Regeneration and confirmation of ZFN-transgenic events

The four ZFN constructs were transformed into A. tumefaciens strain AGL1 and then transformed into poplar clones 717-1, 184 and 353-5. All of the culture media contain kanamycin for transgenic tissue selection, and screening for introduction of Agrobacterium free tissue. After two months in root induction medium, in vitro propagated candidates of transgenic poplars were harvested for GFP signal checking, DNA extraction and PCR confirmation.

Low shoot regeneration and transformation rates with ZFNs

For each ZFN construct and each poplar clone, we co-cultivated ~2,000 explants. In total, we were able to get the 1,188 regenerated shoots. However, only 259 ZFN-transgenic events were confirmed by GFP expression and/or PCR, and all of them were produced from the "pulse" heat shock method. The reduced transformation rate might be a result of ZFN expression. Additionally, we were unable to get any regenerated ZFN802-transgenic events. PCR analysis of the Agrobacterium strain that was used for plant transformation showed an absence of the ZFN transgene, despite multiple efforts to transform it. We suspect this ZFN pair is toxic to the bacteria thus it could not effectively transform plants.

Detection of ZFN-induced mutations using HRM

High resolution melting (HRM) is a PCR-based method for detecting DNA sequence polymorphisms or mutations. It detects variants based on Tm values and the shape of the melt curve. Using this method, we screened all of the 259 ZFN-transgenic events for alterations to the GFP marker gene. PCR amplification of the ZFN transgene was performed, and the products were analyzed by HRM using locus specific primers. Two putative insertions in PtAG2 were detected among the 127 ZFN800-transgenic events by HRM analysis and agarose gel electrophoresis analysis. No insertions were found in ZFN802- or ZFN803-transgenic events. These suggest a mutation rate per allele well below 0.4% per explant, much less than anticipated. Further analysis of the putative mutated loci is underway by cloning and sequencing.

GFP expression to monitor ZFN T-DNA delivery

The constitutive GFP gene in our ZFN constructs helped to non-destructively monitor transformation and chimerism. The GFP signal was recorded during organogenesis. (a) after 2 days on calli induction medium, (b) after 3 weeks on calli induction medium, (c) after 3 weeks on shoot regeneration medium, and (d) after 3 weeks on shoot regeneration medium.

Ongoing work and future plans

- Produce more ZFN transgenic plants for analyzing ZFN-induced mutation efficiency (focus on ZFN800 and ZFN801)
- Heat shock the newly-regenerated ZFN-transgenic plants to induce GFP expression and hopefully obtain desired biallelic mutations
- Screen for ZFN-induced mutations with HRM and/or restriction digestion
- Characterize the nature of HRM-detected mutations by cloning and sequencing
- Analyze forklift morphology of biallelic ZFN-mutated poplars, if found

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