Transgenic Rootstock Protein Transmission in Grapevines

M. Dutt, Z.T. Li, K.T. Kelley, S.A. Dhekney, M. Van Aman, J. Tattersall and D.J. Gray
Mid-Florida Research and Education Center
University of Florida/IFAS
2725 Binion Road
Apopka, FL 32703-8504
USA

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Abstract

Methods to manage uncontrolled flow of transgenes via pollen and seeds are increasing in importance as transgenic crops become more commonplace. In many parts of the world, grapevine scions are grafted onto rootstocks, which are adapted to resist adverse soil conditions and pests and/or to promote vigor. Water, minerals and other important nutrients are transferred in bulk from the rootstock to scion via the xylem. Thus, it is possible that bioactive peptides produced by a transgenic rootstock and deposited into its xylem sap would be similarly transferred to a non-transgenic scion. If the peptide conferred a desirable trait to the non-transgenic scion, such as disease resistance, the issue of unwanted gene flow would be solved, since transgenic pollen and seeds would not be produced during flowering and fruit production. Furthermore, the commercialization of transgenic grapevines would be simplified, since relatively few transgenic rootstock varieties would be needed to protect any non-transgenic scion. As an example, transgenic rootstock technology might be used in the control of Pierce's disease (PD), which is caused by the xylem-limited bacteria, *Xylella fastidiosa*. Antimicrobial peptides produced by a transgenic rootstock may control bacteria in the xylem sap of a non-transgenic scion, thus providing PD resistance. To test this hypothesis, transgenic *Vitis vinifera* 'Thompson Seedless' expressing the Shiva-1 lytic peptide gene was treated as a rootstock. Non-transgenic *V. vinifera* 'Cabernet Sauvignon' and 'Thompson Seedless' were grafted onto the rootstock. Controls consisted of grafted and non-grafted transgenic and non-transgenic vines. Presence of the Shiva-1 peptide in xylem sap of the scion was detected by Enzyme-Linked Immunoabsorbent Assay (ELISA).

INTRODUCTION

Movement of transgenes via pollen and seed is one of the concerns associated with release of genetically modified plants. Transgene flow from crops to related wild species has come under focus in risk-assessment studies (Lavigne et al., 2002) and methods to stop unwanted gene flow are becoming increasingly important as transgenic crops become more commonplace.

In many parts of the world, grapevine scions are grafted onto rootstocks, which are adapted to resist adverse soil conditions and pests and/or to promote vigor (Winkler et al., 1974). Water, minerals and other nutrients are transferred in bulk from rootstock to scion via the xylem (De Boer and Volkov, 2003). Thus, it is possible that bioactive peptides produced by a transgenic rootstock and deposited into its xylem sap would be similarly transferred into a non-transgenic scion. If a rootstock-derived transgenic peptide conferred the desired trait to non transgenic scion, unwanted gene flow would not occur since any pollen or seeds produced would not be transgenic. As an example, transgenic rootstock technology might be used in the control of Pierce's disease (PD), which is caused by the xylem-limited bacteria, *Xylella fastidiosa* (Hopkins, 1977). Antimicrobial peptides produced by a transgenic rootstock may control bacteria in the xylem sap of a non-transgenic scion, thus providing PD resistance. Furthermore, commercialization of transgenic grapevines would be simplified, since relatively few rootstock cultivars would need to be developed and approved.

To test this hypothesis, transgenic *Vitis vinifera* 'Thompson Seedless' expressing the Shiva-1 lytic peptide gene (Jaynes, 1993) was used as a rootstock. Shiva-1 is a small (4.4 kD) synthetic lytic peptide containing 39 amino acids which preserve the lytic activity of the natural antibacterial cecropin B (Jaynes, 2002), and it has potential uses as a bioactive peptide. It retains 40% amino acid homology with cecropin B, while providing broad spectrum antimicrobial activity equaling to cecropin B (Reed et al., 1997). Non-transgenic *V. vinifera* 'Cabernet Sauvignon' and 'Thompson Seedless' were grafted onto the transgenic rootstock. Presence or absence of the peptide in xylem sap of scion was determined by Enzyme-Linked Immunoabsorbent Assay (ELISA).

MATERIALS AND METHODS

Plant Materials and Genetic Transformation

Agrobacterium-mediated transformation of 'Thompson Seedless' somatic embryos was used to produce transgenic grapevines (Li et al., 2001a). Transformation was carried out using a vector containing the Shiva-1 lytic peptide gene under the control of a 35S promoter and a bi-functional marker gene composed of in-frame translational fusion between the enhanced green fluorescent protein (EGFP) gene and a neomycin phospho-transferase (NPTII) gene (Fig. 1) (Li et al., 2001a, 2004). A transgenic grapevine was selected based on high EGFP and Shiva-1 protein expression and was propagated via cuttings. Greenhouse-raised vines were grown for a full year and allowed to become dormant in winter.

Grafting

Whip grafting was carried out in mid-spring of the following year using one year old transgenic 'Thompson Seedless' vines as the rootstock. Non transgenic 'Cabernet Sauvignon' and 'Thompson Seedless' were used as scion. Twelve grafted vines were produced; six from each scion cultivar. From these, two of each cultivar was selected for the experiment based on vine vigor. Grafted vines were allowed to grow for six months before being used for collection of xylem sap. Xylem sap was also collected from non grafted transgenic and non transgenic 'Thompson Seedless' plants which were used as positive and negative controls, respectively.

Xylem Sap Collection

Xylem sap was collected from greenhouse grown grapevines which had been well watered the previous evening. A branch of the scion was cut to within a meter of the graft union. The sap was allowed to flow into an eppendorf tube attached to the end of cut vine (Fig. 2). In vines where it was not possible to attach an eppendorf tube to cut surface, the sap was collected with a micropipette and placed into eppendorf tubes. Typically 0.5 ml of sap was collected within 15 minutes of cutting of the arm. All samples were kept on crushed ice during transport from greenhouse to laboratory.

Enzyme-Linked Immunoabsorbent Assay (ELISA)

Presence or absence of transgenic Shiva-1 lytic peptide in xylem sap was detected by ELISA. It was performed within an hour of collection of xylem sap as per the protocol outlined by Li et al. (2001b) but modified by using half the recommended quantity of EDTA free protease inhibitor tablets. Also, cross absorption antiserum buffer concentration was doubled to a final ratio of 1 part of rabbit anti Shiva-1 antiserum to 250 parts of the negative plant extract in antiserum buffer. Average values in µg protein per ml of xylem sap from 3 independent experiments were plotted in Figure 3.

RESULTS AND DISCUSSION

The flow of xylem sap upwards and out through the cut vine surface (also known as vine bleeding) was due to root pressure. ELISA technique provides a sensitive serological tool for the detection of small antigenic proteins (Shiva-1 lytic peptide in this

case) and macromolecules present in biological systems (Li et al., 2001b). Here we were able to detect the presence of Shiva-1 protein in xylem sap of non-transgenic 'Cabernet Sauvignon' and 'Thompson Seedless' scions that were grafted onto transgenic Shiva-1 producing rootstocks. However, the amount of lytic peptide present varied among plants (Fig. 3). In grafted CS-1, lytic peptides were detected at levels similar to that of non grafted Shiva-1 producing transgenic vines, which were used as positive controls. In the other vines, the peptide was detected at lower levels as compared to control transgenic vines. Various factors, including development of root system in individual plants, nutrient status and ability of an individual plant to transport nutrients through the graft union via xylem could account for such differences. Based on ELISA results from chemically synthesized Shiva-1 protein (results not shown), we estimated that trans-protein levels in vine sap range from 1.2 µg to 1.8 µg per ml of xylem sap.

CONCLUSIONS

In this study, we determined that Shiva-1, a small lytic peptide, is transmitted in xylem sap from transgenic rootstock to non-transgenic scion. This suggests the possibility of protecting non-transgenic scions by use of transgenic rootstocks to inhibit the xylem-limited bacteria *Xylella fastidiosa* and thus control PD. This approach is particularly attractive because it greatly reduces the transgenic varieties needed to only a few rootstocks, conceivably allowing any non-transgenic scion to be protected. The approach also solves concerns about unwanted gene flow. The presence or absence of this trans-peptide in scions was only tested to within a meter of the graft union. Thus, further studies are needed to measure transmission to distal regions of larger vines.

ACKNOWLEDGEMENTS

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Literature Cited

De Boer, A.H. and Volkov, V. 2003. Logistics of water and salt transport through the plant: Structure and functioning of the xylem. Plant Cell Environ. 26:87-101.

Hopkins, D.L. 1977. Diseases caused by leafhopper-borne, rickettsia-like bacteria. Ann. Rev. Phytopathol. 17:277-294.

Jaynes, J.M. 1993. Use of genes encoding novel lytic peptides and proteins that enhance microbial disease resistance in plants. Acta Hort. 336:33-39.

Jaynes, J.M. 2002. Methods of using helical peptides in plants. United States Patent Application 2002002591.

Lavigne, C., Klein, E.K. and Couvet, D. 2002. Using seed purity data to estimate an average pollen mediated gene flow from crops to wild relatives. Theor. Appl. Genet. 104:139-145.

Li, Z., Jayasankar, S. and Gray, D.J. 2001a. Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). Plant Sci. 160:877-887.

Li, Z.T., Jayasankar, S. and Gray, D.J. 2001b. An improved enzyme-linked immuno-absorbent assay protocol for the detection of small lytic peptides in transgenic grape-vines (*Vitis vinifera*). Plant Mol. Biol. Rep. 19:341-351.

Li, Z.T., Jayasankar, S. and Gray, D.J. 2004. Bi-directional duplex promoters with duplicated enhancers significantly increase transgene expression in grape and tobacco. Transgen. Res. 13:143-154.

Reed, W.A., Elzer, P.H., Enright, F.M., Jaynes, J.M., Morrey, J.D. and White, K.L. 1997. Interleukin 2 promoter/enhancer controlled expression of a synthetic cecropin-class lytic peptide in transgenic mice and subsequent resistance to *Brucella abortus*. Transgen. Res. 6:337-347.

Winkler, A.J., Cook, J.A., Kliewer, W.M. and Lider, L.A. 1974. General Viticulture. University of California Press, Berkeley, CA.

Figures

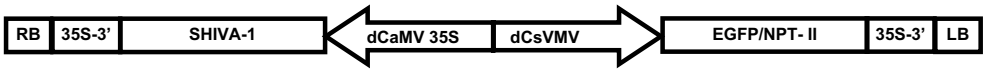

Fig. 1. Physical map of T-DNA region of the transformation vector pSEN containing Shiva-1 and fusion EGFP/NPT-II genes under control of bi-directional promoters. dCaMV35S, doubly enhanced (2 x -419 to -90) CaMV 35S promoter; dCsVMV, doubly enhanced (2 x -443 to -123) CsVMV promoter; 35S-3', the termination site and polyadenination signal of the CaMV 35S transcript; RB, right border; LB, left border.

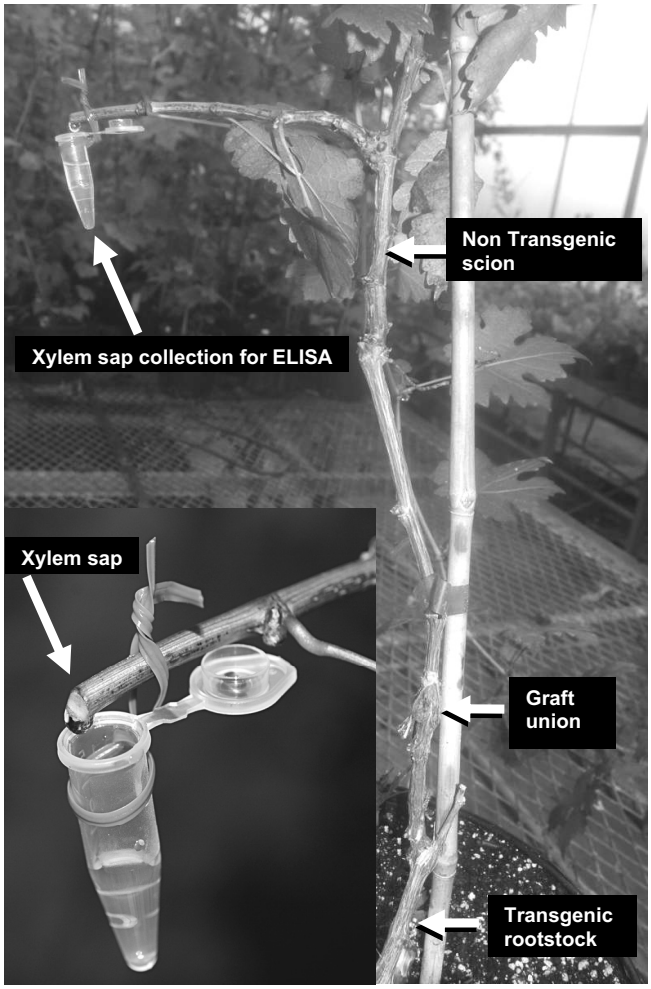

Fig. 2. Collecting xylem sap from a grafted grapevine.

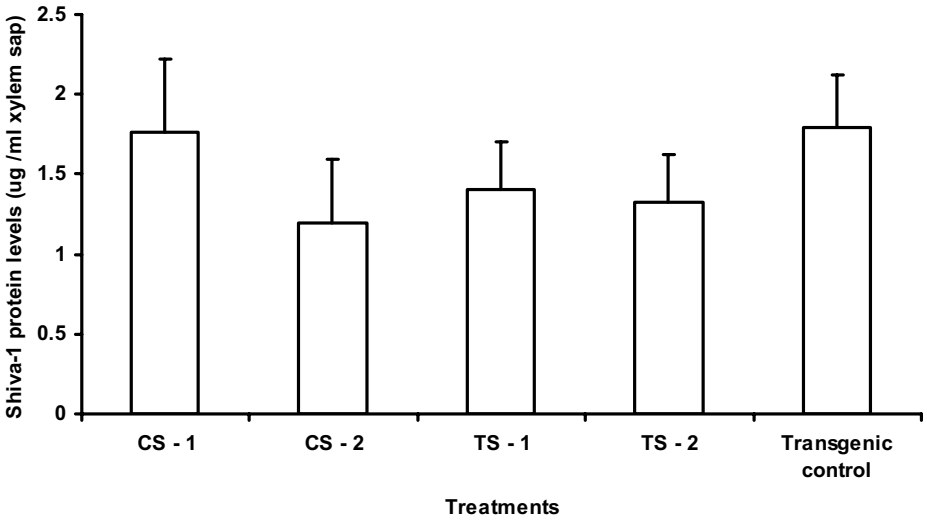

Fig. 3. Quantitative determination of Shiva-1 protein in xylem sap by ELISA. Shiva-1 protein levels were calculated based on absorbance values at 405 nm and measured at 20 min. after the initiation of enzymatic reaction. Transgenic 'Thompson Seedless' Shiva-1 producing vines were used as rootstock. CS-1 and CS-2 are vines containing non-transgenic 'Cabernet Sauvignon' grafted as scions and TS-1 and TS-2 are vines containing non-transgenic 'Thompson Seedless' grafted as scions. Transgenic control consisted of a non-grafted Shiva-1 producing vine. Bars represent average values from 3 independent experiments. The standard error values ranged from 0.2 to 0.45.