CROWN GALL RESISTANT WALNUT ROOTSTOCKS: ANALYSIS OF THE GRAFT UNION FOR TRANSMISSION OF GENETIC AND BIOLOGICAL MATERIALS.

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ABSTRACT

Crown gall disease, caused by the bacterium Agrobacterium tumefaciens, is a significant source of economic losses in walnut orchards and nurseries in California. Profits are reduced in the form of lowered productivity from galled trees, increased susceptibility to other pathogens and/or adverse environmental conditions, and unsalable stock at the level of nurseries. A resistant rootstock was developed as a potential solution to this problem using RNAi technology. These transgenic rootstocks were generated by Agrobacterium-mediated plant transformation that express double stranded RNA corresponding to the common and highly conserved Agrobacterium T-DNA encoded genes ipt and iaaM responsible for tumor formation (Escobar et al., 2001; 2002; 2003). Typically when wild type walnut plants are infected with Agrobacterium these genes are introduced into the infected cells leading to their expression that results in the uncontrolled cell proliferation and the crown gall phenotype. The genetically engineered rootstocks are able to block the expression of these genes resulting in the suppression of tumor formation (Escobar et al., 2001; 2002; 2003). Now that the success of this technology has been demonstrated with a transgenic Paradox hybrid genotype, the next step is to obtain detailed analysis of the elite transgenic material to enable deregulation of these rootstocks that would permit further extensive field testing to establish efficacy under rigorous field conditions at multiple locations and eventual commercial release. A key issue is the stability and movement of the introduced genes and their products across the graft union in bark tissues. The regulations focus on the movement of genetic materials, i.e., seeds and pollen Studies on the long distance movement of genes have been completed and show no movement of genetic materials (genes) and supports the earlier work with tomato (Escobar et al., 2003). Stegemann & Bock et al.(2009) recently documented short distance movement of genetic materials from rootstock into a few cell layers of the scion within the graft union. The movement of gene products (i.e., RNA, protein and metabolites) and to the extent this occurs was the focus of this study. No movement of small RNA was observed past the graft union. The level of the two expressed proteins present on the T-DNA was below the levels of detection. The analysis of metabolites revealed almost 200 different metabolites, only a handful of which appear to be significantly different in transgenic tissues; none of these appear to be related to the activity of the transgenes but appear to be more related to plant stress response and could represent plant environment interaction.

OBJECTIVES

Analysis of walnut graft union for the movement of novel biological materials.

Activity 1: Transcriptome analysis by deep sequencing
Activity 2: Proteome analysis by targeted proteomics
Activity 3: Metabolome analysis using GC-MS
SIGNIFICANT FINDINGS

No movement of small RNA from transgenes was observed past graft union
• Transcriptome was analyzed for movement of small RNA corresponding to the introduced transgenes containing iaaM and ipt sequences.
• No movement of small RNA corresponding to either iaaM or ipt was observed

No movement of proteins was observed past the graft union from transgenic rootstock.
• No protein corresponding to the GUS gene could be detected in bark tissues, the levels if present were below the levels of detection even in the bark tissues obtained from transgenic rootstock.
• Targeted proteomics for the GUS gene product also failed to detect any movement of GUS protein.
• Sensitive GUS florescence assay revealed GUS activity in rootstock and graft union but not in scion tissues

No movement of any transgenic related metabolites was observed from transgenic rootstock to scion
• One hundred and ninety nine biochemical were detected in our 28 samples
• Of these 166 were found in bark tissues
• There were many differences in metabolites when comparing tissues, Paradox vs. Chandler and pellicle vs. kernel
• Very few differences: 7 were observed comparing Chandler to Chandler grafted to transgenic Paradox, 4 when comparing normal vs. transgenic graft union and 3 when comparing normal vs. transgenic Paradox rootstock.
• None of the different metabolites could be traced to the activity of the transgenes and represented stress responses or potentially differences due to the environment.

PROCEDURES

Sample collection: An elite transgenic event, Paradox line J1 19A was selected as the transgenic rootstock as it showed the best resistance to tumor formation. The crown gall resistant paradox rootstock (CGR-PR) line designated J1 19A was created using the binary vector pDE00.0201 (Escobar et al., 2001; 2002; 2003a and Figure 1). This event is planted in a field plot in Yolo County where this event grows as a tree as well as a rootstock grafted to a Chandler scion. We chose two trees, one with two grafted Chandler branches, so has two graft unions, and one tree planted as a rootstock grafted with Chandler as a scion variety. As a control we have three wild type Paradox rootstocks (sensitive to Agrobacterium tumefaciens) grafted to Chandler scion variety in the same block. From each of these trees we have collected three types of bark tissues; the graft union and tissues immediately above and immediately below for each of the two types of graft unions shown in Fig 2 and 3 in which wild type Chandler is used as scion and a transgenic line of Paradox (J1 19A) that shows resistance to Agrobacterium tumefaciens as rootstock. In addition, we have collected the pellicle and kernel from 3 nuts harvested from Chandler grafted to transgenic rootstock and 2 nuts from Chandler grafted to wild type paradox rootstock (Fig 2). In total we are analyzed 28 samples for each of our activities (Table 1). All samples were collected in the field and were flash frozen in liquid nitrogen immediately after harvesting. Collected samples were stored frozen at -80C prior to the analysis.
Activity 1: Transcriptome analysis by deep sequencing: For this activity each of the 28 tissue samples were ground to a fine powder in liquid nitrogen and then total RNA was extracted using a phenol extraction method that incorporates guanidine isothiocyanate (to rapidly inactivate RNase activity). The total RNA was then fractionated into small and long RNAs. We used the small RNA fraction to construct small RNA libraries using an miRNA library construction kit. For library preparation we used TruSeq RNA Sample Prep Kit (Illumina Inc., San Diego, CA). The RNA samples were barcoded using two sets of 12 barcodes which allowed us to use 24 of the 28 RNA samples. The highlighted samples in Table 1 indicate the samples that were not used in the smallRNA-Seq experiments. The steps involved in the library preparation was first the chemical fragmentation of the RNA followed by first strand cDNA synthesis, second strand cDNA synthesis, repair ends, adenylate 3’ ends, ligate adapters, PCR amplification, validate library size, and pool libraries that were sequenced on two HiSeq2000 lanes, each with 12 libraries. The prepared samples were sequenced at the DNA Technology Core facility at the UC Davis Genome center.

Raw, demultiplexed sequence files were produced by the UC Davis DNA Technologies Core. The reads were processed by the UC Davis Bioinformatics Core using in-house developed software to assess overall read quality by sample, to trim 3’ adapter contamination, and to trim low-quality sequences. The trimmed reads were aligned to a sequence dataset containing the iaaM and ipt sequences used for the construction of the transgenes, the previously assembled walnut (Chandler) transcriptome plus the GUS and nptII marker transgenes using BWA’s short read aligner (Li and Durbin, 2009) to generate a table of read counts by transcript.

The Bioconductor package DESeq (Anders and Huber, 2010) was used to normalize the data. Diagnostic plots were also produced. A Kruskal-Wallis test (Kruskal and Wallis, 1952) was applied to the normalized counts for each transgene to determine the significance of differential expression between each of the control and transgenic samples.

Activity 2: Targeted Proteome Analysis: We targeted the analysis to focus on the GUS protein, which is expressed on the same T-DNA (Fig 1). To detect the transgenic protein, a peptide library was prepared from the known sequence. We purchased pure GUS protein produced commercially (Abcam Inc). In order to ensure the purchased GUS protein was in a pure form free from any residual salts we precipitated it. The protein pellet after precipitation was resuspended in 50mM ammonium bicarbonate (AmBiC) until fully dissolved before adding enough acetonitrile so that the final percentage of the sample was 10% and then TCEP (Pierce Bondbreaker) was added to give a 10mM final concentration. The sample was then heated at 90°C for ten minutes on a thermomixer and cooled to ambient temperature for about five minutes. Iodoacetamide (IAA) was then added to give the sample a final concentration of 15mM before incubating in the dark on shaker for one hour. Dithiothreitol (DTT) was then added to quench the iodoacetamide and enough was added to make the final concentration of DTT to be 5mM. The pellet was then trypsinized using Promega sequencing grade trypsin and incubated at 37°C overnight. The peptides were then analyzed on a LTQ (Thermo Fisher) coupled with a Michrom-Bruker HPLC paradigm (Michrom-Bruker, Auburn, CA, USA) using a captive spray ionization source and a reverse phase column. The peptides were eluted using a sixty minute LC/MS method where the primary solvents were 0.1% Formic Acid and 100% Acetonitrile. The raw LC/MS/MS data was then analyzed using custom scripts and software available at the UCD
Genome Center and visualized using Scaffold 3. Peptides and their corresponding transitions with the highest signal intensity were chosen to create a targeted method on Skyline (Macoss Lab, WA). The targeted method chooses peptides seen in the shotgun proteomics data before being run on the TSQ-vantage (Thermo Fisher) coupled with a Michrom-Bruker Advance UPLC system. The method was then refined to include the top four transitions for each peptide and at least three unique peptides per protein to justify the presence of that protein in the walnut tissue sample.

Activity 3: Metabolome analysis using GC-MS: All 28 bark and nut samples (Table 1) were analyzed with the three instruments at Metabolon Inc, and their ion features were matched against a chemical library for identification. The major components of the process are summarized below.

For this experiment, 20 mg of each bark or nut sample was thawed on ice and extracted using an automated MicroLab STAR system (Hamilton Company) in 400 ul of methanol containing internal standards.

UPLC/MS was performed using a Waters Acquity UHPLC (Waters Corporation) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific Inc.) equipped with an electrospray ionization source. Two separate UHPLC/MS injections were performed on each sample: one optimized for positive ions and one for negative ions. Derivatized samples were used for GC/MS and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS operated at unit mass resolving power. Chromatographic separation, followed by full-scan mass spectra, was performed to record retention time, molecular weight (m/z), and MS/MS2 of all detectable ions present in the samples.

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments, as well as their associated MS/MS2 spectra. This library allows the rapid identification of metabolites in the experimental samples with high confidence. Comparison of experimental samples to process blanks (water only) and solvent blanks allows the removal of artifactual peaks.

RESULTS AND DISCUSSION

Transcriptome analysis by deep sequencing: Extraction of total RNA was quite a challenge from walnut bark tissues. Walnut bark tissue has a very highly reactive organic composition which makes it challenging to extract nucleic acid due to presences of polyphenols, carbohydrate and nucleases. We were able to extract nucleic acid with higher purity after trying many different possible procedures. Using Plant RNA concert (Invitrogen) and addition of plant RNA Aid solution (Ambion) and ascorbic acid enabled us to increase the purity however the quality (intactness) of the RNA was still a problem that we then adopted a procedure used in grapevine that was successful in obtaining undegraded high quality RNA. However, using this procedure we were successful with 24 of the 28 samples the 4 samples highlighted in yellow in Table 1 we were unable to make smallRNA (sRNA) libraries. The 24 libraries were each barcoded with a unique barcode and 12 samples were run on a single lane. The bioinformatics pipeline employed
was able to separate the reads for each of the samples and since each of the samples certainly for
the bark samples were in triplicate some statistics could be run on these. The reads were maps to
the iaaM and ipt transgene sequences. As shown in Fig 4A for the iaaM gene there were over
400,000 reads that mapped to the transgene sequences in the transgenic rootstock and in the graft
union tissues but the scion tissues had very few reads in the 10 to 100 read range that was over 3
orders of magnitude less that that observed in transgenic tissues, The bark tissues that came from
the rootstock, graft-union and scion of the Chandler grafted to the wild type paradox rootstock
had also 3 orders of magnitude less reads mapping. The distribution was also similar for the
untransformed tissues (Fig 4A) which was very distinctly different to that observed in the
transgenic tissues. The pellicle and kernel tissues from the nuts harvested from either the tree
with the transgenic rootstock or the wild type rootstock were the same with very few reads
mapping (Fig 4B). It is clear that the siRNA certainly for the iaaM gene is not moving past the
graft union. Shown in Fig 5A and B are the reads that map to the ipt transgene observed in bark
(Fig 5A) and nut (Fig 5B) tissues. The results are very similar to that observed for the iaaM gene
with very high about 3 orders of magnitude of reads that map to the entire ipt gene. However,
past the graft union and in all of the tissues from the wild type rootstock very few reads map.
However, for the ipt gene there appears to be a very short sequence in the 3’ region of the ipt
gene that has reads map to in all tissues including the nut tissues (Fig 5A and B). It appears that
this might be microRNA but this will need to be investigated further. The conclusion of the
sRNAseq is that there appears to be no movement of siRNA form either of the transgenes used to
control Crown Gall past the graft union.

Proteome analysis by targeted proteomics: We have conducted this analysis and have identified
the best peptide from the constructed library described above in the procedures. We were unable
to detect its presence and thus conclude the absence of the GUS protein in all the tissues
mentioned in Table 1. To ensure the sensitivity and accuracy of the assay in detecting the
targeted peptide sequences, we also included a high GUS expressing walnut embryo line that has
been characterized for high expression of GUS protein. The proteomics approach was unable to
detect the physical protein even in this tissue. We then measured the enzymatic activity of the
GUS protein which is a beta-glucouronidase enzyme and since there is a very sensitive
fluorescent substrate available we were able to visualize the activity to determine the presence or
absence of the GUS protein. The outcome of this sensitive assay is shown in Fig 6 where the
transgenic rootstock and graft-union both show very high levels of activity indicating the
presence of the GUS protein in these tissues. However, the scion tissues and any of the bark
tissues obtained from Chandler grafted to wild type rootstock had no activity. We also measured
all 18 bark samples using a fluorimeter and were able to calculate the activity of GUS. Although
these are crude measurements, one can clearly see that the only observable GUS activity resides
in the highest levels in the transgenic rootstock bark tissues, followed by the graft union tissues
from the transgenic trees. The scion bark tissues or the Chandler grafted to wild type showed
very low activity, indistinguishable from background levels. Clearly, it can be concluded that the
GUS protein (the product of another transgene present in our construct) does not move past the
graft union.

Metabolome analysis using GC-MS: For this analysis we shipped the ground-up bark and nut
(pellicle and kernel) samples mentioned in Table 1 to Metabolom (metabolom.com) on dry ice to
be tested for metabolomic compositions. One hundred and ninety-nine biochemicals were
detected in this study. Of these 166 were found in bark tissue. Few differences were observed in the metabolic profile between wild type grafted to transgenic (WT-MUT) and wild type grafted to wild type (WT-WT) bark tissues. When the biochemical profile of the rootstock bark (bottom) of WT-MUT was compared with the corresponding tissue of WT-WT, a difference was observed in only 3 compounds at a statistical significance of \( p = 0.05 \). Similarly, only 4 compounds in the middle bark and 7 in the top bark, showed a variation in the WT-MUT when compared with corresponding tissue from WT-WT. At a significance of \( p = 0.05 \), about 8 biochemicals are expected to be significantly different due to random chance. Although, there were many changes in the biochemical composition of the rootstock when compared to the scion, in most cases the same changes were observed in both groups (MUT-WT and WT-WT).

Comparison of the WT and transgenic nut (seed) showed no difference in biochemical composition. However, there appears to be many differences between the kernel (inner seed) and the outer coat or pellicle. The pellicle has a higher content of antioxidants, alpha and gamma tocopherols, ascorbic acid / dehydroascorbic acid and secondary plant metabolites including anthocyanins and ellagic acid (tannins), than the inner seed. The content of various sugars was higher in the pellicle relative to the inner seed, and amino acid levels, particularly proteinogenic amino acids, was lower in the pellicle relative to the inner seed. This information implies that the pellicle is very rich in important nutrients.

References:

Table 1: 28 tissue samples collected from grafted walnut trees.

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<th>Bark Samples</th>
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Fig 1: Binary vector used to confer crown gall resistance in Paradox walnut rootstocks
Fig 2: Graft-union study design for harvested tissues

Fig 3: Chandler scion grafted to transgenic Paradox rootstock one year after bark was removed for analysis

Fig 4A: Mapping of sRNAseq reads to the iaaM transgene present in different bark tissue samples.
Fig 4B: Mapping of sRNAseq reads to the ipt transgene present in different bark tissue samples

Fig 5A: Mapping of sRNAseq reads to the iaaM transgene present in different nut tissue samples
Fig 5B: Mapping of sRNAseq reads to the ipt transgene in nut tissues

Fig 6: Mapping and measurement of a fluorescent substrate to determine the presence and activity of the GUS protein in different bark tissue samples
Fig 7: Analysis of metabolites in bark and nut tissues. WT-wild type; MUT-Transgenic; TOP-Scion; MIDDLE-graft union; BOTTOM-Rootstock; INNER-Kernel; OUTER-Pellicle