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Lignin Biosynthesis Regulated by the Antisense *4CL* Gene in Alfalfa

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Abstract

Meng J., Li C., Zhao M., Wang C., Ru Y., Cui Z., Han Y. (2018): Lignin biosynthesis regulated by the antisense *4CL* gene in alfalfa. Czech J. Genet. Plant Breed., 54: 26–29.

The antisense *4CL* gene was transfected into alfalfa through *Agrobacterium*-mediated transfer. The test results indicated that the antisense *4CL* gene was successfully integrated into the genome DNA of alfalfa and was stably transmitted to the offspring. Compared to the wild-type plants, the lignin content of T_0 and T_1 generation plants was reduced by 45.77% and 31.97%, respectively; there were no significant differences in height and weight of T_0 and T_1 plants, compared to the wild-type plants. However, the transgenic plant differed from the wild-type plant by softer stems and leaves, larger leaves, fewer flowers and a fewer seeds. The T_0 line was susceptible to disease infection, but significantly improved in the second year. The results suggest that the *4CL* gene from *Amorpha fruticosa* can be used to regulate lignin biosynthesis in transgenic forage crops.

Keywords: antisense recombinant plasmid; 4-coumarate coenzyme A ligase; lignin content; *Medicago sativa*

Lignin is a complex phenolic polymer closely linked with cellulose and hemicellulose, forming an important structural component of the plant secondary cell wall (Vanholme *et al*. 2010). It can enhance the mechanical strength of plants, resistance to pressure, supporting forces and protect against diseases and pest invasion (Kajita *et al*. 1996). *4CL* gene encodes 4-coumarate:coenzyme A ligase (EC 6.2.1.12) and it is possible to achieve genetically directional regulation and modification of biosynthesis of secondary products in plants, thereby improving and modifying the quality of products (Lu *et al*. 2003). The small family of *4CL* genes has been cloned from many plant species such as soybean, parsley, pea, spruce, maize, potato, rice (Voo *et al*. 1995). Studies showed that the 4CL enzyme activity and the lignin content were decreased in most antisense *Pm4CL1* transgenic tobacco lines (Huan *et al*. 2012). Suppressing the activity of *4CL* reduced the lignin content of the plant (Hu *et al*. 1999). Therefore the *4CL* gene is a good candidate gene for regulating lignin biosynthesis.

Alfalfa is one of the most important legume forages in stock farming in China (Yuan *et al*. 2016), but the low digestibility of alfalfa reduces its economic value (Baucher *et al*. 1999). In this paper, we investigate the feasibility of using the antisense RNA technology to regulate and control the lignin content of alfalfa for providing a reference in the breeding industry.

Alfalfa (*Medicago sativa* L. var. Gan Nong No. 1) cotyledons were cut into 0.2 × 0.3 cm pieces as explants. These were transformed with *Agrobacterium tumefaciens*-transformant strain LBA4404 harbouring the binary plasmid vector (named as *p2300-ubi-4fan-4CL*) constructed by our laboratory. The plasmid contains the antisense *4CL* gene fragment (722 bp) obtained from *Amorpha fruticosa*. The construct of the *p2300-ubi-4fan-4CL* is shown as Figure 1. The plasmid was mobilized into *A. tumefaciens* strain LBA4404 using the freeze-and-thaw method (Holsters *et al*. 1978).

Yellowish-green callus initiation was observed at the cut edges and wounded regions of the cotyledon explants after 30 days in a callus inducing medium containing 50 mg/l kanamycin (Figure 2A). Adventitious bud regeneration was observed after culturing the callus 20 days later in a callus differentiation

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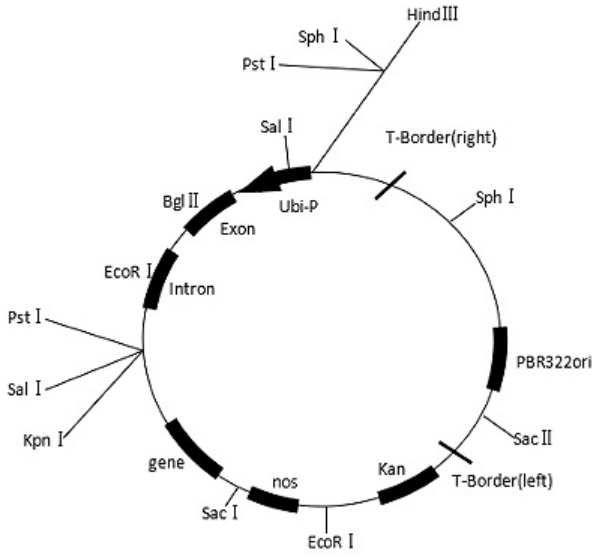

Figure 1. The plasmid vector *p2300-ubi-4fan-4CL* constructed by our laboratory contains an antisense *4CL* gene fragment obtained from *Amorpha fruticosa*

medium containing kanamycin (Figure 2B). When adventitious shoots reached 5 cm in length approximately (Figure 2C), they were carefully cut from the callus and transferred into a rooting medium. Seven days later in the rooting culture, the plantlets began to take roots (Figure 2D). When the length of roots reached around 5 cm, the plantlets were transplanted to soil in an artificial climate box (Figure 2E). Finally, the plantlets were moved outdoors (Figure 2F) under the natural environment after stable growth. The basal medium used throughout the experiments was Murashige and Skoog medium (Murashige & Skoog 1962). Transformants surviving on the medium containing a selective reagent kanamycin were called putative transgenic alfalfa lines (T_0). Subsequently, germinated T_0 seeds produced T_1 plants.

The genomic DNA of T_0 and T_1 transgenic plants and wild-type plants was isolated and used for PCR identification. The primers for PCR amplification were:

Forward primer: 5'-TCGCCTATGACTGGGCACAACAGA-3'

Reverse primer: 5'-AAGAAGGCGATAGAAGGCGATGCG-3'

The PCR target amplifications were sequenced and aligned via basic local alignment search tool (BLAST). PCR-positive T_1 lines were used for reverse transcription PCR (RT-PCR) analysis. Total RNA samples were extracted from leaves of 3 months old T_1 transgenic and wild-type alfalfa plants. The same primers and PCR conditions as described above were used for cDNA amplification. Both PCR in T_0 lines and RT-PCR in T_1 lines (Figure 3) showed that all transformants contained a 722 bp fragment, with size consistent with

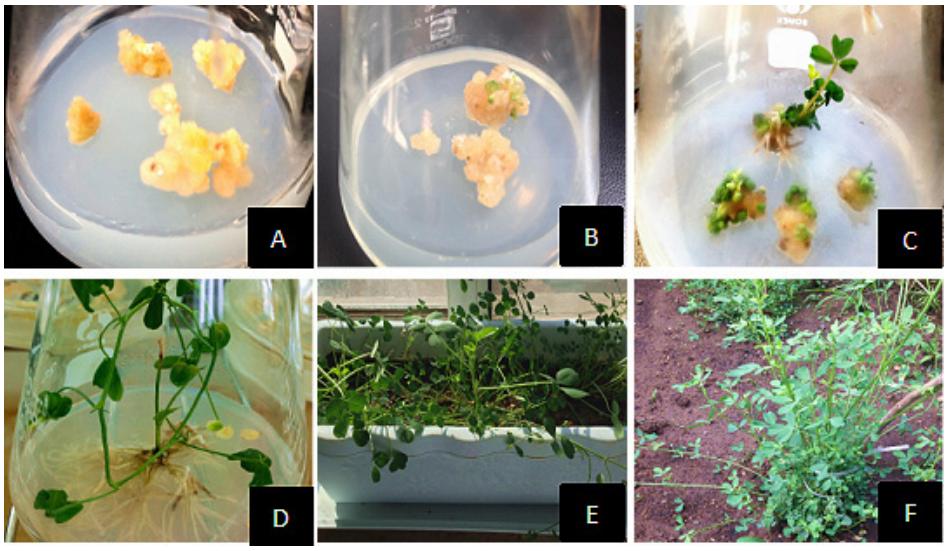

Figure 2. T_0 transgenic alfalfa plants obtained after selection for kanamycin resistance and regeneration: recovered kanamycin-resistant callus after 30-day culture on a screening medium (A), kanamycin-resistant callus dedifferentiated into adventitious buds on a differentiation screening medium (B), three adventive buds (C), subculture on a rooting medium for 20 days, the root elongated by 5 cm (D), transgenic plantlets growing in the pot soil in a greenhouse (E), transgenic plant transferred to the outdoor environment (F)

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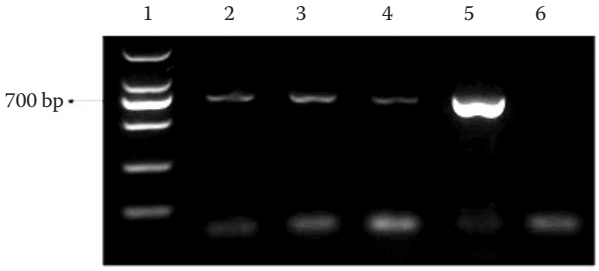

Figure 3. RT-PCR detection of T_1 alfalfa plants: 1 – electrophoregram of a molecular size standard marker; 2–4 – independent T_1 transgenic plants; 5 – positive control (plasmid DNA); 6 – negative control (wild-type DNA)

the target fragment. The sequence analysis indicated that the isolated 722 bp fragment showed 99% homology to the sequence of target fragment (data not shown). It confirmed the target gene was successfully integrated into the T_0 and T_1 alfalfa genome.

Transgenic and wild-type plants aged 5 months were cut in the basal stem for lignin content determination according to the Klason method (Song *et al*. 2011). The lignin content of T_0 transgenic plants was decreased by 45.77% (Table 1), and the lignin content of T_1 lines was decreased by 31.97% (Table 2). Both contents of T_0 and T_1 were significantly lower than that of the wild-type. This result indicated that transferring the target gene inhibited 4CL activity remarkably and decreased the lignin content.

We further observed that the phenotype of the T_0 transgenic alfalfa plants differed from that of wild-type plants. The stems and leaves of transgenic plants were softer than those of the wild-type plants, while the leaf was larger than that of the wild type. Besides, fewer flowers in T_0 line, fewer seeds were harvested. Some black spots appeared on the blade surface due to fungal infection, probably caused by a weakened resistance of the plant. We speculate that soft stem and resistance reduction resulted from the decreased lignin content.

Table 1. Comparison of lignin content between T_0 transgenic and wild-type alfalfa plants

	Lignin contents (% total dry weight)
Wild-type plants (control)	26.59 ± 0.137
T_0 transgenic plants	14.42 ± 0.145

Table 2. Comparison of lignin content between T_1 transgenic and wild-type alfalfa plants

	Lignin contents (% total dry weight)
Wild type plants (control)	24.61 ± 0.132
T_1 transgenic plants	16.74 ± 0.491

Table 3. Comparison of height and weight between T_1 transgenic and wild-type alfalfa plants (means from three determinations ± standard errors)

	Plant height (cm)	Plant weight (g)
Wild-type plants	11.7 ± 0.153	0.34 ± 0.031
T_1 Transgenic plants	12.5 ± 0.651	0.32 ± 0.025

All seven T_0 plants recovered in the second year and the growth of the plants improved compared to that in the first year. Although T_0 transgenic alfalfa plants still had softer and larger leaves than the wild type, the fungal infection on the blade surface disappeared. It might be due to an enhanced adaptive capacity of the T_0 plants. The height and weight of T_1 line plants were determined after culturing for 40 days. The height and weight of 10 T_1 line plants were not evidently different from wild-type plants (Table 3). The results show that the gene did not affect the normal growth of plants.

The results suggest that the *4CL* gene from *A. ruticosa* could be applied to regulate lignin biosynthesis in transgenic forage crops. And we observed some beneficial effects of transgenic alfalfa, i.e. soft and large leaves could be eaten and utilized easily by livestock.

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