EBB1, an AP2/ERF Transcription Factor,

Promotes Transgenic Shoot Development in *Populus*

By

Joseph Ree

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Steven Strauss, Forest Ecosystems and Society Date

_____ _____

Kelly Vining, Forest Ecosystems and Society Date

_____ _____

Katherine Fields, BRR Director Date

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Abstract

Recalcitrance to transformation makes genetic engineering of many valuable plants infeasible for practical use. Transitory host modification during transformation using transgenes offers a possible means of overcoming this obstacle. In prior work in our laboratory, the genes *GA20ox7* and *EBB1* were found to increase regeneration *in vitro*. In this project, I reanalyzed the regenerative properties of these two genes using a model poplar (hybrid *Populus tremula x P. alba* INRA 717-1B4) and a genotype that is recalcitrant to transform, *P. trichocarpa (Nisqually-1):N-1.* Three replicate transformation experiments were performed to compare callus, shoot, and transgenic shoot formation with the two genes and an Empty-Vector control. No shoots were produced by N-1 explants with or without the genes. 717 explants transformed with *p409S:GA20ox7* regenerated poorly, producing a low proportion of transgenic shoots for stem (12.4%) and leaf (8.19%) explants compared to that for empty vector controls (24.4%, 29.5%). 717 explants transformed with *p409S*:*EBB1*, however, had a greater numbers of shoots per stem and leaf explant (37.7% and 54.2% increase, respectively) and a higher proportion (50.8%) of transgenic shoots from 717 leaf explants (44.5%) compared to the Empty-Vector controls (29.5%). Additionally, callus growth on N-1 stem explants was increased by 116% by *EBB1* and 76.8% by *GA20ox7* compared to Empty-Vector controls. I conclude that the *EBB1* transgene shows promise in improving transformation, but it must be removed or reactivated shortly after transformation due to apparent cytokinin toxicity and morphological defects.

Introduction

Biotechnology as a tool

Humankind has used breeding for centuries to domesticate and improve plant and animal species. With the advent of recombinant DNA technology, the process of modifying organisms has broadened the gene pool to enable incorporation of specific traits not feasible with conventional breeding. Conventional breeding uses sexual reproduction, which yields a plethora of genotypes in offspring. Transformation, often called genetic engineering (GE), asexually transfers genetic information with comparatively small alteration to the genome of the organism. An example of a GE plant is papaya cultivars resistant to the *papaya ringspot virus;* the virus almost destroyed the Hawaiian papaya industry (Yi-Jung 2009). GE organisms, termed "transgenic," are commercially grown for traits such as herbicide resistance, pesticide synthesis, disease resistance, and stress tolerance (Ervin 2010).

Agrobacterium and transformation

Agrobacterium tumefaciens, a gram negative soil-dwelling bacterium*,* has been used extensively for genetic transformation of plants. *A. tumefaciens* possesses the ability to insert T (Transfer)-DNA across natural kingdom barriers into host plant cells (Howe 1994). T-DNA is a segment of DNA which is transferred through a plant cell's wall and membrane where it supports transcription transiently in the cytoplasm or is integrated directly into the cell's genome (Bundock 1996). In its natural state, *A. tumefaciens* causes crown gall, a disease that creates tumors on the crown, stem, and roots of host plants (Rao 1982). These tumor cells

secrete nutrients for the benefit of the associated bacteria but with obvious detriment to the plant. For use in GE, the Ti (tumor-inducing)-plasmid, which transfers the genes that cause the disease, has been disarmed: genes related to tumor formation have been excised, while leaving the other genes needed for host infection and gene transfer intact (Kiyokawa 2009). *Agrobacterium*-mediated transformation is often preferred compared to electroporation or biolistic gene delivery due to low cost, high insert DNA integrity, and ease of use (Gelvin 2003).

Infection of plant cells by *Agrobacterium* begins with its detection of phenolic compounds from wounded tissue (Brencic 2004). The detection of the phenolic compounds induces attachment to the plant cell and processing of T-DNA. A channel between cells is formed by a type IV secretion system, in which the T-DNA and virulence proteins are transferred from the *Agrobacterium* to the cytoplasm. Virulence proteins perform many different functions within the plant cells such as cytoplasmic trafficking, nuclear targeting, and facilitating integration of the T-DNA into the genome (Tomlinson 2009). Transformation by *Agrobacterium* results in a variety of inserted T-DNA copies between cells, and most will be untransformed. The fate of each T-DNA varies among cells: either transiently expressed (with eventual degradation in the cytoplasm) or stably inserted into the nuclear genome (at more or less random locations) (Lacroix 2010). Each cell is very different from one another after transformation, thus each transformant tends to have distinct transgenic properties.

Tree biotechnology

A major focus of forest tree biotechnology is the application of genetic information to improve cultivated tree species. GE trees have included those with improved wood quality, disease

resistance, tolerance to adverse environmental conditions, and remediation of contaminated soil (Harry 2010). Many species within the genus *Populus*, including aspens and cottonwoods, are used for industrial production of wood pulp, paper products, and potentially as a biomass crop for biofuels. Notable characteristics of *Populus* include rapid growth, easy vegetative propagation, stable transgene expression, and a genome sequence, together making it a model forest tree for biotechnology (Brunner 2004). Efficient transformation systems have been created for a few poplar genotypes, including extensively used model hybrid species. The hybrid *Populus tremula x P. alba* (clone INRA 717-1B4, hereafter referred to as 717) has been used extensively in many experiments due to its high DNA transformation rates and regenerative properties (Han 2000). *P. trichocarpa (*Nisqually-*1,* hereafter referred to as N-1*)* can be termed a recalcitrant phenotype because of its very low transformation and regeneration rates.

In vitro regeneration of plants

Once a cell is transformed, it must be regenerated into a transgenic plant. This requires the reprogramming of its development. Most plant cells are pluripotent, indicating that they have the ability to differentiate into any type of plant cell or tissue (Gutierrez 2005). Many transformation protocols employ organogenesis, where cells differentiate simple organs like shoot, including many *Populus* genotypes. Transformation of 717 and N-1 uses explants harvested from stem internodes and leaf disks for *Agrobacterium* infection and organogenic tissue regeneration. Cells use available nutrients from surrounding *in vitro* media to proliferate into masses of undifferentiated cells, referred to as calli. High cytokinin concentrations promote

differentiation of callus cells into shoot cells. Auxins can be used to facilitate formation of adventitious roots on harvested shoots, thus allowing regeneration of a whole plant.

Recalcitrance

Certain species or genotypes can be reliably transformed and regenerated *in vitro*. However, most plant species are difficult to transform (Gelvin 2003). These genotypes possess traits that prevent the insertion or expression of foreign DNA, or have blocks to regeneration. Because of its complexity, including *Agrobacterium* compatibility, host defense, gene silencing, and capacity for redifferentiation, transformation capacity is often difficult to improve.

Diverse approaches have been used to overcome poor transformation efficiency. Modification of environmental conditions can influence *Agrobacterium* T-DNA expression and infection rate (Oberpichler 2008). Virulent strains of *Agrobacterium* have been discovered and manipulated to increase infection rates (Gelvin 2000). Crops that had been recalcitrant to regeneration, such as bell pepper, have been made transformable with new protocols of *in planta* transformation (Kumar 2009). Other crops may silence T-DNA expression through anti-viral defenses, thus preventing expression of selectable marker genes (Lakatos 2006). The resulting cell death prevents recovery growth of transgenic tissue.

By targeting genetic mechanisms responsible for recalcitrance, transformation can be improved (Gelvin 2003). For example, overexpression of histone protein gene *HTA1* increased T-DNA integration into the genome in *Arabidopsis thaliana*. Transformation of tobacco was increased by over-expressing a nuclear protein VIP1 from *Arabidopsis* (Tzfira 2002). Improved transformation through host modification or co-delivery of genes can thus improve

regeneration of previously recalcitrant genotypes. We followed the latter strategy in this research. Such genes may have wide-spread effects on plant physiology, including potentially unfavorable traits. If genes are co-delivered to aid transformation, it will be important to remove or inactivate them afterward.

Removal of unwanted genes after transformation

Several mechanisms could potentially be used to silence genes after transformation. Zinc finger nucleases (ZFNs), for example, could be used to remove the gene's function through mutation (Osakabe 2010). ZFNs contain DNA-binding domains and a DNA cleaving domain such as the restriction endonucelase *FokI* that target recognition sequences for *FokI* (Kim 1996). It causes double-stranded breaks, and the repair usually causes mutations, eliminating the function of the gene (Cathomen 2008).

Regeneration genes: GA20ox7 and EBB1

Gibberellic acid (GA) is a hormone that affects many plant processes including shoot elongation, internode length, leaf size, seed germination, cell division, and cell elongation. Genes related to GA biosynthesis have been heavily studied in plants such as rice, wheat, citrus, and aspen due GA's large impact on plant growth. The gene family GA20ox encodes a highly active enzyme in the later steps of gibberellic acid production (Han *et al*. 2010). GA20ox overexpression in rice, poplar, and citrus caused increased internode length, xylem fiber length, and overall biomass in young plants. Han (2010) studied the effect of multiple GA regulating enzymes, including *GA20ox7*, on poplar growth. *GA20ox7* is a member of the heavily studied GA20ox gene family

that acts as a positive regulator of GA synthesis. Han (2010) observed that the speed of regeneration and number of transgenic shoots were increased by *GA20ox7* insertion. We therefore selected *GA20ox7* as a possible tool for improving transformation. We also speculated that recalcitrant plants, such as Nisqually-1, might regenerate transgenic shoots more efficiently if *GA20ox7*-induced higher concentrations of GA increase cell proliferation and subsequent organogenesis of stem tissue.

The gene *EBB1* (Early Bud Break 1) was identified by activation tagging (Busov 2010). During activation tagging, the constitutive *35S* promoter was transformed into poplar cells to create random changes, mostly increases, in gene expression. However, resulting activation can also cause gene silencing, protein modification, and up/down regulation in some cases (Harrison 2007). Plants with overexpressed *EBB1* due to activation tagging had enlarged shoot meristems and greater cell division rates in the apex than wild type plants. *EBB1* also promoted the spontaneous regeneration of shoots from wounded cambium and also caused increased shoot regeneration *in vitro*.

The *EBB1* gene encodes a putative AP2/ERF transcription factor. AP2/ERF is a superfamily of plant-specific transcription factors, of which *EBB1* belongs to a subfamily characterized by strong developmental regulators involved in shoot and root meristem activity. Microarray analysis of gene expression in tissue at stem apex in *EBB1* transgenic plants showed 2,870 genes, with roughly equal number of genes upregulated or downregulated, compared to wild-type. Examples of upregulated genes include those involved in epidermal cell fate specification and regulation of meristem growth. Genes that were downregulated were associated with

synthesis of or response to GA, abscisic acid, light, and temperature The *EBB1* transcriptional factor is similar to the *ESR1* transcription factor gene in *Arabidopsis,* both members of the same family of AP2/ERF transcription factors. ESR1 overexpression induced shoot formation on transformed plant tissue in *Arabidopsis* (Matsuo 2008) similar to that observed with *EBB1* in poplar.

In this project, we tested if the genes *EBB1* and *GA20ox7* promoted improved transformation and regeneration of 717 and N-1 *in vitro* by observing growth of tissue at multiple steps of regeneration.

Methods

Binary vectors

The binary vectors *p409S:EBB1*, *p409S:GA20ox7*, *p409S:Empty-Vector* were provided by Jim Thompson of the United States Department of Agriculture. *P409S*:*EBB1* contains the *EBB1* gene controlled by the *p409S* promoter/terminator sequences and an *NPTII* gene for kanamycin resistance. The binary vector *P409S:GA20ox7* likewise contains the *GA20ox7* gene controlled by the constitutive *p409S* promoter/terminator sequences and an *NPTII* gene for kanamycin resistance. *P409S:Empty* provides an Empty-Vector transgenic control with an *NPTII* gene for kanamycin resistance. All three vectors were introduced into disarmed *A. tumefaciens* strain AGL1 by the freeze-thaw method.

Plant culture

One-month-old 717 and N-1 were cultured on rooting media in Magenta boxes. Boxes containing the shoot cultures were kept under light at stable temperatures until needed for transformation.

Media

All media used were adjusted to pH 5.8 except for LB media at pH 7.0 and induction medium at pH 5.0. All media were autoclaved at 120C° for 20 minutes; temperature sensitive chemicals were added after media was cooled after autoclaving. See table 3 for a list of media used.

Plant transformation

Protocols for 717 and N-1 transformation have been previously described (Han 2000, Ma 2004 respectively). Stem explants were harvested from 717 and N-1 internodes sections about 0.5cm long. Leaf discs were harvested using a sterilized hole punch. The numbers of explants harvested by experiment ranged from 361 to 460 explants for 717 and 233 to 380 explants for N-1 (Table 1 and 2). The explants were washed in *Agrobacterium* induction media at OD 0.6 then placed on callus inducing media (CIM) media to allow *Agrobacterium* to grow and stored in darkness.

After two days in CIM, *Agrobacterium* was removed from the explants with double-distilled, deionized water and a wash solution with antibiotics. The newly washed explants were then placed on CIM+KTC media (antibiotics kanamycin, timentin, and cefotaxime) and placed in darkness for 20 days to promote callus growth. Plates were evaluated for calli, which were

identified by the growth of a tan to green tissue over the original explant (Figure 1). All explants, including those without obvious callus, were placed on shoot inducing media 2SIM+KTC (717) or SIM3+KTC (N-1) to promote differentiation of callus into shoots. N-1 explants were transferred to SIM4+KTC after twenty days and continuously subcultured.

In vitro measurements

After 30 days on SIM, all plates were evaluated for shoot growth. All explants that formed one or more clearly defined shoots (Figure 2) were counted. All explants with shoots were subcultured onto shoot elongation media (SBO.1+KTC), which contained cytokinin 0.1 µM BAP to promote shoot elongation. After 30 days on SBO.1+KTC media, all explants were evaluated for the number of shoots per explant. Each shoot 10mm or greater in length was counted (Figure 3); a reference mark was made on a scalpel at a distance of 10mm from the tip of the blade to measure each shoot. I chose 10mm because the same measurement was used in the preliminary studies by *Busov et al* (2010). The blade of the scalpel was placed as close to the callus as possible without cutting into it and forceps were used to straighten the shoot when needed.

Molecular analysis

Shoot samples were harvested from each explant for DNA isolation and PCR analysis. Two samples were taken for each explant when two or more were available, and one sample was taken if the explant had only one shoot. Samples were collected into 1.5mL centrifuge tubes and frozen in liquid nitrogen and stored at -80C° until use. DNA was isolated using a variation

of a DNA isolation protocol described in Rodgers et al (1996). Before grinding tissue, 200uL extraction buffer and 50uL 5% N-lauroylsarcosine were added to each sample tube. Tissue was ground using sterile plastic tips mounted on a drill press. Samples were then incubated in a 60C° water bath for one hour, and centrifuged at 13,000rpm for 20 minutes at room temperature. After centrifugation, 150uL of the supernatant was added to a fresh 1.5mL centrifuge tube with 150uL ammonium acetate and 300uL isopropanol. Solutions were then mixed by inversion and incubated at -20C° for 15 minutes, and centrifuged at 13,000rpm for ten minutes at room temperature. The supernatant was discarded and the solid pellet of nucleic acid was rinsed with chilled 70% ethanol. The pellet was resuspended in 100uL pH 8.0 Tris-EDTA. The number of samples analyzed by PCR varied from 334 to 433 per experiment (Appendix 1).

DNA from each sample was tested for successful transformation through PCR using primers designed based on the known sequence of *p409S*:*EBB1* and *p409S*:*GA20ox7* (Appendix 2). PCR programs were tested using the original vector and DNA from transgenic *E. coli* and *Agrobacterium*. Electrophoresis was used to separate PCR products on a 2% agarose gel for one hour at 100V. Positive controls and negative controls were included with every run to test for contamination (Figure 4).

Data analysis

We performed three transformations, hereafter referred to as Experiments, which each started on 9/27/10, 10/4/10, and 10/5/10 for 717 and 12/13/10, 12/14/10, and 12/20/10 for N-1. For each experiment, 67 to 169 stem and leaf explants taken from single Magenta boxes for each

gene/genotype, were allocated to 3 to 7 Petri dishes per gene/genotype. Dishes and derived dishes from subculture were placed essentially at random during subsequent culture. To simplify analyses and because of the preliminary nature of this study, explants were assumed to be independent observations for statistical tests, and thus only P-values below 1% were considered as indicative of a possible biological effect. All data subject to ANOVA were visually inspected for approximate normality, however, none were fully normal (even after attempts at transformation), thus prompting further caution in the interpretation of significance values. The development of callus, shoots, and transgenic shoots were scored as presence/absence, while shoots per explant were recorded as integers. Both mean and medians for shoots per explant were also reported due to the presence of outliers. Minitab 16 and Microsoft Excel 2010 software was used to compile all data, conduct ANOVAs, and produce means and charts.

Results

EBB1 vs. Empty-Vector Control

Callus formation was not affected by EBB1 in 717

The pooled percent of 717 leaf explants that formed calli was 80.3% for *EBB1* and 81.4% for Empty-Vector, which was not found to be statistically significant (p=0.533) (Figure 5-A). For pooled 717 stem explants, 71.3% of *EBB1* explants formed callus tissue compared to 74.4% for Empty-Vector which was likewise not significantly different (p=0.212) (Figure 5-B). For percentages by experiment for all analyses see Appendices 4-7.

EBB1 greatly increased regeneration of calli in N-1 stem explants

EBB1 N-1 leaf explants had a greater proportion of callus formation at 5.37% after experiments were pooled, compared to 3.04% for Empty-Vector (Figure 6-A). However, the difference was not statistically significant (p=0.264). *EBB1* stem explant callus formation was statistically significant compared to Empty-Vector (p<0.001). More than double the number of EBB1 N-1 stem explants produced calli (27.0%) compared to Empty-Vector (12.5%) (Figure 6-B).

EBB1increased regeneration of shoots formation after outlier removal

Shoot formation for *EBB1* in Experiment Three was unexpectedly far less than the other experiments (p<0.001, Figure 7A). Shoot regeneration data for *EBB1* leaf explants was therefore adjusted by removing the third experiment as an outlier (called “Adjusted” in figures). The

percent of pooled *EBB1* leaf explants that formed shoot tissue was 59.6% in unadjusted data and 81.2% in adjusted data, which would be statistically significant ($p<0.001$) (Figure 7-A). Empty-Vector did not have this level of variability between experiments; it had a pooled percent of 52.8% explants formed shoots. The difference between Empty-Vector and *EBB1* in stem explants was not statistically significant ($p=0.294$) with 48.9% of *EBB1* and 45.4% of Empty-Vector explants forming shoots (Figure 7-B).

EBB1 increased both mean and median number of shoots per explant

EBB1 induced significantly more shoots per explant in comparison to Empty-Vector for stem ($p<0.001$) and leaf explants ($p<0.001$). *EBB1* also induced numbers of shoots per explant but the data are skewed, with outliers (Figure 8). The pooled mean and median for *EBB1* leaf explants were 6.06 and 5 shoots per explant. Empty-Vector's mean and median shoots per explant were 3.92 and 3 respectively. For stem explants, *EBB1* also had a higher mean and median scores of 5.55 and 5 shoots per explant compared to 4.03 and 4 shoots per explant in Empty-Vector.

A large proportion of regenerated EBB1 shoots were transgenic

The proportion of successfully transformed samples was significantly greater for *EBB1* leaf explants compared to Empty-Vector ($p=0.001$) but not between *EBB1* and Empty-Vector stem explants ($p=0.633$). *EBB1* samples from leaf explants were 44.5% transgenic compared to Empty-Vector at 29.5% (Figure 9-A). This difference was not observed in samples taken from stem explants with 26.4% of samples being transgenic in *EBB1* and 24.4% of samples being transgenic in Empty-Vector (Figure 9-B).

GA20ox7 vs. Empty-Vector Control

Callus formation in 717 explants

Callus regeneration was not statistically significant between *GA20ox7* and Empty-Vector leaf explants ($p= 0.776$). The proportion of callus regeneration between the two constructs was statistically significant for stem explants ($p<0.001$), with Empty-Vector having a higher proportion. Callus formation in *GA20ox7* leaf explants had a pooled percentage of 83.4% compared to 81.4% in Empty-Vector (Figure 5-A). For stem explants, *GA20ox7* had a lower percent of explants forming callus tissue at 60.8% of explants compared to 74.4% in Empty-Vector (Figure 5-B).

GA20ox7 N-1 stem explants regenerated more calli compared to empty-vector

The difference between the proportion of N-1 leaf explants that formed callus tissue between *GA20ox7* and Empty-Vector was not statistically significant (p=0.985). Despite the greater proportion of Empty-Vector stem explants that formed callus tissue in 717, *GA20ox7* N-1 stem explants had a statistically significant greater callus regeneration (p= 0.004). One experiment each of *GA20ox7* and Empty-Vector N-1 leaf explants failed to produce any callus tissue. Pooled percent of leaf explants that formed calli was 4.27% for *GA20ox7* and 3.04% for Empty-Vector (Figure 6-A). Within the stem explant population, 22.1% of *GA20ox7* explants formed callus tissue, compared to 12.5% for Empty-Vector explants (Figure 6-B).

GA20ox7 reduced shoot formation for 717 stem explants

Of 717 leaf explants, 58.5% of the *GA20ox7* and 52.8% of the Empty-Vector explant population formed shoots; the difference was not significant (p=0.058), (Figure 7-A). Empty-Vector 717 stem explants formed a higher proportions of shoots (p=0.001), with 45.4% of Empty-Vector explants compared to 33.8% of the *GA20ox7.*

GA20ox7 had greater numbers of shoots per explant for 717 leaf explants

The difference between number of shoots per explant was not statistically significant between *GA20ox7* and Empty-Vector for 717 stem explants (p=0.253 or leaf explants (p=0.014). The data was seen to be skewed with a tail toward larger numbers of shoots per explants but not as strongly as seen in *EBB1* leaf explants. The mean and median of the number of shoots per explant in 717 leaf explants was 4.65 and 4 shoots respectively for *GA20ox7* compared to 3.92

and 3 shoots for Empty-Vector. In stem explants, the mean and median number of shoots per explant in *GA20ox7* explants had 4.39 and 4 shoots compared to 4.03 and 4 shoots for Empty-Vector.

GA20ox7 regenerated few transgenic shoots

The number of identified transgenic *GA20ox7* samples was far less compared to the other two constructs in either 717 stem explants ($p= 0.034$) or 717 leaf explants ($p<0.001$). Very few *GA20ox7* transgenic shoot samples were observed. Of the tested samples, 8.19% of *GA20ox7* shoot samples from leaf explants were transgenic compared to 29.5% in Empty-Vector. A pooled percentage of 12.4% of samples from *GA20ox7* stem samples compared to 24.4% in Empty-Vector indicated that *GA20ox7* did not regenerate well, to the point of even perhaps having a toxic effect on transformed cells (Figure 9).

Discussion

I found some highly promising trends with respect to the value of the *EBB1* gene for promoting transformation. However, the *GA20X7* gene showed little promise, and the statistical support for the trends observed was complex. Below I discuss our main findings, the limits of the experimental design and statistical analysis, and the significance of my work for future research in this area.

Limits of experimental design

We conducted a preliminary study, thus the number of explants and replicate experiments was limited. The variability between experiments for 717 callus and shoot formation was often large, with p-values for variance among experiments as low as <0.001. This might result from several difficult to control causes. Callus and shoot formation data might have been influenced by the explant source health, which varied over time. Some source plants appeared unhealthy, and displayed signs of chlorosis. Others, however, were more vigorous and fast growing.

Several modifications to experimental design would help to reduce variation among experiments in future work. First, to minimize the effect of uncontrollable, random factors on explant physiology, more repetitions would be helpful. Ideally, these would be organized into larger number of plates with equal numbers of explants and experiments to increase statistical power. A larger number of plates would allow more extensive randomization to limit uncontrollable factors influencing tissue regeneration, such as differences in light, media composition, and explant source. Unfortunately, we were unable to divide harvested explants equally among all constructs in our experiments, thus construct and explant source effects are confounded. All explant sources should be randomized over constructs and replicates within constructs in future work.

N-1 failure to regenerate

Recalcitrant N-1 failed to regenerate more than a single shoot pooled over all explants, and this shoot eventually died upon contact with media after subculture, indicating it most likely was an

escape. The reason for complete failure to regenerate so much as a single transgenic shoot from more than 1000 explants is unknown. Shoots had been regenerated as shown in the established transformation protocol (Ma 2004), however, the rate was very low and highly variable. Other work with this clone has shown that the physiology and source of explants has a large impact on transformability (Song 2006). Thus, more work to adapt the source tissues and current transformation methods should be undertaken prior to further work. However, the beneficial effects of *EBB1* on callus formation in this clone is encouraging. Callus formation for *EBB1* and *GA20ox7* N-1 stem explants was 116% and 78.8% above that of Empty Vector controls, respectively.

PCR results indicate effectiveness of regeneration genes

We anticipated that the genes tested might not only promote callus or shoot growth, but also the recovery of transgenic over non-transgenic tissues. Although PCR can give a high rate of false positives and false negatives, we always included negative controls (water plus PCR cocktail), and many of our samples were tested more than once and rarely gave results different from the original assay. Thus we think that our PCR data presents an accurate description of the true rate of transformation.

EBB1 promoted increased rate of shoot formation per explant, giving rise to an increased proportion of transgenic shoots. However, it is possible that many shoots were from the same event, giving a false perception of increased recovery of independent transgenic events. In contrast to *EBB1*, *GA20ox7* appeared to consistently reduce the proportions of transgenic vs. non-transgenic shoots. PCR efficiency was similar in *EBB1* and *GA20ox7* as they had similar

amplicon sizes (~2.1kb vs. ~1.9kb), GC proportion (58.7% vs. 59.1%), and amplification programs. They also gave a similar success rate in PCR repeated runs of the same DNA samples.

One possible factor that affected the rate of escape may be differences in the effectiveness of selectable markers among genes or genotypes. Selectable markers are used to prevent non-transgenic shoots from growing and regenerating during transformation, but some are more efficient than others, and their use must be customized to individual genes, species, and even genotypes. For example, kanamycin is not as stringent at killing non-transgenic tissues as is hygromycin, thus it can allow more escapes (Long 2011). However, if antibiotics further retard regeneration of highly recalcitrant genotypes such as N-1, it might be beneficial to exclude selectable markers and antibiotics from experiments so that at least some transgenics are recovered. Stringent selectable markers should be highly effective in 717 because most surviving events are unlikely to be escapes.

Increased GA production may promote regeneration of non-transformed cells

GA, a phytohormone, can migrate between cells by diffusion (Feraru 2011). Therefore, the benefit conferred by *GA20ox7* to transformed cells might be shared to some degree by other neighboring cells in competition for space and media. As calli expand, other cells are physically blocked from access to media, thereby inhibiting further growth. On a 717 explant, where growth of transformed calli is expected under antibiotic selection, the advantage of increased GA could therefore also lead to regeneration of non-transgenic shoots, and be detrimental to transgenic cells in competition with neighbors. Thus, the GA20ox7 transgene, even if it promoters regeneration of transgenic tissues, might also stimulate regeneration of non-

transgenic escapes. For readily transformable genotypes like 717, this would be undesirable. However, for highly recalcitrant genotypes like N-1, where hardly any transgenic shoots form, increased GA production might still improve ultimate recovery of transgenic shoots as PCR or reporter genes could be used to identify transgenic events from large populations of regenerated shoots.

Regeneration differences between explant types

Comparisons of data for stem and leaf explants show that developmental differences between cell types affect tissue regeneration. The difference was largest in N-1, where stem and leaf calli had very different behaviors. In contrast, only slight differences in callus and shoot formation were observed between 717 leaf and stem explants. The differences in regeneration could have a number of causes, including their physical attributes, such as the relative surface areas of explants that are exposed to media. Langens-Gerrits (2003) found that larger lily explants regenerated larger bulbs due to increased surface area in contact with media. For an example, a leaf explants that has a diameter of a single hole punch, ~0.6cm, and a stem explant has a length of ~0.5cm and a diameter of ~0.1cm. Moreover, the leaf explant is often placed on the surface of the media and the stem explant is submerged halfway into media. The calculated surface area of the leaf explant for this case is ~0.28cm compared to ~0.18cm for the stem explant. Greater surface area allows more cells access to media and thus more room for calli to grow, possibly explaining slightly greater regeneration data for 717 leaf explants.

The average N-1 and 717 leaf explant was about equal in size, but the ability to grow calli was distinctly different. 717 leaf explants formed many calli across its entire surface in contrast to

sparse calli found only on vascular tissue on the outer rim of N-1 leaf disks. A cuticle may have prevented N-1 leaf cells from acquiring sugar from media. During explant harvest, N-1 plants tended to retain turgor pressure longer compared to 717, which wilted faster upon exposure to the less-humid air outside of Magenta boxes. This might indicate that N-1 leaves secrete a thicker cuticle layer *in vitro* than 717. This might be an avenue of future study to reduce recalcitrance by accounting for physical barriers between explants and energy sources.

A system to use and then remove EBB1

EBB1 can be used as part of a system to regenerate recalcitrant phenotypes, but also confers unwanted traits. Overexpressed *EBB1* plants were observed to have unhealthy morphologies, such as poor rooting ability, stunted leaves, and cytokinin toxicity (Busov 2010). For *EBB1* to be useful as a gene to improve transformation, it must be silenced after serving its purpose. Inducible promoters, such as a heat shock promoter, driving a ZFN or recombinase targeting *EBB1,* would allow programmed silencing of the gene. The end product would ideally be a plant without the *EBB1* phenotype. Through this system a recalcitrant genotype might be enabled to regenerate a high rate of transgenic shoots without lasting detrimental phenotypes.

Conclusion

I found several lines of evidence to suggest that *EBB1* could be a useful tool to promote regeneration of transgenic shoots in poplar. For *GA2ox7*, the data were weak to negative, though it did strongly promote callus regeneration from the recalcitrant poplar genotype

tested. Both genes warrant further study under different experimental conditions, as well in different species and genotypes.

References

Binns AN, Thomashow MF (1988) Cell biology of Agrobacterium infection and transformation of plants. Annu Rev Microbiol 42:575-606

Brencic, A., Eberhard, A., & Winans, S. (2004). Signal quenching, detoxification and mineralization of vir gene-inducing phenolics by the VirH2 protein of Agrobacterium tumefaciens. *Molecular Microbiology*, *51*(4), 1103-1115. doi:10.1046/j.1365-2958.2003.03887.x.

Brunner, A., Busov, V., & Strauss, S. (2004). Poplar genome sequence: functional genomics in an ecologically dominant plant species. *Trends in Plant Science*, *9*(1), 49. doi:10.1016/j.tplants.2003.11.006.

Bundock, P., & Hooykaas, P. (1996). Integration of Agrobacterium tumefaciens T-DNA in the Saccharomyces cerevisiae genome by.. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(26), 15272. Retrieved from Academic Search Premier database.

Busov, V., Y. Yordanov, C. Ma, S. and S.H. Strauss. 2010 *EARLY BUD-BREAK 1* (*EBB1*) is an AP2/ERF domain protein involved in the process of growth resumption after winter dormancy in *Populus. Unpublished*

Busov, V., Y. Yordanov, J. Gou, R. Meilan, C. Ma, S. Regan, and S.H. Strauss. 2010. Activation tagging is an effective gene tagging system in Populus. *Tree Genetics & Genomes,* DOI 10.1007/s11295-010-0317-7

Cathomen T, Joung JK. Zinc-finger nucleases: The next generation emerges. *Mol Ther.*2008;**16**:1200–120

Dharmawardhana, P., Brunner, A.M. and S.H. Strauss. 2010. Poplar as a tree model for horticulture and beyond: A case study of genome-scale changes in gene expression during bud entry and release from dormancy. *Acta Hort. (ISHS)* 859:43-47.

Ervin, D., & Welsh, R. (2010). Genetically Engineering Crops for a Sustainable Agriculture. *Choices: The Magazine of Food, Farm & Resource Issues*, *25*(2), 1-11. Retrieved from Academic Search Premier database

Feraru, E., Feraru, M., Kleine-Vehn, J., Martinière, A., Mouille, G., Vanneste, S., & ... Friml, J. (2011). PIN Polarity Maintenance by the Cell Wall in Arabidopsis. Current Biology, 21(4), 338-343.

Gelvin, S. (2000). AGROBACTERIUM AND PLANT GENES INVOLVED IN T-DNA TRANSFER AND INTEGRATION. *Annual Review of Plant Physiology & Plant Molecular Biology*, *51*(1), 223. Retrieved from Academic Search Premier database

Gelvin, S. (2003). Improving plant genetic engineering by manipulating the host. *Trends in Biotechnology*, *21*(3), 95. doi:10.1016/S0167-7799(03)00005-2.

Gutierrez, C. (2005). Coupling cell proliferation and development in plants. *Nature Cell Biology*, *7*(6), 535-541. doi:10.1038/ncb0605-

Han, K.M., Dharmawardhana, P., Arias, R.S., Ma, C., Busov, V. and S.H. Strauss (2010). Gibberellin-associated cisgenes modify growth, stature, and wood properties in *Populus*. *Plant Biotechnol. J.* 1-17

Han, K.-H., Meilan, R., Ma, C., and Strauss, S.H. (2000) An Agrobacterium transformation protocol effective in a variety of cottonwood hybrids (*genus Populus*). *Plant Cell Report 19*:315-320

Harrison, E., Bush, M., Plett, J., McPhee, D., Vitez, R., O'Malley, B., et al. (2007). Diverse developmental mutants revealed in an activation-tagged population of poplar. *Canadian Journal of Botany*, *85*(11), 1071-1081. doi:10.1139/B07-063.

Harry, D.E. and S.H. Strauss (2010). Biotechnology and Genetic Engineering in Forest Trees University on California, Agricultural Biotechnology information web site

Howe, G.T., Goldfarb, B., and Strauss, S.H. (1994) *Agrobacterium*-mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants. *Plant Cell, Tissue and Organ Culture 36*:59-71

Iwase, A., Mitsuda, N., Koyama, T., Hiratsu, K., Kojima, M., Arai, T., & ... Ohme-Takagi, M. (2011). The AP2/ERF Transcription Factor WIND1 Controls Cell Dedifferentiation in Arabidopsis. *Current Biology*, *21*(6), 508-514. doi:10.1016/j.cub.2011.02.020

Janositz, A. A., & Knorr, D. D. (2010). Microscopic visualization of Pulsed Electric Field induced changes on plant cellular level. Innovative Food Science & Emerging Technologies, 11(4), 592-597. doi:10.1016/j.ifset.2010.07.004

Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci USA.* 1996;**93**:1156–1160

Kiyokawa, K., Yamamoto, S., Sakuma, K., Tanaka, K., Moriguchi, K., & Suzuki, K. (2009). Construction of Disarmed Ti Plasmids Transferable between Escherichia coli and Agrobacterium Species. *Applied & Environmental Microbiology*, *75*(7), 1845-1851. doi:10.1128/AEM.01856-08.

Kumar, A., Reddy, K., Sreevathsa, R., Ganeshan, G., & Udayakumar, M. (2009). Towards crop improvement in bell pepper (Capsicum annuum L.): Transgenics (uid A::hpt II) by a tissue-culture-independent Agrobacterium-mediated in planta approach. *Scientia Horticulturae*, *119*(4), 362-370. doi:10.1016/j.scienta.2008.08.034.

Lacroix, B., Jianxiong, L., Tzfira, T., & Citovsky, V. (2006). Will you let me use your nucleus? How Agrobacterium gets its T-DNA expressed in the host plant cell. *Canadian Journal of Physiology & Pharmacology*, *84*(3/4), 333-345. doi:10.1139/Y05-108.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E., Carrington, J., Yu-Ping, L., et al. (2006). Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO Journal*, *25*(12), 2768-2780. doi:10.1038/sj.emboj.7601164.

Langens-Gerrits, M., Kuijpers, A., De Klerk, G., & Croes, A. (2003). Contribution of explant carbohydrate reserves and sucrose in the medium to bulb growth of lily regenerated on scale segments in vitro. Physiologia Plantarum, 117(2), 245-255. doi:10.1034/j.1399-3054.2003.1170212.x

Long, D., Wu, X., Yang, Z., Lenk, I., Nielsen, K., & Gao, C. (2011). Comparison of three selectable marker genes for transformation of tall fescue (Festuca arundinacae Schreb.) plants by particle bombardment. *In Vitro*n Cellular & Developmental Biology Plant, 47(6), 658-666. Doi:10.1007/s11627-011-9382-3

Ma, C (2004) Transformation protocol for Aspen INRA 717-1B4 (female, Populus tremuludes x P.Alba) and 353-38 (male, P. tremuludes x tremula)

Ma, C., Strauss, S.H., and Meilan, R. (2004) Agrobacterium-mediated transformation of the genome-sequenced poplar clone, Nisqually-1 (*Populus trichocarpa*). *Plant Molecular Biology Reporter* 22:1-9

Matsuo, N., & Banno, H. (2008). The Arabidopsis transcription factor ESR1 induces *in vitro* shoot regeneration through transcriptional activation. *Plant Physiology & Biochemistry*, *46*(12), 1045-1050. doi:10.1016/j.plaphy.2008.07.007.

Oberpichler, I., Rosen, R., Rasouly, A., Vugman, M., Ron, E., & Lamparter, T. (2008). Light affects motility and infectivity of Agrobacterium tumefaciens. *Environmental Microbiology*, *10*(8), 2020-2029. doi:10.1111/j.1462-2920.2008.01618.x.

Osakabe, K., Osakabe, Y., & Toki, S. (2010). Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, *107*(26), 12034-12039.

Phillips, C., Croker, S., García-Lepe, R., Lewis, M., & Hedden, P. (1999). Modification of gibberellin production and plant development in Arabidopsis by sense and antisense expression of gibberellin 20-oxidase genes. *Plant Journal*, *17*(5), 547. Retrieved from Academic Search Premier database.

Rao, S., Lippincott, B., & Lippincott, J. (1982). Agrobacterium adherence involves the pectic portion of the host cell wall and is sensitive to the degree of pectin methylation. *Physiologia Plantarum*, *56*(3), 374-380. doi:10.1111/1399-3054.ep13272814

Rogers, H.J., N.A. Burns, and H.C. Parkes. 1996. Comparison of small-scale methods for the rapid extraction of plantDNA suitable for PCR analysis. Plant Mol. Biol. Report.14:170-183..

Song, J., Lu, S., Chen, Z., Lourenco, R., and Chiang, V. (2006) Genetic Transformation of *Populus trichocarpa* Genotype Nisqually-1: A Functional Genomic Tool for Woody Plants. Plant Cell Physiology 47(11): 1582-1589 first published online October 3, 2006doi:10.1093/pcp/pcl018

Tomlinson, A., & Fuqua, C. (2009). Mechanisms and regulation of polar surface attachment in Agrobacterium tumefaciens. *Current Opinion in Microbiology*, *12*(6), 708-714. doi:10.1016/j.mib.2009.09.014.

Tzfira, T., Vaidya, M., & Citovsky, V. (2002). Increasing plant susceptibility to Agrobacterium infections by overexpression of the Arabidopsis nuclear protein VIP1. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(16), 10435. Retrieved from Academic Search Premier database.

Yi-Jung, K., Huey-Jiunn, B., Yi-Ling, W., Chiung-Huei, H., Tsui-Miao, C., & Shyi-Dong, Y. (2009). Generation of Transgenic Papaya with Double Resistance to Papaya ringspot virus and Papaya leaf-distortion mosaic virus. *Phytopathology*, *99*(11), 1312-1320. doi:10.1094/PHYTO-99-11-1312.

Figures and Tables

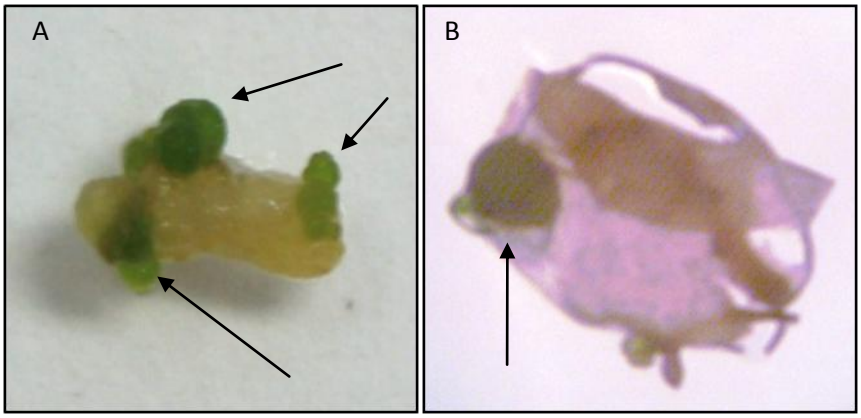

Figure 1: Examples of green calli on (A) a 717 stem explant and (B) a 717 leaf explant. Arrows point to individual calli. Multiple calli can form on a single explant.

Figure 2: Shoot growth on a 717 leaf explant.

Figure 3: A 717 explant at the time the number of shoots was observed. The number of shoots 10mm or greater was recorded.

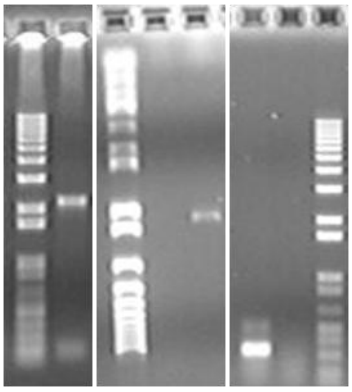

Figure 4: Expected PCR product band lengths in comparison to 1kb ladder. Three individual sets of primers were used for the three constructs to identify successfully transformed samples. Pictured is the expected band size for, from left to right, EBB1, GA20ox7, and kanamycin for Empty Vector.

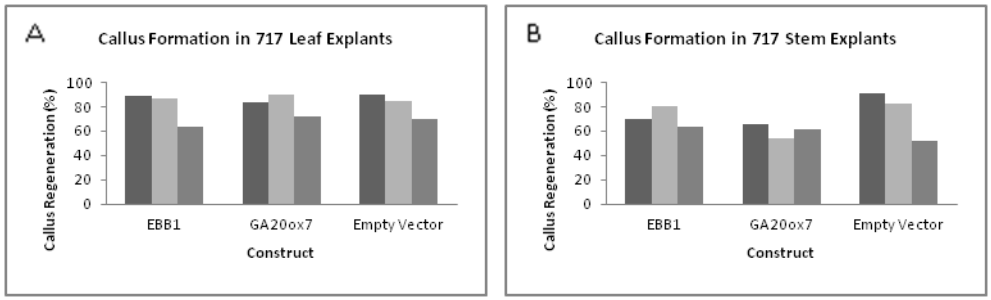

Figure 5: Callus formation on 717 explants by experiment and construct. The percent of explants that showed callus growth is shown for (A) leaf explants and (B) stem explants. Bar shades from left to right correspond to experiment 1 (darkest), experiment 2 (lightest), and experiment 3 (intermediate).

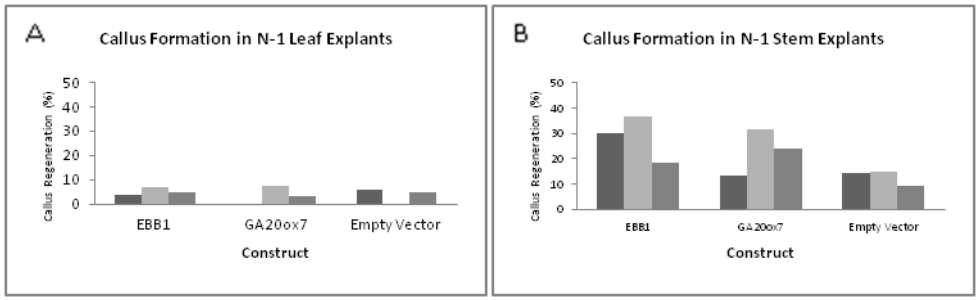

Figure 6: Callus formation on N-1 explants by experiment and construct. The percent of explants that showed callus growth is shown for (A) leaf explants and (B) stem explants. See Figure 5 for bar shade coding.

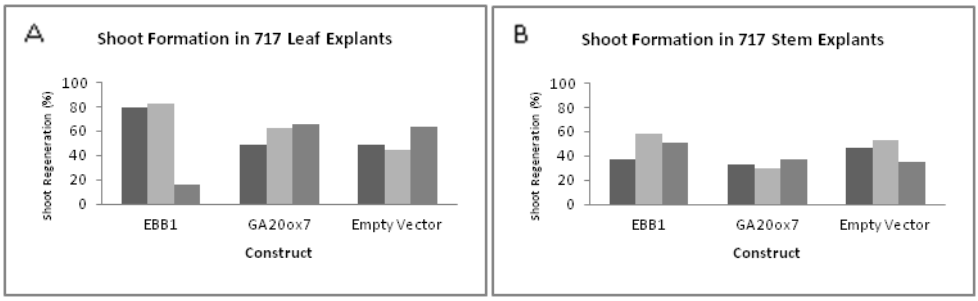

Figure 7: Shoot formation on 717 explants by experiment and construct. The percent of explants that showed shoot growth 60 days after cocultivation is reported for (A) leaf explants and (B) stem explants. See figure 2 for bar shade coding.

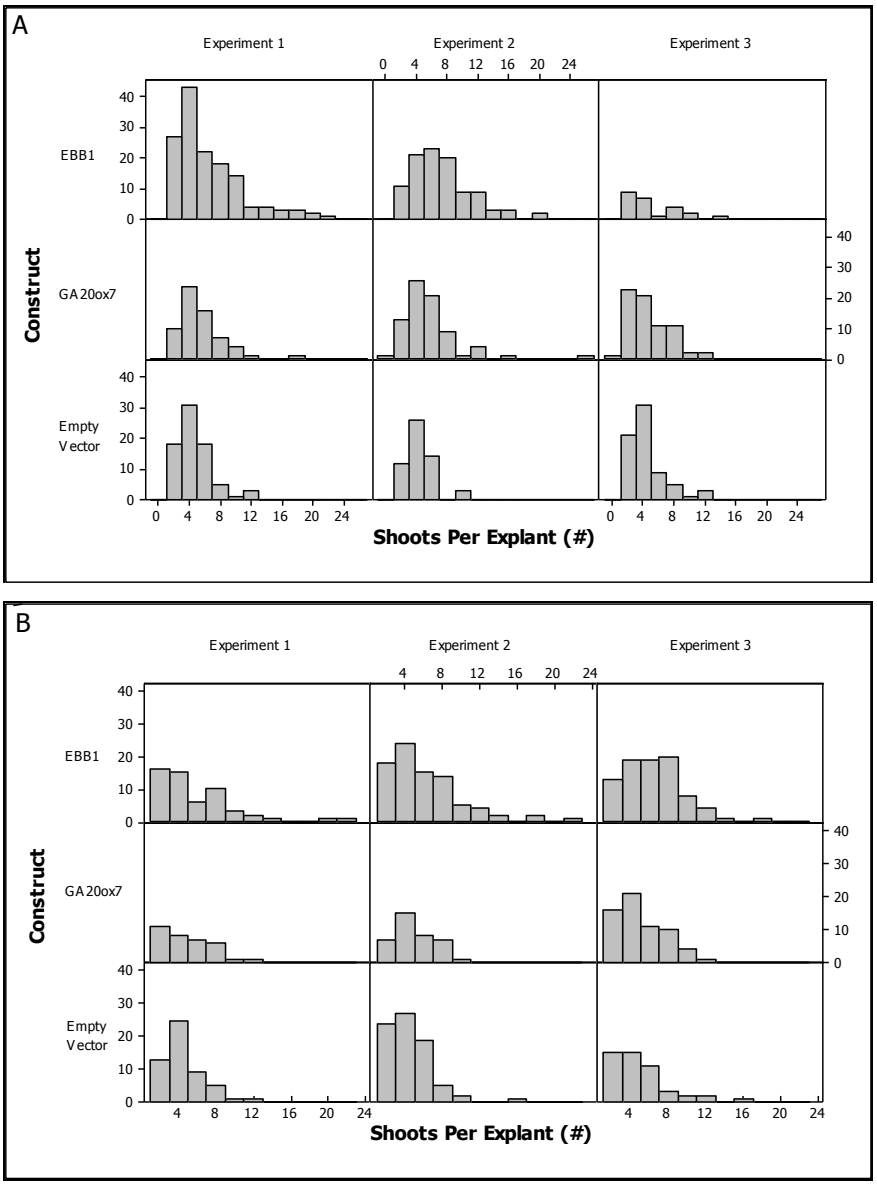

Figure 8: Number of shoots per explant in 717 by experiment and construct. After 30 days on shoot elongation media, the number of shoots 10mm or greater were counted on each explant for (A) leaf explants and (B) stem explants. The y-axis represents the number of explants with a given number of shoots. The x-axis represents the number of shoots on a given explant.

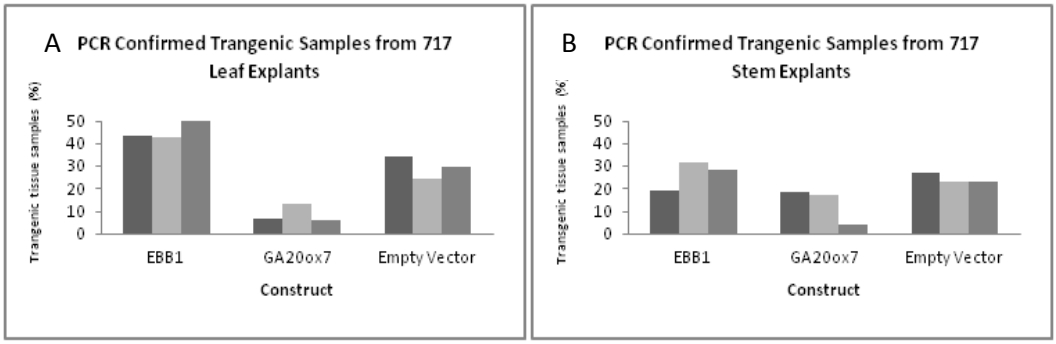

Figure 9: Proportion of transgenic shoots in comparison to escapes. DNA was extracted from each tissue sample and tested for successful transformation using PCR with primers corresponding to each construct. The percent of samples that tested positive for successful transformation are reported for (A) samples from717 leaf explants and (B) samples from 717 stem explants. See figure 2 for bar shade coding.

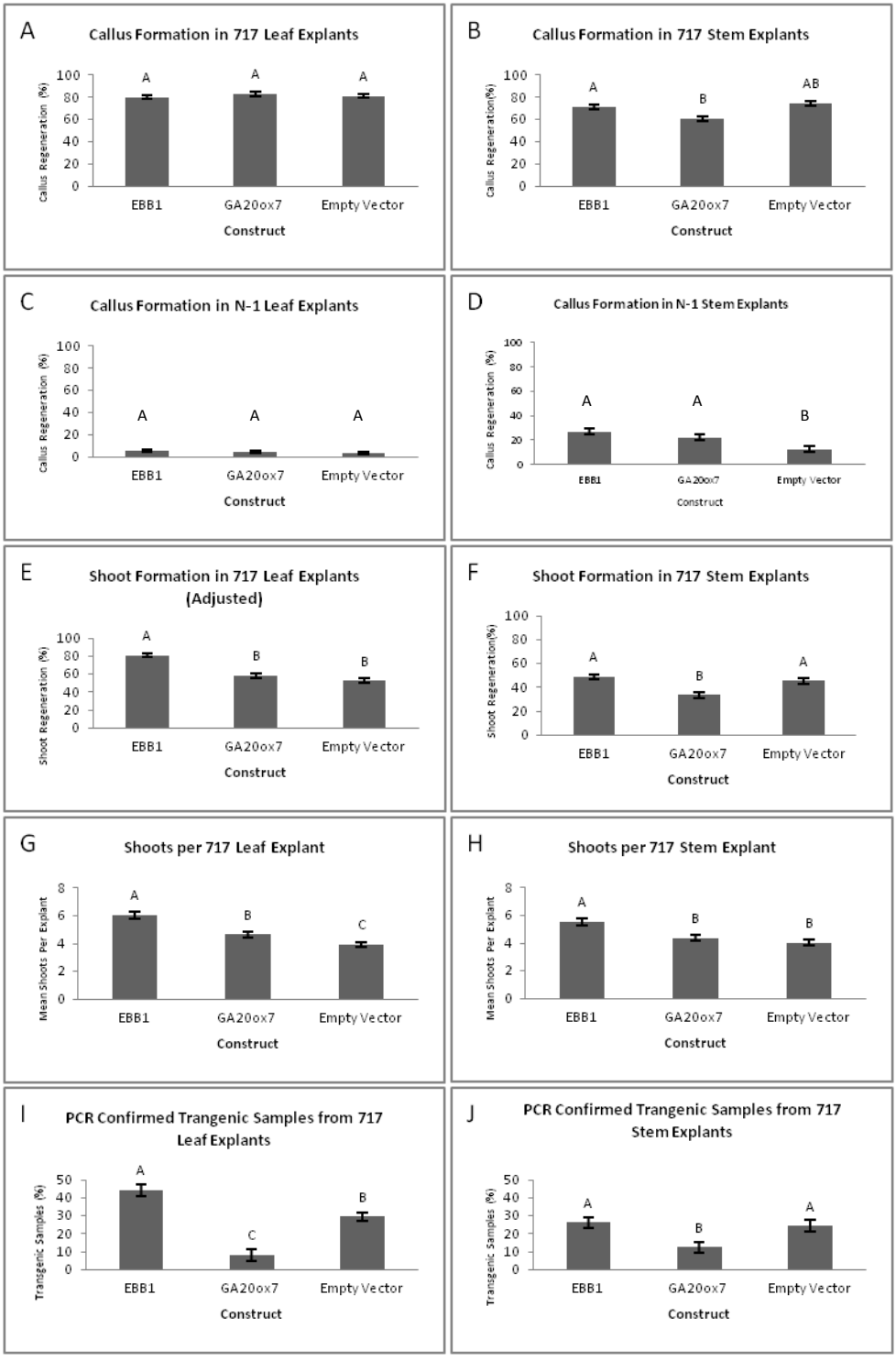

Figure 10: Summarized comparisons between each construct are given for each type of regenerated tissue. Letter subscripts denote statistical relationships. Like subscripts represent statistically nonsignificant differences between the means of two constructs. Different letter subscripts represent statistically significant differences between the means of two constructs. “Adjusted” in figure 10-E indicates that the third experiment was removed from analysis.

Experiment	EBB1	GA20ox7	Empty Vector
One	149/172	104/141	114/114
Two	144/126	129/122	145/133
Three	167/148	169/98	140/134
Combined	460/446	400/361	399/381

Table 1: Population of explants (# of leaf explants/# of stem explants) harvested from one month old 717 trees in Magenta boxes over three experiments.

Experiment	EBB1	GA20ox7	Empty Vector
One	98/119	57/143	67/35
Two	100/95	118/111	138/100
Three	137/156	153/126	124/98
Combined	335/370	328/380	329/233

Table 2: Population of explants (# of leaf explants/# of stem explants) harvested from one month old N-1 trees in Magenta boxes over three experiments.

Media/Antibiotics	Purpose	Genotypes	Growth Regulators/Selectable Markers
Antibiotics	**Selectable markers**	**both**	**Kanamycin 100mg/L Timentin 200mg/L Cefotaxime 300mg/L**
CIM	**Callus growth**	**717**	**NAA 10mM 2ip 5mM**
2SIM	**Shoot growth**	**717**	**TDZ 0.2μM**
SBO.1	**shoot elongation**	**717**	**BAP 0.1μM**
CIM2	**Callus growth**	**N-1**	**NAA 10mM 2ip 5mM**
SIM1	**Shoots growth**	**N-1**	**TDZ 0.2μM**
SIM3	**Shoots growth**	**N-1**	**TDZ 0.6μM**
SIM4	**Shoots growth**	**N-1**	**TDZ 0.01μM**

Table3: Summarized media used in explant regeneration. See Ma (2004) for 717 and Ma (2004) N-1 basal media recipes. Cytokinins include thidiazuron (TDZ), 6-(α,α-dimethylallylamino)-purine (2ip), and 6-benzylaminopurine (BAP). The auxin 1-Naphthaleneacetic acid (NAA) was used.

	A	GA20ox7 vs. Empty Vector		EBB1 vs. Empty Vector		EBB1 vs. GA20ox7	
	717						
	Trait	Experiment	Construct	Experiment	Construct	Experiment	Construct
Leaf Explants	Callus Formation	<0.001	0.776	<0.001	0.533	<0.001	0.360
	Shoot Formation	0.001	0.058	<0.001	0.021	<0.001	0.948
	Shoots per Explant	0.094	0.014	0.0160	<0.001	0.0100	0.009
	PCR Confirmation	8.18	<0.001	0.465	0.001	0.687	<0.001
	B	GA20ox7 vs. Empty Vector		EBB1 vs. Empty Vector		EBB1 vs. GA20ox7	
	717						
	Trait	Experiment	Construct	Experiment	Construct	Experiment	Construct
Stem Explants	Callus Formation	<0.001	<0.001	<0.001	0.212	0.347	0.001
	Shoot Formation	0.407	0.001	0.001	0.294	0.036	0.001
	Shoots per Explant	0.720	0.253	0.372	<0.001	0.775	0.003
	PCR Confirmation	0.290	0.034	0.726	0.633	0.417	0.009

Table 4: Summary of P-values comparing each construct to the other by type of regeneration data taken for 717 explants. The P-values (α=0.05) for individual ANOVA comparisons are reported for (A)717 leaf explants and (B) 717 stem explants. The column labeled 'Experiment' shows tests for differences between the three experiments the column labeled 'Construct' shows tests for differences between constructs.

	A	GA20ox7 vs. Empty Vector		EBB1 vs. Empty Vector		EBB1 vs. GA20ox7	
	N-1						
	Trait	Experiment	Construct	Experiment	Construct	Experiment	Construct
Leaf Explants	Callus Formation	.868	.985	.251	.264	.061	.313

	B	GA20ox7 vs. Empty Vector		EBB1 vs. Empty Vector		EBB1 vs. GA20ox7	
	N-1						
	Trait	Experiment	Construct	Experiment	Construct	Experiment	Construct
Stem Explants	Callus Formation	.640	.004	.006	<0.001	.001	.071

Table 5: Summary of P-values comparing each construct to the other by type of regeneration data taken for N-1 explants. The P-values (α=0.05) for individual ANOVA comparisons are reported for (A) N-1 leaf explants and (B) N-1 stem explants. The column labeled 'Experiment' shows tests for differences between the three experiments the column labeled 'Construct' shows tests for differences between constructs.

Appendices

Experiment	EBB1	GA20ox7	Empty Vector
One	138/77	58/37	61/62
Two	72/69	44/23	61/104
Three	26/81	69/45	88/35
Combined	236/227	171/105	210/201

Appendix 1: Population of harvest samples for DNA isolation and PCR analysis (Leaf/Stem)

Construct	Primer set 1 name	Primer set sections replicated	Primer sequence
pBin/ARS-409s-Empty	NPTII	NPTII (selectable marker-kanamycin)	Forward:ATGCCTGCTTGCCGAATATC Reverse:CCAAGCTCTTCAGCAATATCAC
p409S:EBB1	P409S:EBB1	Promoter:EBB1:Terminator sequences	Forward:AGGAACCTTTGTGACCTCA Reverse:AGACGGAAGCAAAAGCAAAA
P409S:GA20ox7	P4092:GA20ox7	Promoter:GA20ox7:Terminator sequences	Forward:AGGCCCAGAATGTGGGGTTT Reverse:TGGCTTCACCACCTTGTCCA

Appendix 2: Primers used for PCR analysis for each construct. Primers were designed based on transgene sequence.

ANOVA: EBB1 vs. Empty Vector (Stem Explants, Callus Formation)

Source of Variance	DF	SS	MS	F	P
Construct	1	0.21	0.287	1.56	**0.212**
Experiment	2	9.56	5.26	28.6	**<0.001**
Construct*Experiment	2	3.74	1.87	10.2	**<0.001**
Error	853	157	0.184		
Total	858	170			

Appendix 3: Example of ANOVA table used to construct tables 3 and 4

A:Callus Formation on Leaf Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	89.14%	94.40%	90.35%
Two	86.99%	90.98%	84.96%
Three	64.16%	72.45%	70.15%
Pooled Average	**80.30%**	**83.40%**	**81.40%**

B:Callus Formation on Stem Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	70.47%	66.35%	91.23%
Two	80.56%	54.26%	82.76%
Three	64.07%	62.28%	52.14%
Pooled Average	**71.30%**	**60.80%**	**74.40%**

C:Callus Formation on N-1 Leaf Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	4.08%	0%	5.97%
Two	7%	7.63%	0%
Three	5.11%	3.27%	4.84%
Pooled Average	**5.37%**	**4.27%**	**3.04%**

D:Callus Formation on N-1 Stem Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	30.3%	13.3%	14.3%
Two	36.8%	31.5%	15%
Three	18.6%	23.8%	9.18%
Pooled Average	**27%**	**22.1%**	**12.5%**

Appendix 4: Summarized data for percent of explants developing callus tissue within 30 days after cocultivation in 717 (A and B) and N-1(C and D)

A:Shoot Formation on Leaf Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	80.57%	48.94%	49.12%
Two	82.11%	63.11%	44.36%
Three	16.22%	66.33%	64.18%
Pooled Average	**59.60%**	**58.50%**	**52.80%**

B:Adjusted Shoot Formation on Leaf Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	80.57%	48.94%	49.12%
Two	82.11%	63.11%	44.36%
Three	**Removed**	66.33%	64.18%
Pooled Average	**81.20%**	**58.50%**	**52.80%**

C:Shoot Formation on Stem Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	36.91%	32.69%	47.37%
Two	59.05%	29.46%	53.79%
Three	50.90%	37.72%	35%
Pooled Average	**48.90%**	**33.80%**	**45.40%**

Appendix 5: Summarized data for percent of explants developing shoot tissue within 60 days after cocultivation in 717 for (A) leaf explants, (B) leaf explants after removing the third experiment EBB1, and (C) stem explants. “Adjusted” refers to data with experiment 3 removed.

A: Shoots per 717 Leaf Explant

Experiment	EBB1 Mean(Median)	GA20ox7 Mean(Median)	Empty Vector Mean(Median)
One	5.87(5)	4.83(4)	4.04(3.5)
Two	6.74(6)	5.01(4)	3.84(3)
Three	4.38(4)	4.09(3)	3.85(3)
Pooled	6.06(5)	4.65(4)	3.92(3)

B: Shoots per 717 Stem Explant

Experiment	EBB1 Mean(Median)	GA20ox7 Mean(Median)	Empty Vector Mean(Median)
One	5.23(4)	4.32(3.5)	3.82(3)
Two	5.42(5)	4.74(4)	3.96(4)
Three	5.86(6)	4.37(4)	4.37(4)
Pooled	5.55(5)	4.39(4)	4.03(4)

Appendix 6: Summarized data for shoots per explant by mean (median) for (A) 717 leaf explants and (B) 717 stem explants.

A:Transgenic Samples from 717 Leaf Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	43.50%	6.90%	34.40%
Two	43.10%	13.60%	24.60%
Three	53.90%	5.80%	29.60%
Pooled Average	**44.50%**	**8.19%**	**29.50%**

B:Transgenic Samples from 717 Stem Explants

Experiment	Construct		
	EBB1	**GA20ox7**	**Empty Vector**
One	19.50%	18.90%	27.40%
Two	31.90%	17.40%	23.10%
Three	28.40%	4.44%	22.90%
Pooled Average	**26.40%**	**12.40%**	**24.40%**

Appendix 7: Summarized data for percent of shoot samples from explants that were identified to be transgenic from (A) leaf explants and (B) stem explants. All other samples are considered escapes (despite kanamycin resistance).