Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2017

www.elsevier.com/locate/jbiosc

Repression of the DCL2 and DCL4 genes in *Nicotiana benthamiana* plants for the transient expression of recombinant proteins

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Received 5 December 2016; accepted 26 February 2017
Available online xxx

The production of recombinant proteins in plants has many advantages, including safety and reduced costs. However, this technology still faces several issues, including low levels of production. The repression of RNA silencing seems to be particularly important for improving recombinant protein production because RNA silencing effectively degrades transgene-derived mRNAs in plant cells. Therefore, to overcome this, we used RNA interference technology to develop *DCL2*- and *DCL4*-repressed transgenic *Nicotiana benthamiana* plants (ΔD2, ΔD4, and ΔD2ΔD4 plants), which had much lower levels of *NbDCL2* and/or *NbDCL4* mRNAs than wild-type plants. A transient gene expression assay showed that the ΔD2ΔD4 plants accumulated larger amounts of green fluorescent protein (GFP) and human acidic fibroblast growth factor (aFGF) than ΔD2, ΔD4, and wild-type plants. Furthermore, the levels of GFP and aFGF mRNAs were also higher in ΔD2ΔD4 plants than in ΔD2, ΔD4, and wild-type plants. These findings demonstrate that ΔD2ΔD4 plants express larger amounts of recombinant proteins than wild-type plants, and so would be useful for recombinant protein production.

[**Key words:** Dicer-like proteins; RNA interference; Gene repression; Transient gene expression; Vacuum infiltration; RNA silencing; Recombinant protein expression]

Plants have been successfully used for the production of recombinant proteins, such as enzymes and pharmaceutical proteins, and are particularly attractive due to the increased safety and lower costs involved. However, this technology faces several issues, including low levels of production. Many attempts have been made to increase recombinant protein production in plants (1). For example, since proteases are known to be the major factor that lowers the yield of recombinant proteins, recombinant proteins have been expressed as fusion proteins with various carrier proteins (2) and have even been co-expressed with protease inhibitors (3,4) to reduce their degradation in plant cells. It has also been shown that the organelle- or organ-specific expression of recombinant proteins improves their yield (5,6), and so the robust replication and transcription ability of plant viruses has been exploited to use plant virus vectors and their derivatives for the expression of recombinant proteins (7,8). Genetic approaches have also been used for the high-level expression of recombinant proteins. For example, to increase the amounts of transgene-derived mRNA, effective promoters and terminators have been discovered and used in many expression vectors (9); and codon optimization of transgenes that are suitable for the respective platform plants has also been used frequently (10). However, despite these attempts, the expression levels of recombinant proteins frequently remain disappointing.

Plants have a defense system against foreign genetic elements, such as viruses, that is known as RNA silencing. RNA silencing can efficiently and specifically degrade viral RNAs in plant cells, and consequently acts against plant viral vectors and transgenes for recombinant protein production, reducing their yield. Several pathways are involved in RNA silencing (11). Firstly, RNA-dependent RNA polymerases (RDRs) synthesize double-stranded RNAs (dsRNAs) using aberrant single-stranded RNAs, such as viral RNAs and mRNAs as templates. The dsRNAs are then cleaved by Dicer-like proteins (DCLs) to generate small or micro interference RNAs (siRNAs and miRNAs, respectively). These RNAs are captured by Argonaute proteins (AGOs) and incorporated into RNA-induced silencing complexes (RISCs) to induce sequence-specific RNA degradation or inhibition of translation. Mammals, fungi, and insects also have RNA silencing as an antiviral defense system (12).

Four DCLs (*DCL1–4*) have been identified to date, the functions of which have been intensively investigated (11). *DCL1* generates 21-nt-long small RNAs and is mainly involved in the generation of miRNAs, but there is no evidence for its involvement in defense against RNA viruses. *DCL3* generates 24-nt siRNAs that are incorporated into AGO4 and is involved in transcriptional gene silencing (TGS). *DCL2* and *DCL4* generate 22- and 21-nt siRNAs that are incorporated into *AGO1*, which mediates RNA cleavage to start and maintain RNA silencing, and so are involved in viral defense (11).

Previous studies have shown that co-expression of the *Tomato bushy stunt virus* P19 gene silencing suppressor protein with monoclonal antibody (trastuzumab) increases the concentration of the trastuzumab approximately 15-fold in tobacco (*Nicotiana* spp.) plants (13), and P25 of *Potato Virus X* (PVX) and P19 of *Artichoke Mottled Crinkle virus* also improve recombinant protein yield (14). It has also been reported that the production of siRNAs for the target gene in agroinfiltrated leaves starts as early as 2 days post

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.02.019

Please cite this article in press as: Matsuo, K., and Matsumura, T., Repression of the DCL2 and DCL4 genes in *Nicotiana benthamiana* plants for the transient expression of recombinant proteins, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.02.019

infiltration and reaches peak abundance by day 5 in *Nicotiana benthamiana* (15,16). Therefore, it appears that the repression of RNA silencing could improve recombinant protein production in plants.

Many studies on the repression or knockout of gene silencing-related genes in plants have been previously published. In particular, many kinds of RNA silencing-related gene knockout *Arabidopsis thaliana* mutants have been reported (17–20). In addition, *DCL2*-repressed *Oryza sativa* have been developed, which contain larger amounts of *O. sativa* endornavirus (OsEV)-derived siRNA than the wild-type plants (21). Several RNA silencing-related gene-repressed *N. benthamiana* plants have also been reported (22–26), in which the contributions of *RDR6* and *DCL2–4* to gene silencing under plant viral or viroid infection, and the high accumulation of viral or viroid RNA have been described. *RDR6*-repressed *N. benthamiana* plants accumulated higher amounts of green fluorescence protein (GFP) than wild-type plants when the PVX vector was used for GFP expression (23), and *DCL4*-repressed plants accumulated slightly more GFP than wild-type plants when a PVX-GFP mutant that lacked the p25 silencing suppressor was used for GFP expression (24). By contrast, there were no significant differences in GFP expression levels between wild-type, *DCL2*-repressed, and *DCL3*-repressed *N. benthamiana* plants (24). The effects of DCLs against viroid infection have also been investigated using *DCL*-repressed *N. benthamiana* plants (25,26). This has shown that *NbDCL2* and *NbDCL4* double-repressed plants produce much lower amounts of siRNAs against a viroid (25). Furthermore, the combined activity of the *NbDCL2* and *NbDCL3* pathways potently suppress viroid infection, but, *NbDCL4* appears to obscure the *NbDCL2* and *NbDCL3* effect on viroid infectivity (26).

Given this relationship between the repression of RNA silencing and the improvement of recombinant protein production in plant cells, we used RNA interference technology to develop *DCL2*- or *DCL4*-repressed transgenic *N. benthamiana* plants (ΔD2 and ΔD4 plants) and *DCL2* and *DCL4* double-repressed *N. benthamiana* plants (ΔD2ΔD4 plants) in which mRNAs derived from the respective target *DCL* genes were greatly decreased compared with the wild-type plants. We then estimated the recombinant protein productivity of these transgenic plants using vacuum infiltration, which is widely used for the transient expression of large amounts of recombinant proteins in plants (27). This is the first report on the use of *DCL*-repressed *N. benthamiana* plants for the production of recombinant proteins.

MATERIALS AND METHODS

Isolation of *DCL2* and *DCL4* gene fragments from *N. benthamiana* Total RNA was extracted from leaf samples of *N. benthamiana* using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and then treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) with 50 pmol of the random hexamer primer. Isolation of the *NbDCL2* and *NbDCL4* genes was performed based on the reported *DCL* gene sequences (GenBank accession numbers FM986781 and FM986783).

A partial sequence of the *NbDCL2* gene was amplified using cDNA from *N. benthamiana* with the primer set NbD2-F (5′-GAAGCAATTACAACAAAGAAATGCGTCG-3′) and NbD2-R (5′-GCATAATCTAGCACTACATCTCCAAG-3′) using KOD-FX Neo (Toyobo, Osaka, Japan). A partial sequence of the *NbDCL4* gene was amplified with the primer set NbD4-F (5′-CCAACAACTTCTACGCAGTAG-3′) and NbD4-R (5′-CATCTATTTCATCAGCAAGTGGAATAAAGAT-3′). Thermal cycling was performed as follows: denaturing at 94°C for 3 min, followed by 35 cycles of denaturing at 98°C for 10 s, annealing at 56°C for 30 s, and extension at 68°C for 1 min. The polymerase chain reaction (PCR) products were cloned into a pTA2 vector using TArget Clone -Plus- (Toyobo).

Escherichia coli DH5α competent cells (Takara Bio) were transformed with the plasmid vector and incubated overnight on Lysogeny broth (LB) agar plates containing ampicillin (50 μg/mL) at 37°C. Several of the resulting colonies were then cultured overnight in liquid LB medium containing ampicillin (50 μg/mL) at 37°C. Plasmids were prepared from the overnight cultures using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI, USA) for sequencing.

Construction of double-stranded RNA expression vectors and production of transgenic plants To generate a dsRNA expression vector, a section of the *NbDCL2* gene with restriction enzyme recognition sites (*Xba*I, *Bam*HI, *Nhe*I, and *Sac*I), NbinvDCL2(B-X) and NbinvDCL2(X-S), was PCR amplified using the following primer sets: [NbD2-F(Bam) (5′-TAGTTAGGATCCGAAGCAATTACAACAAAGAAATGCGTCG-3′) and NbD2-R-XX (5′-TAGTTATCTAGACTCGAGGCATAATCTAGCACTACATCTCCAAG-3′)] and [NbD2-F(Xba) (5′-TAGTTATCTAGAGAAGCAATTACAACAAAGAAATGCGTCG-3′) and NbD2-R-SS (5′-AGTTAGAGCTCACTAGTGCATAATCTAGCACTACATCTCCAAG-3′)]. A section of the *NbDCL4* gene was also amplified using the following primer sets: [NbD4-F(Bam) (5′-TAGTTAGGATCCCCAACAACTTCTACGCAGTAGG-3′) and NbD4-R-XX (5′-GTTATCTAGACTCGAGCATCTATTTCATCAGCAAGTGGAATAAAG-3′)] and [NbD4-F(Xba) (5′-TAGTTATCTAGACCAACAACTTCTACGCAGTAGG-3′) and NbD4-R-SS (5′-GTTAGAGCTCACTAGTCATCTATTTCATCAGCAAGTGGAATAAAG-3′)].

Smith et al. (28) reported that an intron sequence containing a self-complementary RNA vector could effectively induce RNA interference (RNAi) and that the spliceable intron could enhance the silencing efficiency. Therefore, RNAi vectors containing the intron sequence of a β-1,2-xylosyltransferase gene from *A. thaliana* (*AtXTint*) were constructed for the generation of stable *NbDCL2*- and *NbDCL4*-gene-repressed *AtXTint* was PCR amplified from the genomic DNA of *A. thaliana* using the primers AtXylt-int1(F) (5′-GTGAAGAGGTTTGTGCATTTTACTCATTG-3′) and AtXylt-int1(R) (5′-TCCACCCACTGCAGCCAAACAAAAAG-3′) (29,30). *A. thaliana* genomic DNA was purified from a leaf sample using a DNeasy Plant Mini Kit (Qiagen). An AtXTint1 DNA fragment with *Bam*HI and *Nhe*I recognition sites [AtXTint(B-N)] was prepared using the primers AtXTint1-F(Bam) (5′-TAGTTAGGATCCGAGGTTTGTGCATTTTACTCATTGATCTG-3′) and AtXTint1-R(Nhe) (5′-TAGTTAGCTAGCCACTGCAGCCAAACAAAAAGC-3′) using AtXTint1 as a template. The prepared DNA fragments [AtXTint(B-N), NbinvDCL2(X-B), NbinvDCL2(X-S), NbinvDCL4(X-B), and NbinvDCL4(X-S)] were digested with the corresponding restriction enzymes and successively cloned into the binary vector pBE2113 (31) to construct dsRNA expression vectors (pNbDCL2i and pNbDCL4i) (Fig. 1).

To construct a vector for double repression of the *NbDCL2* and *NbDCL4* genes, sections of the *NbDCL2* and *NbDCL4* genes were amplified using the following primer sets: [NbD2-F(Bam) and NbD2-R(Spe) (5′-TAGTTAACTAGTGCATAATCTAGCACTACATCTCCAAG-3′)] and [NbD4-F(Nhe) (5′-TAGTTAGCTAGCCAACAACTTCTACGCAGTAGG-3′) and NbD4-R-XX]. The prepared DNA fragments [NbinvDCL2(B-S) and NbinvDCL4(N-X)] were digested with *Nhe*I and *Spe*I, respectively, and then ligated. Using the resultant DNA fragment as a template, NbD2D4(B-X) and NbD2D4(X-S) were then amplified using the following primer sets: [NbD2-F(Bam) and NbD4-R-XX] and [NbD2-F(Xba) and NbD4-R-SS]. The prepared DNA fragments [AtXTint(B-N), NbinvD24(B-X), and NbinvD24(X-S)] were digested with the corresponding restriction enzymes and successively cloned into the binary vector pBE2113 (pNbDCL24i; Fig. 1). *Agrobacterium tumefaciens* strain LBA4404 was then transformed with the respective RNAi vectors and used for leaf disc transformation to

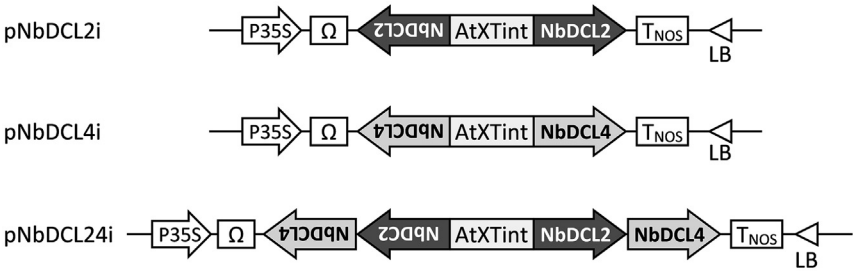

FIG. 1. RNAi vectors for *NbDCL2* and *NbDCL4* gene silencing. Parts of the respective genes were cloned into the binary vector pBE2113 for the construction of inverted repeat structures. An *Arabidopsis thaliana* β-1,2-xylosyltransferase intron sequence (AtXTint) was placed between the respective gene sequences to effectively induce RNAi in the plants. P35S, cauliflower mosaic virus 35S promoter; Ω, 5-untranslated sequence of tobacco mosaic virus; T_{NOS}, terminator of nopaline synthase; LB, left border sequence.

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