TRANSFORMATION AND EXPRESSION OF AN **AUXIN BIOSYNTHESIS GENE IN TOBACCO AND HYBRID POPLAR**

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The objective of this research is to increase wood/fiber production by elevated endogenous auxin in hybrid poplar trees transformed with an auxin synthesis gene. Indoleacetic acid (IAA) is a plant hormone that stimulates cambium activities and influence wood/fiber development (Roberts *et al.,* 1988). Phenotypic modifications have been described in transgenic plants over-expressing the *Agrobacterium turnefaciens* T-DNA genes responsible for auxin biosynthesis (Klee and Estelle, 1991). Two of the *Agrobacterium tumefaciens* Ti-plasmid T-DNA genes have been shown to encode a biosynthetic pathway for IAA production that is different from plants. The *iaaM* gene, which encodes an enzyme converting tryptophan to indole-3 -acetamide (IAM) (Thomashow *et al.* 1986) is subsequently hydrolyzed to IAA by the gene product of *iaaH.* Although IAM is not a standard intermediate in plant auxin biosynthesis, the expression of both *iaaM* and *iaaH* is not normally required for synthesis of IAA. Research in petunia suggests that overexpression of *iaaM* alone leads to high IAA production via hydrolysis (Klee *et al.,* 1987). We therefore constructed two gene cassettes designated as pZKY99 and pZKY100, which contain 19SCaMV-iaaM, nos-nptll and 35SCaMV-GUS within the T-DNA region. The only difference between the two plasmids was the reversed transcription orientation of the *iaaM* gene. The combination of GUS reporter gene and *nptll* selectable marker with *iaaM* gene in T-DNA region makes the constructs uniquely different from other available IAA-gene containing constructs (Klee *et al.,* 1987; Sitbon *et al.,* 1992). The gene constructs were successfully introduced into tobacco, the model transformation system, and into hybrid poplar *(Populus trichocarpa x P. deltoides,* clone '53-246') using an *Agrobacteriurn*

Optimization of tissue culture and transformation conditions

To date our focus has been on development of a reliable method of micropropagation of *Populus trichocarpa X P. deltoides,* and on the methods to improve subsequent transformation. To optimize the transformation conditions, three disarmed *Agrobacteriurn turnefaciens* strains, LBA4404, EHA105 and C58, containing the binary vector p35SGUSINT, were tested for their efficiency to infect `53-246.' Tested vector p35SGUSINT contains selectable marker *nptll* gene for kanamycin (1(n) resistance and GUS gene for histochemical localization of ß-glucuronidase. Three independent transformation experiments all indicated that EHA105, an agropine type of supervirulence strain (Jin *et al.,* 1987), gave the highest GUS transient expression in 30-d-old calli, where up to 67% poplar tissues were GUS positive. Therefore, pZKY99 and pZKY100 were all transformed into EHA105 for further use in the transformation of IAA gene in hybrid poplar. Transformation conditions, such as Agrobacterial concentration, inoculation and cocultivation, were also determined.

Agrobacterial concentration. To examine the effect of bacterial titer on gene transfer efficiency, explants were inoculated with concentrations ranging from 10^5 to 10^{10} bacteria/ml for 3 hr.

Lower concentration of bacteria up to the level of 10^8 gave higher transformation efficiency. Thus, we routinely used a fresh overnight culture at a concentration of 107-108bacteria/mL.
Inoculation: To study the influence of exposure time to bacteria, stem internodes were cut longitudinally and inoculated by immersion in 15-25 mL of a culture at a concentration of 108 cells/mL for 1, 2, 4, 20, 24 and 28 hrs. There was little difference in transformation efficiency up to 4 hr. Overnight exposure (20 hr) significantly reduced transformation efficiency. A 28-hr inoculation totally inhibited callus formation and shoot regeneration, probably due to the extensive bacterial contamination that inhibited explant growth. Three to four hr inoculation in a liquid culture with a slow agitation (50 rpm) was routinely used.
Co-cultivation: Inoculated explants were blotted dry with a sterile filter paper to remove excess bacteria and then transferred to a callus induction medium (CIM, WNA-see below + 0.5 mg/L 2,4-D) without any antibiotics for a co-cultivation period of 1-8 d. Up to 3 d of cocultivati0n resulted in the highest percentage of shoot regeneration on Kn-containing medium.
Plant regeneration and selection: After co-cultivation, explants were washed with WNA medium (Cole and Ernest, 1991) containing 250 mg/L of cefotaxime for 2 hr and then transferred t0 a shoot-induction-medium (SIM; WNA + 0.5 mg/L zeatin) supplemented with cefotaxime t0 eliminate bacterial carry over. Ten d later, 50 ug/mL of Kn was introduced to the SIM plates t0 select for transformed tissues. The frequency of adventitious shoot formation and embryo-like structures was determined 30-35 days after infection. Elongated adventitious shoots were then excised and transferred to hormone free, root inducing medium (WNA with 1% sucrose, 250 ug/mL cefotaxime) with 25 ug/mL Kn. Regenerated plantlets were transplanted to a sterile Bact0 soil mix in a Magenta GA7 vassel when roots were appeared, and transferred to the pots in the greenhouse.

Expression of *iaaM* gene in tobacco plants

To overcome the lack of morphogenesis in putatively transformed hybrid poplar, pZKY99 and pZKY100 were tested for their T-DNA intergration and gene expression in tobacc0 transformation system. Approximately 40 putative transformants were rooted from Kn-containing RIM with a uniform, and unique morphological aberrations. Several morphological changes attributed to overproduction of auxin were apparent in transgenic tobacco, such as apical dominance, epinastic leaf growth and a massive adventitious root formation. These morphological changes were expressed more dramatically in pZKY99 plants than in th0se transformed with pZKY100, with changes so abnormal that none of pZKY99 transformed plants completed their life cycle. Histochemical analysis of ß-glucuronidase (GUS) in the tissues indicated its strong expression in roots, stems and leaves of pZKY99 plants, but not in pZKYl00 transformants. The result may be attributed to the pararell transcriptional orientation of *iaaM* and GUS in pZKY100 construct. In spite of predominant morphological modifications, Southern and Northern hybridizations using non-radioactive digoxigenin-labeling system did not reveal the integration and expression of *iaaM* gene in both pZKY99 and pZKY100 plants.

Biochemical validation of plant transformation

Biochemical verification of plant transformation with genes that regulate auxin overproduction requires that the transformed plants have higher levels of auxin (IAA) or its

precursor or catabolites (breakdown products), providing evidence of increases in pool size or its turnover. The successful insertion of *iaaM* gene produces an unambiguous biochemical signal, IAM, a compound that typically does not accumulate in higher plants, but a compoundthat, if present, can be converted by plants to IAA (Klee et al., 1987). The typical precursor of auxin in higher plants is indole-3-acetaldehyde. Therefore, the presence of IAM validates that plant transformation has indeed occurred. Auxin and its metabolites occur in low concentrations in plant tissues. We have established protocols to analyze IAA by capillary gas chromatography-mass spectrometry (GC-MS) using selectable ion minitoring. It was demonstrated that tobacco plants transformed with the pZKY99 gene cassette produce high levels of IAM, up to 36 μmol g^{-1} dry weight of stem and leaf tissues, confirming that genetic transformation had occurred. IAA levels were also increased by transformation but levels were still very low. Therefore, the primary effect of transformation was on the production of IAM, whereas mechanisms, such as turnover, catabolism, and conjugation, may function to keep the pool size of IAA relatively stable.

Hybrid poplar was also transformed with the same constructs and 75% of the calli were GUS positive, but the expression declined over the time. About 500 plantlets from independent transgenic plants of hybrid poplar have been regenerated and planted in soil. Although none of these plants showed positive reactions on GUS histochemical and enzymatic assays, further DNA and biochemical analyses are being evaluated.

Keywords: IAA, hybrid poplar *(Populus trichocarpa* x *P. deltoides),* genetic transformation

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