Cloning of cDNA encoding F5H from *Brassica napus* and regulation of lignin biosynthesis by expressing antisense gene in transgenic tobacco

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Abstract

The whole coding sequences of F5H were obtained by RT-PCR from *Brassica napus* cv. *Zhongshuang 9*. All the sequences had the conserved functional domains and showed high identity with those of *Brassica napus* cv. *Westar*. From the sequences analysis, a new member of *F5H* gene distinguished from the published members was found. The result suggested there were at least 3 members of *F5H* gene in *Brassica napus.* Antisense gene expression binary vector was constructed by inserting one *F5H* gene after the xylem-specific-expression promoter C4H from *Arabidopsis thaliana*. With *Agrobacterium*-mediated transformation, many independent T_0 antisense *F5H* transgenic tobacco plants have been obtained. The growth conditions of the transgenic plants and the wildtype control were identical before flowering. In the reproductive growth stage, the transgenic plants grew slowly and were slender with less leaves, but there were no obvious differences in florescence and seeding. Thin transverse sections were cut from the basal-stem of some plants in uniform growth with a freezing microtome for histochemical coloration. The results of Wiesner reaction and Maüle reaction indicated lignin distribution of transgenic plants changing. Most mature transgenic plant roots had less fibres and obvious taproot.

Key words: *Brassica napus;* Lignin; Ferulate 5-hydroxylase (F5H); RT-PCR; *Agrobacterium*-mediated transformation

Introduction

Brassica napus is a major oilseed crop grown worldwide. A natural lodging event resulted in a yield reduction of 13-20% and more sensitive to pathogen infect (Goodman et al., 2001). Lignin is crucial for structural integrity of the cell wall and strength of the stem, and therefore plays a role in protecting plants against lodging and pathogens (Boerjan et al., 2003).

In dicotyledonous angiosperms, lignin is composed of two major monomeric phenolic constituents: guaiacyl (G) and syringyl (S) units. Increased G content leads to more highly condensed lignin composed of a greater proportion of biphenyl and other carbon-carbon linkages, whereas S subunits are commonly linked through more labile ether bonds at the 4-hydroxyl position (Li et al., 2000). Softwood gymnosperm lignin essentially lack S residue and therefore are highly condensed.

Ferulate 5-hydroxylase (F5H) is a rate-limiting enzyme determining syringyl-substitued lignin precursors (John *et al.*, 2004). Earlier studies have demonstrated that lignin of the *F5H* mutant lacks the sinapic acid-derived components typical of wild-type lignin (Chapple *et al.*, 1992), while over-expression of *F5H* increases lignin syringyl monomer content (Meyer *et al.*, 1998; Franke *et al.*, 2000). We intend to acquire more condensed lignin to impart dramatic resistance against lodging and pathogen by down-regulation of F5H activity. Here we report regulation of lignin biosynthesis by expressing antisense *F5H* gene under the control of the lignification-associated cinnamate-4-hydroxylase promoter in tobacco.

Materials and Methods

Plant material

Leaf and petiole tissues were collected from *Brassica napus* cv. *Zhongshuang No.9* planted in Oil Crops Research Institute, Chinese Academy of Agricultural Sciences.

Tobacco was *Nicotiana benthamiana*, planted in chamber under 16-h light/25°C and 8-h dark/23°C cycle.

Cloning of cDNA encoding F5H from Brassica napus

Gene specific and degenerate primers were designed on the basis of comparing the published plant F5H sequences (*Brassica napus:* AF214007, AF214008, AF214009; *Arabidopsis thaliana*: At4g36220; *Eucalyptus gunnii*: AJ249093; *Liquidambar styraciflua*: AF139532), primarily referred to the sequences of *Brassica napus* and *Arabidopsis thaliana*. The primers are as following:

The forward primer: 5′-ATGGAGTCTTCTATATCACAAACACTAAG-3′;

The reverse primer: 5′-TTA(a,g)A(c,g)AG(a,c)ACA(a,g)AT(a,c)AGGCG(t,c)GTG-3′.

Vector construction and tobacco transformation

The plant expression vector was pBI121, which carried an nptII gene as a selection marker, the 35S promoter was substituted with *Arabidopsis thaliana* C4H promoter provided by Dr. Yang. The sequence encoding GUS of pBI121 was replaced by the *F5H* sequence in reverse orientation. The recombined plasmid was transformed into *Agrobacterium tumefaciens* LBA4404 by freezing-thawing methods. *Nicotiana benthamiana* tissue was transformed and regenerated into

whole plants as common methods.

Another primer corresponding to the NOS terminator region were used for detecting the regenerate tobaccos, that is: 5′-GATTGCTTTGATATTGTCACGGGT-3′.

Analysis of transgenic tobacco

In the reproductive growth stage, the lower stems of some plants in uniform growth were cut to 20μm transverse sections with freezing microtome for histochemical coloration. For Wiesner staining, sections were stained with 2% (w/v) phloroglucinol (dissolved in 95% alcohol) in 12% HCl. For Maüle staining, sections were immersed in 1% (w/v) potassium permanganate solution for 5 min at RT, then washed twice with 3% hydrochloric acid until the color turned from black or dark brown to light brown.

Results and Discussion

Sequence analysis of the cDNAs encoding F5H

The whole coding sequences were obtained by RT-PCR from *Brassica napus* cv. *Zhongshuang 9*. All the deduced amino acid sequences of the F5H clones contained the motifs known to be conserved (Meyer *et al.*, 1996). Immediately following the inferred initiator methionine was a 17-amino acid sequence containing nine hydroxy amino acids. The subsequent 15-amino acid sequence was rich in hydrophobic amino acids. An RRRR putative stop transfer sequence followed the hydrophobic stretch immediately. Following the stop transfer sequence was the sequence PPGPRGWP, which obeyed the consensus for the proline-rich sequence found in many P450s. The most obvious of these conserved regions was the heme-binding domain between residues Pro-450 and Gly-460, including Cys-458, the presumed heme-binding cysteine.

The clones could be classified to 3 groups. The sequences in one group showed over 99% identity each other at protein level (Nuances of amino acids were some similar amino acids replacements), so randomly choose one clone each group to analyze at protein level. The NO.1 clone only showed 94% identity with the NO.2 as those of Brassica *napus* cv. *Westar* (Remesh *et al.*, 2000). The NO.1 clone contained a 1563 bp open reading frame coding 520 amino acids, while the NO.2 and the NO.3 both contained a 1560 bp open reading frame coding 519 amino acids. The similarity scores of the NO.3 clone with the other two were all 96%. The result suggested there were at least 3 members of *F5H* gene in *Brassica napus*. So a new member of *F5H* gene might be found in this study.

Antisense plant expression binary vector was constructed by inserting the NO.1 clone after xylem-specific-expression promoter C4H from *Arabidopsis thaliana*. The C4H promoter sequence was identity with the sequence reported by Mizutani M et al (1997), contained three cis-acting elements (box P, YTYYMMCMAMCMMC; box A, CCGTCC; and box L, YCYYACCWACC), which were conserved among the genes involved in the core reactions of the phenylpropanoid pathway of several plant species. These elements might be involved in coordinating C4H gene expression with regulation of the PAL and 4CL in response to wounding and light.

Analysis of antisense-F5H-*transgenic tobacco*

With *Agrobacterium*-mediated transformation, many independent T_0 antisense *F5H* transgenic tobacco plants had been obtained. The growth of the transgenic plants and the wildtype control were almost identical before flowering. In the reproductive growth stage, the transgenic plants grew slowly and were slender with less leaves, but there were no obvious differences in florescence and seeding.

In the reproductive growth stage, thin transverse sections were cut for histochemical coloration (as showed in Fig 1). Wiesner reaction is the method to detect the total extent of lignin in the rough through shade of color, and Maüle reaction is the way to determine the content of S lignin (presented in red). In Wiesner reaction, the vascular bundle of some transgenic plants presented different shade of mauve strip, the vascular bundle near the marrow of some other transgenic plants showed visible deep mauve. However, the vascular bundle of the wildtype control were with even mauve. In Maüle reaction, the vascular bundle of some transgenic plants exhibited red strips and inter-phase yellow strips, the vascular bundle near the marrow of some other transgenic plants showed visible deep brown yellow and the periphery of the vascular bundle showed visible deep red, while the vascular bundle of the wildtype control were yellow changing to red from the periphery to the middle part gradually.

Most mature transgenic plant roots were with less fibres and obvious taproot than the control (Fig 2).

The *F5H* mutants exhibit a characteristic red fluorescence under UV, whereas wild-type plants have a blue-green appearance (Chapple *et al.*, 1992). In the present study, all the T_0 antisense *F5H* transgenic tobacco plants were not different from the wildtype in any growth stage to UV. This indicated the F5H activity existed in the transgenic tobacco plant.

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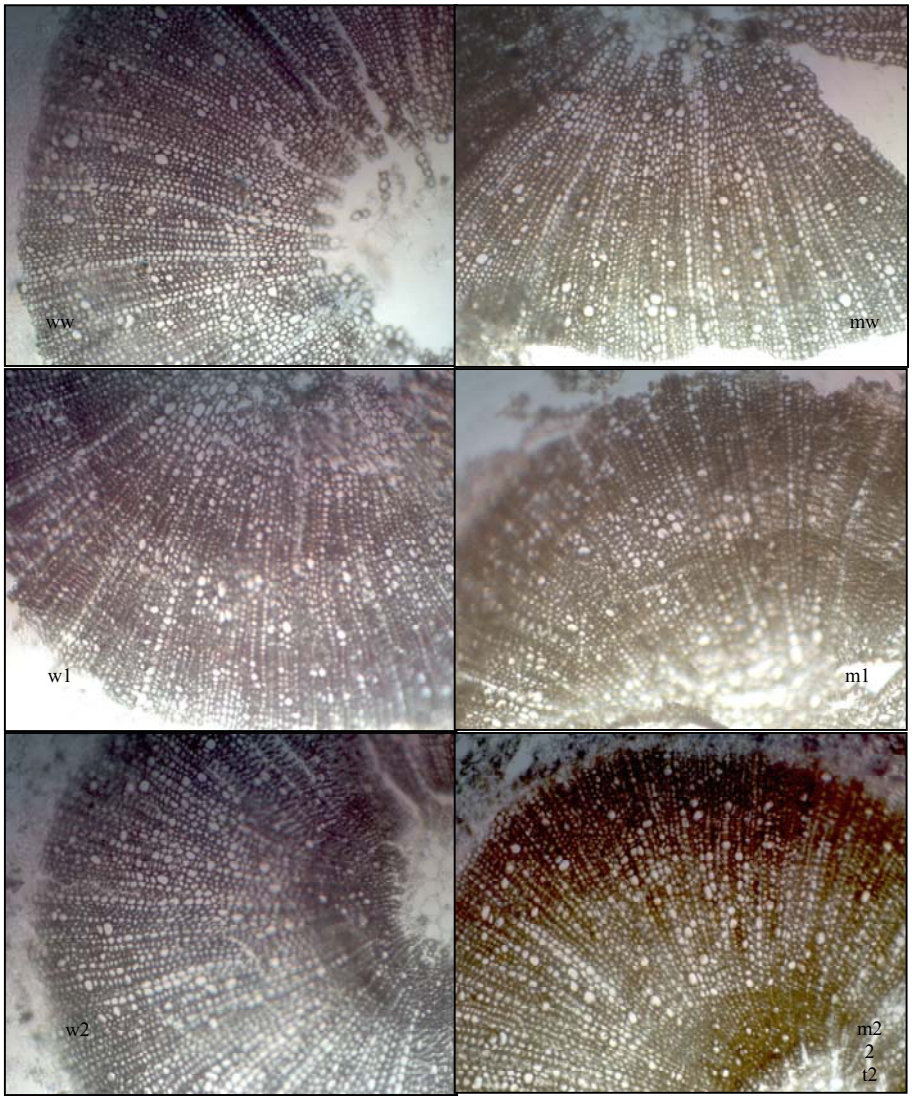

Fig 1. Histochemical coloration of trangenic and untransformed tobaccos

ww: untransformed tobaccos in Wiesner reaction; w1 and w2: trangenic tobaccos in Wiesner reaction;
mw: untransformed tobaccos in Wiesner reaction; mi and m2: transgenic tobaccos in Maüle reaction

Fig 2. Root difference between transgenic tobaccos and untransformed tobaccos

The plants with tally are the transgenic tobacco plantlets, others are the CK.