# The lignin biosynthesis regulation and its relationship with *Sclerotinia* and lodging resistances for *Brassica napus*

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**Abstract**

*Sclerotinia sclerotiorum* and lodging are two important factors affecting oilseed rape production in China. Though traditional breeding strategies can enhance *Sclerotinia* resistance to some degree, s*clerotinia* resistance breeding has still not made substantial progress because of absence of resistant resources. For the study of lodging, it is just at its beginning stage. So it is of a great significance to explore a new road for the improvement of disease and lodging resistances in *Brassica napus*. In this paper, we first carried out correlation analysis on stalk lignin content with *Sclerotinia* and push resistances with 29 cultivars of *B. napus*. The results indicated that lignin content was positively correlated with both s*clerotinia* and push resistances significantly, with correlation coefficients of 0.6971 and 0.618 respectively. A *4-CL* cDNA was isolated from lignified stem tissues of *Populus tomentosa*. Phylogenic analysis showed that the deduced protein was classified into classⅠof 4-CL proteins, and phylogenically more related to *Populus tremuloides* 4-CL1 involved in lignin biosynthesis. A 4-CL recombinant protein with a $His_{\times 6}$ tag at amino terminal was expressed in *E. coli*. Enzyme analysis showed that the recombinant protein possessed catalytic activity and could activate the three lignin biosynthesis associated substrates PA, CA and FA, with a preferential order PA, FA and CA. The recombinant protein showed no activity to SA. Two plant expression vectors, pBI121:*4CL* and pBI-*C4H:4CL*, were constructed and transformed into *B. napus* cultivars, Zhongshuang 9 and 37301, by *A. tumefaciens*-mediated transformation method. A total of 56 plants with Kan resistance were obtained. PCR and Southern blotting analysis of the putative transgenic plants indicated that the target gene was integrated into the *B. napus* genome. Western blotting analysis was also conducted on the transgenic lines with 4-CL antibody prepared from the purified 4-CL recombinant protein. The results showed that the heterogeneous *4-CL* gene was expressed in some of the transgenic lines, and in these lines the lignin content increased and s*clerotinia* and lodging resistance were improved.

**Key words**: *Sclerotinia sclerotiorum,* lodging; 4-coumarate:CoA ligase, *brassica napus*

## Introduction

*Sclerotinia sclerotiorum* is taken as the most important fungal disease for rapeseed in China, accounting for about 30-50% of total loss every year (Fangqiu L, et al.,2001). Great attempts have been made to produce cultivars resistant to *S. sclerotiorum* in many rapeseed breeding programmes. Among theses attempts, physiological resistance and escape mechanism are taken as two primary ways to tackle with the disease. Many strategies including detoxification, defense activation and fungal inhibition have also been exploited to engineer *sclerotinia* resistance (Grison,et al.,1996; Thompson et al.,1995). But no attempts have been made to exploit structural resistant substances such as lignin to combat back *S.sclerotinia* as yet. Lodging is another important factor affecting rapeseed production, and is estimated to bring approximately 16% of yield loss each year (Islam N, et al.,1994). More importantly, lodging can affecting the soil and canopy moisture crucial to infection from *S. sclerotiorum* to occur especially at flowering (Kim, et al.,1999). Two strategies have been utilized to improve lodging-resistant performance of crop, namely reducing height and strengthening stem. As reduced height is somewhat conflictive with high yield breeding, it seems to be expedient to strengthen stem in lodging resistance. In this paper, we carried out correlation analysis on stalk lignin content with *sclerotinia* and push resistance of *B. napus.* Besides, we attempt to explore the possibility of improving *sclerotinia* and lodging resistances for rapeseed by increasing lignin content through the over-expression of a heterogeneous *4CL* gene. Here we report our results.

## Materials and methods

Total RNA was isolated from stem tissues of poplar (*Populus tomentosa*). The first cDNA strand was synthesized according to the manufacturer's manual. A *4-CL* cDNA was amplified with a pair of primers, viz. sense primer: ATGAATCCACAAGAAGAATTCATC, antisense primer: TTATATGCCTGCCAACTTTTCT TTCAG. DNA and protein sequence were analyzed by DNAMAN software with parameters as default on the PC computer. The phylogenic analysis was performed with maximum parsimony methods by PHYLIP software.

The *4CL* cDNA was cloned into pET 32(a+) vector and transformed into *E. coli* strain $DE_3$ ($BL_{21}$). The construct was sequenced to confirm the fidelity of *4CL* insert. The bacterial cells were induced with 0.2 mM of IPTG for 4 hours and the recombinant protein with an amino terminal $His_{\times 6}$ tag was purified using His-Bind Resin as described by the manufacturer. The purified recombinant protein was used to prepare the 4CL antibody against rabbit and enzyme activity was measured with spectrophotometric assay (Knoblochk,et al.,1977).

The *4CL* cDNA with introduced *BamHI* and *BstXI* (a *SacI* site sequence also in it) sites at 5′ and 3′ ends respectively was

cloned into the corresponding sites of pBI121 vector digested with *BamHI* and *SacI*. The chimeric construct was named as pBI121:*4CL*. A C4H promoter was isolated from *Arabidopsis thaliana* and cloned into pBI121:*4CL* replacing the corresponding 35S. The resultant construct was designated as pBI-*C4H:4CL*. pBI121:*4CL* and pBI-*C4H:4CL* expression vectors were mobilized respectively into *A. tumefaciens* strain LBA4404 by the electrotransporation. The cotyledonary petiole cells of 4-days grown rapeseed were transformed with the *A. tumefaciens*-mediated method. For analysis of transgenic plants, the parts of 35S or C4H promoter and *4CL* insert were taken as template for PCR amplification. For Southern blotting high stringency washes (0.5×SSC, 0.1% SDS, at 66°C) were performed. Western blotting analysis was also conducted on the transgenic plants with the 4-CL antibody prepared above.

At the end of anthesis, stalk lignin content was determined by the Klason method (Jin Z, et al.,2003). The *Sclerotinia* resistances of stalk and leaf were represented with the length and area of the pathological spots respectively. The stalk strength was measured with the prostrate tester as described by Xiao (2002).

## Results

29 cultivars of *B. napus* were chose for correlation analysis of stalk lignin content with *Sclerotinia* and lodging resistance. The variance analysis indicated that lignin contents, spot length and pushing resistance were all significantly different among 29 cultivars tested. Accordingly the difference was caused mostly by cultivars themselves other than environment. The correlation analysis showed that lignin content was positively correlated with both *Sclerotinia* and push resistance significantly, with correlation coefficients of 0.6971 and 0.618, respectively. The results suggested that lignin played a significant role for *B. napus* in *Sclerotinia* and lodging resistances.

A 1621bp of *4-CL* cDNA with a complete coding region was isolated from Chinese poplar (*Populus tomentosa*). The molecular mass of the deduced protein was 58KD and isoelectric point was 5.9. The protein sequence contained two characteristic regions of all known 4CL, namely boxⅠ(SSGTTGLPKGV) and boxⅡ(GEICIRG) in it. For phylogenic analysis, 33 of the known plant 4CL protein sequences were retrieved and aligned by Clustal software. The retrieved 4CL sequences included *Arabidopsis thaliana* 4-CL1 (U18675), *A. thaliana* 4-CL2 (AF106086), *A. thaliana* 4-CL3 (AF106088), *A. thaliana* 4-CL4(AAM19949), *Capsicum annum Ca* 4CL(AAG43823), *Glycine. max* 4-CL1 (AF279267), *G. max* 4-CL2 (AF002259), *G. max* 4-CL3 (AF002258),*G. max* 4-CL4 (X69955), *Lithospermum erythrorhizon* 4-CL1 (D49366), *L. erythrorhizon* 4-CL2(D49367), *Lolium perenne* 4-CL1 (AF052221), *L. perenne* 4-CL2 (AF052222), *L. perenne* 4-CL3(AF052223), *Nicotiana tabacum* 4-CL (D43773), *N. tabacum* 4-CL1 (U50845), *N. tabacum* 4-CL2 (U50846), *Oryza sativa* 4-CL1 (X52623), *O. sativa* 4-CL2 (L43362), *Petroselinum crispum* 4-CL1 (X13324), *P. crispum* 4-CL2 (X13325), *Pinus taeda* 4-CL1 (U12012), *P. taeda* 4-CL2 (U12013), *Populus hybrida* 4-CL1 (AF008184), *P. hybrida*4-CL2 (AF008183), *Populus tremuloides* 4-CL1 (AF041049), *P. tremuloides* 4-CL2 (AF041050), *Rubus idaeus* 4-CL1 (AF239687), *R. idaeus* 4-CL2 (AF239686), *R. idaeus* 4-CL3 (AF239685), *Solanum tuberosum* 4-CL1 (M62755), *S. tuberosum* 4-CL2 (AF150686), *Vanilla planifolia* 4-CL(X75542). The resulting data matrix was subsequently analyzed using PHYLIP software with 1000 replicates. The most parsimonious unrooted tree was constructed with *Pinus taeda* 4CL1 as an outgroup. The result showed that there were two major 4CL classes evolved within the angiosperms, consistent with the previous observations(Lindermayr, et al.,2002). The *P.tomentosa* 4CL was classified into the class I cluster, and phylogenically more related to the *Populus tremloids* 4CL1 which was confirmed to be involved in the lignin biosynthesis (Hu ,et al., 1998).

The *4-CL* recombinant protein was expressed in protokaryotic *E. coli* system. Since the stop codon (TAA) of the *4CL* insert wasn't mutated, the fusion protein produced was to have only a $His_{\times 6}$ tag at its amino terminal. After induction with IPTG of the bacteria strain harboring the recombinant vector, an about 70 kDa of recombinant protein was produced compared to that containing the empty expression vector (pET32). 4CL-6×His tag fusion protein were purified from ultrasonic lysate of cells for preparation of 4-CL antibody by rabbit. For assay of recombinant 4-CL protein activity, four of biochemical substrates involved in lignin biosynthesis were chose in the experiment. The extract from bacteria expressing the recombinant protein was tested for its abilities to utilize different substituted cinnamic acids as substrates. Among them, 4-coumaric acid (PA), ferulic acid (FA) and cinnamic acid (CA) can be easily converted into corresponding CoA-esters, with a preferential order PA, FA and CA. The protein showed no activity to SA, as reported for many heterogeneously expressed 4-CL except Arabidopsis 4-CL4 and soybean 4-CL1 (Lindermayr, et al., 2002; Hamberger, et al., 2004).

**Table1 The reactivity of the 4-CL recombinant protein to the four different substrates**

| Enzyme activity | PA | CA | FA | SA |
|---|---|---|---|---|
| (pkat/mg protein) | 4992.07±79.64 | 2777.94±108.27 | 3644.89±128.26 | 0 |

The enzyme activity is equal to average value plus standard difference

Two different constructs harboring the sense *4-CL* cDNA driven by 35S and C4H promoters respectively were transferred into *B. napus* cultivars Zhongshuang 9 and 37301. 51 $T_0$ plants including 12 transformed with the pBI121:*4CL* construct and 39 transformed with the pBI-*C4H:4CL* one were regenerated from 37301. 5 plants including 2 transformed with the pBI121:*4CL* construct and 3 transformed with the pBI-*C4H:4CL* one were regenerated from Zhongshuang 9. PCR analysis showed that all positive plants came from 37301 and no positive one was obtained from Zhongshuang 9. Southern blotting analysis on the putative transgenic plants indicated that the target gene was integrated into the genome of *B. napus*. Western hybridization was conducted to detect the expression of inserted target gene. As showed in figure1 (d), in some of the selected transgenic lines the target 4CL protein was expressed while in other lines and non-transgenic plants, the

heterogeneous protein was not expressed or undetectable.

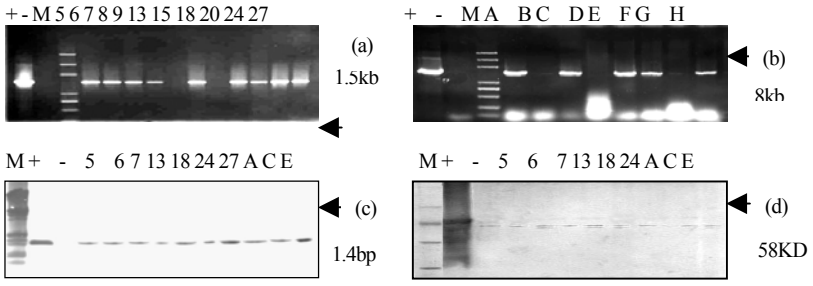


Figure1 (a),(b): PCR analysis; (c):Southern blotting; (d): Western blotting. M: marker, '+':positive control, '-':negative control, numbers denoting the plants transformed with the pBI-*C4H:4CL* construct, A-H denoting the plants transformed with the pBI:*4CL* construct.

In our study, $T_0$ transgenic plants from 37301 were propagated with each line composed of 5-7 individual plants. Just at the end of anthesis, the transgenic lines were tested for their stalk lignin content, *sclerotinia* and pushing resistance. The result showed that among the selected lines, klason lignin contentin the line 6 and 18 were increased compared to those of non-transgenic lines with 13.41% and 11.48% increase respectively. Lignin contents for other lines did not show significantly different. The spot areas of lines 6, 18 had significantly decreased and accounted for 66.42%, 72.08% of non-transgenic lines respectively. The pushing resistance of these four transgenic lines also increased compared to the non-transgenic lines with 27.58% increase for lines 6, 22.84% increase for lines 18. Compared to transgenic lines 6 and 8, s*clerotinia* and lodging resistances for some transgenic lines such as lines 7 decreased instead, which might be caused by silencing of the endogenous genes.

**Table 2 lignin content analysis of transgenic lines**

| | CK | 5 | 6 | 7 | 18 | 24 | A | C | E |
|---|---|---|---|---|---|---|---|---|---|
| Lignin content | 9.32±0.69 | 9.61±0.83 | 10.57±0.52* | 9.27±0.61 | 10.35±0.59* | 9.84±0.74 | 9.18±0.43 | 9.31±0.87 | 9.92±0.58 |

*: 5% probility level

## Discussions

Lignin is an important protective substance for plant combating back many environmental adversities like wind, rain, pathogens, etc.. Many studies showed that lignin content was related to disease resistance for plants (Jingjiang et al., 1999). Lignin can prevent intrusion and spread of pathogen and also restrain them by such metabolic intermediates as phenolics in lignin biosynthesis (Shuwen et al., 2002). As a mechanic component part of plant stalk, lignin also plays a very important role in maintaining plants erect in air. Lignin seems inherently related to lodging resistance of crops. Our result further showed that lignin content was positively related with *Sclerotinia* and lodging resistances significantly,

Nowadays, many genes in lignin biosynthesis have been cloned and utilized to manipulate lignin content and composition (Dianjing et al., 2001; Sewal ,et al., 1997). Among these genes, *4-CL* are commonly classified into small gene families, which usually included 2-4 *4CL* members, based on the previous study (Lindermayr et al., 2002; Hu, et al., 1998; Hamberger et al., 2004; Lee et al., 1997). However in our study, only one *4-CL* cDNA was isolated from *P. tomentosa* based on the homology-sequence cloning, and the deduced protein was phylogenically more related to the *P. tremloids* 4CL1 involved in lignin biosynthesis, which hinted that the *P.tomentosa* 4-CL might have a similar role as that of *P. tremloids* 4CL1. Since the recombinant 4-CL couldn't utilize SA as substrate for lignin biosynthesis, there might be other uncharacterized 4-CL isoforms involved the activation of SA into the corresponding CoA ester. The question need be further explored.

Since 4-CL plays a pivotal role in directing the photosynthetic intermediate into lignin biosynthesis. Many attempts have been made to manipulate the expression of *4CL* gene in order to decrease the lignin content for improving digestibility of forages and pulping properties of trees (Dianjing et al., 2001; Sewalt et al., 1997). To our knowledge, all the known 4-CL transgenic examples are to decrease 4CL level by anti-RNA and co-suppression technologies, and no study on increasing lignin content by over-expression of *4-CL* gene for disease and lodging resistance of crops have been reported. In this paper, a heterogeneous *4CL* gene was over-expressed in *B. napus*, and the transgenic plants obtained grew and developed normally with phenotypes just like those of non-transgenics. The altered 4-CL level in transgenic plants appeared to have no or little effects on the biosynthesis of other important secondary products which affect growth and development of plants. Compared to non-transgenics, transgenic plants showed slow expansion of spot after inoculated with *S. sclerotiorum* and greater pushing resistance. Therefore, *4-CL* gene could be taken as an appropriate target gene for disease and lodging engineering. For expression of *4-CL* gene in *B.napus*, two plant vectors pBI121:*4CL* and pBI-*C4H:4CL* were constructed driven by 35S and C4H promoter respectively. Different to 35S, the C4H promoter-driven *4CL* can be induced by environmental cues such as damage, pathogens, development(Mizutani, et al.,1997). On intrusion of *S. sclerotiorum*, the downstream target gene could be induced and co-expressed with other endogenous genes, i.e. *PAL*, *C4H* and *4-CL*. So the C4H promoter was more effective than 35S in driving *4CL* gene in controlling pathogens and lodging, and even in regeneration of transgenic plants (data not showed). As of different expression of the exogenous *4-CL* gene, transgenic lines showed *sclerotinia* and lodging resistance at

a range of levels, and some lines (i.e. lines 7) were even more susceptible than the non-transgenic counterparts, which might be caused by interaction of the endogenous gene and transgene at DNA-DNA or DNA-RNA level.

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