A new method for functional analysis of plastid *EMBRYO-DEFECTIVE PPR* genes by efficiently constructing cosuppression lines in *Arabidopsis*

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**Research**

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Abstract

Background: Pentatricopeptide-repeat proteins (PPRs) characterized by tandem arrays of a degenerate 35-amino-acid repeat (PPR motif) can bind a single strand RNA and regulate organelle gene expression at the post-transcriptional level, including RNA cleavage, splicing, editing and stability etc. PPRs are conserved in all eukaryotes and extremely expanded in higher plants. Many knockout mutants of PPR genes are embryonically lethal. These genes are named EMB PPRs and functional analysis of them is hindered by the difficulty in obtaining their knockout mutants.

Results: Here, we report a new method for functional analysis of plastid EMB PPRs by efficiently constructing their cosuppression lines in Arabidopsis. When we overexpressed a mutated full length or truncated coding sequence (CDS) of EMB PPRs, such as EMB2279, EMB2654 and EMB976 (all belong to the P family PPRs) in the wild-type (WT) background, a large portion of T_1 plants displayed chlorosis phenotypes, which are similar to those of the weak allele mutants, knockdown lines or partially complementary lines. RT-PCR analysis showed that overexpression of the truncated EMB PPRs led to significant and specific downregulation of their corresponding endogenous mRNAs. However, when these EMB PPRs were overexpressed in the PTGS (Post transcriptional Gene Silencing) deficient mutant, rdr6 (RNA-dependent RNA polymerase 6), none of the T_1 plants displayed chlorosis phenotypes. These results indicate that the chlorosis phenotype results from post transcriptional silencing of the corresponding endogenous gene (also known as sense cosuppression).

Conclusions: Overexpression of an appropriately truncated EMB PPR CDS in WT leads to gene silencing in a RDR6-dependent manner, and this method can be employed to study the unknown function of EMB PPR genes. By this method, we showed that EMB976 is required for splicing of chloroplast clpP1 intron 2 and ycf3 intron 1.

Background

It is a common phenomenon that knockout mutants of plant essential genes display embryo or seedling lethal phenotype [1]. In the Arabidopsis genome, there are about 510 EMBRYO-DEFECTIVE (EMB) genes. Among them, 34 genes belong to the pentatricopeptide-repeat (PPR) gene family [1, 2]. PPR proteins characterized by tandem arrays of a degenerate 35-amino-acid repeat (PPR motif) are conserved in all eukaryotes and extremely expanded in higher plants. It has been known that over 450 PPR proteins are present in Arabidopsis and divided into two subfamilies, designated P and PLS, according to the characteristics of the PPR motifs [3]. Most PPRs are involved in regulation of organelle gene expression, including RNA cleavage, splicing, editing and stability, via directly binding to their RNA targets [4]. A set of EMB PPRs have been reported to be required in RNA processing of the chloroplast housekeeping genes. For example, EMB2261 is specifically involved in chloroplast rps14 mRNA editing [5]. EMB2750 (PPR2) is specifically involved in chloroplast 23S rRNA processing [6]. EMB3140 (PDM3) is specifically involved in chloroplast tm4 and clpP1 intron1 splicing [7]. EMB2279/SOT5 is specifically involved in chloroplast rpl2 and trnK intron splicing [8]. EMB2654 and atPPR4 are coordinately and specifically involved in trans-splicing of plastid rps12 intron1 [9, 10]. And more recently EMB976/PDM4 is reported to be specifically involved in chloroplast group II intron splicing including clpP1 and ycf3 [11]. Since the targets of these PPRs are usually housekeeping genes in chloroplasts, it is understandable that knockout mutants of these PPRs are embryonically lethal. Thus, generation of weak allele mutants, viable knockdown lines or partially complementary lines of EMB PPRs is required for their functional analysis. Currently, techniques such as antisense, RNAi, artificial microRNA are employed to make knockdown lines [12, 13]. Complementation of emb2654 heterozygous lines with a cDNA carrying the WT coding sequences under the control of the seed-specific ABSCISIC ACID-INSENSITIVE3 (ABI3) promoter is employed to generate the partially complementary lines [10]. In addition, CRISPR/CAS9 techniques are also widely used for constructing knockout or knockdown mutants [14, 15]. However, these techniques are sometimes not efficient to get wanted transgenic plants. For example, small interfering RNA (siRNA) and artificial microRNA (amiRNA), which are about 21~22 nucleotide (nt), often target more than one endogenous genes if the gene sequence is very conserved among its gene family [13]. And the knockdown lines generated by RNAi or artificial microRNA sometimes display very mild phenotypes, which is not helpful for phenotypical study. Constructing partially complementary lines is a time-consuming process due to making transgenic plants in heterozygotes at first [10]. CRISPR/CAS9 techniques usually cause off-target effects, and identification of the genome-edited lines is relatively costly [14].
The gene cosuppression often occurs when a homologous coding sequence is overexpressed in plants [16-18]. This phenomenon is controlled by the post transcriptional gene silencing (PTGS) pathway [12], which is initiated by the conversion of single-stranded RNAs (over accumulated aberrant RNAs) into double-stranded RNAs by RNA-dependent RNA polymerase 6 (RDR6). The double strand RNAs are subsequently sliced into 21-22 nt small interfering RNAs (siRNA) by dicer-like 2 (DCL2) or DCL4. siRNAs are loaded into the RNA-induced silencing complex (RISC) to cleave the target RNAs [19, 20]. The PTGS pathway is well conserved among eukaryotes. Based on this pathway, antisense, RNA interfering (RNAi) and artificial microRNA techniques are developed and widely used to construct knockdown lines for a lot of model organisms to facilitate their gene function studies [12].

Here, we reported a method that can efficiently make cosuppression lines via overexpressing an appropriately truncated PPR CDS in the WT background. In addition, we dissected the biological function of a PPR protein, EMB976, in Arabidopsis using this method.

Results

Overexpression of the mutated EMB2279/SOT5 and EMB2654 CDS in WT leads to RDR6-dependent gene silencing

We previously reported that a weak allele of emb2279-2/sot5 mutant exhibits a virescent phenotype, which is caused by a point mutation that significantly reduces splicing efficiency of the seventh intron of SOT5 and generates two additional mRNA variants [8]. The smallest transcript that lacks 22-base pairs (bp) at the 3' end of the seventh exon is predicted to produce a truncated SOT5 protein with only 6 PPR motifs, named SOT5-m1 (Fig.1), whereas the largest transcript that contains the seventh intron is predicted to produce a mutated protein with 10 PPR motifs, named SOT5-m2 (Fig.1). While the wild type SOT5 encodes a protein with 11 PPR motifs (Fig.1 and Table 1). To test whether the two predicted proteins are functional or not in plants, we cloned SOT5-m1 and SOT5-m2 CDS and transformed them under the control of the cauliflower mosaic virus 35S promoter into WT plants. We obtained 30 and 42 positive T1 transformants for 35S:SOT5-m1 and 35S:SOT5-m2 constructs, respectively. Our results showed that 80% of the 35S:SOT5-m1 transgenic lines exhibited severe leaf chlorosis at the early growth stage, and these leaves were gradually turned into pale green later; and 48% of 35S:SOT5-m2 transgenic lines displayed relatively mild leaf chlorosis and virescence (Table 1 and Fig. 2a). This chlorosis and virescent phenotype was similar to that of the sot5 mutant. Thus, we suspected that the transgenic lines with chlorosis were the cosuppression lines, in which expression of the endogenous SOT5 was silenced. Then, we analyzed expression levels of SOT5 in T1 transformants with chlorosis.

Indeed, RT-PCR and quantitative PCR (qPCR) analyses showed that the level of the endogenous SOT5 transcripts (En-SOT5), detected by the specific primer pair spanning 5'UTR (the red arrows highlighted on EMB2279/SOT5 cDNA in Fig.1), was significantly decreased in 35S:SOT5-m1/Col-0 transgenic line2 and line3 (less than 40% of the WT expression level), whereas the level of total SOT5 transcripts (including endogenous and exogenous SOT5 in transgenic lines), detected by the primer pair spanning intron 7 (Fig. 2b) or by the primer pair in CDS (black arrows highlighted on EMB2279/SOT5 cDNA in Fig.1) was significantly increased (Fig. 2c), compared with that in non-transgenic WT plants. Consistently, in 35S:SOT5-m2/Col-0 transformants, the expression level of endogenous SOT5 was also significantly decreased (less than 60% of the WT expression level, Fig. 2b and 2c), while the content of total SOT5 transcripts was significantly increased (Fig. 2d). Meanwhile, splicing efficiency of the plastid rpl2 gene, a SOT5 target, was dramatically reduced in these transformants (Fig. 2b and 2e). In contrast, splicing efficiency of the plastid atpF gene, a non-target gene of SOT5, was not altered in all transgenic plants (Fig. 2b and 2e). These results indicate that overexpression of the mutated SOT5 CDS in WT leads to suppression of the endogenous gene expression, probably through the post transcriptional gene silencing (PTGS, also known as sense cosuppression) pathway.

To confirm this hypothesis, we transformed 35S:SOT5-m1 and 35S:SOT5-m2 constructs into the rdr6-11 mutant. RDR6 is a key component in the PTGS pathway and the rdr6-11 mutant presented elongated and curled downward leaves (Fig. 3a) [21, 22]. It has been demonstrated that overexpression of homologous genes in this mutant was not able to trigger the PTGS pathway [21-23]. We obtained twenty-five 35S: SOT5-m1/rdr6 and eighteen 35S: SOT5-m2/rdr6 T1 transformants, and found that none of the transformants exhibited the chlorosis phenotype (Fig. 3a). RT-PCR analysis showed that the endogenous SOT5 transcript level was not decreased, although the total SOT5 mRNA level was significantly increased in the transformants (Fig.3b-3d).
Consequently, no splicing defect of plastid rpl2 was detected in these transformants (Fig. 3e). Taken together, our results indicate that overexpression of the mutated SOT5 CDS in the WT background leads to PTGS in a RDR6-dependent manner.

We then asked whether the above result could be repeated with other EMB PPR genes. To address this question, we overexpressed a truncated CDS of EMB2654 (EMB2654-11M) which encodes a truncated protein with only 11 PPR motifs into the WT background (Fig. 1 and Table 1). While the wild type EMB2654 has 18 PPR motifs. It has been reported that EMB2654, a P family PPR protein, was required for trans-splicing of the plastid gene rps12 intron 1 [10]. Interestingly, all the 47 T1 transgenic lines exhibited leaf chlorosis (Table1 and Fig. 4a), indicating the silence of endogenous EMB2654. RT-PCR analysis showed that expression of the endogenous EMB2654 was down-regulated (about 20% of the WT expression level) while the total mRNA of EMB2654 was significantly increased in these transformants (Fig. 4b-d). Indeed, the splicing efficiency of rps12 intron 1 was significantly decreased in the cosuppression lines, while the splicing efficiency of the non-target gene, clpP1 intron2 was not significantly decreased (Fig. 4e). However, when the truncated EMB2654-11M CDS was overexpressed in rdr6-11, no leaf chlorosis was observed among 30 transformants (Fig. 4f). RT-PCR results showed that the endogenous EMB2654 mRNA was not decreased, and the total EMB2654 mRNA was significantly increased in these transformants (Fig.4g-4i). Consistently, splicing efficiency of the rps12 intron 1 was not decreased in these transformants (Fig. 4j). Thus, these results confirm that overexpression of the truncated EMB2654 CDS in the WT background leads to PTGS in a RDR6-dependent manner.

**Functional analysis of EMB976 via its cosuppression lines**

EMB976 is a functionally unknown PPR protein, which belongs to P subfamily containing 22 PPR motifs and is predicted to be localized in chloroplasts. Its knockout mutant has been demonstrated to be embryonically lethal [2]. To study its physiological role in plant growth, we constructed two plasmids named EMB976-7M and EMB976-14M, which encode the truncated protein with 7 and 14 PPR motifs, respectively (Fig.1 and Table 1), and transformed them into the WT background. Our results showed that four of eight 35S:EMB976-14M/Col-0 T1 transformants displayed virescent leaves (Fig. 5a and Table 1), whereas all of the thirteen 35S:EMB976-7M/Col-0 transformants had the same phenotype as WT (Table 1). To confirm whether gene silencing occurred in 35S:EMB976-14M/Col-0, we checked expression levels of the endogenous EMB976 in the transformants with the virescent phenotype. As expected, the endogenous EMB976 was significantly down-regulated (about 40% of the WT expression level) while the total EMB976 mRNA was significantly increased in these transformants (Fig.5b and 5c), indicating that the phenotype of the transgenic lines is caused by the silencing of EMB976. Since the P family PPR proteins were often involved in organelle RNA stability and splicing, we further examined the intron splicing of chloroplast genes in these 35S:EMB976-14M/Col-0 cosuppression lines. Indeed, RT-PCR analysis showed that the precursors of ndhA, clpP1 intron 2 and ycf3 intron 1 were significantly and specifically accumulated in these lines (Fig. 5d). qPCR analysis showed that the splicing efficiency of clpP1 intron 2 and ycf3 intron 1 was not decreased, compared with that of ycf3 intron 2 (Fig. 5e), suggesting chloroplast clpP1 intron 2 and ycf3 intron 1 were the possible targets of EMB976. However, further experiments are required for verification of the results. Again, no yellow young leaves were observed among the transformants when EMB976-14M was overexpressed in rdr6-11 (Fig. 5f). This result was consistent with that of RT-PCR analysis. In these transformants, the endogenous EMB976 transcripts was not significantly decreased while the total EMB976 transcripts was strongly increased (Fig. 5g-5i). Consistently, the splicing efficiency of clpP1 intron 2 and ycf3 intron 1 was not decreased, compared with that of ycf3 intron 2 (Fig. 5j). Thus, we conclude that gene silencing triggered by overexpression of truncated EMB genes is dependent on RDR6.

**Table 1 The cosuppression phenotype and frequency of transgenic plants expressing various constructs in WT**
| Plasmid construct | Length of encoding protein (aa) | Number of PPR motifs | Phenotype of cosuppression | Number of total T<sup>1</sup> transformants | Number of T<sup>1</sup> transformants with visible chlorosis | Frequency of cosuppression<sup>b</sup> | Severity of chlorosis in cosuppression lines |
|------------------|-----------------------------|----------------------|---------------------------|---------------------------------------------|------------------------------------------------|-----------------|-----------------------------------------------|
| *SOT5*          | 978                         | 11                   | yellow inflorescence and cauline leaves albino young leaves at seedling stage partial albino leaves at seedling stage | 35                                          | 20 57%                                           | strong          | very mild                                    |
| *SOT5-m1*       | 712                         | 6                    |                           | 30                                          | 24 80%                                          |                 | strong                                       |
| *SOT5-m2*       | 1006                        | 10                   |                           | 42                                          | 20 48%                                          | mild            | mild                                         |
| *EMB2654*       | 822                         | 18                   | chlorosis leaves at seedling stage | ND                                          | ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND | mild            | mild                                         |
| *EMB2654-11M*   | 550                         | 11                   |                           | 47                                          | 47 100%                                          |                | mild                                         |
| *EMB976*        | 1038                        | 22                   |                           | ND                                          | ND ND ND ND ND ND ND ND ND ND ND ND ND ND ND |                 | mild                                         |
| *EMB976-7M*     | 443                         | 7                    | WT-like yellow young leaves at seedling stage | 13                                          | 0 0%                                           |                | ND                                           |
| *EMB976-14M*    | 747                         | 14                   |                           | 8                                           | 4 50%                                           |                | mild                                         |

<sup>a</sup>The pGWB2 plasmid containing the mutated or truncated CDS indicated by the name.

<sup>b</sup>cosuppressed T<sup>1</sup> transformants/total T<sup>1</sup> transformants. ND: not determined.

**Discussion**

**Overexpressing mutated *EMB* PPR<sub>s</sub> CDS in WT background leads to specific silencing of the endogenous genes**

Since it’s hard to get knockout mutants for *EMB* genes, obtaining weak alleles or constructing viable knockdown lines of *EMB* genes is valuable for their functional study. For example, by characterization its weak allele, the PPR protein EMB2279 was disclosed to be specifically involved in chloroplast *tmk* and *rpl2* intron splicing [8]. And by the help of its partial complementation line, EMB2654 was revealed to be specifically involved in trans-splicing of chloroplast *rps12* intron1 [10].

In this study, we found a simple way to make cosuppression lines of *EMB* PPR gene by overexpressing its appropriately truncated CDS fragment in WT background. In the cosuppression lines, the over-accumulated transgene's transcripts are converted into double-stranded RNAs by RDR6. Then the double strand RNAs are subsequently sliced into 21-22 nt siRNA by dicer-like 2 (DCL2) or DCL4. siRNAs are loaded into the RNA-induced silencing complex (RISC) to cleave the target RNAs [20]. The silenced gene is specifically targeted by its exogenous (transgene) sequence in the cosuppression lines. In our study, the sequence of transgene is long enough (more than or close to 2/3 of WT full length CDS, Table 1), theoretically, the targeting is more specific than that of siRNA or amiRNA. As expected, by RT-qPCR analysis, we found the expression level of *EMB2654* or *EMB976* was not significantly decreased in 35S:*SOT5-m1*/*Col-0* or 35S:*SOT5-m2*/*Col-0* cosuppression lines (Additional file 1a and 1b). The expression level of *EMB2654* was not significantly decreased in 35S:*EMB976-14M*/*Col-0* cosuppression lines and vice versa (Additional file 1c and 1d). These results indicate that the targeting is very specific by cosuppression.

Besides the *EMB* genes encoding chloroplasts localized PPR proteins, we also overexpressed an alternative splicing variant of *EMB2784*/*PRPL4*, which encodes a plastid ribosomal protein L4 into WT background. Compared with the major splicing variant *PRPL4* CDS, the *gPRPL4* CDS retains its unique intron with a premature termination codon (PTC) and leads to a truncated PRPL4 protein. Interestingly, among 9 T<sup>1</sup> transformants, there were 6 transformants exhibited chlorosis leaves (Additional file 2a). RT-PCR analysis showed that in these transformants with chlorosis leaves, the endogenous *PRPL4* was significantly decreased (asterisk in Additional file 2b) and the exogenous *gPRPL4* was increased (arrowhead Additional file 2b).
Meanwhile, RT-qPCR analysis confirmed that \textit{PRPL4} was specifically decreased in the \textit{35S:gPRPL4/Col-0} cosuppression lines when compared with the expression level of \textit{PRPL28 (PLASTID RIBOSOMAL PROTEIN L28, Additional file 2c and 2d)}. These results indicated that overexpression of a mutated CDS of \textit{PRPL4} in WT also leads to cosuppression with a high frequency. Taken together, it seems that this method is universal and robust for constructing knockdown lines for the chloroplast localized EMB proteins. It is likely that dysfunction of the chloroplast-localized proteins often leads to visible chlorosis phenotypes, which makes us easy to know whether the target gene is silenced in transgenic lines. In the future, we will test more genes using this method and try to work out an optimal experimental system to construct cosuppression lines efficiently for \textit{EMB} genes in Arabidopsis.

The length of truncated PPR CDS is critical for obtaining cosuppression lines with strong mutant phenotype efficiently

We found that cosuppression hardly occurred in the transgenic lines if the length of truncated PPR CDS overexpressed in WT plants was less than half of the full PPR CDS, such as \textit{EMB976-7M} (Table 1). This result is consistent with the report that overexpression of exogenous \textit{Chalcone Synthase} (\textit{Chs}) with a premature translational termination codon (PTC) significantly decreased the frequency of cosuppression, compared with WT \textit{Chs} in Petunia [24]. And Mallory et al. (2009) also reported that when the transgene sequence only shared 557 nt of homology with the endogenous AGO1 mRNA, the cosuppression frequency was significantly lower than that using the transgene sequence shares more than 3 kb of homology with the endogenous AGO1 mRNA [23]. The transcripts of transgene with PTC were probably degraded by the nonsense-mediated mRNA decay (NMD) pathway, which can recognize and degrade aberrant transcripts harboring PTC, and thereby prevent the production of truncated proteins which might be deleterious [25]. However, if the PTC occurs close to the 3'-UTR, the mRNA will escape from the NMD pathway, and thus can accumulate and trigger the RDR6-dependent PTGS pathway [26, 27]. So, theoretically, the full length of CDS will lead to a highest cosuppression frequency. However, we found that when the full length of SOT5 was overexpressed in WT background, only very mild chlorosis (cosuppression) phenotypes were observed at late developmental stage (Additional file 3), although the cosuppression frequency is high enough (Table 1). In contrast, overexpression of the truncated (more than 2/3 of WT full length CDS, Table 1) or mutated SOT5 CDS in WT led to a high cosuppression frequency and the strong mutant phenotype. It is likely that the absence of endogenous SOT5 protein due to PTGS can be partially compensated by the truncated or mutated SOT5 protein in the cosuppression lines. These results are consistent with the report that the developmental defects of cosuppressed plants were more pronounced in a non-functional AGO1 protein transformants than in a functional AGO1 protein transformants [23]. Thus, we suggest that the appropriate truncation of PPR CDS is critical for constructing cosuppression lines with strong mutant phenotype efficiently.

\textbf{EMB976 is required for splicing of chloroplast clpP1 intron 2 and ycf3 intron 1}

We investigated the role of functionally unknown EMB976 in plant growth by overexpressing the truncated \textit{EMB976-14M} CDS in WT. Our data showed that the \textit{35S:EMB976-14M/Col-0} cosuppression lines displayed a leaf virescent phenotype and splicing efficiency of \textit{clpP1} intron 2 and \textit{ycf3} intron 1 was significantly deceased in these cosuppression lines. ClpP1 is a core component of ClpP protease complex, which plays critical roles in chloroplast protein homeostasis [28, 29]. Ycf3 is involved in assembly of photosynthetic complex I, which is a key complex in light reaction of photosynthesis [30]. The absence of these critical proteins due to the decreased mature RNAs may explain why the knockout mutant (\textit{emb976}) is embryonically lethal. Interestingly, a recent work showed that EMB976/PDM4 is involved in splicing of group II introns and rRNA processing in chloroplasts [11]. They found the precursors of the chloroplast \textit{ndhA}, \textit{petB}, \textit{ycf3}, \textit{petD} and \textit{clpP1} were present and accumulated in a high level in the \textit{pdm4} knockout mutant but absent in the wild type, which is consistent with our results. The role of EMB976 for group II intron splicing might be independent on its role for rRNA processing. It's probable that rRNA processing defect is an indirect effect of absence of the chloroplast ClpP protease complex since it was reported that in the weak allele, \textit{clpR4-3} mutant, the plastid rRNA processing was also defective [31].

\textbf{Conclusions}

Overexpression of an appropriately truncated \textit{EMB PPR} CDS in WT leads to generation cosuppression lines with strong mutant phenotype efficiently, and this method can be employed to study the unknown function of \textit{EMB PPR} genes. By this method, we
found that EMB976 is required for intron splicing of plastid clpP1 and ycf3.

Materials And Methods

Plant materials and growth conditions

The Arabidopsis ecotype Columbia-0 (Col-0) was used as WT in this study. The rdr6-11 mutant used in this study was previously described [22]. Seeds were surface-sterilized by 75% ethanol and stratified at 4°C for 3 days, and then sown onto half-strength Murashige and Skoog (MS) agar medium with 1% sucrose. Transformants were screened on 1/2 MS agar medium containing 50 μg/ml Kanamycin. About two week old seedlings were transferred into soil. Plants were grown in phytotron under long-day conditions (8 h light/16 h dark) with light intensity (100 μmol photons m\(^{-2}\) s\(^{-1}\)) at 22°C.

Plasmid construction and transformation

The mutated SOT5 CDS (SOT5-m1 and SOT5-m2) was amplified from sot5 cDNA. The truncated EMB2654-11M CDS (1-1635bp) was amplified from WT cDNA. The truncated EMB976-7M CDS (1-1332bp) and EMB976-14M CDS (1-2244bp) were amplified from WT cDNA, respectively. In order to produce a truncated protein in the transgenic lines, we introduced a stop codon in the reverse primer when we amplifying EMB2654-11M, EMB976-7M and EMB976-14M sequences. The genomic PRPL4 (gPRPL4) was amplified from WT gDNA. The primer sequences were listed in Additional file 4. These amplified sequences were inserted into the pENTR SD/D-TOPO entry vector (Invitrogen). After sequencing, the corrected CDS fragments were recombined into the pGWB2 destination vector as previously described [8]. The destination vectors were transformed into Col-0 or rdr6-11 mutants using Agrobacterium strain GV3101 and the floral-dip method. Transformants were screened on 1/2 MS agar medium containing 50 μg/ml Kanamycin.

RNA isolation and RT-qPCR

The chlorosis or albino leaves of each cosuppression lines were sampled for RNA extraction. Total RNAs were extracted from leaves of various lines according to the manufacturer's instructions (Promega Denaturing Solution, Z5651). Downstream DNAse I treatment was performed according to the manufacturer's instructions (Invitrogen/Gibco DNA-free DNase Treatment & Removal Reagents, AM1906) and reverse transcription (RT) steps were conducted according to the manufacturer's instructions (Promega Reverse Transcription System, A3500). PCR was performed using the gene-specific primers that are listed in Additional file 4 and according to the manufacturer's instructions (TaKaRa Taq, R001WZ; The Applied Biosystems MicroAmp® Fast Optical 96-Well Reaction Plate-0.1mL, 4346906; ThermoFisher Scientific QuantStudio™ 3 Real-Time PCR Systems). The data set was normalized using ACTIN2 as a reference. The method to quantify the transcript level and splicing efficiency of plastid genes was previously described [8, 30]. The expression of endogenous EMB2279 genes was detected by the primer pair located in 5'UTR (Fig. 1). The total EMB2279 (including endogenous and transgenes) mRNA was detected by the primer pair located in the coding sequence. The expression of endogenous EMB2654 or EMB976 was detected by the primer pair located in the last half of its CDS (Fig. 1). The primers are listed in Additional file 4. In the figures of qPCR, the values are means of three technique replicates (bars indicate SD), asterisks indicate significant differences between wild type (WT) and transgenic plants (Student's t test, *, P < 0.05 and **, P < 0.01). At least two T\(_1\) transgenic lines were analyzed for each construct.

Abbreviations

PPR: Pentatricopeptide-repeat proteins; EMB: embryonic lethal; WT: wild-type; PTGS: post transcriptional gene silencing; RDR6: RNA-dependent RNA polymerase 6; DCL2: dicer-like 2; DCL4: dicer-like 4; RISC: RNA-induced silencing complex; RNAi: RNA interfering; CDS: coding sequence; EMB2279: EMBRYO DEFECTIVE2279; SOT5: SUPPRESSOR OF THF1; EMB2654: EMBRYO DEFECTIVE2654; EMB976: EMBRYO DEFECTIVE976; SOT5-m1: SOT5 mutated protein 1; SOT5-m2: SOT5 mutated protein 2; EMB2654-11M: truncated EMB2654 protein with 11 PPR motifs; EMB976-7M: truncated EMB976 protein with 7 PPR motifs;
EMB976-14M: truncated EMB976 protein with 14 PPR motifs; RPL4: PLASTID RIBOSOMAL PROTEIN L4, PRPL28: PLASTID RIBOSOMAL PROTEIN L28, rpl2: plastid ribosomal protein l2, rps12: plastid ribosomal protein s12; bp: base pairs; nt: nucleotide

Declarations

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Authors’ contributions

Weihua Huang conceived and designed the experiments; Jingli Chen, Haojie Zhu and Weihua Huang performed the experiments; Weihua Huang and Jirong Huang supervised the experiments; Jingli Chen, Weihua Huang and Jirong Huang wrote the paper.

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Availability of data and materials

All data generated or analyzed during this study are available in this published article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

![Diagram](attachment:image.png)

**Figure 1**
Figure 1

Schematic diagram of the EMB PPR CDS constructs made in this study and their coding proteins. a Schematic diagram of the EMB PPR CDS constructs. The EMB PPR CDS is indicated by black box. The UTR regions are indicated by black lines. The black arrows show the position of the primer pair to detect the total genes (including endogenous and transgenes). The red arrows show the position of the primer pair to detect the endogenous genes. b Schematic diagram of the corresponding EMB PPR proteins. The protein structures were predicted by PROSITE (https://prosite.expasy.org/). Pentagons mean PPR motifs in each construct. The amino acid (aa) numbers indicate the size of WT or truncated PPR proteins.
The phenotypes of transgenic plants overexpressing the mutated SOT5 CDS in WT background. 

a) Chlorosis leaf phenotype of transgenic plants overexpressing SOT5-m1 and SOT5-m2 constructs in WT background (bar = 1.5 cm).

b) RT-PCR analysis of expression levels of the endogenous SOT5, total SOT5 (including endogenous SOT5 and transgene), and two plastid genes in the typical cosuppression lines.

c) RT-qPCR analysis of the endogenous SOT5 transcript level in the cosuppression lines.

d) RT-qPCR analysis of the total SOT5 transcript level in the cosuppression lines.

e) Splicing efficiency of plastid rpl2 and atpF (as a control) in the cosuppression lines. For RT-qPCR, the values are means of three technique replicates (bars indicate SD). Asterisks indicate significant differences between wild type (WT) and transgenic plants (Student’s t test, *, P < 0.05 and **, P < 0.01). Two to three T1 transformants were analyzed for RT-PCR and RT-qPCR.
Figure 2

The phenotypes of transgenic plants overexpressing the mutated SOT5 CDS in WT background. a Chlorosis leaf phenotype of transgenic plants overexpressing SOT5-m1 and SOT5-m2 constructs in WT background (bar = 1.5 cm). b RT-PCR analysis of expression levels of the endogenous SOT5, total SOT5 (including endogenous SOT5 and transgene), and two plastid genes in the typical cosuppression lines. c RT-qPCR analysis of the endogenous SOT5 transcript level in the cosuppression lines. d RT-qPCR analysis of the total SOT5 transcript level in the cosuppression lines. e Splicing efficiency of plastid rpl2 and atpF (as a control) in the cosuppression lines. For RT-qPCR, the values are means of three technique replicates (bars indicate SD). Asterisks indicate significant differences between wild type (WT) and transgenic plants (Student’s t test, *, P < 0.05 and **, P < 0.01). Two to three T1 transformants were analyzed for RT-PCR and RT-qPCR.
Figure 3

No chlorosis leaf phenotypes appeared when the mutated SOT5 CDS were overexpressed in rdr6-11 background. a Phenotype of transgenic plants overexpressing SOT5-m1 and SOT5-m2 in rdr6-11 (bar = 1.5 cm). b RT-PCR analysis of mRNA levels of endogenous and total SOT5, and two plastid genes in transgenic lines. c RT-qPCR analysis of the endogenous SOT5 transcript level in transgenic lines. d RT-qPCR analysis of the total SOT5 expression level in transgenic lines. e Splicing efficiency of plastid rpl2 and atpF (as a control) in transgenic lines. For RT-qPCR, the values are means of three technique replicates (bars indicate SD). Asterisks indicate significant differences between wild type (WT) and transgenic plants (Student’s t test, *, P < 0.05 and **, P < 0.01). Two to three T1 transformants were analyzed for RT-PCR and RT-qPCR.
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Figure 4

The phenotypes of transgenic plants overexpressing the truncated EMB2654-11M are dependent on RDR6. a Overexpression of the truncated EMB2654-11M in WT background leads to chlorosis phenotype (bar = 1.5 cm). b RT-PCR analysis of the endogenous and total EMB2654 transcripts level in the cosuppression lines. c RT-qPCR analysis of the endogenous EMB2654 transcripts level in the cosuppression lines. d RT-qPCR analysis of the total EMB2654 transcripts level in the cosuppression lines. e RT-qPCR analysis of the splicing efficiency of plastid rps12 intron1 in the cosuppression lines. f Phenotype of transgenic plants overexpressing EMB2654-11M in rdr6-11 (bar = 1.5 cm). g RT-PCR analysis of the endogenous and total EMB2654 transcripts level in the transformants. h RT-qPCR analysis of the endogenous EMB2654 transcripts level in the transformants. i RT-qPCR analysis of the total EMB2654 transcript level in transgenic lines. j Splicing efficiency of plastid rps12 intron1 in the transformants. For RT-qPCR, the values are means of three technique replicates (bars indicate SD). Asterisks indicate significant differences between wild type (WT) and transgenic plants (Student's t test, *, P < 0.05 and **, P < 0.01). Two to three T1 transformants were analyzed for RT-PCR and qRT-PCR.
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Figure 5

Functional analysis of EMB976 via overexpressing truncated EMB976 CDS in WT a Yellow leaf phenotype of T1 transgenic plants overexpressing the truncated EMB976-14M (bar = 1.5 cm). b RT-qPCR analysis of mRNA levels of the endogenous EMB976 in transgenic lines. c RT-qPCR analysis of mRNA levels of total EMB976 in transgenic lines. d RT-PCR analysis of precursor transcripts of chloroplast genes with specific primers. The number after the decimal point indicates the intron of the gene. e Splicing efficiency of clpP1 intron 2 and ycf3 intron 1 in the cosuppression lines. f Phenotype of T1 plants overexpressing truncated EMB976-14M in rdr6-11 (bar = 1.5 cm). g RT-PCR analysis of mRNA levels of endogenous EMB976, total EMB976, clpP1 and ycf3 precursors in the transformants. h RT-qPCR analysis of mRNA levels of endogenous EMB976 in the transformants. i RT-qPCR analysis of mRNA levels of total EMB976 in the transformants. j RT-qPCR analysis of splicing efficiency of plastid clpP1 intron 2, ycf3 intron 1 and ycf3 intron 2 in the transformants. For RT-qPCR, the values are means of three technique replicates (bars indicate SD). Asterisks indicate significant differences between wild type (WT) and transgenic plants (Student's t test, *, P < 0.05 and **, P < 0.01). Two to three T1 transformants were analyzed for RT-PCR and RT-qPCR.
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