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Transgenic American chestnuts show enhanced blight resistance and transmit the trait to T1 progeny

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ABSTRACT

American chestnut (*Castanea dentata*) is a classic example of a native keystone species that was nearly eradicated by an introduced fungal pathogen. This report describes progress made toward producing a fully American chestnut tree with enhanced resistance to the blight fungus (*Cryphonectria parasitica*). The transgenic American chestnut 'Darling4,' produced through an *Agrobacterium* co-transformation procedure to express a wheat oxalate oxidase gene driven by the VspB vascular promoter, shows enhanced blight resistance at a level intermediate between susceptible American chestnut and resistant Chinese chestnut (*Castanea mollissima*). Enhanced resistance was identified first with a leaf-inoculation assay using young chestnuts grown indoors, and confirmed with traditional stem inoculations on 3- and 4-year-old field-grown trees. Pollen from 'Darling4' and other events was used to produce transgenic T1 seedlings, which also expressed the enhanced resistance trait in leaf assays. Outcrossed transgenic seedlings have several advantages over tissue-cultured plantlets, including increased genetic diversity and faster initial growth. This represents a major step toward the restoration of the majestic American chestnut.

1. Introduction

1.1. Background

The American chestnut (*Castanea dentata* [Marsh.] Borkh.) was ecologically and economically significant throughout the eastern United States until it was nearly eliminated by an invasive fungus (*Cryphonectria parasitica* [Murr.] Barr.) that causes chestnut blight. The blight was first identified in 1904 [1] and had spread through the American chestnut's range by the 1950s [2,3]. Treatment and control efforts attempted while the blight was spreading were generally unsuccessful. However, surviving American chestnut stump sprouts have left interested parties with a partially intact gene pool and therefore some potential for restoration. The backcross breeding program implemented by The American Chestnut Foundation aims to produce a chestnut tree with primarily American chestnut growth and nut characteristics, while retaining blight resistance traits from the Chinese chestnut (*Castanea mollissima*) [3]. However, the Chinese chestnut's blight resistance is quantitative, involving three primary resistance loci (of up to seven total) [4], which makes breeding a challenge. Another approach to producing an American chestnut tree with enhanced blight resistance is to directly transfer resistance-enhancing genes via *Agrobacterium*-mediated transformation [5]. In contrast to traditional breeding, genetic transformation offers complete control over which genes are inserted into a host organism. In addition to some transgenic agricultural crops which have become almost ubiquitous in the US, a variety of transgenic trees has been reported for several purposes including timber production and wood properties [6], fruit production [7], freeze tolerance [8], and disease

Abbreviations: AC, American chestnut; AMP, antimicrobial peptide; BP, base pairs; CC, Chinese chestnut; GFP, green fluorescent protein; GOI, gene of interest (here, putative resistance-enhancing gene); OD, optical density; OxO, oxalate oxidase; PDA, potato dextrose agar; qPCR, quantitative polymerase chain reaction (typically on genomic DNA, without reverse transcription); RT-qPCR, reverse-transcription quantitative polymerase chain reaction; T1, first transgenic generation after pollination, offspring of T0 (original transgenic parent).

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resistance [9–11]. American chestnut specifically has previously been transformed with marker genes and regenerated into sexually mature trees [12]. Transformation of American chestnut with putative blight resistance genes has also been reported [13–15]. The basic transformation method used in the current study has been optimized and several variations have been tested [13,16], resulting in a repeatable protocol that has been effectively implemented by many researchers and students.

1.2. Transgene selection and co-transformation

Putative resistance-enhancing transgenes are typically accompanied by additional marker genes to help with identification and selection of transformed tissue early in the transformation process, after which these genes are unnecessary. In many *Agrobacterium* transformation protocols, the marker genes are incorporated into a single plasmid construct along with the gene of interest (GOI). Alternatively, *co*-transformation involves transforming target tissue with two separate plasmid constructs, then selecting for tissue that has been transformed by both constructs. The primary advantage of this system is that it allows at least some of the marker genes to be bred out in subsequent generations, but it also simplifies creation and testing of new GOI constructs, and permits the use of smaller constructs.

The transgenic lines in the current study contain one or both of two GOIs: oxalate oxidase (OxO) alone [17], or a stacked combination of OxO and a synthetic antimicrobial peptide called ESF39 [9,18,19]. Oxalate oxidase, which degrades oxalic acid into hydrogen peroxide and water, is a member of a gene family found in most monocots. Oxalic acid is a toxic component produced by *C. parasitica* and a fundamental part of this necrotroph's virulence against the host [20,21]. Consequently, degrading this molecule by means of the oxalate oxidase enzyme may directly prevent blight symptoms. Furthermore, hydrogen peroxide is a signal molecule in the host disease response pathway [22], so the activity of oxalate oxidase may protect the host at multiple levels. Antimicrobial peptides (AMPs) are small molecules with broad antifungal and antibacterial properties, and they are found in a wide variety of organisms. ESF39 is a synthetic AMP, designed with a structure similar to a magainin-type AMP, modified to ensure safety to eukaryotes and enhanced activity against prokaryotes [18,19].

1.3. Partial blight resistance

While the various genes described above should in theory be able to impart blight resistance characteristics to the host plant, there are a large number of factors that control whether and to what extent a gene is expressed. Additionally, according to the classic "disease triangle," variations in the environment, the host, and the pathogen all affect the outcome of a disease. Given all these factors, complete and durable resistance to blight is unlikely to be achieved quickly or simply. Indeed, there are up to seven genes or gene families that contribute to the natural blight resistance of the Chinese chestnut [4]. These genes are quantitative, meaning that any one of the genes on its own can only be expected to impart partial resistance to the host. The same pattern may be expected in transgenic plants: a single GOI may enhance resistance beyond the level found in a wild-type host, but complete or durable resistance may require multiple genes. Therefore, screening procedures used in this study were chosen specifically to observe partial or enhanced resistance, rather than a qualitative pass/fail (or survival/mortality) test.

1.4. Pollination and transgene inheritance

Some drawbacks to a basic transgenic plant production system are a direct result of the tissue culture clonal propagation. These include energy deficiencies due to the lack of the large energy stores in a seed, physiological challenges and non-apically dominant (branch-like) growth, and a notable lack of genetic diversity. Outcrossing and growing trees directly from nuts instead of tissue culture shoots could solve all of these problems. While this is not possible with our chestnut transformation and propagation process as it currently stands, it is possible to produce transgenic nuts through a cross between transgenic trees with existing wild-type trees. However, not all of these nuts will be transgenic: since a single-copy transgenic event is effectively heterozygous for the transgene allele, approximately half of the offspring should contain the transgene (potentially more than half from multiple-copy events). Also, in contrast to a traditional breeding program in which there are multiple quantitative resistance-enhancing genes, any offspring from a transgenic cross that DO contain the transgene(s) should in theory exhibit the same trait as the parent.

2. Materials and methods

2.1. Transgene constructs and host tissue

The two vectors used in a co-transformation may vary between experiments, but in the experiments described here they fall into two categories. First, the Gene of Interest (GOI) vector contains one or more putative resistance-enhancing genes as well as a selectable marker for antibiotic or herbicide resistance. The two GOI plasmid vectors in this experiment were pTACF3 and pTACF7 (Fig. 1), both of which contain genes used in previous transformations. Plasmid TACF3 is based on pVspB-OXO, which has been previously transformed into American chestnut [13] and poplar [17], and the GOI is controlled by the same promoter (vegetative storage protein B from *Glycine max*) as used in these transformations. Plasmid TACF3 was compared to another OxO-containing plasmid in a recent American chestnut co-transformation experiment [15]. Plasmid TACF7 contains the oxalate oxidase gene and VspB promoter from pVspB-OXO, as well the synthetic antimicrobial peptide pSE39, which has been transformed into elm [10,23] and poplar [9], and is controlled by the ACS2 vascular promoter (isolated from American chestnut [24]) used in these elm and poplar studies. Next, the marker vector typically contains an easily identifiable visual marker, such as constitutively expressed GFP, which was used in this experiment under control of the CaMV-35S promoter (Fig. 1) [15]. In the current study, both of these constructs were transformed into American chestnut somatic embryos from the line known as WB275-27 [13].

2.2. Agrobacterium mediated co-transformation and regeneration

Agrobacterium transformation was carried out according to previously described protocols [5,14,16], including a key desiccation step [25], with minor changes to allow for co-transformation with multiple constructs. Specifically, the *Agrobacterium* inoculum used during the co-cultivation step consisted of a mixture of two *Agrobacterium* cultures (both from strain EHA105) containing different vectors as described above. Each culture was quantified according to Optical Density (OD, absorbance at 650 nm), and they were combined prior to embryo co-cultivation. (The total quantity of *Agrobacterium* remained the same as that used in single-vector transformations.) The cultures containing the GOI and Marker vectors were mixed at a 2:1 ratio based on OD, with the GOI vector

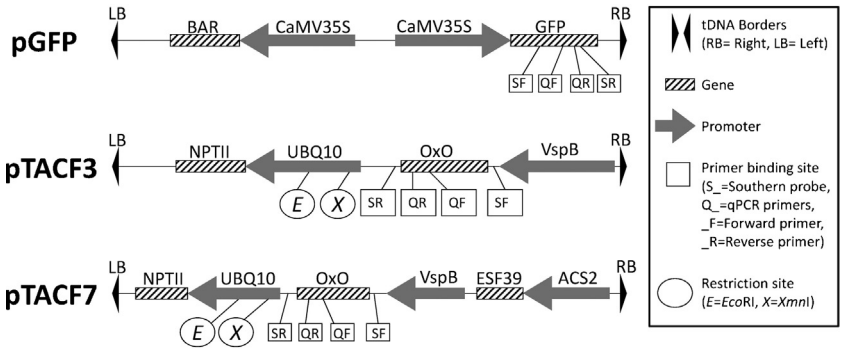

Fig. 1. tDNA maps of each transgene construct. Maps are for positional and directional reference only; length is not to scale. Genes, promoters, primer binding sites, and restriction sites are indicated. Abbreviations: BAR = gene for bialaphos resistance, CaMV35S = cauliflower mosaic caulimovirus 35S promoter, NPTII = neomycin phosphotransferase II selectable marker gene, UBQ10 = constitutive Ubiquitin 10 promoter from *Arabidopsis thaliana*, VspB = vascular storage protein B promoter.

at a higher concentration to decrease the frequency of Marker construct-only transformations.

After transformation, embryos were maintained on a selection medium containing the antibiotics carbenicillin (200 mg/L) and cefotaxime (100 mg/L, both to kill *Agrobacterium*) as well as the herbicide Finale® (1 mg/L phosphinothricin, to kill non-transformed embryogenic tissue). Putative transgenic embryos were selected based on GFP expression as observed under a stereomicroscope [13] (starting with discreet foci on an embryo, some of which developed into entirely transgenic embryos, which were then multiplied into embryo clumps, see Fig. 2.)

2.3. Tissue culture and acclimatization

Once foci had developed into entire fluorescing embryo clumps, they were moved their own Petri dishes and treated as unique transformation events through subsequent multiplication and regeneration steps, as described by Polin et al. [13]. Since each of these putative lines originated from a single embryo, mixed or multiple-line cultures were extremely unlikely. The embryos were stimulated to grow into shoots, rooted, and potted in Fafard Superfine Germinating Mix (Conrad Fafard, Inc., Agawam, MA) in D27 DeePots (Stuewe & Sons, Inc., Tangent, OR) for acclimatization [5,14,26]. New plantlets were maintained in a high-humidity growth chamber for approximately 6–20 weeks, and then moved to a greenhouse for experimentation and/or acclimation to field conditions.

2.4. Initial confirmation of transgene integration

The first step in confirming transgene integration was an embryo's survival on selection medium. This indicated the selectable marker genes (neomycin phosphotransferase II [NPTII] and bialaphos resistance [BAR]) from both constructs had been integrated into the genome and were being expressed in the embryo. Visual selection for GFP confirmed that all embryos contained and expressed GFP from the Marker construct, eliminating the possibility of non-transgenic "escapes." After *Agrobacterium*-free, single-event embryogenic lines had been established, further confirmation of GOI construct integration was accomplished with standard PCR ([27], AN/AZ primers, see Table 1 for sequences).

2.5. Transgene copy number (Southern hybridization, qPCR)

Southern hybridization is a traditional method of confirming transgene presence in the host genome, determining transgene copy number, and distinguishing unique transgenic events. Genomic DNA for Southern hybridization was extracted with the "Carlson/Qiagen Method"[28], and digested separately with *Eco*RI and *Xmn*I (both cut only once in the tDNA of each GOI construct, Fig. 1). The use of two separate restriction enzymes enhances accuracy by reducing the chance that closely spaced transgene copies will appear to be a single insert. Digested DNA was separated on a 0.8% agarose gel at 48 V, and blotted to a positively charged nylon membrane (Amersham Hybond −N+, Buckinghamshire, UK) with the Genie electrophoretic blotter (Idea Scientific, Minneapolis, MN). A 966 bp probe (Table 1) was designed to contain the coding region of the OxO gene (present in both GOI constructs, Fig. 1), and labeled with 32P-dCTP (Stratagene Prime-It II Random Primer Kit). Hybridization was performed in Stratagene QuikHyb buffer (Agilent, Santa Clara, CA) for 2 h at 65 °C, and washed according to Stratagene's instructions. The blot was exposed to Kodak BioMax MS film for 16–18 h at −80 °C, and developed with Kodak GBX film developing solutions.

More recently, quantitative real-time PCR (qPCR) has allowed copy number determinations to be carried out more quickly and at smaller scales [29,30]. Genomic DNA for the current qPCR experiments was extracted using the Qiagen Plant Mini kit, which worked more efficiently in this application than DNA from the CTAB-based extraction procedure referenced above. At least two separate DNA extractions were performed on different individual plants from each transgenic event and amplified independently to achieve a minimum of two biological replicates for each sample type. Primers (Table 1) were designed to amplify <200 bp sections of the OxO, ESF39, and GFP genes, as well as two reference genes (glyceraldehyde 3-phosphate dehydrogenase and elongation factor 1alpha). SYBR Green reagents (BioRad, Hercules, CA) were used to assemble triplicate reactions (technical replications) according to the manufacturer's instructions, modified to a final single reaction volume of 9 μL. Amplification was done in a BioRad iCycler-48, and analyzed with BioRad CFX Manager software (v1.6). Initial template concentration, and thus copy number, was calculated with the ΔΔct method, comparing target amplification to that of two internal reference genes. All technical and biological replicates were combined in the ΔΔct calculation.

2.6. Transgene expression

Qualitative transgene expression was observed at several points through the transformation and regeneration process, and any measure of expression served as further confirmation of *intact* transgene integration. (Hybridization and PCR could theoretically give a positive result even with a partial transgene insertion.) As mentioned above, GFP (Marker construct) expression was observed visually, and only embryos that expressed GFP were selected for regeneration. When transgenic plants were being multiplied as

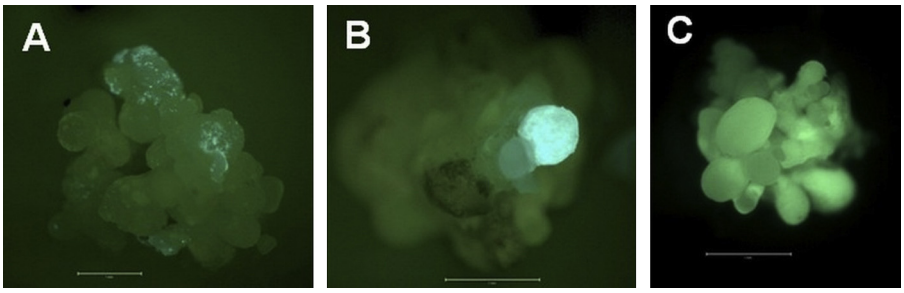

Fig. 2. GFP expression in somatic embryos after transformation. The first visible results of a successful transformation are individual transformed foci (A) which grow into fully transformed single embryos (B) and eventually entire embryo clusters (C). Bar in each photo = 1 mm.

Table 1
PCR primer details.

Name	Usage	Forward (F) sequence	Reverse (R) sequence	Product (bp)
AN/AZ	PCR hybridization probe	caacaaccagtgccatagac	tccgttcagtgaaaagaacaa	966
IDT1	qPCR expression copy #	cagcggcaaacttggacttgagaa	tgcacttccagttcaacgtcggta	192
ESF39	qPCR expression copy #	agtgatggtgatggtgatggctct	acaatatgggtaacctgcgctcct	196
GFP I1	qPCR copy #	atcaaagccaacttcaagacccgc	agggcagattgtgtggacaggtaa	140
ef1	qPCR reference gene	cggttactgagtactagccttg	ctgccgaagaccttattgaaag	84
GAP	qPCR reference gene	gctgcactaccaattgtcttg	tcattgaaggaccatcgacag	129

shoots in tissue culture, OxO enzyme activity was measured with an OxO assay [17,31]. This test is effective in confirming presence of OxO and determining which tissues primarily express the OxO gene, but precise quantitative comparisons are limited.

Quantitative PCR again served as a relatively quick and effective test at this point in the procedure. In contrast to copy number determination, expression tests were done with Reverse Transcription quantitative PCR (RT-qPCR), to measure relative quantities of mRNA present in various samples. Tissue was collected and processed separately from at least two individuals of each event in all expression studies. RNA was extracted from chestnut *stem* tissue, to observe transgene expression directly where *C. parasitica* is active. Larger (woody) stem sections were ground in the presence of liquid nitrogen with a cryogenic impact mill (6870 Freezer/Mill, Spex SamplePrep LLC, Metuchen, NJ) and younger/smaller samples were ground under liquid nitrogen with a chilled mortar and pestle. RNA was extracted according to Chang et al. [32], and an aliquot was quantified on a NanoDrop 2000 spectrophotometer. All RNA was stored at −80 °C. One microgram of RNA was treated to remove residual genomic DNA (TurboII kit, Ambion), and then reverse-transcribed to cDNA (iScript cDNA Synthesis Kit, BioRad). Amplification of cDNA was performed with the same qPCR cycler and reagents described above. Again, ΔΔct calculated with two reference genes (same software as copy number analysis above; see Table 1 for primer sequences).

2.7. Blight screening via leaf and stem

Leaf assays were performed according to Newhouse et al. [33]. Briefly, young excised-leaf midveins were lightly wounded with a scalpel, *C. parasitica* was introduced to the wound, leaves were stored in a dark, humid chamber for 5 days, and resulting necrotic areas were measured and compared between leaf types and known controls. Additionally, stem assays were performed on events that had grown to sufficient size (stem >1.5 cm diameter at 2–4 cm above ground level) in field plots. Since these stems were still smaller than those typically used for inoculations [34], a slightly modified protocol was used. First, wounds were smaller and shallower, made with a thin wire hook (Dritz "Knit Picker," Prym Consumer Co., Spartanburg, SC, available from sewing and craft stores). The Knit Picker was pressed into the outer stem bark and pulled down to create a 5 mm-long scratch of consistent depth. *C. parasitica* (Strain SG2-3 or EP155), grown on PDA, was introduced to the wound on a 3 mm agar plug, which was secured to the stem with Parafilm® for 1 week. Resulting cankers were measured bi-weekly through the summer (14 weeks post-inoculation).

2.8. Production and analysis of T1 nuts

Pollen from transgenic plants (T0 generation) was collected in two ways. First, transgenic trees have been grown in a high-light growth chamber, resulting in flower production on approximately one third of the plantlets in less than a year (for example, prior to the 2012 pollination season, 6 of 14 Hinchee1 trees produced pollen in 11 months or less, as reported by Baier et al. [35]). Pollen from these growth chamber trees was collected, filtered, desiccated, and frozen for up to 6 months prior to the typical pollination season. Second, field-planted transgenic trees greater than three years old were allowed to produce catkins, which were carefully bagged before ripening to prevent unintended release of transgenic pollen. These catkins were collected after they had ripened in the bag. In both cases, transgenic pollen was used to pollinate female flowers on wild-type (non-transgenic) American chestnut mother trees. These female flowers had been bagged before ripening, and were re-bagged immediately after pollination to contain the transgenic pollen and exclude pollen from nearby trees. Before the resulting burs ripened (in late summer), larger bags made of aluminum window screen were secured around each pollination bag to prevent animal disturbance and to keep the resulting nuts (T1 generation) contained. After ripening, nuts were collected and stored in slightly moist peat moss at 4 °C for >4 months. Open-pollinated non-transgenic control nuts from the same mother trees were collected and stored in the same manner.

2.9. Metabolite analysis

During cold stratification, a subset of the nuts was selected for metabolomics analysis. A sterilized, sharpened 12 gauge dispensing needle was used to remove a core of each of these nuts, the core was stored on dry ice until analysis, and the hole in the nut was sealed with silicone adhesive. Nut cores (7–32 mg fresh weight) were ground in liquid nitrogen and were twice extracted for their metabolites with 2.5 mL 80% ethanol overnight.

Extracts of each type were then combined prior to drying a 1.0-mL aliquot in a nitrogen stream and metabolite conversion to their trimethylsilyl (TMS) derivatives and analyzed by gas chromatography–mass spectrometry (GC–MS) using electron impact (70 eV) ionization, following protocols described previously [36]. Peaks were quantified by area integration and the concentrations were normalized to the quantity of the internal standard (sorbitol) recovered, amount of sample extracted, derivitized, and injected. A large user-created database (>1900 spectra) of mass spectral electron ionization fragmentation patterns of TMS-derivatized compounds was used to identify the metabolites of interest to be quantified. Unidentified metabolites were denoted by their retention time as well as key mass-to-charge (m/z) ratios. This analysis was performed on twelve nuts from a Hinchee1 × wild-type American cross, as well as six non-transgenic open-pollinated American chestnut and six Chinese chestnut controls, all produced in 2012. (Analysis of nuts from the 2013 pollination season, including Hinchee1 and two additional transgenic events, is in progress.) Of the twelve nuts from the Hinchee1 cross, six contained at least one copy of the GOI construct, and six were non-transgenic. These non-transgenic nuts were combined with the wild-type American and Chinese controls to form a non-transgenic standard panel.

A total of 41 major primary and secondary metabolites were quantified in all nut types (see Section 3.6). Mean metabolite concentrations were compared with two-sample t tests between the Hinchee1 transgenic nuts and the non-transgenic nuts from the same cross, since these groups contain only full siblings and are therefore more appropriate for close comparison. Additionally, total ranges (minimum and maximum) of each metabolite in the transgenic nuts were compared to the total range found in the standard panel of non-transgenic controls. After germination (initial radicle elongation), all nuts were potted, maintained in a greenhouse, and then planted outdoors. A subset of the surviving trees was tested for transgene presence, copy number, and expression in the same manner as the original transgenic events.

3. Results and discussion

3.1. Confirmation of co-transformation

Co-transformation is one method to stack, or pyramid, genes from different vector constructs in single transgenic events. Both pTACF3 and pTACF7, which contain putative blight resistance-enhancing genes with the NPTII selectable marker gene, were successfully transferred into American chestnut host tissue along with the GFP and BAR genes in pGFP. GFP presence and expression was visually confirmed early in the transformation process (Fig. 2), while oxalate oxidase enzymatic assays and PCR confirmed the presence of the GOI construct at both embryo and shoot stages. Three confirmed co-transformed events were produced containing pTACF3 and pGFP; these have been designated Darling1 (also called AN-2XG1), Darling4 (AN-2XG4), and Darling5 (AN-2XG5). Two events were produced containing pTACF7 and pGFP: Hinchee1 (KS-2PG1) and Hinchee2 (KS-2PG2). One GFP-only event was retained and brought through the regeneration process as a non-GOI control; this was called AN-2G3. Some of these events have been described briefly and studied in separate experiments looking at mychorrizal colonization of transgenic American chestnut events [37,38]. The Darling4 event has been planted and studied most extensively; not all tests explained here have been performed on the other events simply because fewer trees of those events were produced or planted. Transformation efficiency in these experiments was approximately 20 stable events per gram of somatic embryo tissue [14], but recent modifications to this procedure (unpublished) have resulted in nearly 10-fold efficiency increases.

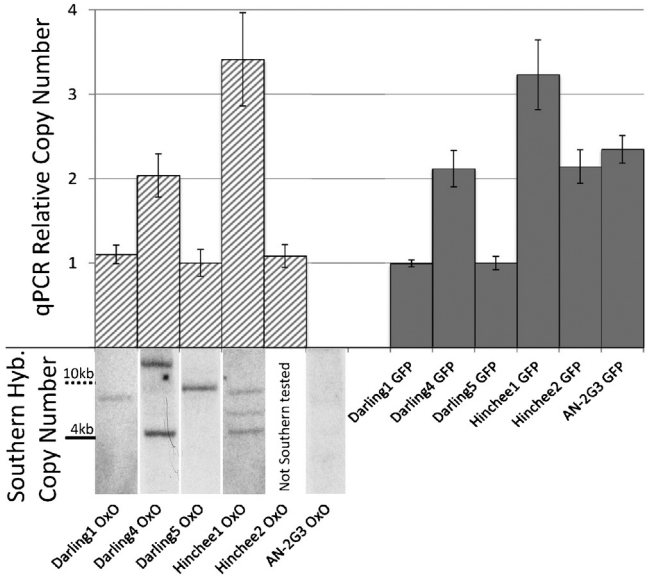

Fig. 3. Transgene copy number via qPCR and Southern hybridization. Copy number data from qPCR are shown in the form of column graphs in the upper section: Relative height of each column indicates the number of inserts in a given event. OxO data (diagonal striped bars in graph) are on the left: Three of five events contain a single copy of the OxO transgene. GFP data (solid gray bars) are on the right: two of five events have a single copy, and four of five events have the same copy numbers as for the OxO gene. Southern hybridization results for the OxO transgene are shown in the lower left section: copy number is indicated by number of bands in each lane. Southern results directly correlate with qPCR results. Dashed line across Southern blot lanes indicates 10 kilo-base pair (kbp) size marker; solid line indicates 4 kpb marker. Southern blot results shown are from restriction digests with *Eco*RI; the number of bands in each lane after *Xmn*I digests (not shown) indicated the same copy numbers.

3.2. Transgene copy number

Single transgene insertions are desired, so insert copy number was determined for all the events. Three of the five co-transformed events (Darling1, Darling5, and Hinchee2) were found to have a single copy of OxO from the GOI construct. For four of the five events, this was initially tested by Southern hybridization (indicated by the number of bands exposed onto film, *XmnI* results not shown, Hinchee2 not tested) and confirmed by quantitative PCR (indicated by relative bar height on a column graph). Fig. 3 compares results of both methods; see figure caption for more detail. Copy number of the ESF39 gene (part of the pTACF7 construct) was tested with qPCR, and as expected, matched the copy number of OxO (also present in the same pTACF7 construct). Copy number of pGFP was also tested with both qPCR (Fig. 3) and Southern hybridization (not shown), and again, results from the two techniques matched. Interestingly, copy numbers of the GOI and the GFP were the same in four of the five co-transformed events. Event Hinchee2 was the only exception, with a single copy of the GOI and two copies of the marker, which confirms that the two transgenes can insert independently.

Transgene copy number is an important consideration for gene expression, future breeding, and any potential de-regulation of transgenic plants. It is not surprising that three of the five co-transformed events featured in this study received a single copy of the GOI construct. *Agrobacterium*-mediated transformation commonly results in single- or few-copy transgenic events (as opposed to biolistic systems, in which higher-copy events are common [39,40]). Co-transformation events with single copies of both constructs are desirable for simpler inheritance patterns in breeding

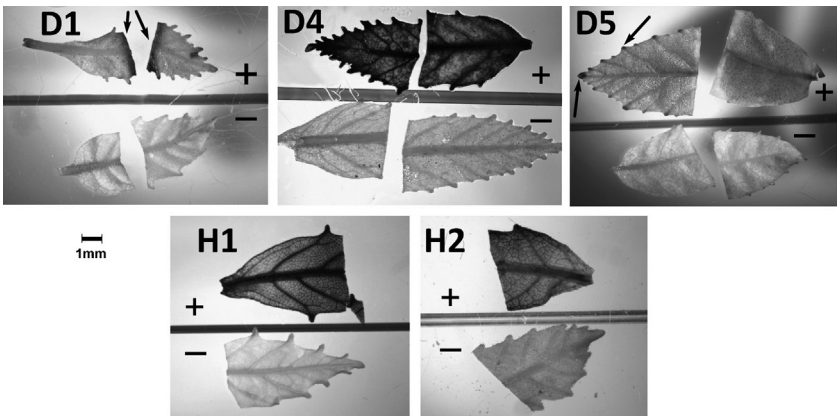

Fig. 4. Oxalate oxidase assay on tissue culture leaves. The upper leaf in each pair (indicated with "+" on figure) has been treated with oxalic acid, while the lower leaf (indicated with "–") has not. Dark staining indicates the presence of oxalate oxidase activity. D# = Darling transgenic event (top row), H# = Hinchee transgenic event (bottom row). While this assay is not strictly quantitative, note that Darling4 has the highest overall level of expression and Darling5 has the lowest, which correlates with inoculation results.

programs, and for potentially more predictable gene expression patterns. Not only can the Marker construct potentially be bred out more easily when a single copy is present, but inheritance patterns of new plants containing fewer copies of the GOI are more predictable. In the context of future chestnut restoration, this means genetically diverse transgenic chestnuts could more easily be included in a restoration project, since breeding with diverse wild-type trees is simpler with single copy events than it would be with multiple-copy events.

3.3. Transgene expression

Simply inserting a gene is not enough to enhance blight resistance; in order for a meaningful change it also has to be expressed in a consistent and beneficial manner. Transgene expression in all events was also confirmed by multiple tests. GFP expression was strong in transformed embryos: since this was used as part of the selection process, all events clearly contained and expressed GFP. Expression of the marker gene is not important after this initial selection, but RT-qPCR results (not shown) confirmed that it continues to be expressed in woody tissue. Expression of the GOI is not quite as obvious, but it was tested and confirmed at several time points on numerous trees. First, the colormetric oxalate oxidase enzyme assay confirmed vascular expression of the OxO gene in tissue culture-derived leaves. This test has limited quantitative discriminatory power, but as shown in Fig. 4, staining intensity indicated that Darling4 produced more OxO than Darling1 or Darling5, especially in the vascular tissue. Differences in spatial expression patterns are likely due to insert position effects; these differences are also reflected in lower overall expression of the OxO gene in these two events. Transgene expression was also tested with RT-qPCR on stem tissue from <1-year-old plantlets. According to this test, transgene expression varied somewhat between events, and also within events over time (not shown), but all transgenic events were found to express both GOI (Fig. 5) and Marker construct genes. Multiple non-transgenic controls were tested (only WB275-27 is shown) and none of these showed meaningful amplification with transgene primers.

The RT-qPCR expression measurements, along with inoculation screening described in section 3.4, confirmed that the transgenes were not only present, but active in host tissues. This transgene activity was maintained for at least four years in the field (Fig. 5), and we have no reason to believe it will decrease over time. While decreases in transgene expression are possible due to gene silencing and other factors, other studies have shown that expression can remain fairly stable under field conditions [41,42]. More than three hundred transgenic trees from the five primary events featured in this study have been planted at a total of six field sites across New York State. Preliminary observations indicate that growth of transgenic trees is comparable to non-transgenic tissue culture controls (WB275-27), and mycorrhizal colonization of transgenic trees is comparable to that of non-transgenic controls and related seedling trees [38].

3.4. Blight assays

The ultimate test of the transgenic American chestnut is whether it can survive infections with chestnut blight. Leaf assays predicted that at least two of the events in this study (Darling4 and Hinchee1) may have enhanced blight resistance compared to non-transgenic American chestnut controls (Fig. 6). Experiments on Darling4 leaves have been replicated more than other events, and lesion size on this event is consistently intermediate between

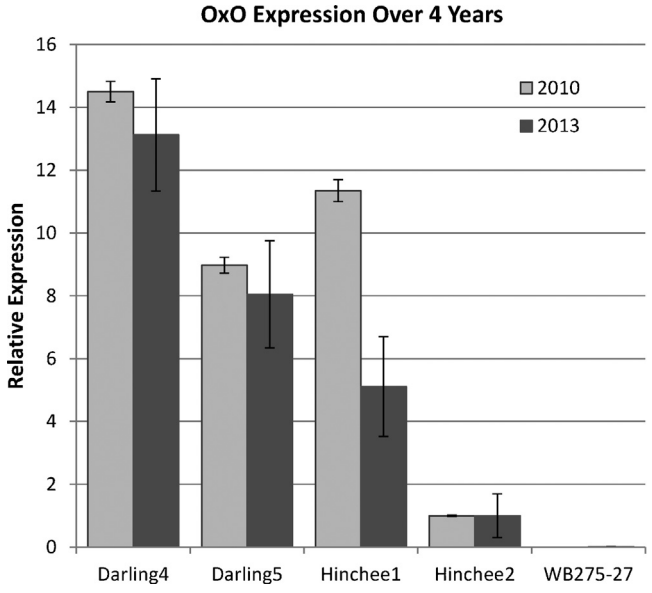

Fig. 5. Oxalate Oxidase expression on first-year plantlets (2010) and four-year-old field trees (2013). Relative expression (as measured by RT-qPCR) varies somewhat between events, but all tested events are still expressing the OxO transgene after four growing seasons outdoors.

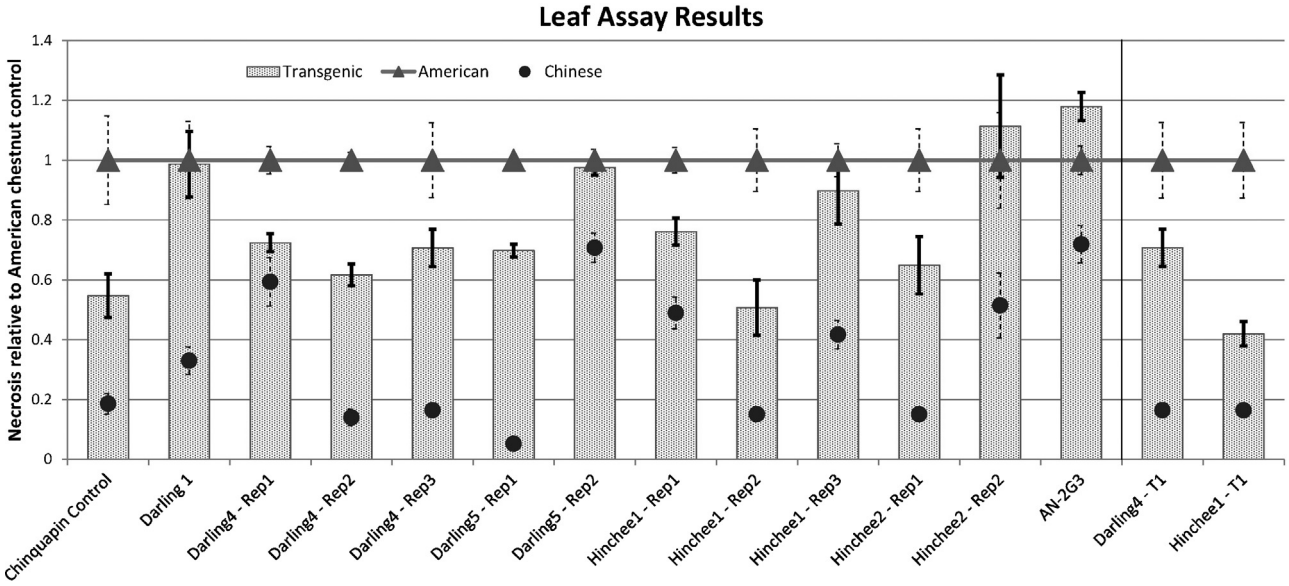

Fig. 6. Leaf assay results. Each column shows mean leaf lesion size relative to the susceptible American chestnut and resistant Chinese chestnut controls in that experiment. American chestnut average lesion size (line with triangles) has been normalized to 1.0 for ease of comparison between experiments. Average lesion size of transgenic events is indicated by the gray columns: if any column falls below the American line, average lesion size for that event is smaller than American chestnut, which indicates it may be less susceptible to blight than American chestnut. Lesion size on blight-resistant Chinese chestnut controls is indicated by the round dot superimposed on each column. Note that Chinese chestnut shows consistently smaller lesions than American chestnut, and that the known-intermediate control (Allegheny chinquapin, far left) shows a lesion size intermediate between American and Chinese chestnuts; both of these results correlate well with commonly observed results in the field. Most transgenic events were tested in multiple experiments, indicated by replicate (Rep) numbers below each column. The two columns to the right of the vertical line are from the T1 generation (seedling offspring of a transgenic parent); all others are T0. Error bars indicate+/− one standard error of the mean; where no error bar is visible, it is smaller than the symbol size.

susceptible American and resistant Chinese chestnut controls. In each case the lesion size on Darling4 is significantly different than the American control according to *t*-tests ($P < 0.05$). Hinchee1 also shows a trend of intermediate resistance, but the average lesion size is not consistently significantly different than the American control. Darling1 has not shown enhanced resistance in any test (replicated experiment not shown). Both Hinchee2 and Darling5 showed enhanced resistance in initial experiments, but in replicated experiments they were not significantly different than susceptible controls.

All transgenic events in this report have been screened with stem inoculation assays for at least one season with at least one strain of *C. parasitica*. Darling4 was tested for two years with two strains (Fig. 7), and it shows at least intermediate resistance between American chestnut (AC) and Chinese chestnut (CC) controls in all but one experiment (SG2 in 2013; this experiment also showed less necrotic variation between the AC and CC controls than in past years, possibly due to higher than average rainfall.) As shown in the *Graphical Abstract*, relative stem canker sizes and leaf lesion sizes on AC, Darling4, and CC were very similar. Stem inoculations of Darling5 showed no enhanced resistance (canker sizes were similar to the susceptible control), which generally correlates well with the OxO enzyme assays (Fig. 4), RT-qPCR results (Fig. 5), and leaf assays (Fig. 6), all of which indicate a lower level of transgene activity in this event. Inoculations on Darling1, Hinchee1, and Hinchee2 were performed on smaller stems (1–1.5 cm diameter), and these results were generally similar to leaf assay results: there was a trend of smaller cankers compared to susceptible controls. These differences, however, were not significant compared to susceptible controls according to *t*-tests ($P > 0.05$). The control event AN-2G3, which contains only marker genes, was not significantly different from non-transgenic susceptible controls in any experiment.

3.5. Transgene transmission through controlled pollination

One key factor for the ultimate success of American chestnut restoration is reliable inheritance of the enhanced blight resistance trait. Transgenic pollen has been used successfully to pollinate wild-type mother trees for three years in a row. To our knowledge, this is the first report of pollination and nut harvest from non-plantation-type transgenic forest trees. Pollen from Darling4, Hinchee1, and Hinchee2 trees (plus four additional events not described here) has been used for pollinations, and all tested events have produced viable T1 transgenic nuts. Transgenic pollinations have produced a total of 177 nuts over the past three years. Of those, 43 are Darling4, 83 are Hinchee1, three are Hinchee2 (the remainder include separate OxO-containing events produced by Zhang [15], and other events not previously described in the literature). Not all of these have been tested for transgene presence, but of those that have, integration of the GOI was confirmed by colormetric OxO enzyme assays, PCR on cotyledon tissue from pre-germinated nuts, PCR on germinated leaf tissue, or some combination of these tests. As expected, not all tested nuts were transgenic, since the transgene is effectively in a heterozygous state in only one parent. Darling4 and Hinchee1 both have multiple copies of the GOI, so if the transgenes were randomly and independently inserted into the genome, more than half of the offspring would be expected to have at least one transgene copy (3/4 from the two-copy Darling4, 7/8 from the three-copy Hinchee1). However, at least in our relatively small sample, this was not the case: in T1 populations from each of these parents, approximately half or less of the offspring had at least one copy of the OxO gene. (2 of 9 tested Darling4 trees contained OxO, as did 29 of 55 tested Hinchee1's.) Therefore it is likely that the multiple transgene copies in these events were inserted in tandem or are otherwise linked. Copy number analysis of this population is in progress, which will

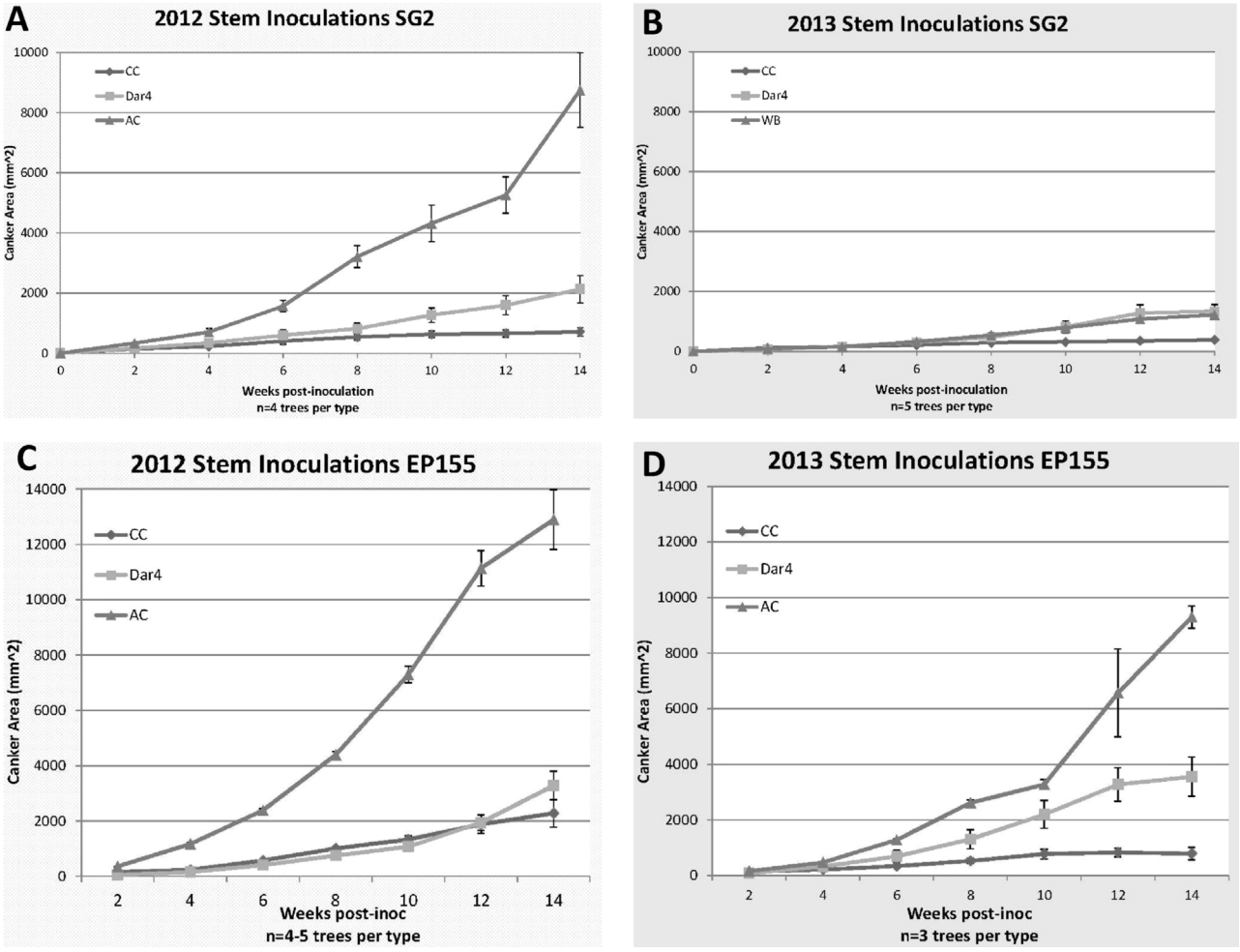

Fig. 7. Stem inoculation results over two years with two fungal lines. CC = Chinese chestnut, Dar4 = Darling4 transgenic American chestnut, AC = susceptible American chestnut. Lines show the average growth of cankers over 14 weeks, from approximately mid-June to late August. SG2 (sections A and B) is a moderately virulent line of *C. parasitica*, EP155 (C and D) is a more highly virulent line, so overall canker sizes are larger on EP155 inoculations. 2013 (B and D) was apparently an unusual year for *C. parasitica* inoculations; cankers in other experiments (not shown) were smaller than expected as well, even on trees known to be very susceptible to blight.

allow a more precise analysis of transgene insertion and inheritance patterns.

Several individual transgenic plants from the T1 generation were tested for transgene expression. Since these individuals are not clonally produced like their tissue culture-derived parents, each individual must be analyzed separately. Results of RT-qPCR indicated that the relative transgene expression in tested transgenic offspring (two T1 individuals from each Darling4 and Hinchee1) were approximately similar to those of the T0 transgenic parent (Fig. 8). Further, a few individual transgenic T1 plants (one each of Darling4 and Hinchee1) produced enough leaves for a leaf assay, and while there were not enough leaves to replicate the experiment, results correlated with those of the transgenic parents, both of which typically showed intermediate blight resistance between American and Chinese chestnut controls (Fig. 6; T1 events at far right).

One major advantage to growing T1 transgenic seedlings (as opposed to T0 plantlets produced in tissue culture) is the seed itself. Our observations indicate that tissue culture-derived chestnut plantlets tend to grow slower than seedlings the first year, after which growth rates are more comparable. Additionally, many tissue culture-derived plantlets initially lack strong apical dominance, instead featuring lateral (branch-like) growth tendencies. Trees grown directly from seed, whether they are transgenic or not, tend to initially grow faster and straighter than their tissue-cultured counterparts. This does not mean that tissue culture propagation should be abandoned altogether; in some situations it is the most efficient way to get trees in the ground. However, the option to produce seeds expands the potential for restoration by enhancing genetic diversity and simplifying distribution.

3.6. Metabolomics

Because chestnuts are traditionally a food crop for both humans and wildlife, it will be essential to test the transgenic nuts themselves for the presence of any compounds that might be significantly different than those found in non-transgenic nuts. One way to do this is through metabolomics analysis, which is the identification of a wide variety of metabolites down to near-trace levels. When mean metabolite values were compared between transgenic and non-transgenic nuts from the Hinchee1 cross, there were only two that showed significant differences ($P > 0.05$). These were pentoside conjugates of ferulic acid and coumaric acid, which are likely cell wall bridging components between hemicellulose and lignin. These components are typically released as cell walls are hydrolyzed during hypocotyl emergence, and they would be synthesized as new cell wall synthesis occurs. Therefore, the

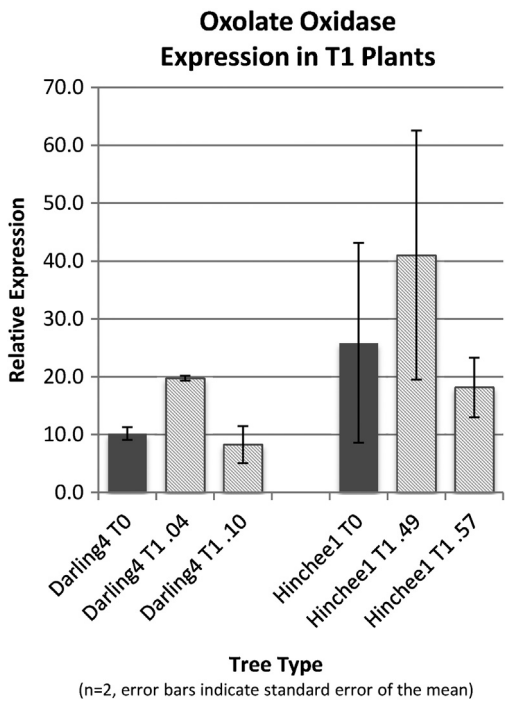

Fig. 8. Oxalate oxidase expression in T1 plants. Seedlings from the T1 generation and T0 parent trees were both analyzed with RT-qPCR in the summer of 2013. Height of columns indicates relative OxO expression levels. Transgenic offspring clearly express the OxO transgene, though relative expression levels may vary between individuals.

differences between transgenic and non-transgenic samples likely reflect more on the timing of this experiment (toward the end of cold stratification period as germination may have begun) than any difference due directly to the transgenes. Additionally, declines in some of the abundant fatty acids and lipid-related metabolites, sterols, organic acids, higher-order sugars and sugar alcohols in the transgenic nuts further support the interpretation that these nuts were more advanced in the germination process versus the controls, as these metabolites would be consumed by the growing hypocotyl. Furthermore, being cell wall components, these differentially produced compounds are present in all other plant-based food sources, so they should not be a concern for consumers.

One meaningful way to analyze a transgenic product is to compare it to the full spectrum of closely related non-transgenic products that are currently available. In this case, an important comparison is between transgenic American chestnuts and a non-transgenic standard panel consisting of both American and Chinese chestnuts (since nuts from both of these species are eaten by people and wildlife). Most (30 of 41) tested metabolite ranges in the transgenic nuts were nested entirely within the range produced by the standard panel of non-transgenic nuts, and in all of the remaining 11 samples, the majority of each range overlaps (Fig. 9). These similarities suggest that transgenic chestnuts should be safe for consumption by anyone who can safely eat non-transgenic chestnuts.

4. Conclusion

Production of a blight-resistant transgenic American chestnut tree would be a noteworthy success for historical, ecological, economic, and technological reasons. While the most resistant of the events described here is not as resistant as the Chinese chestnut, significantly enhanced blight resistance to an intermediate level (as seen in Darling4) is a major step toward that goal. New transgenic events are currently being produced that express the OxO gene at higher levels, and leaf assay results indicate they may be considerably more resistant than Darling4, approaching or even exceeding the blight resistance of Chinese chestnuts [15]. Additionally, genes isolated from Chinese chestnut are currently being evaluated for potential use as stacked transgenes, and insights into the chestnut genome [43] will continue to facilitate research in this direction. The fact that transgenes are inherited and expressed in seedling offspring means that blight resistance can be stable across generations, and it would also facilitate many other aspects of a restoration program. While there are still numerous considerations to be addressed before restoration can begin on a large scale [44], an American chestnut tree with enhanced blight resistance is an absolute necessity before restoration can occur. We believe, based on the data presented here, that transgenic American chestnuts expressing OxO can meet that need.

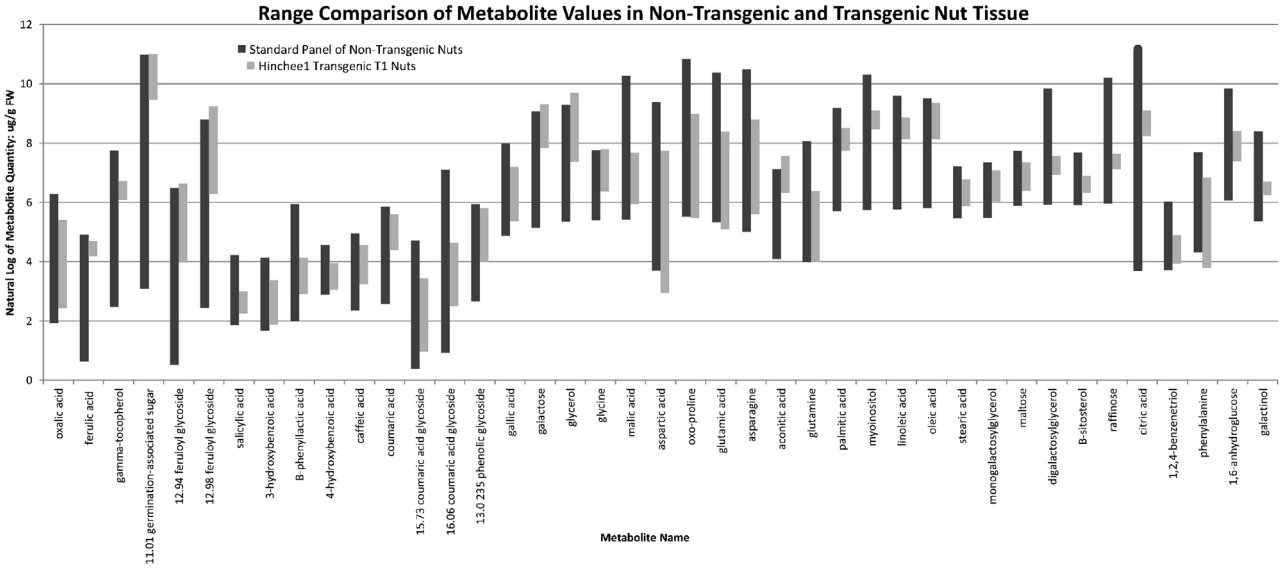

Fig. 9. Metabolite range comparisons. Total ranges of all 41 tested metabolites are compared for Hinchee1 transgenic and combined non-transgenic nut tissues. For most of the metabolites, the range found in transgenic nut tissue is entirely nested within the non-transgenic range. All metabolite values were natural log (ln) transformed to allow all samples to be shown on a single graph with a reasonable scale.

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