121-PDS-ihpRNA-mediated silencing of PDS gene

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Long introm-spliced hairpin RNA (ihpRNA) constructs which contained inverted repeats of the phytoene desaturase (PDS) separated by an intron, had been shown to very effective in triggering PDS silencing in Brassica napus. Using the PDS gene as a target control, it was shown that the RCA-mediated long ihpRNA construct was signicantly effective in triggering gene silence in B. napus.

Keywords: Brassica napus ; RNAi ; ihpRNA ; PDS)

1. RCA-mediated construction of PDS ihpRNA

To investigate whether RCA effectively generates iphRNA constructs, the *B. napus* phytoene desaturase (*PDS*) gene coding sequence of 450 bp in size (from the initial codon ATG) was amplified using the primer pair PDS-F1/PDS-R1 (Fig. 1A), added an ‘A’, and inserted into the pKan-*Bsa*I using the T-A approach. Following *Bsa*I digestion, PDS fragments with two different sticky ends respectively were obtained (Fig. 1A), which then were ligated to adaptor 1 and adaptor 2, forming closed circular DNA molecules. Agarose gel electrophoresis of the ligation product showed that almost all of the *Bsa*I-digested PDS DNA fragments were connected to both adaptors, generating adaptor1::PDS DNA::adaptor2 closed circular ligation products (Fig. 1B). RCA were performed using the ligation products, *Phi29*, and the loop2-specific primer pair loop2-F/loop2-R. Large DNA molecules containing inverted repeat units of double-stranded DNA were observed (Fig. 1C). The RCA products were confirmed by *Bam*HI and *Sac*I digestion, which generated a 1.1-kb of linear fragment that was approximately two-fold greater in size than the unamplified *PDS* control, and by a further digestion for the 1.1-kb fragment with *Bsa*I, which produced a 500-bp fragment, including the *PDS* control and partial sequences of loop1, and a 600-bp fragment, including 450-bp PDS control fragment and 128-bp adaptor 2 (Fig. 1D). These results indicated that a pool of single inverted repeat units for *PDS* were formed by RCA. Finally, *Bam*HI- and *Sac*I-digested fragments of RCA products were inserted into eukaryotic expression vector pBI121, generating a 121-PDS-ihpRNA construct (Fig. 1E).

Figure 1. RCA-mediated construction of PDS ihpRNA. (A) Amplification and cloning into pKan-*Bsa*I. A 400-bp PDS fragment was amplified using the PDS-F and PDS-R primers (lane 1), followed by T-A cloning. *Bsa*I digestion indicates that a 450-bp PDS fragment was inserted into the pKAN-*Bsa*I vector (lane 2). (B) Formation of closed circular molecules. *Bsa*I-digested PDS fragment is shown in lane 1, ligation products between two adaptors and the *Bsa*I-digested PDS fragment are shown in the upper band in lane 2, and free adaptors are shown in the lower bands in lane 2. (C) Rolling circular amplification. Closed circular DNA was amplified using Phi29 DNA polymerase and loop2-specific primers loop2-F and loop2-R, resulting in a large double DNA molecules containing multiple-unit inverted repeats (lane 1). (D) Verification of inverted DNA repeats. The PDS control DNA is shown in lane 1. RCA products were digested with *Bam*HI and *Sac*I, generating a 1.1-kbp fragment in lane 2. *Bam*HI and *Sac*I-digested fragments were further cut using *Bsa*I, generating a 500-bp fragment and a 600-bp fragment (lane 3). (E) Construction of the pBI121-*PDS* ihpRNA. *Bam*HI and *Sac*I-digested PDS fragment of the RCA products was inserted into eukaryotic expression vector pBI121, generating the *PDS* ihpRNA. The construct was confirmed by *Bam*HI and *Sac*I digestion (lane 1).

2.Testing of eukaryotic expression 121-PDS-ihpRNA construct

The 121-PDS-ihpRNA eukaryotic expressional construct was initially transferred into *Agrobacterium* strain GV3101 by electroporation, which was then transformed into *B. napus* cultivar zhongshuang 6 [1] via *Agrobacterium*-mediated gene transformation [2] to generate transgenic PDS-ihpRNA plants. Currently, this method of genetic transformation is considered to be of high efficiency (about 17%) [3]. The binary vector pBI121 harbors the neomycin phosphotransferase gene (*NPTII*) resistance selection marker (Figure 2A). The selection marker-resistant regenerated T_0 transformants were initially subjected to kanamycin-based selection and then rooted well in selective medium. The selected resistant T_0 transformants were then verified by PCR-based screening using the corresponding primer pairs NPTII-F and NPTII-R, which were specific for the *NPTII* gene in the binary construct (Figure 2A). A total of five transgenic plants (approximately 17% transformation efficiency) were obtained, all displaying photo-bleaching phenotypic characteristics (Figure 2B). To verify whether the *PDS* gene was silenced, qRT-PCR corresponding to the *PDS* target gene in five RNAi T_0 lines was investigated, which confirmed that the mRNA expression level of the target *PDS* had significantly decreased (Fig. 2C). These results suggested that the PDS ihpRNA construct effectively silenced the *PDS* target gene. Due to the complex secondary hairpin structures formed by ihpRNA constructs, special primers (avoiding specificity to the stem of the hairpin structure) were designed to efficiently amplify the hpRNA target sequence in the binary pBI121 vector. The primer pairs loop2-F and 121-F or loop2-R and 121-R were used to identify the target gene sequences of the ihpRNA lines (Fig. 2D). For the five PDS RNAi lines, loop2-F and 121-F or loop2-R and 121-R primer pair successfully amplified bands of the

desired size, and the amplification products were sequenced, demonstrating that the *PDS* ihpRNA expression cassette was successfully integrated into the *B. napus* plants (Fig. 2D). This result indicated that these two pairs of primers could be employed to rapidly identify the candidate target genes of the ihpRNA lines.

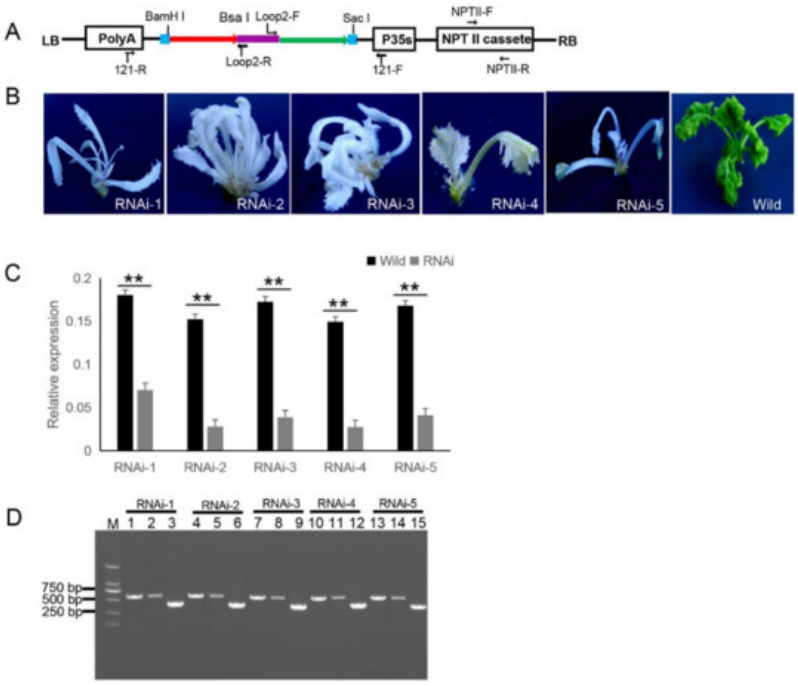

Figure 2. 121-PDS-ihpRNA-mediated silencing of the *PDS* gene. (**A**) Map of the binary vector 121-PDS-ihpRNA. NPTII-F and NPTII-R primers were used in the identification of positive transgenic plants, the primer pairs loop2-F/121-F and loop2-R/121-R were utilized to rapidly screen target genes. Blue line is the adaptor1 sequence. Purple line is the adaptor2 sequence. Red line with the arrow is the sense strand of dsDNA. Green line with the arrow is the anti-sense strand of dsDNA. (**B**) Interfering phenotypes of 5 PDS-ihpRNA transgenic plants. RNAi-1, RNAi-2, RNAi-3, RNAi-4, and RNAi-5 were PDS-ihpRNA transgenic plants. Wild was the transgenic negative plant. (**C**) qRT-PCR analysis of the *PDS* target gene mRNA level in the five hpRNA lines shown in (**B**) using the primer pair PDS-F2/PDS-R2. Data shown as mean ± s.d.,**, $p < 0.05$. (**D**) Integration into the *Brassica napus* genome for the *PDS* ihpRNA expression cassette. For the five PDS RNAi lines, loop2-F and 121-F or loop2-R and 121-R primer pair could amplify the bands with the desired size, demonstrating that the PDS ihpRNA expression cassette was successfully integrated into the *B. napus* plants. Lanes 3, 6, 9, 12, and 15 show the 450-bp PDS control; lanes 1, 4, 7, 10, and 13 exhibit the PCR products of loop2-F and 121-F; lanes 2, 5, 8, 11, and 14 depict the PCR products of loop2-R and 121-R.

References

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