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EXPRESSION OF FOUR TRANSGENES IN ONE TOBACCO LINE

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Abstract

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Pyramiding of four transgenes in one tobacco line led to a complex resistance against two viruses (TSWV, PVY) and one bacterial pathogen (*Pseudomonas syringae* pv. *tabaci*), as well as the herbicide Glean®. Expression analyses of the corresponding transgenes (*Np*, *CP*, *ttr* and *ahas 3R*) were performed with selected tobacco lines of the cross 2002. The RNA gel blot hybridization analyses of all tested tobacco plants showed an expression of the *ahas 3R* gene, but no signal was detected for the other three transgenes. Expression of the *Np*, *CP* and *ttr* genes was detected by RT-PCR analyses, suggesting low level of accumulation of the corresponding mRNAs in the transgenic tobacco lines. Involvement of RNA silencing as a mechanism of resistance to plant pathogens is discussed.

Key words: Np, *CP*, *ttr*, *ahas 3R*, transgene expression, RNA hybridization, RT-PCR

Introduction

Pyramiding of genes for resistance to various pathogens aims to enhance the protection of plants to broad spectrum of diseases. This leads to improvement of crop production, but also gives opportunities to investigate the mechanisms of resistance and the interactions between plants and pathogens. The most important and crucial step in the combination of different transgenes in one genome is selection of plants that expressed genes for resistance and thus provide increased protection to various stress factors.

The RNA silencing is a part of a natural protection mechanism of plants against viral pathogens. It has become a powerful tool for engineering resistant plants in the last two decades. Plant protection to viral pathogens based on degradation of their RNA by RNA interference mechanism is also known as post-transcriptional gene silencing (PTSG) (Metzlaff et al., 1997; Hamilton et al., 1998; Waterhouse et al., 1998; Waterhouse et al., 1999). Various approaches have been developed based on sense/antisense RNA, hairpin RNA and artificial miRNA precursors (Duan et. al., 2012). A sense RNA-mediated protection against PVY (Potato virus Y) of tobacco plants transformed with the viral coat protein (CP) cistron was reported (Van der Vlugt et al., 1992). Tobacco plants expressing CP protein demonstrated virus specific resistance to Tobacco etch virus (TEV) by targeting specific RNA sequences for inactivation (Lindbo et al., 1993). Resistance of *Nicotiana benthamiana* against Turnip mosaic potyvirus (TuMV) was achieved by PTGS (Jan et al., 1999). RNA silencing has been used for protection of barley from Barley yellow dwarf virus (BYDV) (Wang et al., 2000).

Resistance to multiple viruses based on PTGS was reported for different transgenic plants (Jan et al., 2000; Bucher et al., 2006; Praveen et al., 2006; Zhu et al., 2009; Yu et al., 2011; Wu et al., 2010; Lin et al., 2011; Lin et al., 2012). Transformation of *Nicotiana benthamiana* with full-length CP gene of turnip mosaic virus (TuMV) linked to a 218 bp N gene segment of the TSWV (Tomato Spotted Wild Virus) conferred resistance to both viruses (Jan et al., 2000). Nuclear run-on and RNA gel blot hybridization experiments confirmed that resistance was achieved by RNA-mediated PTGS. Resistance to both TSWV and ToLCTWV in transgenic *Arabidopsis* and tomato plants via PTGS have been obtained by fusion of a partial N gene with the C2 gene segment (Lin et al., 2011). The study demonstrated that linkage of gene segments from two viruses with distinct

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genomic organization (one DNA and the other RNA) can confer multiple virus resistance in transgenic plants via gene silencing. A fusion of different viral CP gene fragments in transgenic watermelon contributed to multiple virus resistance (Lin et al., 2012). Low level accumulation of transgene transcripts in resistant plants and small interfering (si)RNAs specific to CMV and WMV were detected in the resistant R1 plants by RNA gel blot analysis, indicating that the resistance was established by RNA-mediated PTGS.

Recently, involvement of RNA silencing mechanism of plant defence against non-viral pathogens was investigated (Pumplin and Voinnet, 2013). RNA-mediated gene regulation model was indentified in the interactions between *Arabidopsis thaliana* and the bacterial pathogen *P. syringae* pv. *tomato*. RNA silencing has also been reported as a resistance mechanism against the fungal pathogen *Verticillium dahliae* in *Arabidopsis* (Ellendorff et al., 2009).

In our previous report, four transgenes (*Np*, *CP*, *ttr* and *ahas 3R*) that conferred resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and the herbicide Glean®, respectively were combined into the genomes of tobacco lines (cross 2002) by a sexual crossing approach (Christova and Batchvarova, 2015). The resistance to all three pathogens and the herbicide Glean® in these lines, containing four transgenes in a single tobacco genome, was confirmed in both F1 and F2 progenies of the cross 2002. In the present study, expression of the four transgenes was analyzed by RNA gel blot hybridization and RT-PCR. RNA gel blot hybridization analyses of transgenic tobacco lines of the cross 2002 showed expression of the *ahas 3R* gene, but the accumulation of the other three transgenes was below detection limits of the method. Expression of the *Np*, *CP* and *ttr* genes was proven by RT-PCR analyses, implying low accumulation level of the corresponding RNAs.

Materials and Methods

Plant material

Pyramiding of four transgenes that conferred resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and the herbicide Glean® into one tobacco genome (cross 2002) by a cascade of sexual crosses was previously reported (Christova and Batchvarova, 2015). Tobacco plants of F1 generation of the cross 2002 that showed resistance to all pathogens and had four transgenes (*Np*, *CP*, *ttr* and *ahas 3R*) pyramided in their genome were selected for expression analyses.

Total RNA extraction

Total RNA was extracted from leaf tissue using RNeasy Plant Mini Kit (Qiagen, GmbH), according to the manufacturer's instructions.

RNA gel blot hybridization analyses

Total RNA (10 µg) was separated on 1.2% formaldehyde agarose gel and transferred on a Hybond N+ membrane (GE Healthcare Life Sciences). Prehybridization for 1 h was performed using DIG hybridization solution (DIG Easy Hyb, Roche Diagnostics, GmbH). Four hybridization procedures were performed subsequently on the same membrane using specific for each gene DIG-dUTP labeled probe (50 ng/ml). The hybridization probes were prepared by PCR amplification of *Np*, *CP*, *ttr* and *ahas 3R* genes, respectively by using PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH). Detection was done with the DIG Luminescent Detection Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions.

RT-PCR analyses

RT-PCR analyses were performed using Ready-To-Go™ RT-PCR Beads (GE Healthcare Life Sciences) according to the manufacturer's instructions following the two step protocol. To eliminate potential genomic DNA contamination, the total RNA samples were treated with DNaseI prior to RT-PCR experiments. Each RT-PCR reaction was performed using 1–2 µg total RNA and the first strand cDNA was synthesized using random hexamer primers (0.5 µg/ml). Resulted first strand cDNA samples were directly used for PCR amplification by adding specific for each transgene primers for 40 cycles (*Np* gene: 5'-AGC TAT CAA GCC TTC TGAAGGTC-3' and 5'-ACC TTG AGT TTG AGG AAG ATCAG-3'; *CP* gene: 5'-TCG ATG CAG GAG GAA GCA CTA-3' and 5'-TTC CGT CGC GCA GAT TAC GAA-3'; *ttr* gene: 5'-CTT CGC CCA TTA TCG CCA TGG TC-3' and 5'-CAG CCC GCG TTT GTG TTT TAC TG-3'). For each RNA sample, a control RT-PCR reaction where the reverse transcriptase was heat inactivated prior to adding RNA template was prepared. The RT-PCR products were visualized by Ethidium bromide staining after electrophoresis in 1.5% TAE agarose gel.

Results and Discussion

RNA gel blot hybridization analyses of F1 tobacco lines of the cross 2002

F1 tobacco lines of the cross 2002 with pyramided genes for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® were selected for analyses of transgenes expression. The tobacco lines were resistant to all tree pathogens and the herbicide and the integration of transgenes *Np*, *CP*, *ttr* and *ahas 3R* into tobacco genome was proven by PCR amplification (Christova and Batchvarova, 2015).

RNA gel blot hybridizations analyses of selected tobacco plants were performed with DIG-labeled DNA of the transgenes. Two RNA membranes were prepared and were hybridized with the four labeled probes.

A positive signal for the expression of *ahas 3R* gene was detected in all tested F1 plants of the cross 2002 while hybridization signal was absent in nontransgenic control tobacco Nevrokop 1146 (Figure 1). These results indicate that the intact *ahas 3R* gene was successfully transferred by sexual crosses in all analyzed plants and their resistance to the herbicide Glean® is conferred by its expression.

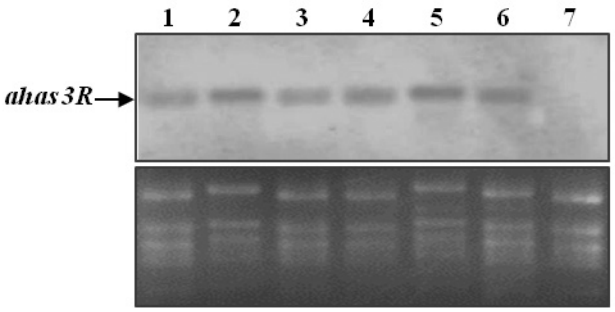

Fig. 1. RNA gel blot hybridization analyses of *ahas 3R* gene of plants from the cross 2002

RNA gel blot hybridization was performed using DIG labeled ahas 3R DNA probe

1–6 –plants of the cross 2002; 7 – nontransgenic tobacco cultivar Nevrokop 1146

In the lower panel, Ethidium bromide stained gel for loading control was shown

In contrast to *ahas 3R* gene, no signal was detected by RNA hybridization analyses for *Np*, *CP* and *ttr* genes in the selected plants of the cross 2002, suggesting low expression of the transgenes, that was below the detection limits of the method (data no show). Nevertheless, the analyzed plants were resistant to TSWV, PVY and *P. syringae* pv. *tabaci*. Moreover, all of them showed positive PCR signal for all three corresponding transgenes, indicating their successful transfer by the sexual crossing (Christova and Batchvarova, 2015).

A diseases resistance of transgenic plants with introduced viral genes based on RNA degradation has been established earlier (Van der Vlugt et al., 1992; Waterhouse et al., 1998; Waterhouse et al., 1999). Other authors reported lack of RNA expression of the integrated viral genes in transgenic plants that were resistant to the corresponding virus (Van der Vlugt et al., 1992; Malnoe et al., 1994; Savenkov and Valkonen, 2001). It was found that the resistance of the transgenic plants is conveyed mainly at RNA level and is associated with low expression of the transgenes (Lindbo et al., 1993; Smith et al., 1994; Mueller et al., 1995; Dawson 1996; Goodwin et al., 1996; Pang et al., 1996; Sijen et al., 1996).

RT-PCR analyses of F1 tobacco lines of the cross 2002

The expression of *Np*, *CP* and *ttr* genes in plants of the cross 2002 was investigated by RT-PCR analyzes. This method allowed verification of a very low expression level of transgenes. RT-PCR of each transgene was performed using gene-specific primers and 40 cycles of amplification. Results of the analyses showed amplifications of all three genes in the analyzed F1 plants of the cross 2002 (Figure 2). No PCR product was detected in the control nontransgenic tobacco cultivar Nevrokop 1146. The lack of PCR products in the control RT-PCR reactions with inactivated reverse transcriptase ruled out the possibility for contamination of the RNA samples with genomic DNA. Results of the RT-PCR analyses revealed the expression of *Np*, *CP* and *ttr* genes, although it was too low to be detected by RNA blot hybridization.

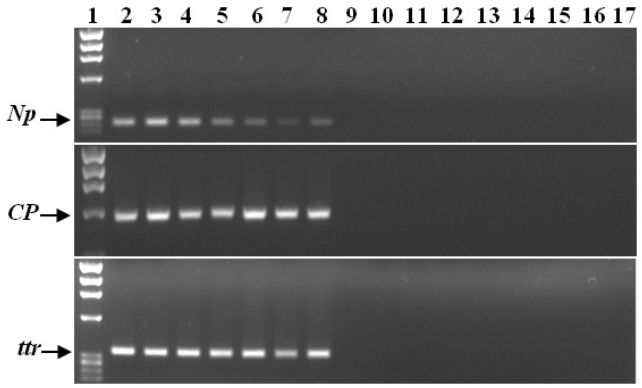

Fig. 2. RT-PCR of *Np*, *CP* and *ttr* genes of plants from the cross 2002

1 – DNA ladder (λ Hind III/ ϕX Hae I); 2–8 – plants of the cross 2002; 9 – nontransgenic control tobacco cultivar Nevrokop 1146; 10–17 – No Template Control (NTC) reactions with inactivated reverse transcriptase of the samples 2 to 9

RT-PCR approach has been extensively used by other authors for investigation of low transgene expression. Expression of the *CP* gene in the resistant to PVY transgenic potato has been proven by RT-PCR analyses (Saker, 2003). Activation of gene silencing mechanism in transgenic peanut lines, containing multiple copy insertions of the *N* gene of TSWV, was suggested based on the results from Northern blot, RT-PCR and ELISA analyses (Yang et al., 1998). *CP* gene ex-

pression of TEV was analyzed in transgenic peanut lines by RT-PCR, real-time PCR and ELISA (Mehta et al., 2013).

Succesful pyramiding of genes for resistance to multiple plant viruses have been reported in various plants species (Bucher et al., 2006; Jan et al., 2000; Lin et al., 2011; Lin et al., 2012). The N gene sequence fragments of the four major tomato-infecting tospoviruses, TSWV, Groundnut ringspot virus (GRSV), Tomato chlorotic spot virus (TCSV) and Watermelon silver mottle virus (WSMoV), have been constructed in a single small chimaeric hairpin RNA vector (Bucher et al., 2006). Due to simultaneous RNA silencing, as demonstrated by specific siRNA accumulation, the transgene expression of these cassettes rendered up to 82% of the transformed plant lines heritably resistant to all four viruses.

RNA gel blot hybridization analyses of transgenic *N. benthamiana* showed that a multiple viral resistance to PVY, TSWV and TuMV was based on PTGS (Jan et al., 2000). A complex resistance to TSWV and ToLCTWV in transgenic *Arabidopsis* and tomato plants via PTGS have also been demonstrated (Lin et al., 2012). The involvement of RNA mediated PTGS in the transgenic plants resistance to both PVY and TSWV conferred respectively by *CP* and *Np* genes have been well documented. This mechanism is further supported by the low expression levels of both transgenes in resistant tobacco plants observed in the present study.

The role of RNA silencing as a mechanism of plant defence to non-viral pathogens was established in recent years (Navarro et al., 2008; Mosher and Baulcombe, 2008; Pumplin and Voinnet, 2013). RNA-mediated gene regulation was investigated in the plant-pathogen interaction between *A. thaliana* and *P. syringae* pv. *tomato* (*Pst*) (Pumplin and Voinnet, 2013). The *P. syringae* pv. *tabaci* - *ttr* gene encodes an acetyltransferase that is produced by the bacteria to acetylate the tabtoxin and thus protect itself (Wencewicz and Walsh, 2012). It is believed that the similar mechanism applies when the *ttr* gene is expressed in transgenic plants but to our knowledge there is no experimental evidence confirming it. However, the low expression level of the *ttr* gene, proven by RT-PCR in the present study, suggests involvement of RNA-mediated process in the resistance mechanism to *P. syringae* pv. *tabaci*. Further studies are warranted to elucidate the exact mechanism of this resistance in transgenic plants.

Conclusion

Our results demonstrated the different level of the expression of four transgenes in one tobacco genome that leads to a complex resistance to three pathogens and one herbicide. Resistance to the herbicide Glean® is a result of accumulation of the *ahas 3R* gene product in the selected lines, whereas resistance to TSWV and PVY is a result of RNA silencing of the *Np* and *CP* genes. The data, presented here contributes to the design and implementation of effective strategies for pyramiding of multiple resistance genes with different modes of action. The selected in this study transgenic tobacco lines will be useful tool for future experiments for elucidation of the mechanism of transgenic resistance to pathogens as well as expression stability of multiple transgenes combined in a single tobacco genome by sexual crossing.

References

Bucher, E., D. Lohuis, M. Pieter, J. A. van Poppel, C. Geerts-Dimitriadou, R. Goldbach and M. Prins, 2006. Multiple virus resistance at a high frequency using a single transgene construct. *J. Gen. Virol*., **87**: 3697–3701.

Christova, P. and R. Batchvarova, 2015. Pyramiding of four transgenes in one tobacco line by sexual crosses. *Bulg. J. Agric. Sci.*, **21**: 829–834.

Dawson, W. D., 1996. Gene silencing and virus resistance: a common mechanism. *Trends in Plant Science*, **1**: 107–108.

Duan, Ch.-G., Ch.-H. Wang and H.-Sh. Guo, 2012. Application of RNA silencing to plant disease resistance. *Silence*, **3**: 5.

Ellendorff, U., E. F. Fradin, R. de Jonge and B. P. Thomma, 2009. RNA silencing is required for *Arabidopsis* defence against *Verticillium* wilt disease. *J. Exp. Bot.* **60**: 591–602.

Goodwin, J., K. Chapman, S. Swaney, T. D. Parks, E. A. Wernsman and W. G. Dougherty, 1996. Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. *Plant Cell*, **8**: 95–105.

Hamilton, A. J., S. Brown, H. Yuanhai, M. Ishizuka, A. Lowe, S. A.-G. Alpuche and D. Grierson, 1998. A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. *Plant J.*, **15**: 737–746.

Jan, F.-J., C. Fagoaga, S.-Z. Pang and D. Gonsalves, 2000. A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *Journal of General Virology*, **81**: 2103–2109.

Jan, F.-J., S. Z. Pang, C. Fagoaga and D. Gonsalves, 1999. Turnip mosaic potyvirus resistance in *Nicotiana benthamiana* derived by post-transcriptional gene silencing. *Transgenic Res.*, **8**: 203–213.

Lin, Ch.-Yi, H.-M. Ku, Yi-H. Chiang, H-Y. Ho, T.-A. Yu and F.-J. Jan, 2012. Development of transgenic watermelon resistant to Cucumber mosaic virus and Watermelon mosaic virus by using a single chimeric transgene construct. *Transgenic Res.,* **21**: 983–993.

Lin, Ch.-Yi, H.-M. Ku, W.-S. Tsai, S. K. Green and F.-J. Jan, 2011. Resistance to a DNA and a RNA virus in transgenic plants by using a single chimeric transgene construct. *Transgenic Res.*, **20**: 261–270.

Lindbo, J. A., R. L. Silva, W. M. Proebsting and W. G. Doughtery, 1993. Introduction of a highly specific antiviral state in

transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell*., **5**: 1749–1759.

Malnoe, P., L. Farinelli, G. Collet and W. Reust, 1994. Small-scale field tests with transgenic potato, cv. Bintje, to test resistance to primary and secondary infections with PVY. *Plant Mol. Biol*., **25**: 963–975.

Mehta, R., T. Radhakrishnan, A. Kumar, R. Yadav, J. R. Dobaria, P. P. Thirumalaisamy, R. K. Jain and P. Chigurupati, 2013. Coat protein-mediated transgenic resistance of peanut (*Arachis hypogaea* L.) to peanut stem necrosis disease through Agrobacterium-mediated genetic transformation. *Indian J. Virol.*, **24** (2): 205–213.

Metzlaff, M., M. O'Dell, P. D. Cluster and R. B. Flavell, 1997. RNA-mediated RNA-degradation and chalcone synthase A silencing in petunia. *Cell,* **88**: 845–854.

Mosher, R. A. and D. C. Baulcombe, 2008. Bacterial pathogens encode suppressors of RNA-mediated silencing. *Genome Biology*, **9** (10): 237–237.5.

Mueller, E., J. Gilbert, G. Davenport, G. Brigneti and D. C. Baulbombe, 1995. Homology-dependent resistance: transgenic virus resistance in plants related to homology-dependent gene silencing. *Plant J.,* **7**: 1001–1013.

Navarro, L., F. Jay, K. Nomura, S. Y. He and O. Voinnet, 2008. Suppression of the microRNA pathway by bacterial effector proteins. *Science,* **321**: 964–967.

Pang, S.-Z., F.-J. Jan, K. Carney, J. Stout, D. M. Tricoli, H. D. Quemada and D. Gonsalves, 1996. Post-transcriptional gene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. *Plant J.*, **9**: 899–909.

Praveen, S., A. K. Mishra and G. Antony, 2006. Viral suppression in transgenic plants expression chimeric transgene from tomato leaf curl virus and cucumber mosaic virus. *Plant Cell Tissue Organ. Cult*., **84**: 47–53.

Pumplin, N. and O. Voinnet, 2013. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. *Nature Reviews*/*Microbiology* **11**: 745–760.

Saker, M. M., 2003. Production of biosafe transgenic potato plants with coat protein gene for potato virus Y. *Arab J. Biotech.*, **6** (1): 125–138.

Savenkov, E. I. and J. P. T. Valkonen, 2001. Coat protein gene-mediated resistance to *Potato virus A* in transgenic plants is suppressed following infection with another potyvirus. *Journal of General Virology,* **82**: 2275–2278.

Sijen, T., J. Wellink, J. B. Hiriart and A. van Kammen, 1996. RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. *Plant Cell*, **8**: 2277–2294.

Smith, H. A., S. L. Swaney, T. D. Parks, E. A. Wernsman and W. G. Dougherty, 1994. Transgenic plants virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. *Plant Cell*, **6**: 1441–1453.

Van der Vlugt, R. A. A., R. K. Ruiter and R. W. Goldbach, 1992. Evidence for sens RNA-mediated protection of PVY-N of tobacco plants transformed with the viral coat protein cistron. *Plant Mol. Biol.*, **20**: 631–639.

Wang, M. B., D. C. Abbott and P. M Waterhouse, 2000. A single copy of a virusderived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. *Mol. Plant Pathol.,* **1**: 347–356.

Waterhouse, P. M., M. W. Graham and M.-B. Wang, 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl. Acad. Sci.*, **95**: 13959–13964.

Waterhouse, P. M., N. A. Smith and M.-B. Wang, 1999. Virus resistance and gene silencing: killing the messenger. *Elsevier Science*, **4**: 452–457.

Wencewicz, T. A. and Ch. T. Walsh, 2012. Pseudomonas syringae Self-Protection from Tabtoxinine-β-Lactam by Ligase TblF and Acetylase Ttr. *Biochemistry*, **51** (39): 7712–7725.

Wu, H. W., T. A. Yu, J. A. J. Raja, S. J. Christopher, S. L. Wang and S. D. Yeh, 2010. Double-virus resistance of transgenic oriental melon conferred by untranslatable chimeric construct carrying partial coat protein genes of two viruses. *Plant Dis.,* **94**: 1341–1347.

Yang, H., C.·Singsit, A.·Wang, D. Gonsalves and P. Ozias-Akins, 1998. Transgenic peanut plants containing a nucleocapsid protein gene of tomato spotted wilt virus show divergent levels of gene expression. *Plant Cell Reports*, **17**: 693–699.

Yu, T. A., C. H. Chiang, H. W. Wu, C. M. Li, C. F. Yang, J. H. Chen, Y. W. Chen and S. D. Yeh, 2011. Generation of transgenic watermelon resistant to Zucchini yellow mosaic virus and Papaya ringspot virus type. *W. Plant Cell Rep*., **30**: 359–371.

Zhu, C.-X., Y.-Z. Song, G.-H. Yin and F.-J. Wen, 2009. Induction of RNA-mediated multiple virus resistance to Potato virus Y, Tobacco mosaic virus and Cucumber mosaic virus. *J Phytopathol*., **157**: 101–107.

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