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BRIEF COMMUNICATION

Transformation of tobacco and birdsfoot trefoil by lupin leghemoglobin I cDNA clone

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Abstract

The full length cDNA clone of leghemoglobin I gene from *Lupinus luteus* was placed under dual promoter into the plant expression vector pCB1399 and the resulting vector (pCB1415) was transfered into the *Agrobacterium* strain LBA4404 (pAL4404). The binary system LBA4404 (pAL4404, pCB1415) was then used for transformation of *Nicotiana plumbaginifolia* and *Lotus corniculatus*. In both species kanamycin-resistant plants have been selected and regenerated. The synthesis of LbI protein in transformed plants has not been shown.

The leghemoglobins from legume nodules are oxygen-binding proteins that provide an optimum concentration of O_2 to bacteriod in order to maintain nitrogenase and respiratory activites. It is well established that the leghemoglobin apoprotein is synthesized by the plant (Baulcombe and Verma 1978). In various legumes we can find several molecular species of leghemoglobins. In soybean, *e.g.*, four major leghemoglobin proteins are present (Brisson and Verma 1982, Bojsen *et al.* 1983). Two leghemoglobins (LbI and LbII) and their amino acid sequences were found in yellow lupin (Szybiak-Strozycka *et al.* 1978). Here we present the transfer of lupin LbI cDNA into the new expression vector (pCB1399) and using the resulting construction (named pCB1415) for the transformation of *Lotus corniculatus* and *Nicotiana plumbaginifolia*.

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Abbreviations: BAP-benzylaminopurine; kb-kilobase; NAA-1-naphthaleneacetic acid; nptII-neomycin phosphotransferase II.
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LB medium for *E. coli* (Miller 1972) and complete LK medium (Langley and Kado 1972) for *Agrobacterium tumefaciens* strains were used. Cloning and Southern blot analysis were performed by using standard methodologies (Maniatis *et al.* 1982, Sambrook *et al.* 1989). ^{32}P-dCTP and *Multiprime DNA Labelling System* (*Amersham*) were used for radioactive labelling of DNA. Plasmid and plant DNA were isolated by the method of Sambrook *et al.* (1989) and Amasino *et al.* (1984). *A. tumefaciens* strain LBA4404 was transformed with pCB1415 according to Holsters *et al.* (1978).

Leaf disc method (Horsch *et al.* 1985) was used for transformation of diploid *in vitro* cultivated *Nicotiana plumbaginifolia* (cv. Viviani). Transformed tissues were selected on MS medium (Murashige and Skoog 1962) containing 1 μM NAA, 0.5 μM BAP, 500 mg dm^{-3} ticarcillin and 200 mg dm^{-3} kanamycin sulphate. For transformation of *Lotus corniculatus* (cv. Viglassky) modification of leaf disc method was used. Root segments (approx. 1 cm long) were incubated 10 min in bacterial overnight-grown cultures and then placed on agar plates with mineral B5 medium (Gamborg *et al.* 1968). After two-day cultivation period segments were transferred on selection and regeneration plates (B5 medium supplemented with 0.05 mg dm^{-3} BAP, 500 mg dm^{-3} ticarcillin and 200 mg dm^{-3} kanamycin sulphate).

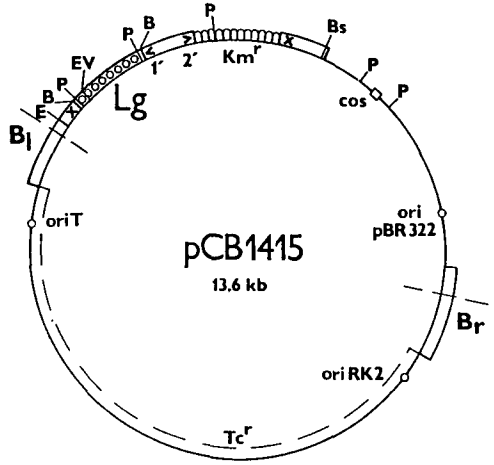

Fig. 1. Physical map of binary plasmid pCB1415 containing full length cDNA of lupin lbI gene and nptII gene under bidirectional promoter. Lg, lbI cDNA (approx. 0.8 kb); < >, bidirectional promoter; x, polyadenylation signal; B_l and B_r, left and right border sequences; Km^r, kanamycin resistance (nptII gene); Tc^r, tetracycline resistance; oripBR322 and oriRK2, replication origins of plasmids pBR322 and pRK2; oriT, origin of conjugal transfer; cos, bacteriophage lambda cos sequence. Restriction sites: B, BamHI; Bs, BstI; E, EcoRI; EV, EcoRV; P, PstI.

The full length cDNA clone of leghemoglobin I gene from *Lupinus luteus* (Konieczny 1987) was inserted into the new plant expression vector pCB1399. This vector contains the replicators and T-DNA borders from plasmid pGA472 (An *et al.* 1985) and the expression part of plasmid pAP2034 (Velten and Schell 1985). Its

bidirectional promoter expresses in plants, in two opposite directions, the foreign genes cloned into the unique BamHI site and the nptII gene. The BamHI ends on our lbI cDNA clone were generated in two steps. First, the PstI fragment of lbI cDNA

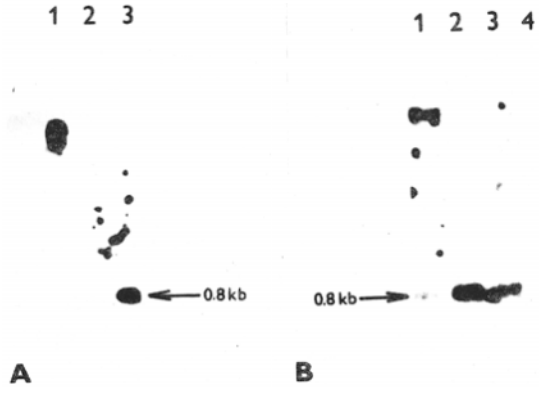

Fig. 2. Southern blot analysis of pCB1415 transformed *L. corniculatus* (*A*) and *N. plumbaginifolia* (*B*) lines. 10 μg of plant DNA was digested with BamHI, separated electrophoretically on agarose gel (0.8 %), transferred to nylon membranes and probed with 0.8 kb BamHI fragment of pCB1415 specific for lbI cDNA (see Fig. 1). *A*: *1* - untransformed *Lupinus luteus* (two genomic leghemoglobin genes hybridized); *2* - untransformed *L. corniculatus*; *3* - transformed *L. corniculatus* clone. *B*: *1*, *2* and *3* - transformed *N. plumbaginifolia* clones; *4* - untransformed *N. plumbaginifolia*. An approx. 0.8 kb BamHI fragment indicative of the presence of lbI cDNA is marked.

was isolated (approx. 0.8 kb) and then subcloned into the plasmid pUC-4K (Vieira and Messing 1982), replacing thus the nptII gene between two polylinkers. In the next step, the lbI cDNA insert was re-isolated from the intermediate plasmid using BamHI and ligated into the BamHI site of plasmid pCB1399. The final construction (named pCB1415, Fig. 1) was transferred into the *A. tumefaciens* strain LBA4404 carrying disarmed Ti plasmid pAL4404 (Hoekema *et al.* 1983). The resulting binary system LBA4404 (pAL4404, pCB1415) was used to transform *L. corniculatus* and *N. plumbaginifolia*. In both cases kanamycin-resistant plants have been selected and cloned. Selection of transgenic plants for resistance to kanamycin was found in *L. corniculatus* reliable and conclusive both through organogenic regeneration from stem segments and on rooting medium. Presence of lbI cDNA in the genomes of transgenic plants was confirmed by DNA-DNA hybridization experiments (Fig. 2). Using specific LbI antibodies the synthesis of LbI protein has not been shown.

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