Transformation Improvement in Poplar (*Populus trichocarpa*): Effects of antioxidant and auxin treatments

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Introduction

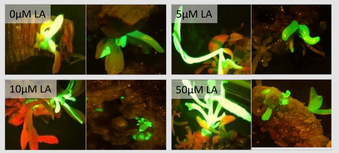
- Robust *in vitro* tissue culture methods for diverse genotypes are critical to expanding the use of gene editing and other biotechnologies in agriculture. However, methods are highly genotype and species dependent, and many genotypes remain nearly impossible to regenerate and/or transform.
- We are undertaking genome-wide association studies (GWAS) of *in vitro* regeneration and transformation in *Populus trichocarpa* to gain insights into the causes of genotype variability. In preparation for these GWAS, we have undertaken preliminary experiments to optimize *in vitro* treatments across a subpopulation of genotypes and to identify treatments that are highly heritable and therefore offer the best opportunity for genetic discovery.
- To facilitate high-throughput screens of diverse treatments across many genotypes, we have developed a phenomics system that applies deep learning and hyperspectral imaging to obtain statistics on the growth of specific *in vitro* tissues (callus and shoot) and to determine whether they are transgenic as indicated by reporter proteins (e.g. GFP, DsRed).
- **We have found that application of developmental regulators that we have studied, including *WUSCHEL* genes and others, have given inconsistent or negative effects on shoot regeneration thus we do not report them here. Instead we report on three factors that have important and genotype dependent effects: Lipoic acid, auxin types, and pre-culture.**

Plant materials

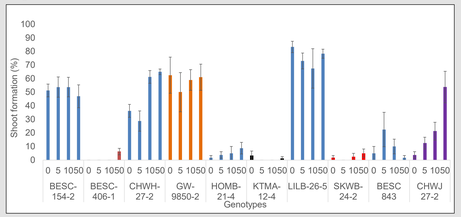
Clones from a *P. trichocarpa* association mapping population (Department of Energy Bioenergy Science Center, Oak Ridge National Laboratory), representing genetic diversity across the Pacific Northwest, were grown in a greenhouse as sources of explants (**Figure**). All of these ~1,300 genotypes have been resequenced, identifying ~28 million SNPs per genotype. **These explants were transformed with *Agrobacterium* harboring a GFP reporter plasmid with a selectable marker for geneticin resistance** (except where noted, stem sections were cultured for three weeks in the dark on callus induction medium (CIM: MS with 0.01mg/L 2,4-D and 0.5mg/L BAP), then cultured for four weeks under light on shoot induction media (SIM: MS supplemented with 1.32 mg/L TDZ). Experiments shown feature 2-5 replicate plates, each with 20 explants, for each of up to 20 genotypes.

Lipoic acid promotes regeneration in most genotypes, but of unclear benefit for transformation

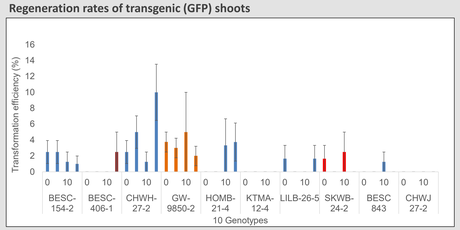
In both CIM and SIM, the antioxidant lipoic acid (LA) was added at concentrations of 0, 5, 10, and 50 µM. LA is an antioxidant that is used in diverse plant species to reduce browning and enhance in vitro shoot regeneration. Shoots were phenotyped by fluorescent microscopy. Results are shown for ten randomly selected genotypes (**Figures**). Many shoots were escapes from geneticin selection, as shown by the difference between rates of total shoot regeneration (**top-right**) and regeneration of shoots with visible GFP (**bottom-right**). In some genotypes (e.g. CHWH-27-2), LA may enhance total shoot regeneration but have no effect on the ability of explants to be transformed. **Our results show that LA enhanced shoot regeneration in most but not all genotypes, but generally did not increase transformation rate. No single concentration of LA produced an optimal response across genotypes.**



Regeneration rates of all shoots



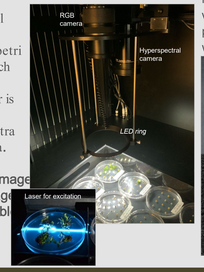
Regeneration rates of transgenic (GFP) shoots



Hyperspectral imaging and machine vision enable automated screens of regeneration phenotypes

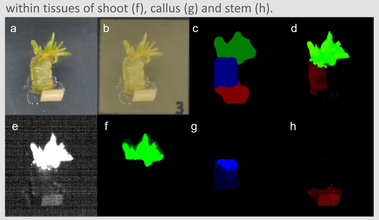
High-throughput imaging

The *macroPhotArray* (Middleton Spectral Vision) was designed to collect RGB and hyperspectral images over trays with 21 petri dishes. A ring of LED lights illuminates each plate as RGB images are taken. During hyperspectral imaging, a 488nm line laser is used to excite fluorescent proteins (e.g., chlorophylls and GFP), and emission spectra for each pixel are collected by the camera. We developed a pipeline to quantify fluorescent proteins in hyperspectral images, recognize specific tissues in RGB images, and cross-analyze these results to enable statistical insights into transformation and regeneration rates.



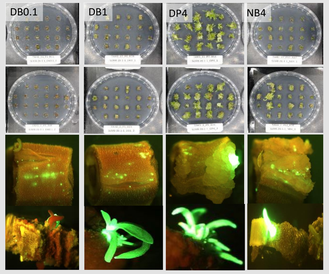
Outputs from phenomics workflow

a) RGB image of explant; b) RGB image of explant with grid superimposed; c) Results from deep segmentation of RGB data; d) False-color reconstruction of hyperspectral data with green and red channels used to visualize GFP and chlorophyll, resembling a view from under a fluorescent microscope; e) Pure channel for pixelwise test-statistics of GFP; f-h) Measurement of GFP signal within tissues of shoot (f), callus (g) and stem (h).



Auxins with strong and highly genotype-dependent influences on regeneration

Transformed explants were cultured on one of four CIM treatments (top-left table) for three weeks followed by seven weeks of SIM incubation. Shoot regeneration was phenotyped using our automated phenomics workflow (described above). Statistics for GFP expression in specific tissues were used to determine if these tissues are transgenic (demonstrated in below figures). **Higher levels of 2,4-D, together with 2ip (treatment DP4), promoted transgenic shoot regeneration in most genotypes, but inhibited regeneration in a few.** Among these notable exceptions to the trend, HOMB 21-4-1 appears to regenerate most efficiently with BAP and moderate levels of 2,4-D (0.01mg/L, media DB1), while BESC 154-2 shows a unique preference for media NB4, which lacks 2,4-D.

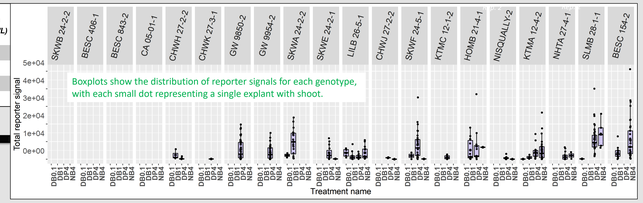


CIM options studied

Mixture ID	2,4-D (mg/L)	NAA (mg/L)	BAP (mg/L)	2ip (mg/L)
DB0.1	0.001	0	0.5	0
DB1	0.01	0.01	0.5	0
DP4	0.1	0.1	0	1
NB4	0	0	1	0

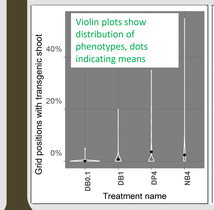
Shoot regeneration rates for each plate are calculated from the number of explant positions on the plate with significant GFP signal in shoot tissue

GFP signal in shoot observed among genotypes

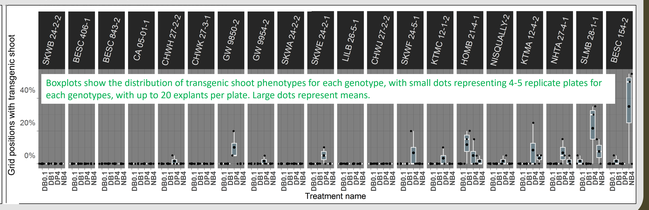


Rates of transgenic shoot regeneration

Means & distributions



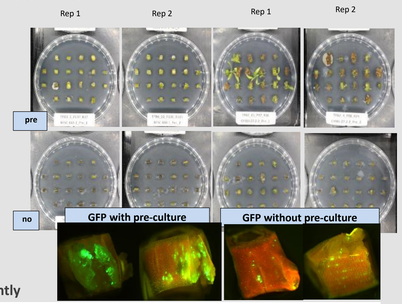
Genotypes viewed independently



CIM pre-culture generally has positive but highly genotype dependent effects

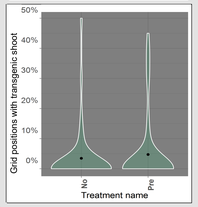
Pre-culture is widely used to promote transformation. However, its effects in *P. trichocarpa*, and how much this effects depends on genotype, is unknown.

Prior to co-cultivation with *Agrobacterium*, stem explants were pre-cultured on CIM with acetosyringone for two days in dark conditions (**Pre**). This treatment group was compared to a control group which underwent co-cultivation immediately upon excision of explants from plant material (**No**). After three weeks of post-transformation CIM incubation and seven weeks of SIM incubation, phenotyping was performed with our automated phenomics workflow. **Results show that in most genotypes, the pre-culture treatment led to positive effects on regeneration of callus (not shown) and shoots (below figure). However, the extent of these effects are highly genotype-dependent,** with a strong inhibitory effect of the pre-culture on genotype CHWH-27-2-2.



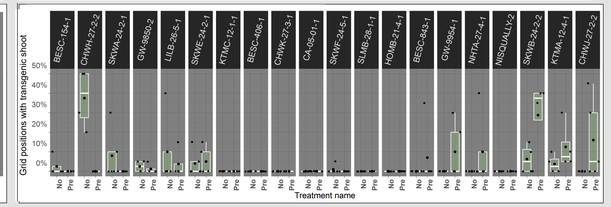
Means & distributions

Violin plots show distribution of phenotypes, dots indicating means



Genotypes viewed independently

Boxplots show the distribution of transgenic shoot phenotypes for each genotype, with small dots representing 4-5 replicate plates for each genotypes, with up to 20 explants per plate. Large dots represent means.



Outlook

- This poster demonstrated how automated imaging with visible and hyperspectral cameras, followed by convolutional neural network/machine vision analysis, enables high throughput and high precision phenotyping for in vitro experiments
- This capability provides new opportunities for *in vitro* optimization for gene editing and transformation, and genetic discovery in GWAS and related methods, by enabling sterile and precise high-throughput phenotyping of complex traits
- Our results also demonstrate how extensive genetic variation in the regeneration and transformation response of wild cottonwoods are, and that *in vitro* treatments interact strongly with plant genotype. None of the methods we have studied to date show evidence of genotype-independent *in vitro* influences.
- However, this genetic variation also provides us with a powerful opportunity for GWAS to uncover the genetic causes of this variation. Carefully conducted GWAS is likely to uncover control points for genetic variation in stress response, hormone signaling, and other processes that will inform treatments, including use of gene reagents, to overcome recalcitrance of difficult genotypes.

Acknowledgements

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