

IMPROVING OUR UNDERSTANDING OF SUBSTANCE TRANSPORT ACROSS GRAFT UNIONS

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ABSTRACT

The goal of this project is to determine whether it is possible to mobilize proteins from rootstocks to scions via xylem transport. Xylem-mobile gene products may provide a means for control of Pierce's disease (PD). Multiple lines of transgenic grapevines containing NPT-II, endochitinase, and GUS/NPT-II fusion genes are being used to investigate the movement of transgene products across graft unions into non-transgenic scions. These transgenic lines produce proteins that differ in molecular weight [29 kDa (NPT-II), 42 kDa (endochitinase), and 97 kDa (GUS/NPT-II fusion product)] as well as concentration. Lines were chosen for either high or low levels of expression in order to determine whether the root concentration of each protein would affect levels found in non-transgenic scions. To determine transgenic protein concentrations in root tissues, protein assays were conducted using in-vitro grown transgenic vines corresponding to the lines grown in the field. ELISA assays were used to determine NPT-II protein concentrations ($\mu\text{g/g}$ protein). Chitinase activity was determined according to a fluorescence assay. As results of these assays, selected transgenic lines were chosen for use as rootstocks with non-transgenic Chardonnay scions. More than five grafted vines of each desired combination were created via either chip-budding or approach grafting. Non-transgenic scion tissues will be assayed for the presence of rootstock-produced transgenic proteins to enhance our understanding of substance transport across graft unions. Initial testing indicates that the 97 kDa GUS/NPT-II fusion product does not move across graft unions.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a Gram-negative xylem-limited bacterium known to cause PD of grapevines. One potential approach to the control of PD is to use transgenic proteins that travel with the xylem fluid and control the proliferation of *Xf*. Protein size and concentration are important factors that may affect xylem transport.

It has generally been shown that proteins are transported mainly via the phloem for long distance distribution within the whole plant, while protein transport via the xylem tissue has not been studied in as much detail. However, some studies reported that proteins can be transported within the xylem system although distance mechanisms have not been reported. Ten to twenty proteins, including peroxidases, chitinase and serine proteases, were detected in xylem sap from vegetables (Buhtz et al. 2004). The proteins detected in the xylem sap ranged from 10 to 100 kDa in weight. A polygalacturonase-inhibiting protein (36.5 kDa) was detected in the xylem sap of scions grafted on PGIP-transgenic grapevines (Agüero et al. 2005). Some disease related proteins (10 to 60 kDa) were also detected in xylem sap of diseased tomatoes (Rep et al. 2002). Xylem sap proteins are likely related to plant defense systems, including repair, as well as pathogen and stress resistance (Buhtz et al. 2004, Rep et al. 2002).

The protein concentration in the xylem sap is likely the most important factor to consider in the use of transgenic proteins to control *Xf*. Although the total protein in xylem sap is relatively small, it is also true that there are not very many proteins (Buhtz et al. 2004, Rep et al. 2002). Also the sap protein concentration likely fluctuates depending on the circumstances of plant growth and the environment (Rep et al. 2002, Oda et al. 2003).

With so much to be learned about xylem protein transport, utilization of transgenic rootstocks and non-transgenic scions will be effective to help delineate some of the features of xylem protein movement. Our hypothesis is that cellular proteins, especially those secreted to the extracellular spaces, can move into the xylem sap and be transported across a graft union and into the scion, depending on either protein size or concentration in the rootstock. Three transgenic proteins, neomycin phosphotransferase II (NPT-II, 29 kDa), endochitinase (42 kDa), and the GUS/NPT-II fusion protein (97 kDa) under control of constitutive promoters (35S, *Arabidopsis* ubiquitin, and *Nos* promoters), were transformed into grapevines. The relation between protein concentration in the rootstock and the resultant concentration in a non-protein producing scion will be examined. The GUS/NPT-II gene fusion is available only in the cultivar Chancellor, whereas all other transformed vines used in this project were developed from 'Chardonnay' (clone 95) and 'Merlot.'

We report here results concerning the selection of transgenic vines for either high or low root tissue concentrations of the mentioned proteins, and for creating grafted vines for further experiments to learn whether protein size and concentration in the rootstock affect levels found in the scion.

OBJECTIVES

1. Selection of transgenic lines with either high or low levels of transgenic proteins in root tissues.
2. Development of graft combinations between the transgenic lines selected (#1 above) and non-transgenic scions.
3. Study substance transport across the graft union, especially in relation to xylem transport.

RESULTS

Selection of transgenic lines with either high or low levels of transgenic proteins in root tissues

NPT-II protein levels in roots of in-vitro cultures lines were determined using an ELISA assay (Agdia, Elkhart, Indiana). Endochitinase activities were measured using the umbelliferyl fluorescence assay (Carsolio et al. 1994). Transgenic grapevines analyzed were chosen from the following three groups: 1. A series of lines of Chardonnay producing NPT-II along with one of three antimicrobial peptides; 2. Multiple lines of Chardonnay and Merlot producing both NPT-II and endochitinase; 3. Two lines of Chancellor with GUS/NPT-II gene fusion producing a fused protein product. All of these lines produce transgenic products under control of constitutive promoters described previously (Reisch et al. 2004).

Expression of NPT-II protein in roots of transgenic lines varied between 0.1 and 1.9 $\mu\text{g/g}$ protein (Figure 1), while no expression was found in either non-transgenic Chardonnay or Merlot.

Seven of nine lines of Chardonnay showed endochitinase activity ranging from 21.5 to 32.3 nM/min/ μg protein. One line (CdEN33) exhibited low activity and also showed very poor growth among in-vitro, greenhouse, and field grown vines (Figure 2). Merlot endochitinase-transformed vines varied greatly for chitinase activity, ranging from 65 nM/min/ μg protein (line MEN9) to under 5 nM/min/ μg protein (MEN7).

Two lines of Chancellor transformed with GUS/NPT-II gene fusion producing a fused protein product (97 kDa) were evaluated using a histochemical GUS detection assay (Jefferson et al. 1987). One line (Chan 1055) strongly expressed GUS activity in all tissues, while the other line (Chan 1134) showed no GUS expression, even though the gene was present.

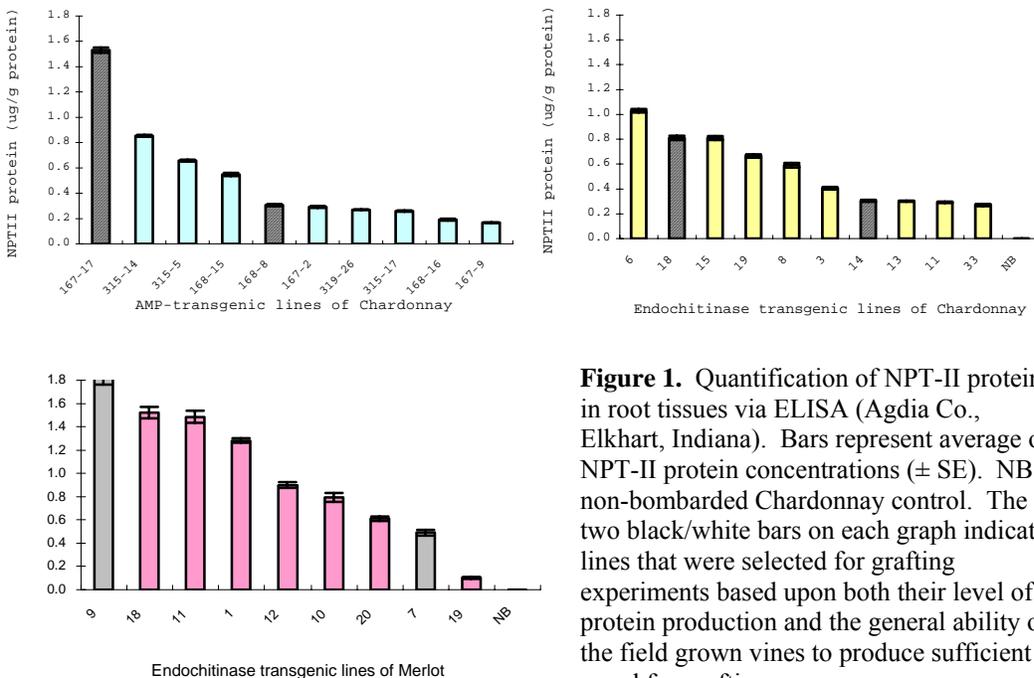


Figure 1. Quantification of NPT-II protein in root tissues via ELISA (Agdia Co., Elkhart, Indiana). Bars represent average of NPT-II protein concentrations (\pm SE). NB: non-bombarded Chardonnay control. The two black/white bars on each graph indicate lines that were selected for grafting experiments based upon both their level of protein production and the general ability of the field grown vines to produce sufficient wood for grafting.

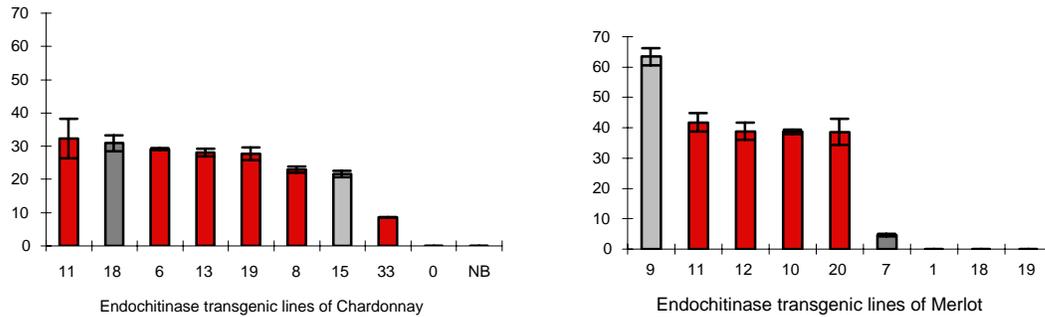


Figure 2. Root endochitinase activities according to the umbelliferyl fluorescence assay (Carsolio et al. 1994). Bars represent the average of endochitinase activities (\pm SE). EN: endochitinase. NB: non-bombarded Chardonnay control. The two black/white bars on each graph indicate lines that were selected for grafting based upon level of endochitinase production.

Development of graft combinations between selected transgenic lines and non-transgenic scions

Transgenic lines to be grafted with non-transgenic Chardonnay scions were selected considering not only the level of protein produced but also the availability of healthy wood for bench-grafting. With regards to cultivars and type of transgenic proteins, two lines of each were chosen, based on having either high or low transgenic protein levels, in addition to non-transgenic controls.

The selected transgenic lines were grafted with non-transgenic Chardonnay using chip-budding and green stem-approach grafting techniques. One line of Chardonnay and two lines of Merlot were suitable for both NPT-II and endochitinase analyses. A total of ten rootstock/scion combinations were grafted, including negative controls, and more than five grafted vines of each combination were established. Xylem sap and leaf tissues from scions will be analyzed for presence of transgenic proteins.

Transport of GUS/NPT-II fusion products

GUS/NPT-II fusion products (97 kDa) were strongly expressed in phloem vascular tissues as well as xylem parenchyma cells in Chancellor line 1055. In a three-month old graft union of a transgenic rootstock and non-transgenic scion, GUS expression could only be detected in transgenic rootstock tissues, but not in scion tissues (Figure 3A, 3B). It appears that the GUS/NPT-II fusion protein is not transported from transgenic rootstock cells to non-transgenic scion cells, nor can it be detected in the xylem vessel elements. This result is not surprising given the very large size of the protein.

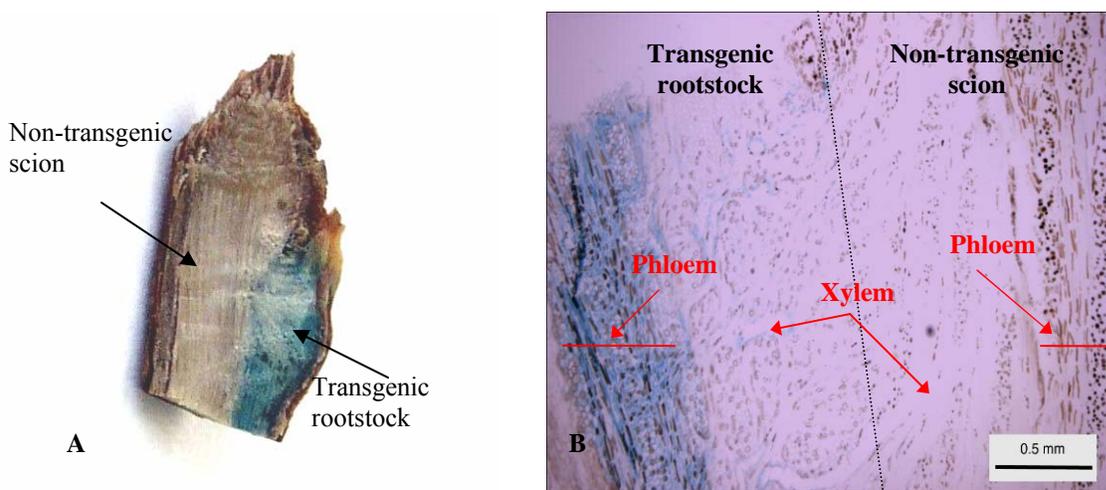


Figure 3A and 3B. GUS expression at the graft union between a transgenic rootstock (Chancellor 1055) and non-transgenic scion (Chardonnay) three months after grafting. GUS protein was detected by histochemical methods using vertical stem sections and visualized by the blue color of tissues.

CONCLUSIONS

1. Transgenic rootstocks with either high or low expression of three different proteins were grafted with non-transgenic Chardonnay scions and more than five grafted vines of each combination were established.
2. Grafted vines are growing in a greenhouse for further analysis. Initial data show that 97 kDa protein is not transported across the graft union. Enhancing our understanding of substance transport across graft unions will be of great use in designing strategies to deploy rootstocks for control of PD.

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