EXPRESSION AND STABILITY OF TRANSGENES IN ASPEN-POPULUS

M.R. Ahuja and M. Fladung

Abstract:-- We have employed *Populus* as a model system to investigate questions regarding stability and expression of foreign genes in transgenic trees. Several clones of European aspen *(P. tremula)* and hybrid aspen *(P. tremula* x *P. tremuloides)* were genetically transformed, using *Agrobacterium as* a vector system, with different gene constructs, in particular *rolC.* In order to test the expressive control of promoters, we have employed two types promoters: 35S from cauliflower mosaic virus, and light-inducible rbcS from potato, for control of *roIC* gene expression in transgenic aspens. The *35S-rolC* chimeric gene caused marked alterations in growth characters, including stem height, internode length, leaf size and pale-green leaf color, and physiology in the transgenic aspens, as compared to untransformed controls. On the other hand, rbcS-rolC recombinant gene caused only minor alterations in leaf size, and the leaf color was similar to 35S-rolC transgenic aspens. However, in the second and third years growth cycles, the leaf color was somewhat similar to the controls. During the three year growth, deviations from the 35S-rolC syndrome, including leaf abnormalities, chimeras, and revertants to normal state were observed in the transgenic aspens. In order to study position effect on *rolC,* a heterologous transposable element *Ac* from maize was introduced into aspens along with the *rolC* gene, under the expressive control of 35S and rbcS promoters. Transgenic aspens carrying *35S-Ac-rolC* and *rbcS-Ac-rolC* were morphologically similar to untransformed controls, because in the presence of *Ac,* the expression of *rolC* is inhibited due to position effect. However, following *Ac* excision during leaf development, there is restoration of *rolC* expression in the form of pale-green spots on the green leaf background. The presence of transgene was confirmed by PCR amplification of *rolC* and *Ac* coding sequences, and the copy number determined by the Southern hybridization, and gene expression was determined by northern blot analysis. These observations suggest that type of promoter/construct seems to affect growth, development and physiology of the transgenic aspens conditioned by the *rolC* gene. It also appears that *rolC* expression is variable during growth cycles.

Keywords: *Populus, rolC,* transgenic aspen, phenotypic alterations, gene expression

INTRODUCTION

Genetic engineering seems to offer prospects for forest tree improvement at an accelerated rate in plants. As compared to annual plants, forest trees have long generations cycles, with vegetative phases extending from one to several decades, and therefore may require special considerations for genetic manipulation. Therefore, it is relevant to ask if foreign genes would be stably integrated and expressed in the forest trees on a short-term or long-termbasis (Ahuja, 1988a, 1988b). Genetic stability of transgenic trees is important for their subsequent utility in the commercial forestry.

BFH, Institute of Forest Genetics, Sieker Landstrasse 2, 22927 Grosshansdorf, Germany

Recent studies with several annual model plant systems have revealed that transgene expression may be variable and unpredictable (see Finnegan and McElroy, 1994; Pazkowski, 1994; Flavell et al. 1995; Meyer, 1995). Therefore, it is important to find out how model promoters and coding genetic sequences from other totally unrelated organisms are stably integrated in the genome and expressed at the biochemical/molecular and phenotypic levels in forest trees.

We have employed *Populus* as a model system to address these and related questions. Poplars and aspens *(Populus* spp.) can be relatively easily regenerated in tissue culture (Ahuja, 1986, 1987, 1993; Chun, 1993; Ernst, 1993) and can be genetically transformed using the *Agrobacterium-vector* system (Fillatti, et. al. 1987; Klopfenstein et al. 1993; Confalonieri et al. 1994; Fladung et al. 1997b).

MATERIALS AND METHODS

By employing a leaf disc co-cultivation methodology (Fladung et al. 1997b), three clones of European aspen *(Populus tremula)* and one of hybrid aspen *(P. tremula* x *P. tremuloides)* were genetically transformed by *Agrobacterium-mediated* binary vector system. A number of gene constructs, in particular those carrying the *rolC* gene from *A. rhizogenes,* were included in this study. *Ro1C* is a dominant pleiotropic gene, which affects plant growth, phenotype, and physiology of transgenic plants. Of course, the phenotypic effects of the *rolC* gene are different, depending upon its heterologous promoter. Two types of promoters were include in the study to monitor *rolC* expression in the transgenic aspens. These included a 35S promoter from Cauliflower mosaic virus, and light-inducible rbcS promoter from potato. In addition to the *rolC* chimeric gene, the construct also carried a linked gene for kanamycin resistance *(npt II),* which served as a selectable marker for screening putative transformants (Fladung et al. 1997b).

Methods for DNA and RNA isolation from the leaf tissues for the PCR and Southern blot hybridization have been previously described (Fladung and Ahuja, 1995, Faldung et al. 1997b).

RESULTS AND DISCUSSION

Phenotypes of transgenic plants. Several thousand transformants have been produced in tissue culture and maintained on a kanamycin-containing medium in the growth chambers. From these, more than 1500 transgenic aspens have been grown in the greenhouse during the past three years to investigate transgene expression and stability (Ahuja and Fladung, 1996; Fladung et al. 1996, 1997a, 1997b,). During the first year of growth, transgenic aspens carrying *35S-rolC* gene much smaller leaves, compacted internode, and the stem was relatively reduced in height, as compared to untransformed controls. On the other hand, transgenic aspens carrying the rbcS-rolC gene exhibited slightly smaller leaves, as compared to controls. But their stem height and internode lengths were rather similar to controls. One trait shared by the *35S-rolC* and rbcS-rolC transgenic aspens was the pale-green leaf color, as compared to green leaf color in the untrasformed controls. However, during the second and third year growth cycles, the leaf color in the transgenic aspens after flushing were pale-green, but later on the leaf color turned green as is in the untransformed controls.

In addition to phenotypic alterations in the transgenic aspens, there were also changes in the hormone metabolism. Hormone levels were different in transgenic conditioned by 35S-rolC and rbcS-rolC , and untransformed controls (Fladung et al. 1997a). In particular, the 35S-rolC transgenic aspens showed significantly lower levels of abscisic acid (ABA) at the predormant and dormant bud stages in fall, as compared to controls of similar age. The *35S-rolC* transgenic aspens showed flushing at least two weeks earlier than controls in spring (Fladung et al. 1996, 1997a), suggesting possible involvement of ABA in the flushing process.

During the three year growth cycles, deviations from the typical *rolC* phenotype, in particular 35S-*rolC* transgenic aspens were observed. These included leaf abnormalities, chimeras, and revertants to normal state. Some of these phenotypic changes may be due to epigenetic events, possibly involving gene inactivation due to methylation, while others could be due to impairment or loss of the transgene. We are in the process of examining the mechanisms of transgene inactivation.

In order to study position effect on the *rolC* gene, a transposable element *Ac* from maize was inserted between the *rolC* gene and the promoter. In one gene construct, both *Ac* and *rolC* genes were regulated by the 35S promoter, while in the second construct these gene were controlled by the rbcS promoter. Transgenic aspens carrying the *355-Ac-rolC* or *rbeS-Ac-rolC* chimeric genes were morphologically similar to untransformed controls, in terms of leaf size and green color, internode length, and stem height. However, following *Ac* excision during leaf development, there is a restoration of *rolC* expression in the form of pale-green spots on the green leaf background (Fladung et al. 1977b). There was variation in the size and shape of pale-green sectors in different transgenic clones. These pale-green spots on the leaves are apparently caused by the excision of *Ac* from its original position in the recombinant gene in the periclinal chimeric leaves of *35SAc-rolC* transgenic aspen (Fladung and Ahuja, 1997).

Molecular characterization. Most putative transgenic aspen clones that exhibited the *rolC* phenotype also tested positive for the *rolC* gene by PCR analysis. However, the copy number of the *rolC* gene, as revealed by the Southern blot non-radioactive hybridization (Faldung and Ahuja, 1995) varied between one and three in the transgenic aspens (Ahuja and Faldung, 1996; Fladung et al. 1997b). The levels of *rolC* expression seemed to be affected by the copy number of the transgene. Those with more than one copy of the *rolC* gene were severely dwarfed, showed much smaller leaves, and seemed to be less viable.

Some of the revertant/chimeric shoots showed the presence of the *rolC* gene, while others exhibited its absence (Fladung et al. 1997b). Northern blot analysis of those revertants still carrying the *rolC* gene either showed the presence of the RNA transcript, or a lack of the transcript in those shoots. Whether, the transgene is inactivated in specific leaf layers, possibly due to the methylation of the promoter or the coding sequence is under study.

Most of the 35S-A*c-rolC* transgenic aspens carried both the *rolC* and *Ac* coding sequences, as determined by the PCR analysis. However, at least, three clones which were phenotypically similar to untranformed aspen, carried only the *rolC* gene, but not the *Ac* element. Southern blot analysis showed the presence of one copy of the *rolC* gene and one of *Ac* in most of the 35S-A*c-rolC* transgenic aspens. In the northern blot experiments, a specific *rolC* specific transcript was detected

only in the light-green sectors of the leaf , but not in the green areas of the leaf (Faldung and Ahuja, 1977).

CONCLUSIONS

These observations suggest that the type of promoter/construct controlling *rolC* expression seems to affect the growth and differentiation in transgenic aspens. It also appears that *rolC* expression is variable in transgenic aspens, as judged by the morphology, but the genetic basis of varaibility needs to be confirmed by molecular analysis.

ACKNOWLEDGEMENTS

Supported by the a project AIR2-CT94-1571 from the European Union (EU) Brussels, and the Deutsche Forschungsgemeinschaft, Bonn.

LITERATURE CITED

Ahuja, M.R. 1986. Aspen. In: Evans, D.E., W.R. Sharp, and P.J. Ammirato (eds). Handbook of Plant Cell Culture. Vol. 4. Macmillan Publishing Company, New York, pp. 626-651.

Ahuja, M.R. 1987. In vitro propagation of poplar and aspen. In: Bonga, J.M. and D.J. Durzan (eds). Cell and Tissue Culture in Forestry. Vol. 3. Martinus Nijhoff Publishers, Dordrecht, pp. 207-223.

Ahuja, M.R. 1988a. Gene transfer in forest trees. In: Hanover, J.E. and Keathley, D.E. (eds). Genetic Manipulation of Woody Plants. Plenum Press, New York, pp. 24-41.

Ahuja, M.R. 1998b. Molecular genetics of transgenic plants. In: Hallgren, J.E. (ed). Molecular Genetics of Forest Trees. Swedish University of Agriculture Sciences, Umeá pp. 127-145.

Ahuja, M.R. 1993. Regeneration and germplasm preservation in *aspen-Populus.* In: Ahuja, M.R. (ed). Micropropagatiopn of Woody Plants. Kluwer Academic Publishers, Dordrecht, pp. 187-194.

Ahuja, M.R. and M. Faldung. 1996. Stability and expression of chimeric genes in *Populus.* In: Ahuja, M.R., W. Boerjan, and D.B. Neale (eds). Somatic Cell Genetics and Molecular Genetics of Trees. Kluwer Academic Publishers, Dordrecht, pp. 89-96.

Chun, Y.W. 1993. Clonal propagation in non-aspen poplar hybrids. In: Ahuja, M.R. (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Dordrecht, pp. 209-222.

Confalonieri, M., A. Balestrmi, and S. Bisoffi. 1994. Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens.* Plant Cell Reports 13:256-261.

Ernst, S.G. 1993. *In vitro* culture of non-aspen poplars. In: Ahuja, M.R. (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Dordrecht, pp. 195-207.

Fillatti, J.J., B.H. McCown, J. Selmer, B.E. Haissig, and L. Comai. 1987. *Agrobacterium*-mediated transformation and regeneration of poplar. Mol. Gen. Genet. 206:192-199.

Finnegan, J. and D. McElroy. 1994. Transgene inactivation - plants fight back. Bio/Technology 12:883-888.

Fladung, M. and M.R. Ahuja. 1995. 'Sandwich method for non-radioactive hybridization. Biotechniques 18:3-5.

Fladung, M. and M.R. Ahuja. 1997. Excision of the maize transposable element *Ac* in periclinal chimeric leaves of *35S-Ac-rolC* transgenic *aspen-Populus.* Plant Mol. Biol. 33:1097-1103.

Fladung, M., K. Grossmann, and M.R. Ahuja. 1997a. Alterations in hormonal and developmental characteristics in transgenic *Populus* conditioned by the *rolC* gene from *Agrobacterium rhizogenes.* J. Plant Physiol. 150:420-427.

Fladung, M., S. Kumar, and M.R. Ahuja. 1997b. Genetic transformation of *Populus* genotypes with different chimeric gene constructs: transformation efficiency and molecular annalysis. Transgenic Research 6:111-121.

Fladung, M., H.J. Muhs, and M.R. Ahuja. 1996. Morphological changes in transgenic *Populus* carrying the *rolC* gene from *Agrobacterium rhizogenes.* Silvae Genet. 45:349-354.

Flavell, R.B., M. Metzlaff, M. O'Dell, and P.J. Dale. 1995. Instability of transgenes in plants and its implications in plant breeding. In: Porc. Induced Mutations and Molecular Techniques for Crop Improvement. International Atomic Energy Agency, Vienna, pp. 13-22.

Klopfenstein, N.B., H.S. McNabb, E.R. Hart, R.B. Hall, R.D. Hanna, S.A. Heuchelin, K. Allen, N.Q. Shi, and R.W. Thornburg. 1993. Transformation of *Populus* hybrids to study and improve pest resistance. Silvae Genet. 42:86-90.

Meyer, P. (ed). 1995. Gene Silencing in Higher Plants and Related Phenomenon in other Eucaryotes. Springer Verlag, Berlin.

Paszowski, J. (ed). 1994. Homologous Recombination and Gene Silencing in Plants. Kluwer Academic Publishers, Dordrecht.