TRANSFORMATION IN LOTUS CORNICULATUS: TOWARDS LOW-LIGNIN PASTURE THROUGH ANTISENSE RNA

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ABSTRACT

We have developed a rapid and reproducible transformation system for bird's-foot trefoil (*Lotus corniculatus* L.) by using *Agrobacterium*-mediated T-DNA transfer and the incorporation of the antisense gene for cinnamyl alcohol dehydrogenase (CAD) from *Aralia cordata* into *Lotus* for lignin reduction. The presence of the transferred antisense gene in regenerated plants has been confirmed by PCR analysis.

KEY WORDS

Agrobacterium tumefaciens, antisense RNA, CAD, lignin degradation, *Lotus corniculatus*

INTRODUCTION

One of the major limitations for cattle production on tropical pasture grasses is poor digestibility if compared to temperate grasses. It is believed that the low digestibility of tropical pasture is due to the high lignin content. Modification of the lignin content of plants appears to be feasible using genetic engineering strategies such as antisense, sense or ribozyme-mediated suppression of genes encoding enzymes involved in lignification. Cinnamyl alcohol dehydrogenase (CAD) catalyzes the reduction of hydroxycinnamic aldehydes (para-coumaryl, coniferyl and sinapyl) to the corresponding alcohols (monolignols) in lignin biosynthesis. Consequently, CAD is an important target to modify lignin content and quality through gene technology. In this communication we report a protocol which has been used to regenerate transformed bird's-foot trefoil using *A. tumefaciens* and the introduction of a heterologous antisense gene for CAD from *Aralia cordata* (Hibino et al., 1995).

MATERIALS AND METHODS

Plant material. Bird's-foot trefoil (*Lotus corniculatus*) seeds, cv. Viking, were obtained from Snow Brand Seed Company, Sapporo, Japan. The sterilized seeds were placed on MS medium containing 3% sucrose and 0.3% gelum gum and incubated at 27°C under continuous light. Cotyledons were dissected from 10 to 14 day-old seedlings. The two ends of the cotyledons were cut off and only the middle portion was used as explants for transformation.

Bacteria strains and plasmids. *Agrobacterium tumefaciens,* strain LBA4404, harboring the binary vector pBI121, was used in initial experiments. For the antisense approach, the disarmed strain EHA101 of *A. tumefaciens,* containing the plasmid pGAH which codes for CAD from *A. cordata* and parts of the flaking sequences, introduced backwards downstream of the 35S CaMV promoter was applied. The system also carries the kanamycin (kan) as well as the hygromycin (hyg) resistance gene for the selection of transgenic plants (Hibino et al., 1995).

Transformation and regeneration of transformants. Cotyledonary explants were inoculated by swirling for 30 min in a culture of bacteria. After an additional one week of co-cultivation on B5 medium in the dark, explants were subcultured on the regeneration B5 medium supplemented with 0.5 mg/l BAP, 500 mg/l claforan and 100 mg/l kan when the plasmid pBI121 was used. If, on the other hand, the plasmid pGAH, coding antisense *A. cordata* CAD cDNA, was used, the same basal B5 medium supplemented with 500 mg/l claforan and 100 mg/l kan was applied, but with 25 mg/l hyg added. Subsequently, the cultures were subcultured on the same medium every two weeks. After 30 days, shoots that had regenerated from callus were cut off and transferred to half strength B5 salts supplemented with 0.1 mg/l NAA for rooting. Well developed plantlets were transferred to pots and grown to maturity in the greenhouse.

GUS assay and PCR analysis. Histochemical GUS assay of the regenerated plantlets was carried out according to the method of Jefferson et al., (1987). PCR analysis, for further confirmation of the integration of GUS, HPTII and antisense genes was performed following the instructions given by Kaneyoshi et al., (1994) and Hibino et al., (1995), respectively.

RESULTS AND DISCUSSION

The frequency of shoot formation was 7.0% in the segments inoculated with pBI121. The quantitative measurement of GUS gene expression, directed by the CaMV promoter, revealed GUS expression in all three main parts of the plant, i.e., leaf, stem and root. However, the intensity of GUS expression differed strongly in those parts. The highest activity was found in leaves. PCR analysis uncovered that all regenerated plants examined showed clear bands corresponding to the relevant sequences of both, the GUS and the NPTII gene.

Transformation of bird's-foot trefoil with the pGAH construct (containing CAD) yielded over 30 kan- and hyg-double-resistant transformants. The frequency of shoot formation in explants inoculated with pGAH was generally higher than in explants treated with pBI121. Handberg and Stougaard (1992) pointed out that in *L. japonicus* transformation frequencies selected on hyg are relatively high and appeared to be independent of the *Agrobacterium* genetic background used. This is in agreement with the results of our transformation study. The presence of the CAD antisense construct in double-resistant transformants, as analyzed by PCR, was found to be higher than 50%. Currently, all transformed plants are potted, transferred to the greenhouse and under investigation for total lignin content.

REFERENCES

Handberg, K. and J. Stougaard. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. Plant J. **2**: 487-496.

Hibino, T., K. Takebe, T. Kawazu, D. Shibata and T. Higuchi. 1995. Increase of cinnamylaldehyde groups in lignin of transgenic tobacco plants carrying an antisense gene for cinnamylalcohol dehydrogenase. Biosci. Biotech. Biochem. **59**: 929-931.

Kaneyoshi, J., S. Kobayashi, Y. Nakamura, N. Shigemoto and Y. Doi. 1994. A simple and efficient gene transfer system of trifoliate orange (*Poncirus trifoliata* Raf.). Plant Cell Rep. **13**: 541-545.