Role of the BANYULS(*BAN*) gene from *Arabidopsis thaliana* in transgenic Alfalfa expression of anthocyanins and proanthocyanidins

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Introduction Condensed tannins (CTs) are flavonoid oligomers, many of which have beneficial effects on animal (bloat safe) and human health. The *BAN* gene encodes anthocyanidin reductase (ANR), an enzyme proposed to convert anthocyanidins to their corresponding 2,3-cis-flavan-3-ols (Xie *et al*., 2003). Ectopic expression of *BAN* in Alfalfa transgenic foliage results in accumulation of CTs. Thus, it has been assumed that the *BAN* gene also acts in starter units for the condensation of tannins in Alfalfa.

Material and methods Transformation was performed in two genotypes (Rgsy27,P1) of *M. sativa*. The binary vector pBI121.BAN was constructed by removing the *gus* gene and inserting the *BAN* gene into the BamHI and SacI sites of pBI121.1 to put *BAN* expression under control of the CaMV35S promoter and NOS 3' terminator. pBI121.BAN was transformed into *Agrobacterium tumefaciens* EHA105 and LBA4404 by the triparental mating method. To confirm chromosomal insertion of the *BAN* transgene, genomic DNA was extracted from leaves of putative transgenic Rgsy27 and P1(cv.Adriana) plants (Sambrook *et al*., 1989). The NPTII selectable marker gene in the binary vector was used as a probe after labeling with ^{32}P-dCTP using the Ready –TO-GO DNA labeling beads (dCTP)Kit. For RT-PCR analysis of *BAN* transcript levels, total RNA isolated from leaf tissue using Nucleo Spin RNA Kit. CTs in the leaves of transgenic plants were visualized by staining tissues in a solution of methanol:6M HCl (1:1) containing 2%(w/v) DMACA for 3 min (Li *et al*., 1996), then washing 3 times with MilliQ water. For quantitative analysis of CTs in transgenic plants, we used DMACA-HCl and Butanol-HCl assays at A643, A550 and A725.

Results We found a simple and efficient method for regeneration-transformation of Medicago including the perennial *M. sativa* var.Rgsy27 (2n=4x=32) and *M. sativa* var.Adriana (P1)(2n=4x=32). Development of this method (using 4 new media M1,M2,M3,M4 with low levels of 2,4-D and BAP) produced transgenic plants on all explants within 1.5-2 months which remained at the same ploidy level and maintained fertility.
The presence and structure of the transgene in the plants were analysed by genomic southern blots in the Rgsy27 and P1 genotypes. In each case both an internal fragment of the construct as well as one of the T-DNA borders were probed. The expected size of the internal fragments characteristic for each construct was detected in the plants analysed except for a few transgenic plants where the *BAN* fragment was not present. The number of T-DNA borders detected in these plants, representing the number of T-DNA copies integrated in the plant genome, varied from one to probably three or more. In several of the transformants we found aberrant sized *BAN* bands. This was probably due to the loss of one of the T-DNA BamHI sites, indicating that truncation of some of the T-DNA copies had occurred in these plants. The qualitative expression of the *BAN* gene was studied by RT-PCR and was compared with the expression of LAR and EF-1 genes. The strain LBA4404 was much better than EHA105 for transformation of both genotypes.

The *BAN* gene encodes anthocyanidin reductase (ANR), an enzyme proposed to convert anthocyanidins to their corresponding 2,3-cis-flavan-3ols. Ectopic expression of *BAN* in Alfalfa transgenic foliage results in accumulation of CTs. The percent of tannin in the dry matter of control and transgenic plants were: for P1(control) = 0.418, Rgsy27(control) = 0.259 , P1+*BAN* = 0.600 and for Rgsy27+*BAN* = 0.474.

Conclusions Preliminary results of our work indicated that in forage legumes BANYULS(*BAN)* could not autonomously induce tannin. However, when tissues are committed for tannin accumulation, the *BAN* gene can strongly increase CT levels.

References

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