run-off experiments showed that transgene RNA transcripts were actively transcribed from all tested transgenic plants, either from the resistant lines 1 and 19 or the susceptible line 12 (Fig. 1c). Some level of variability in the intensity of CP-gene was likely due to the handling variance among samples under this experiment, which was also evidenced in the controls (Actin and NPT II) (Fig. 1c). Taken together, these results showed that the reduced levels of steady-state transgene RNA transcripts in the GLRaV-2 resistant *N. benthamiana* plants were likely due to PTGS.

We have shown here that following introduction of the CP gene of GLRaV-2 into *N. benthamiana*, some of the transgenic plants were protected against GLRaV-2 infection via the mechanism of PTGS. Interestingly, not all the plants in a given transgenic line were resistant. The resistance was also variable from generation to generation. Our results in *N. benthamiana* suggest that development of

**Table 1** Evaluation of $T_1$ transgenic *Nicotiana benthamiana* lines for resistance to GLRV-2 infection

| First screening experiment | | | | |
|---|---|---|---|---|
| Line # | *n* [a] | % showing reactions of | | |
| | | Susceptible [b] | Tolerant [c] | Resistant [d*] |
| 19 | 15 | 0 | 0 | 100 |
| 17 | 12 | 0 | 8 | 92 |
| 20 | 19 | 16 | 0 | 84 |
| 21 | 14 | 7 | 21 | 72 |
| 4 | 31 | 13 | 16 | 71 |
| 2 | 36 | 19 | 17 | 64 |
| 3 | 38 | 29 | 11 | 60 |
| 1 | 39 | 36 | 8 | 56 |
| 7 | 32 | 16 | 28 | 56 |
| 6 | 36 | 12 | 44 | 44 |
| 5 | 33 | 18 | 39 | 43 |
| 9 | 36 | 25 | 33 | 42 |
| 16 | 33 | 18 | 39 | 43 |
| 8 | 37 | 60 | 24 | 16 |
| 11 | 13 | 85 | 15 | 0 |
| 10 | 14 | 93 | 7 | 0 |
| 12 | 17 | 94 | 6 | 0 |
| 15 | 32 | 94 | 6 | 0 |
| 14 | 17 | 100 | 0 | 0 |
| 13 | 14 | 100 | 0 | 0 |
| Control [e] | 15 | 100 | 0 | 0 |
| Second screening experiment | | | | |
| 1 | 19 | 26 | 32 | 42 |
| 4 | 15 | 60 | 7 | 33 |
| 19 | 13 | 77 | 0 | 23 |
| 5 | 17 | 82 | 18 | 0 |
| 12 | 16 | 88 | 12 | 0 |
| 13 | 18 | 72 | 28 | 0 |
| Control [e] | 24 | 96 | 4 | 0 |

[a] Number of $T_1$ plants in each line tested

[b] Susceptible, typical symptoms were observed two to four weeks post inoculation

[c] Tolerant, symptom expression was delayed and attenuated

[d] Resistant, plants remained asymptomatic throughout the test

[e] Non-transgenic plant control

[*] The average percentage of resistant plants when combining the data obtained in the first and second screening experiments for $T_1$ lines 1, 4, and 19 was 52, 59, and 64%, respectively

**Table 2** Evaluation of $T_2$ transgenic *Nicotiana benthamiana* lines for resistance to GLRaV-2 infection

| First screening experiment | | | | |
|---|---|---|---|---|
| Line # | *N* [a] | % showing reactions of | | |
| | | Susceptible [b] | Tolerant [c] | Resistant [d*] |
| 1–22 | 12 | 25 | 25 | 50 |
| 1–30 | 8 | 50 | 25 | 25 |
| 1–31 | 10 | 50 | 30 | 20 |
| 1–35 | 10 | 40 | 60 | 0 |
| 1–41 | 7 | 14 | 29 | 57 |
| 4–139 | 11 | 36 | 36 | 28 |
| 4–149 | 7 | 14 | 72 | 14 |
| 4–152 | 8 | 100 | 0 | 0 |
| 4–174 | 8 | 50 | 0 | 50 |
| 19–650 | 10 | 70 | 10 | 20 |
| 19–657 | 12 | 50 | 17 | 33 |
| 19–659 | 8 | 12 | 25 | 63 |
| 19–660 | 8 | 13 | 74 | 13 |
| Control[e] | 12 | 100 | 0 | 0 |
| Second screening experiment | | | | |
| 1–8 | 10 | 70 | 20 | 10 |
| 1–14 | 11 | 55 | 27 | 18 |
| 1–17 | 12 | 58 | 17 | 25 |
| 1–20 | 14 | 50 | 29 | 21 |
| 1–24 | 13 | 92 | 0 | 8 |
| 4–33 | 12 | 50 | 42 | 8 |
| 4–36 | 14 | 57 | 14 | 29 |
| 4–43 | 14 | 93 | 7 | 0 |
| 4–46 | 14 | 58 | 27 | 15 |
| 19–130 | 13 | 68 | 25 | 17 |
| 19–133 | 14 | 100 | 0 | 0 |
| 19–141 | 11 | 73 | 18 | 9 |
| Control[e] | 13 | 100 | 0 | 0 |

[a] Number of $T_2$ plants in each line tested

[b] Susceptible, typical symptoms were observed 2–4 weeks post inoculation

[c] Tolerant, symptom expression was delayed and attenuated

[d] Resistant, plants remained asymptomatic throughout the test

[e] Non-transgenic plant control

[*] The range of resistant plants when combining the two screening experiments for three $T_2$ lines tested varied as follows: line 1 (0–57%), line 4 (0–50%), and line 19 (0–63%)

transgenic virus resistance could be a practical way to control GLRaV-2 in grapevines, and that GLRaV-2 may be a good model system for investigating the factors affecting the variability of resistance to closteroviruses.

Reports on the use of PDR to develop resistance among closteroviruses have so far been limited to

attempts with CTV (Dominguez et al. 2000, 2002; Febres et al. 2003; Batuman et al. 2006; Fagoaga et al. 2006). In general, results have shown that resistance to CTV in transgenic citrus plants is variable. The work on Mexican lime showed delayed symptom expression in plants transformed with the CP gene (Dominguez et al. 2002) and very strong resistance with plants expressing the p23 gene (Fagoaga et al. 2006). However, reactions among the transgenic lines were variable. For example different lines of p23 expressing transgenic citrus plants showed both resistant and susceptible reactions to CTV infection (Fagoaga et al. 2005 and Fagoaga et al. 2006). In another work, transgenic plants expressing p23 and the 3′UTR sequence provided resistance to CTV in *N. benthamiana* but not in citrus (Batuman et al. 2006). These results with CTV actually show similarities with our work on GLRaV-2 in that the number of resistant plants obtained for a given transgenic line was variable and in general the percentage of resistant plants were lower in the $T_2$ than the $T_1$ generation.

Overall, the results obtained so far for CTV and for GLRaV-2 show that the inheritance of closterovirus-derived resistance is variable and the level of resistance is less than those obtained for other viral groups, such as potyviruses (Batuman et al. 2006; Fagoaga et al. 2006; Tenllado et al. 2004). Perhaps, reason for the difficulty in generating PDR resistance to CTV in citrus and now GLRaV-2 in *N. benthamiana* may be due to the existence of three distinctive RNA silencing suppressors in these large RNA genomes (~20 kb) (Lu et al. 2004). Interestingly, the three RNA silencing suppressors in CTV have three different functions: p23 targeting the intracellular space, CP the intercellular, and p20 both levels (Lu et al. 2004). Furthermore, the strength and number of silencing suppressors in the genome of closteroviruses may accentuate the effects of other factors such as plant age, gene dosage, and environmental conditions (Pang et al. 1996). The latter may account for the differences in level of resistance even among clones taken from a parent plant that showed immunity (Fagoaga et al. 2005; Fagoaga et al. 2006). Because GLRaV-2 is easily transmissible and induces severe symptoms in *N. benthamiana* (Goszczynski et al. 1996), it may serve as a good model for systematically examining the effect of silencing suppressors in closteroviruses.

Grapevine leafroll viruses and CTV, for example, cause much damage to two of the most widely grown fruit crops, grape and citrus. Transformation of grapevines has been achieved and in fact transgenic grapevines with the current GLRaV-2 CP construct were produced (Xue et al. 1999; Krastanova et al. 2000). Unfortunately, evaluation of virus resistance in these plants has been delayed. Nevertheless, the potential benefits in developing multiple virus resistance in grapevines against closteroviruses through genetic engineering might be significant since at least nine different viruses in the family of Closteroviridae are implicated as the causal agents of grapevine leafroll disease (Alkowni et al. 2004; Fauquet et al. 2005). Recent work has demonstrated that linking segments of genes from different viruses could induce multiple virus resistance (Bucher et al. 2006; Jan et al. 2000). Although silencing suppressors have yet to be identified in any of the closteroviruses that are associated with the grapevine leafroll disease, it is tempting to speculate that multiple silencing suppressors may also be involved in these virus genomes, including GLRaV-2. To be successful in developing virus resistance through PDR strategy, the new generation of gene construct design needs to fully consider the involvement of multiple gene silencing suppressors in these viruses. Nevertheless, the success in the present study in transgenic *N. benthamiana* plants against GLRaV-2 may represent the first step towards an eventual control of the leafroll disease in grapevines.

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