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Vector construction and transformation of 4CL1 gene into Chinaberrytree (*Melia azedarach* L.)

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Abstract. The gene 4CL1 was isolated from Chinese red pine (*Pinus massoniana* Lamb) and ligated into vector pPTN289 to perform transformation vector pPTN289-4CL1. This construction was transformed into *Agrobacterium tumefaciens* strain C58, and then transformed into Chinaberrytree (*Melia azedarach* L.). The transgenic Chinaberrytree was screened on selection medium (MS + 0.5mg/l 6-BA + 1mg/l vitamine B5 + 30g/l sucrose + 8g/l agar + 500mg/l Cefotaxime + 1mg/l PPT) and then extracted total DNA and tested the existence of interested gene using PCR method. The result shows that two tested Chinaberrytree samples are positive with 4CL1 gene as same as the positive control, while the nagative control (wild Chinaberrytree) is nagative with 4CL1 gene.

Keyword: 4-coumarate: coenzyme A ligase; 4CL1; lignin; *Pinus massoniana* Lamb; *Melia azedarach* L.

1. Introduction

4CL1 gene encodes 4-coumarate: CoA ligase enzyme (EC 6.2.1.12), which has about 58.5 kDa, and plays a pivotal role in the biosynthesis of plant secondary compounds, especially lignins in plant [1-3]. Lignins occur in cell walls of true vascular plants, ferns, and club mosses and so on. Lignins are generally distributed with hemicelluloses in the spaces of intercellulose microfibrils in primary and secondary walls, and in middle lamellae as a cementing component to connect cells and harden the cell walls of xylem tissues [4]. Therefore, 4CL1 gene are generally used as exogenous gene to transform into plants to increase lignin biosynthesis ability, improving wood quality [3,5].

4CL1 gene has researched and isolated from several plants, such as: quaking aspen (*Populus tremuloides* Michx.), *Populus tomentosa*, Arabidopsis, Pinus and so on [3-5]. The isolated 4CL1 then was transform into plant to create wood quality- improved breed. 2003, Hai Lu et al in Beijing Forestry University was isolate 4CL1 gene from Chinese white poplar (*Populus tomentosa*) and then successfully transformed

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into tobacco with the xylem- specific expression promoter GRP1.8. The result shows that, the content of lignin in the stem was increased 25% in comparison with the control plants (wild tobacco) [3].

With the purpose to supply the materials and create wood hi-quality genetically modified forest tree breeds, we has constructed the transform vector pPTN289- promoter 35S-4CL1 and then transformed into Chinaberrytree. Tested the existence of 4CL1 gene in transformed Chinaberrytree using PCR method with specific primers.

2. Materials and methods

2.1. Materials

In vitro Chinaberrytree (*Melia azedarach* L.) from Breeding and Biotechnology Center- Vietnam Forestry University.

4CL1 gene isolated from Chinese red pine (*Pinus massoniana* Lamb) in cloning vector pBT-4CL1 (with *Nco*I/*Xba*I sites at two ends of 4CL1 gene).

Vector pPTN289-GUSplus with *bar* selection gene.

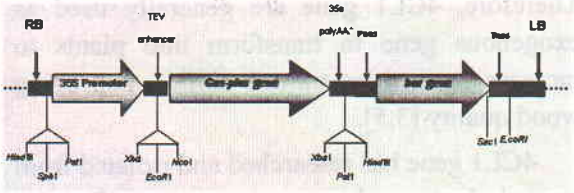

Figure 1. T-DNA region of standard binary vector pPTN289.

E.coli strain TOP10, *Agrobacterim tumefaciens* strain C58 from Invitrogen.

Two specific DNA primers for amplifying 4CL1 were designed based on the 4CL1 gene sequence of Chinese red pine. Forward Primer (4CL1P1):5'TATCCATGGCGCATGGCCAA CGGAATCA 3' ; Reverse Primer (4CL1P2): 5'CGCCGCTCTAGATTTCATTTTGCTGCA GTC 3' (two primers were conjugated with *Nco*I and *Xba*I restriction sites).

Chemicals: restriction enzyme (*Nco*I/*Xba*I) from Fermentas; T4 DNA ligase from Invitrogen; Gel purification Kit from Bioneer (Korea); Dream *Taq* polymerase from Fermentas; Spectinomycin, Cefotaxime, Phosphinothricin (PPT) ... from Merck, Sigma, Wako, Invitrogen ...

2.2. Methods

2.2.1. Construction of transformation vector pPTN289-4CL1

Vector pPTN289 and pBT-4CL1 were double digested with *Nco*I and *Xba*I restriction enzymes, and then purified using Gel purification Kit (Bioneer, Korea). The purified 4CL1 gene was ligated into vector pPTN289 using T4 DNA ligase. The ligation mixture was transformed into *E.coli* strain TOP10 using heat-shock method (42°C in 90 seconds).

Recombinant *E.coli* TOP10 was screened as follow: transformed *E.coli* were growed on LB medium (with Spectinomycin 50mg/l) in 12h (or overnight), 37°C and then extracted plasmids, tested using PCR method and *Nco*I/*Xba*I double digestion.

2.2.2. Mobilize vector pPTN289-4CL1 into Agrobacterium tumefaciens strain C58

Vector pPTn289-4CL1 was mobilized into *Agrobacterium tumefaciens* strain C58 using electroporation method (25μF, 200Ω, 2kV).

Agrobacterium tumefaciens strain C58 was then growed on LB medium (with Rifamycin 100mg/l and Spectinomycin 50mg/l) and extracted plasmid, tested using PCR method and *Nco*I/*Xba*I double digestion.

2.2.3. Transform vector pPTN289-4CL1 into Chinaberrytree (Melia azedarach L.) and tested the existence of 4CL1 gene using PCR method

Leaf disc transformation of Chinaberrytree *(Melia azedarach L.)* was then conducted following procedure: stem pieces of *in vitro* Chinaberrytree were growed on CB5.1 medium (MS + 0.5mg/l 6-BA + 1mg/l vitamine B5 + 30g/l sucrose + 8g/l agar) 2 days, and then infected by *Agrobacterium tumefaciens* strain C58 contains pPTN289-4CL1 in 30 minutes. After that, Chinaberrytree stem pieces were co-cultivated in 2 days on CB5.1 medium (in darkness), and then purged using Cefotaxime 500mg/l and cultured on selection medium (CB5.1 + 500mg/l Cefotaxime + 1mg/l PPT). The Chinaberrytree buds which can grow on selection medium were tested the existence of interested gene 4CL1 using PCR method.

3. Results and discussion

3.1. Construction of transformation vector pPTN289-4CL1

When designed two primers for amplifying 4CL1, we conjugated with *Nco*I (in forward primer) and *Xba*I (in reverse primer) restriction enzyme sites. Therefore, cloning vector pBT-4CL1 contains *Nco*I and *Xba*I sites in the two ends of 4CL1 gene. As the same, vector pPTN289 contains *Nco*I and *Xba*I sites in the two ends of GUS-plus gene.

After digested vector pBT-4CL1 and pPTN289 using *NcoI/XbaI*, we received results as in Figure 2A. In line 1, vector pBT-4CL1 was digested into 2 bands, the first band (about 2.7kb) is the linear backbone of vector pBT, and the another band (about 1.6kb) is 4CL1 gene. In line 2, vector pPTN289 was also digested into 2 bands, the first one (about 12kb) is the linear backbone of vector pPTN289, and the another one (about 2.3kb) is GUS-plus.

We used Gel purification Kit (Bioneer, Korea) to purified the linear vector pPTN289 (without GUS-plus) and 4CL1 gene. The result is showed in Figure 2B.

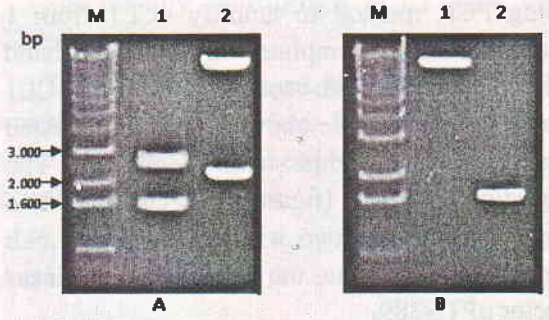

Figure 2. pPTN289 and pBT-4CL1 *NcoI/XbaI* double digestion and purification.

A. Double digestion *NcoI/XbaI*
Line M: 1kb DNA ladder; line 1: pBT-4CL1; line 2: vector pPTN289
B. Purification
Line M: 1kb DNA ladder; line 1: vector pPTN289; line 2: 4CL1 gene.

Because we used the same restriction enzymes (*Nco*I and *Xba*I) for double digestion, both 4CL1 gene and linear vector pPTN289 have the same solenoid ends. Therefore, when we mix them together (in certain ratio, temperature and under the catalysis of enzyme T4 DNA ligase), 4CL1 gene might be ligated into the linear vector pPTN289 to form circle recombination vector pPTN289-4CL1.

The ligation reaction mixture as follow (total volume 15µl): deionized water 4.5 µl; T4 DNA ligase buffer 10X 1.5 µl; 4CL1 µl; pPTN289 3 µl; T4 DNA ligase 1 µl. The mixture was incubated at 14°C overnight.

The ligation product was transformed into *E.coli* strain TOP10 using heat-shock method (42°C in 90 seconds). Transformed *E.coli* were spreaded on solid LB medium with

Spectinomycin 50mg/l at 37°C overnight. Selected some colonies for liquid cultivation, extracted plasmid and tested the existence of 4CL1 gene using PCR method and *NcoI/XbaI* double digestion.

The result in the figure 3A shows that, after using PCR method to amplify 4CL1 from 1 plasmid strain as template, we obtained 1 band which is about 1.6kb- specific length of 4CL1 gene. To confirm the above result, we digested the obtained plasmid with *NcoI/XbaI*. The digestion product (figure 3B) contains two bands, one of the two which has about 1.6kb length is 4CL1 gene, the another one is linear vector pPTN289.

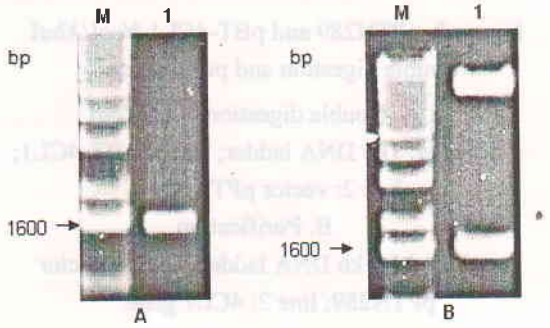

Figure 3. PCR amplified 4CL1 (A) and *NcoI/XbaI* double digestion (B).

Thus, we can concluse that constructed successfully transformation vector pPTN289-4CL1 and transformed into *E.coli* TOP10.

3.2. Mobilize vector pPTN289-4CL1 into Agrobacterium tumefaciens strain C58

Transformation vector pPTN289-4CL1 was mobilized into *Agrobacterium tumefaciens* strain C58 using electroporation (25μF, 200Ω, 2kV). Transformation product were spreaded on solid LB medium with Rifamycin 100mg/l and Spectinomycin 50mg/l, incubated at 28°C in 72 hours.

Selected some colonies for liquid cultivation, extracted plasmid and tested the existence of 4CL1 gene using PCR method and *NcoI/XbaI* double digestion.

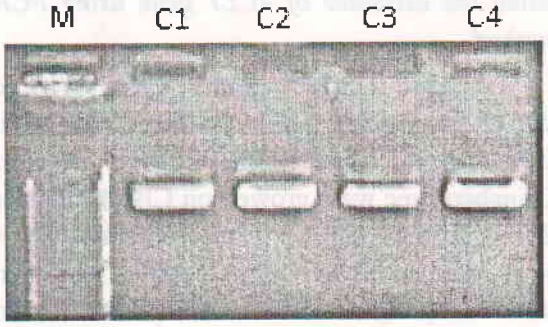

Figure 4. Plasmids from transformed *Agrobacterium tumefaciens*
Line M: 1kb DNA ladder;
Line 1- line 4: A. tumefaciens C1-C4.

Figure 4 is plasmids of 4 transformed *A. Tumefaciens* strains C1, C2, C3, C4. Used C1 and C3 plasmids as templates for PCR (specific primers 4CL1P1 and 4CL1P2), 4CL1 gene was amplified as in figure 5 (line 1 and 2). Double digestion results as in figure 5 (line 3 and 4).

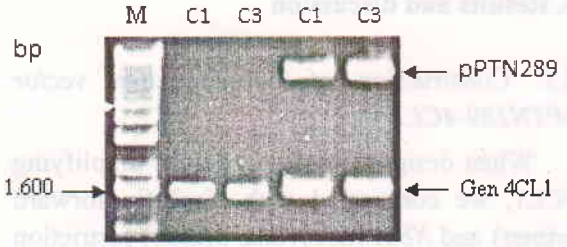

Figure 5. Testing the existence of 4CL1 using PCR method (line 1 and 2) and *NcoI/XbaI* double digestion (line 3 and 4).

3.3. Transformation of vector pPTN289-4CL1 into Chinaberrytree and tested the existence of 4CL1 gene using PCR method

4CL1 gene was transformed into Chinaberrytree follow the previously reported procedure (*Agrobacterium tumefaciens*-mediated method as in 2.2.3). After 4 weeks, we received some transformed Chinaberrytree

buds can grow on selection medium as in figure 6.

Figure 6. Transformed Chinaberrytree on selection medium.

We selected two 4 weeks Chinaberrytree samples, extracted total DNA (Keb Llanes et al., 2003) and tested the existence of 4CL1 using PCR method (with 4CL1P1 and 4CL1P2 specific primers). The result in figure 7 shows that, two Chinaberrytree samples are positive with 4CL1 gene, as same as positive control (pPTN289-4CL1 plasmid), while negative control (wild Chinaberrytree) is nagative with 4CL1 gene.

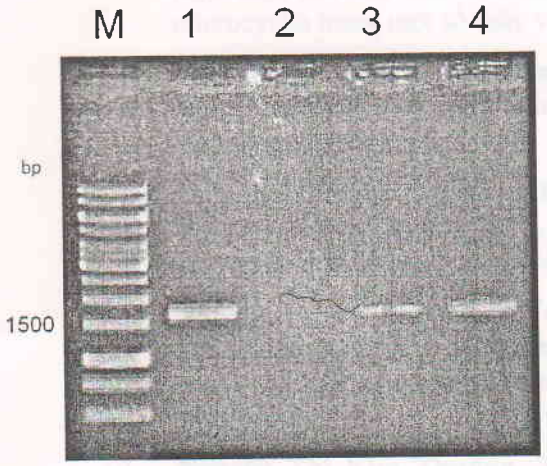

Figure 7. Test the existence of 4CL1 using PCR
M: 1kb DNA ladder; line 1: (+) control; line 2: (-) control; line 3 and 4: transformed Chinaberrytree.

4. Conclusion

Successfully constructed transformation vector pPTN289-4CL1.

Successfully mobilized vector pPTN289-4CL1 into *Agrobacterium tumefaciens* strain C58.

Successfully transformed 4CL1 gene into Chinaberrytree.

Tested the existence of 4CL1 gene in transgenic Chinaberrytree. Result shows that, there are two Chinaberrytree samples were positive with 4CL1 gene.

Acknowledgements

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Thiết kế cấu trúc vector và chuyển gen 4CL1 vào cây Xoan ta (*Melia azedarach* L.)

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Gen 4CL1 được phân lập từ Thông đuôi ngựa (*Pinus massoniana* Lamb), sau đó gắn vào vector chuyển gen pPTN289 đã được loại bỏ gen chỉ thị GUS để hình thành cấu trúc vector pPTN289-4CL1. Cấu trúc này được biến nạp vào vi khuẩn *Agrobacterium tumefaciens* chủng C58 bằng phương pháp xung điện. Gen 4CL1 được biến nạp vào mảnh thân Xoan ta (*Melia azedarach* L.) nhờ chủng *Agrobacterium tumefaciens* thu được ở trên. Chồi Xoan ta chuyển gen được sàng lọc trên môi trường CB5.1 (MS cải tiến có bổ sung chất chọn lọc PPT 1mg/l). Cây Xoan ta chuyển gen được chứng minh bằng kỹ thuật PCR sử dụng cặp mồi đặc hiệu cho gen 4CL1. Kết quả thu được 02 dòng Xoan ta chuyển gen.

Từ khóa: 4-coumarate: Coenzyme A ligase; 4CL1; lignin; Thông đuôi ngựa; Xoan ta.