Extensive Natural Variation in Callus and Shoot Regeneration in relation to *Agrobacterium*-Mediated Transformation of Wild Black Cottonwood *(Populus trichocarpa)*

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Abstract

The capacity for plant regeneration and transformation (RT) is notoriously variable among species and genotypes of plants. In many cases, transformation is impossible or impractical. The reasons for this extraordinary biological variation, however, are largely unknown. As part of a major project to use GWAS (genome wide association studies) to map genes controlling RT in poplar, we are studying variation in RT among resequenced wild genotypes of black cottonwood—for which low levels of linkage disequilibrium facilitate GWAS-based gene identification.

We tested both direct and indirect regeneration pathways using two different types of explants, petioles and leaves, from 20 genotypes of greenhouse-grown plants based on our previously published protocol (1). We found that indirect regeneration, where callus proliferation preceded shoot induction, strongly promoted shoot regeneration, but that the effect varied widely between petiole and leaf explants.

We also studied various influent factors on transient and stable transformation on 3-5 selected genotypes of *in vitro* grown plants, using both leaf and stem explants. We discovered pre-culture for one day on callus induction medium (CIM) greatly increased both transient and stable GFP expression. Auxin-rich media and acetosyringone (AS) in CIM during co-cultivation enhanced GFP expression, both during transient and stable transformation phases and in both leaf and stem explants, of all tested genotypes. Further analysis and recovery of transgenic shoots is under study.

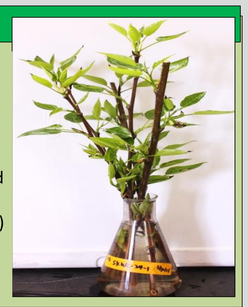
Project overview

To explore the diversity of *in vitro* RT responses in *P. trichocarpa* to inform GWAS analysis we will:

- Expore a diversity of RT methods to maximize in vitro trait heritability (see right panel for partial list)
- Develop new phenomic tools—generalizable machine-vision methods-- to rapidly and precisely determine in vitro phenotypes (in progress)
- Using GWAS, precisely map alleles associated with variation in RT frequency

- ✓ Agar use, type, active charcoal
- ✓ Hormones (auxins, cytokinins)
- ✓ Explant source and type
- ✓ Acetosyringone induction
- ✓ Preinduction (auxin pulse)
- ✓ Agrobacterium strain)
- ✓ Cocultivation-antibiotic selection

Methods - Shoot regeneration



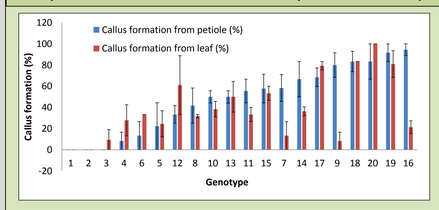
- Cuttings of gentotypes cloned from wild populations from throughout the Pacific Northwest were grow in in a greenhouse
- Leaf discs and petiole segments were used
- Two regeneration systems were tested: indirect vs. direct
- 8-15 explant per plate; 3 plates per genotype
- Explants were cultured on callus induction medium (CIM) for 20d in dark (MS supplemented with 2μM 2iP and 10μM NAA)
- Explants were transferred onto shoot induction medium 1 (SIM1) for 20d under light (MS supplemented with 1μM TDZ)
- Then explants were subcultured on SIM2 (MS supplemented with 0.01μM TDZ)

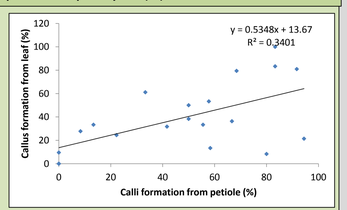
Results

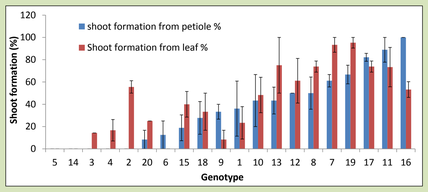
Explants showed highly variable developmental responses on CIM (after 20 d in dark during indirect regeneration: Petiole and leaf explants produced variable forms of callus (A, B); Leaf explants formed extensive roots (C); Petiole and leaf explants regenerated shoots (D, E)

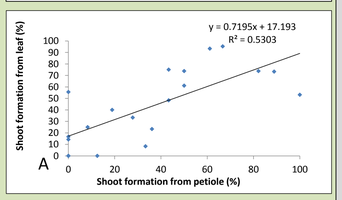
Callus formation varied widely among genotypes under indirect organogenesis, and was weakly correlated among tissues and with shoot formation.

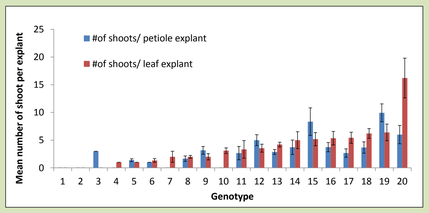
We tested one kind of CIM and 2 kinds of SIM on 20 genotypes based on our previous study of transformation of the sequenced black poplar Nisqually-1 (1). `

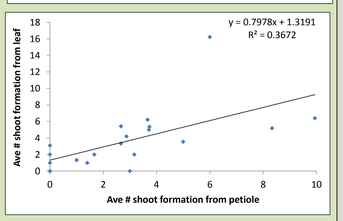






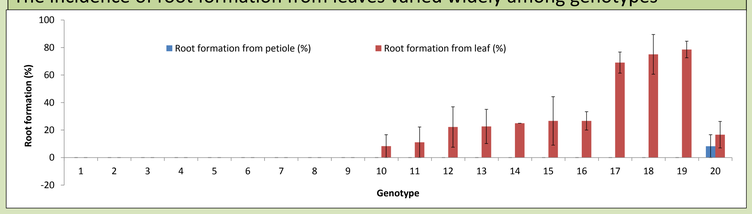






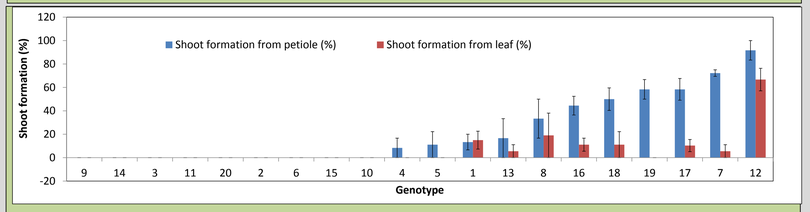
Leaf but not petiole explants frequently produced roots on CIM

The incidence of root formation from leaves varied widely among genotypes

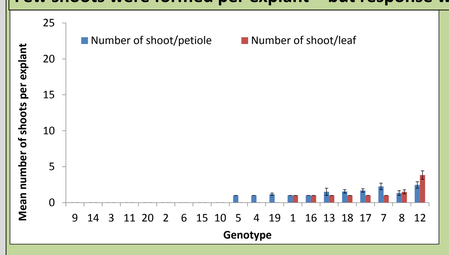


Clone bank of wild black cottonwood genotypes in Corvallis, Oregon

Direct organogenesis: Half of the tested genotypes formed shoots



Few shoots were formed per explant – but response was correlated among tissue explants



Direct shoot regeneration from leaf (left) and petiole (right) explants

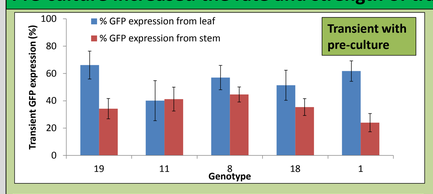
Methods - Transformation

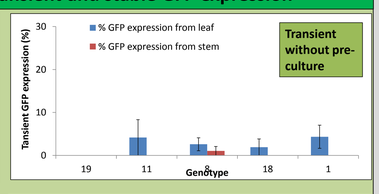
- Three to five genotypes were randomly selected
- Plants were grown in WPM hormone-free medium (example of source plants shown to right)
- Leaf and stem (including petiole) explants
- GMUbi1500::eGFP (kan selection) was transformed
- Two to four plates per genotype and 20-30 explants per plate
- Transient and stable GFP expression checked under GFP microscope (3d and 20d on CIM containing 75mg/L kanamycin
- Four CIMs tested:
  1. CIM1 (WPM+ 5.4μM NAA+ 0.22μM BAP)
  2. CIM2 (MS+ 2μM NAA + 10μM 2ip)
  3. CIM3 (MS+ 2μM NAA + 10μM 2ip + 0.45μM 2,4-D)
  4. CIM4 (MS+ 2μM NAA + 10μM 2ip + 0.9μM 2,4-D)
- Pre-culture vs. no pre-culture with CIM
- Actosyringone (AS) vs. no AS in cultivation medium

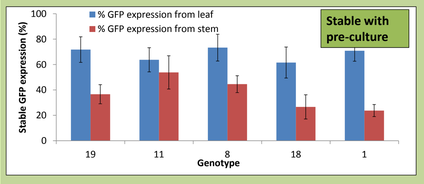
In vitro plants growing in WPM

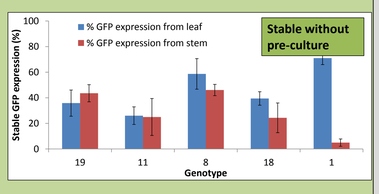
Results

Pre-culture increased the rate and strength of transient and stable GFP expression

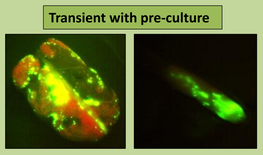






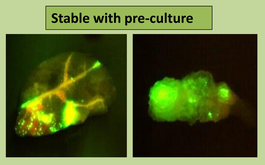


Transient with pre-culture

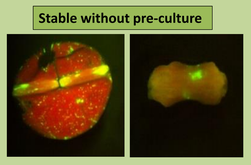


Transient without pre-culture

Stable with pre-culture

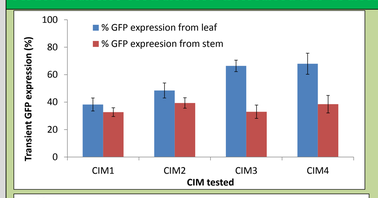


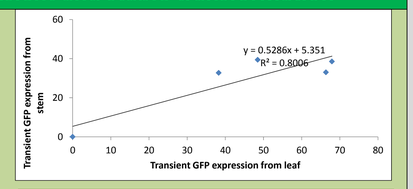
Stable without pre-culture

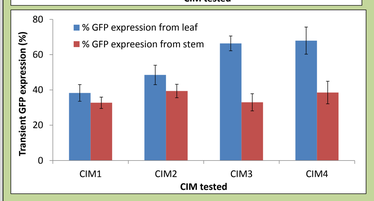


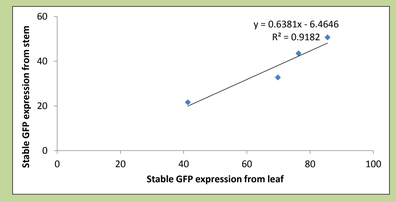
Pre-culture for one day on CIM greatly increased transient GFP expression from 0-4% to 40-66% for leaf explants, and from 0-1% to 23-44% for stem and petiole explants. This suggests that cells on CIM became more susceptible to *Agrobacterium* transfection.

Rich-auxin medium enhanced transient and stable transformation

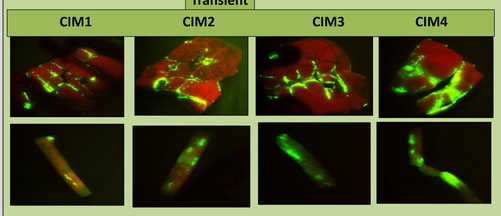




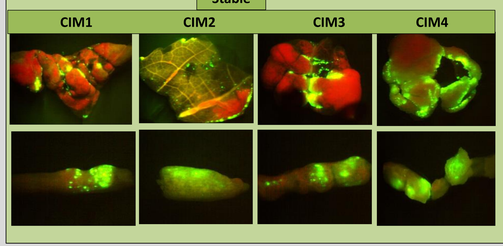




Transient

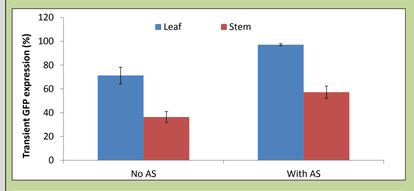


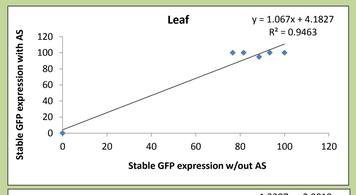
Stable

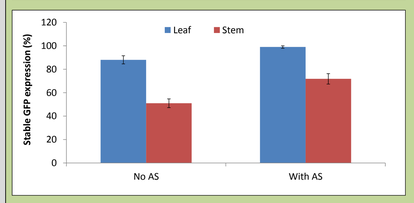


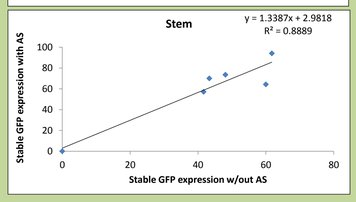
To determine whether transformation was influenced by the co-cultivation medium, we tested 4 different CIMs along with preculture treatment. The highest rate of transient and stable GFP expression was found in explants co-cultivated in CIM3 and CIM4, both of which were supplemental with 2,4-D (in addition to NAA and 2iP). This is perhaps because in auxin rich medium, the rate of actively dividing cells in S-phase is significantly increased, the stage in which cells are more prone to integrate foreign DNA (2). Responses were also correlated among tissues taken from the same genotypes.

AS enhanced transient and stable GFP expression in auxin-rich media

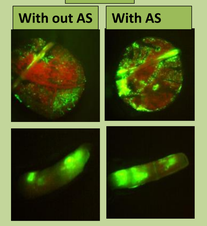








Transient



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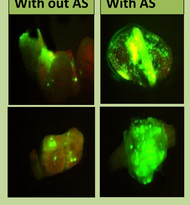

AS in auxin-rich CIM during co-cultivation moderately enhanced GFP expression, both during transient and stable transformation phases, and in both leaf and stem explants of all tested genotypes. The responses of the two tissues were weakly correlated among the five genotypes tested.

Summary

- Callus, root, and shoot regeneration from tested 20 genotypes of *P. trichocarpa* varied widely, confirmed the important impact of natural genetic variation on competence for response to regeneration and transformation treatments
- The indirect shoot organogenic pathway showed far superior rates of callus and shoot formation in all genotypes
- The effect of pre-culture was dramatic: Pre-culture for one day on CIM greatly increased transient GFP expression for leaf and stem explants
- Pre-culture also markedly improved the rate and intensity of stable GFP expression
- Auxin-rich media had greatly enhanced rates of transient GFP expression in leaf explants, but this benefit was not seen in stem explants
- Acetosyringone was moderately beneficial for transformation in leaf and stem explants
- Leaf explants responded better than stem explants in all tested media in transformation tests

References

1. Ma C, Strauss SH, Meilan R. 2004. Agrobacterium-mediated transformation of the genome-sequenced poplar clone, Nisqually-1 (*Populus trichocarpa*). Plant Molecular Biology Reporter 22:1-9
2. Leandro Penäa, Rosa M. Peã Rez, Magdalena Cervera, Joseãa. Juaã Rez and Luis Navarro. 2004. Early Events in Agrobacterium-mediated Genetic Transformation of Citrus Explants. Annals of Botany 94: 67±74

Acknowledgements

This project is supported by NSF I/UCRC Center for Advanced Forestry (NSF 15-548) and the TBGRC industrial cooperative at Oregon State University. We thank the Biological Energy Sciences Consortium at Oak Ridge National Laboratory for use of their poplar collections.