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ORIGINAL RESEARCH

Isolation and characterization of a novel semi-lethal *Arabidopsis thaliana* mutant of gene for pentatricopeptide (PPR) repeat-containing protein

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Abstract A novel *Arabidopsis thaliana* mutant of one member of the pentatricopeptide repeat (PPR) gene family has been identified among T-DNA insertion lines. Tagging of the At1g53330 gene caused the appearance of a semi-lethal mutation with a complex phenotypic expression from embryo lethality associated with the abnormal pattern of cell division during globular to heart transition to fertile plants with just subtle phenotypic changes. The PPR protein At1g53330.1 was predicted to be targeted to mitochondria by TargetP and MitoProt programs. Complementation analysis confirmed that the phenotype is a result of a single T-DNA integration. A thorough functional analysis of this mutant aimed at finding a particular organelle target of At1g53330.1 protein will follow.

Keywords *Arabidopsis thaliana* · Embryonic defect · Mitochondria · Pentatricopeptide repeat · Semi-lethality · T-DNA mutagenesis

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Introduction

The completion of the genome sequence of *Arabidopsis thaliana* L. (Heynh.) (The Arabidopsis Genome Initiative 2000) opened a significant possibility for transition from structural genomics to functional genomics in higher plants. Elucidation of the function of all *Arabidopsis* genes is the goal of ''*Arabidopsis* 2010 Project'' finished by construction of a virtual plant that will enable examination of every aspect of the plant's development (Chory et al. 2000). Many undescribed and often unsuspected genes have been uncovered using bioinformatic tools (Wierling et al. 2002; Svensson et al. 2004). However, such outputs must be regarded as hypothetical in the absence of experimental evidence (Bouché and Bouchez 2001).

Various experimental tools permit investigation of a gene function at the subcellular, cellular, organ or organismal levels. One of the most powerful approaches for determining the biological function of specific genes is the isolation and analysis of mutations, preferably with the T-DNA tag in the gene of interest (Krysan et al. 1999). Establishment of large insertion mutant collections enabled development of reverse genetics strategies as an essential component of functional genomics programs (Azpiroz-Leehan and Feldmann 1997; Krysan et al. 1999; Sussman et al. 2000). However, determining gene function through insertional mutagenesis often fails due to multigene families and unrelated proteins with overlapping function (Blanc et al. 2000). Such multigene families, present as clustered and/or dispersed copies, are particularly frequent in the *Arabidopsis* genome (The Arabidopsis Genome Initiative 2000).

One of the largest gene families is characterized by tandem arrays of pentatricopeptide repeats (PPRs) composed of characteristic 35 amino acid motifs that make up the major

part of each of these proteins. The motif was found in a few animal and fungal proteins (Coffin et al. 1997; Manthey et al. 1998) but the family has greatly expanded in higher plants. The *Arabidopsis thaliana* genome contains about 450 members (Small and Peeters 2000). They represent a considerable proportion (almost 1%) of the *Arabidopsis* proteins for which no obvious function can be assigned by sequence similarity (Aubourg et al. 2000). PPR genes are fairly evenly distributed throughout the 10 chromosome arms, nevertheless the most dense accumulation of PPR genes is located on the long arm of chromosome 1 (around 23 Mb) (Lurin et al. 2004). The vast majority of these proteins are predicted to be targeted to either mitochondria or chloroplasts by the programs TargetP (Emanuelsson et al. 2000) and Predotar (Small et al. 2004), although these programs are not always agreed on which organelle. So far, no evidence was found for PPR proteins targeted to both mitochondria and chloroplasts, suggesting their specific roles in the organelles (Small and Peeters 2000). A very small portion of PPR proteins is predicted as untargeted. They are probably localized outside the organelles of the plant cells (Lurin et al. 2004). Because the PPR motif plays a role in binding to macromolecules such as RNA (Small and Peeters 2000), it is assumed that PPR proteins participate on post-transcriptional RNA modification (Lurin et al. 2004) or may work as coordinators of nuclear and mitochondrial genes expression (Mili and Piñol-Roma 2003).

The functional data available for PPR proteins are rather coherent. Only a few articles describe the functional analysis of individual PPR genes. In *Arabidopsis*, the experimental evidence has been done mainly on chloroplast targeted PPR proteins, such as PGR3 (Yamazaki et al. 2004) or CRR2 (Hashimoto et al. 2003), which are required for regular chlorophyll fluorescence, and HCF152 which affects accumulation of the plastidial cytochrome b_6f complex (Meierhoff et al. 2003). Some genes with PPR motifs isolated from the maize, such as *CRP1* (Fisk et al. 1999) or *PPR2* (Williams and Barkan 2003), show structural similarity to *Arabidopsis* genes. They are also required for the right processing and translation of specific chloroplast RNAs. Recently, Kotera et al. (2005) discovered a significant involvement of CRR4 protein with the PPR motif in editing RNA in chloroplasts.

The known and well-characterized mutants of genes for PPR proteins targeted to mitochondria were mainly induced on non-*Arabidopsis* species. They usually function as restorers of fertility, like *Rf1* in a petunia (Bentolila et al. 2002), *Rf1* in rice (Komori et al. 2004) and *Rfo* in a radish (Brown et al. 2003; Koizuka et al. 2003). All these genes share the ability to prevent expression of proteins encoded by the mitochondrial cytoplasmic male sterility (CMS) inducer gene (Wise and Pring 2002). Oguchi et al. (2004) discovered a subfamily of PPR proteins that are not related to any organelles. These proteins are characterized by a fragment, which was found to be sufficient to regulate circadian rhythmic expression.

In this paper, we describe the isolation and characterization of a semi-lethal mutant in *Arabidopsis thaliana* with the T-DNA insert integrated into a gene coding a member of the PPR family proteins. Special attention is given to the genetic analysis of the mutation and detailed phenotypic analysis from the embryonic stage to seed maturation.

Material and methods

Mutant isolation and growth conditions

The mutant plant was isolated among T_2 *Arabidopsis thaliana* Columbia (Col-0) insertion lines (marked CB_xxxx, where xxxx means number of the line) containing T-DNA from the plasmid pPCVRN4 (Koncz et al. 1994). T_0 plants were transformed by floral dip method in accordance with Clough and Bent (1998). The plants were grown aseptically for up to 20 days in plastic square Petri dishes (120 × 120 mm) with 1% agar MS medium (Murashige and Skoog 1962) and held in a vertical position (75°) under standard conditions (120 μmol m^{-2} s^{-1}, 20°C, 16 h light and 8 h dark). To synchronize germination, the plates with the seeds were kept for 3 days at 4°C. Each plate contained approximately 100 seeds in five rows. Each row represented one individual insertion line. To select plants containing the T-DNA insert, the medium was supplemented with 15 mg l^{-1} hygromycin. Three weeks later the seedlings were transferred to non-sterile conditions and grew to maturity in a controlled climate cultivation chamber, at 20–22°C, irradiation of 70 μmol m^{-2} s^{-1}, and under 16 h light/8 h dark cycles.

Genetic analysis

The mutant was back-crossed to the wild-type plant (wt) Col-0 and Landsberg *erecta* (L*er*) to reduce the number of possible background mutations or other T-DNA inserts, and for later mutant gene mapping (see below). Mutant plants segregated in F_2 were screened for the presence of transgene sequences using the PCR method. The DNA was isolated according to Edwards et al. (1991). The primers ''hpt1'' and ''hpt2'' which specifically amplify the region of 295 bp are described in Scheid et al. (1991). PCR reactions were performed in a 25 μl reaction mixture containing a buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 200 μM of each dNTP, 0.16 μM of each primer hpt1 and hpt2, 1 U of Taq polymerase (*Promega*) and about 50 ng

DNA. The samples were denatured at 94°C for 5 min and amplified using 35 cycles (94°C for 30 s, 55°C for 45 s, 72°C for 2 min) followed by elongation at 72°C for 8 min. The PCR products were analyzed on 2% agarose gels stained by an ethidium bromide in TAE buffer.

The number of T-DNA inserts was screened in T_2 plants, i.e., progeny of a selfed T_1 plant simultaneously with F_2 plants after crossing a mutant with wt (Col) plant. Seeds from the T_1 or F_1 plants were placed on a MS medium containing 15 mg l^{-1} hygromycin. The ratio of resistant and sensitive seedlings was used to estimate the number of independent T-DNA insertions according to Mendelian principles (Ondřej et al. 1999).

For the Southern analysis *Arabidopsis* genomic, DNA (about 1 μg) from the mutant of CB_1265 line and wt plants was digested with 10 units of *Hin*dIII enzyme. The samples were separated on a 2.5% agarose gel and transferred to the nylon membrane (*Boehringer Mannheim*, Germany). The probe used was a 295 bp region of hygromycin phosphotransferase gene (*HPT*) amplified from pPCVRN4 vector sequences with ''hpt1'' and ''hpt2'' primers (Scheid et al. 1991). Cycling conditions were identical as described above. The digoxigenine probe labeling and the immunodetection were done using a DIG luminiscent detection kit (*Boehringer Mannheim*, Germany) as recommended by the supplier. The quality of the labeling was proved by dot-blot hybridization (Sambrook et al. 1989). The chemiluminiscent alkaline substrate CSPD® produced a light signal, which was detected by exposing the membrane to an X-ray film for 12 h.

Embryo test

Embryo-lethal mutations were determined in T_2 and T_3 populations of hygromycin resistant plants by the presence of siliques containing normal and defective embryos in seeds in a ratio of 3:1 (Müller 1963). Three middle contiguous siliques from each T_2 plant were scored for embryo-lethal mutations before the seed coat of normal seeds became brown (Gichner et al. 1994). The seeds from each positively determined heterozygous mutant plant were harvested separately. The next progeny of each mutant plant was checked for a ratio 3:1 in at least 10 heterozygous plants. A 1:2 ratio of wt homozygotes and heterozygotes was tested among the progeny of heterozygous plants in T_2 generation. Immature siliques phenotypes of embryos in seeds were observed and scored under a Nikon SMZ-2T dissecting microscope.

Microscopic study

Embryos in different stages of development were dissected from seed in immature siliques and subjected to a clearing treatment (Mayer et al. 1991) modified by Kyjovská et al. (2003) and viewed with a differential interference contrast (DIC) Olympus BX-60 microscope equipped with Nomarski optics. At least 60 seeds in wt and mutant lines were analyzed. Photographs were taken using an Olympus camera and Lucia 4.21 software (Laboratory Imaging™).

Gene mapping

We have localized the mutant gene position using simple sequence length polymorphisms (SSLPs) markers (Bell and Ecker 1994) on the population of 36 to 48 F_2 plants after crossing them with L*er* wt. In case of mapping the fertile form we have chosen only plants with visible morphological changes (recessive homozygotes). When considering the embryonic-lethal form, owing to the lethality of recessive homozygotes, mapping could not be performed by a mutant allele. Therefore dominant homozygotes were used for mapping by means of wt allele.

Microsatellites were amplified from genomic DNA isolated from 2 week old whole seedlings (Edwards et al. 1991) in 20 μl reactions under standard cycling conditions described for SSLP mapping (Bell and Ecker 1994). Oligonucleotide sequences and other information about markers are accessible via TAIR (http://www.arabidopsis.org). The samples were separated on 3–4% agarose gels. The recombination rate (r, %) between the gene of interest and a DNA marker was converted into the map distance (D, cM) by the Kosambi's mapping function $D=25\ \ln(100+2r/100-2r)$ (Kosambi 1944).

Amplification of T-DNA tagged plant DNA fragments by iPCR

To determine the sequences of plant DNA adjacent to the T-DNA, insert we have modified a method described in Mathur et al. (1998). Plant tissues were harvested prior to flowering and DNA was purified with cetyl-trimethyl-ammonium bromide (CTAB) precipitation (Roger and Bendich 1988). 2–3 μg of total plant DNA was digested with 50 U of restriction endonuclease *Hind*III or *Xba*I, for at least 2 h at 37°C in a volume of 100 μl. After testing 10 μl aliquots by agarose gel electrophoresis, the samples were phenol/chloroform extracted and precipitated with i-propanol (Sambrook et al. 1989). After self-circularization by ligation 0.5 μg of plant DNA was subjected to PCR amplification using primer RB1 (5′-CAA AGC GAA CCA CCA GCT TAC CCG TCC ATC GGC-3′) facing the T-DNA right border, and either primer lb2 (5′-GAC CCT TAC CGC TTT AGT TCC GTA GCT AGC ACT TC-3′) at the left border, or primer PC3 (5′-CCT TGC GCC CTG AGT GCT TGC GGC AGC-3′) at the *Xba*I site. PCR

reactions were performed in 20 µl using Long and Acurate (LA) PCR polymerase (Top-Bio) as recommended by the supplier. DNA samples were denatured at 95°C for 2 min, and amplified using 35 cycles (94°C for 30 s, 65°C for 30 s, 72°C for 8 min) followed by elongation at 72°C for 10 min. The PCR products were resolved on agarose gels and isolated using an UltrafeeDA kit (Millipore). When no amplified DNA fragment was detected, a second PCR amplification was performed using 1 µl from a 500-fold diluted first PCR reaction mixture in combination with the nested primers RB2 (5′-TGC CTC TAC CGA CAG TGG TCC CAA AG -3′), lb4 (5′-AGA GGT ATA ACT GGT AGT ATG AG-3′) or PC4 (5′-CTT GCG GCA GCG TGA AGC TAG CTT C-3′). The isolated PCR fragments were used directly as templates for sequencing on CEQ™2000 sequencer (Beckman Coulter). Sequence analyses were performed using the GCG and BLAST computer program packages, as described for Genbank database searches with ESTs (Newman et al. 1994).

Complementation of the CB-1265 mutant

The At1g53330 sequences were amplified by PCR from *A. thaliana* wt Col-0 genomic DNA. The primers were designed to amplify either intact gene including promoter and terminator sequences or the full-length coding DNA sequence (CDS). The CDS was amplified using primers *PPRcds5′ApaI* (5′-CGG GGC CCA TGT CCG CCG TGA AAT C-3′) and *PPRcds3′XbaI* (5′-CTT CTA GAC TAG CAT TGT GGC ATT GCT G-3′). The 1.43 kb PCR product digested with *Apa*I and *Xba*I was cloned in between a cauliflower mosaic virus 35S promoter and a polyA signal within vector pLV-68, a derivative of pRT100 (Töpfer et al. 1987). This PPR expression cassette was than cloned as an *Asc*I-*Pac*I restriction fragment into a binary vector pLV-07 (Vrba and Matoušek 2005) resulting in pLV-76. The intact gene was amplified using primers *PPRwt5′EcoRI* (5′-CGG AAT TCG TCC ATT ACA AAC CCT TC-3′) and *PPRwt3′BglII* (5′-CCA TCT CAA GAT CTA CGC ACG-3′) and the resultant 2.3 kb PCR product digested with *Eco*RI and *Bgl*II was ligated into *Eco*RI and *Bam*HI digested vector pLV-07 resulting in binary vector pLV-77. Binary vectors pLV-76 and pLV-77 were then introduced into the *Agrobacterium tumefaciens* strain LBA 4404 by the freeze and thaw method (Holsters et al. 1978). A floral dip method (Clough and Bent 1998) was used to transform the mutants or their heterozygotes identified by an embryo test (Müller 1963). Transformed plants were selected on agar plates containing 0.5 × MS medium with hygromycin (20 mg l^{-1}) together with kanamycin (50 mg.l^{-1}). The phenotype of resistant plants was scored 3 weeks after plating. Selected plants were grown further on soil to maturity.

Expression studies

The expression of the At1g53330 gene was evaluated by RT-PCR or Northern hybridization (verification of complementation). Total RNA was isolated from young seedlings, rosette leaf or flower tissue with RNeasy kit (Quiagen). 25 ng of mRNA per 10 µl reaction were reverse transcribed with 25 U of M-MLV reverse transcriptase (Top-Bio) with primers *PPRcDNA-F* (5′-TGA AGG AAG CAC TGA AGA TGA A-3′) and *PPRcDNA-R* (5′-CGG AAT CAT TCT CAA CAC AGA A-3′). The PCR conditions were 94°C for 1 min, followed by 30 cycles (94°C for 15 s, 55°C for 15 s, 72°C 30 s) and finally 72°C for 5 min. The RT-PCR products were validated by size verification after electrophoresis on agarose gels.

A Northern analysis with the probe obtained by PCR with primers *PPRcDNA-F* and *PPRcDNA-R* was carried out, as described by Sambrook et al. (1989), using high stringency hybridization conditions (6 × SSC, 5 × Denhardt, 1% SDS, 20 µg ml^{-1} salmon sperm DNA, 50% formamide at 42°C overnight) and moderate stringency washing conditions (2 × SSC, 0.1% SDS at 65°C, 2 × 15 min, followed by 1 × SSC, 0.1% SDS at 65°C, 15 min).

Results

Mutant isolation

The mutant line (marked CB_1265) was isolated after the screening of 2500 *Arabidopsis* individual insertion T_2 lines for morphological abnormalities (mainly visible on roots) during the first 14 days following germination.

The deviations in growth and development of the CB_1265 mutant plants appeared already at the initial growth. First, the germination was completely inhibited (about 30–40% of the T_3 seeds in each line) or distinctly delayed in comparison with wt plants. The next development of delayed mutant seedlings varied further on an agar medium. According to its phenotype deviations, three classes of mutant plants were observed within the CB_1265 T_2 line as well as in the offspring of fertile mutant plants. (i) Seedlings with marked changes of phenotype (about 10%) had inhibited shoot apical meristem, developed dwarfed cotyledons and short roots with abundant hairs (Fig. 1a). The plants died usually 2 weeks after germination. (ii) Other mutant plants (about 30%) from the same line developed normal cotyledons, but later on they elongated distinctly in comparison to wt and became rather fragile. The rosette leaves were slightly narrower and curved (Fig. 1c). The main roots were shorter (60% of the length of wt roots) and developed long lateral roots. After transfer to non-sterile conditions, the plants formed normal

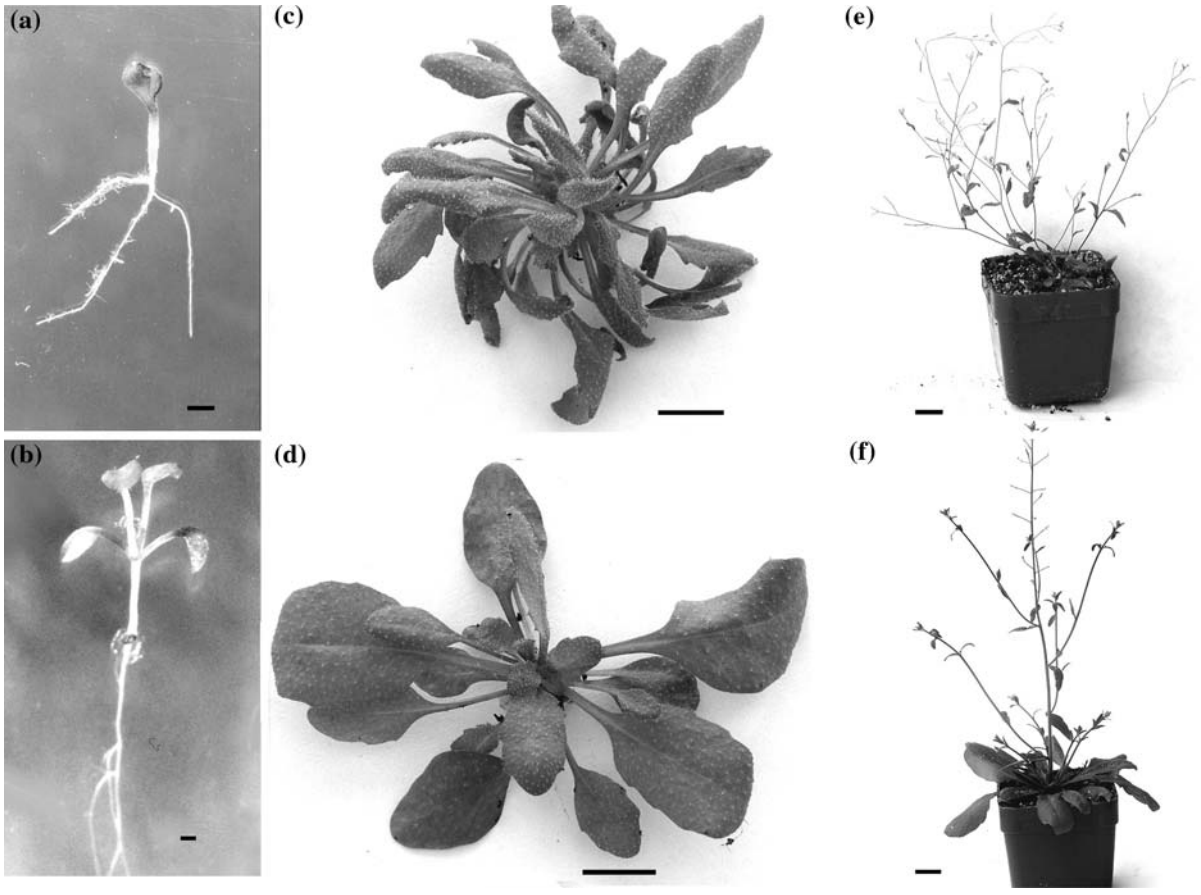

Fig. 1 The phenotype of the CB_1265 mutant at different growth stages compared to wild-type (wt) (Col-0). 14 days old seedlings of mutant **a** and wt **b** plants, Bar=1 mm; 30 days old rosettes grown under short days conditions (8 h light/16 h dark) of mutant **c** and wt **d** plants, Bar=1 mm; 45 days old mature mutant **e** and wt **f** plants, Bar=10 mm

flowers and produced shorter fertile siliques. The plants usually produced shoots with reduced apical dominancy and elongated internodes in comparison to wt (Fig. 1e). (iii) The last group of mutant plants (about 20–30%) had very subtle morphological changes and was identified just from late-germinating seeds.

The range of phenotypic changes was also evaluated in the following generations (T_4–T_6) of selfed fertile mutants as well as after back-crossing with wt plants. As seen in Table 1, the complex phenotypic expression remained also in the progenies of the mutant plants although a minor decrease of embryo-lethals after back-crossing was observed.

Microscopic study

Because of the lethality of some mutants described in the previous section, we have also provided microscopic studies in order to find a developmental defect in the embryonic stage. Unlike the immature green seeds in wt siliques, the mutant immature seeds remained white or pale green and were often shriveled. Microscopic studies after the clearing treatment revealed the defect at the globular stage of embryogenesis characterized by an abnormal pattern of cell division (Fig. 2c–e) in comparison with the organized cell division in wt embryos (Fig. 2a, b). In the globular to heart transition stage, cell expansion in the apical region of immature embryo was observed.

Genetic analysis

The Southern blot analysis revealed that the mutant plants from the CB_1265 T_2 as well as back-crossed lines contained just one insert of T-DNA (data not shown). The results of hygromycin-resistance tests (hyg^R:hyg^S=90:47) correspond to the 2:1 ratio (χ^2=0.03) and are also in agreement with the presence of one T-DNA insert for embryonic-lethal mutation. In the progeny of heterozygous plants, dominant homozygotes and heterozygotes were determined in a ratio of 19:8, decreasing of heterozygotes in comparison with the expected 1:2 (χ^2 = 16.67) and also with a 1:1 ratio (χ^2=4.48). The ratio between wt and mutant immature seeds in siliques of heterozygous T_2 plants corresponds to a 1:1 ratio, while in F_1 or F_2 after backcrosses

Table 1 Phenotypic analysis of CB_1265 mutant plants grown on agar medium 14 days after germination of wt control plants

	T_2	T_3 a[b]	T_3 b[c]	F_2[d] CB_1265×Col-0	F_2[e] Col-0×CB_1265
No. of identified mutant plants[a]	187	55	73	58	80
Not-germinated seeds (Embryo lethals)	60 (32%)	26 (47%)	31 (42%)	14 (24%)	14 (17%)
Seedling lethals	23 (12%)	5 (9%)	7 (10%)	8 (14%)	11 (14%)
Fertile plants with strong phenotype	71 (38%)	14 (26%)	27 (37%)	20 (35%)	35 (44%)
Fertile plants with minor phenotypic changes	33 (18%)	10 (18%)	8 (11%)	16 (27%)	20 (25%)

[a] All mutant plants were checked for the presence of *HPT* gene from the pPCVRN4 (Koncz et al. 1994) vector plasmid

[b] An offspring of T_2 mutant plant with strong phenotype

[c] An offspring of T_2 mutant plant with minor phenotypic changes

[d] F_2 generation after back-cross of CB_1265 mutant line with pollen from Col-0 wt plant

[e] zF_2 generation after back-cross of Col-0 wt plant with pollen from CB_1265 mutant line with strong phenotype

with wt Col-0 the expected 3:1 ratio was found, as shown in Table 2.

Gene mapping

To be sure that the ambiguous mutant phenotype was due to a mutation at a single locus, we have mapped simultaneously fertile recessive homozygotes without strong embryonic defects and heterozygotes with respect to lethal embryonic defects. In both cases, linkage with nga 280 marker was detected (r=5.8±2.6 %, D=5.8±3.5 for mapping recessive homozygotes); (r=8.4±3.2 %, D=8.5±4.3 for mapping heterozygotes). The location of the mutation was assigned to chromosome 1 in position 91.0±4.9 cM in a genetic map derived from recombinant inbred lines (Lister and Dean 1993).

Isolation of plant DNA adjacent to the T-DNA insertion site

Segregation analysis of the mutant phenotype and the hygromycin resistance revealed that the T-DNA insert in CB_1265 line is tightly linked to the mutant locus. To determine the genomic region flanking T-DNA borders we have provided an inverse PCR (iPCR) approach followed by sequencing the products. The number of obtained iPCR fragments confirmed the presence of one T-DNA insertion. We have amplified one 1.3 kb DNA fragment after digestion with *Hind*III and 0.6 kb fragment after digestion with *Xba*I at the left border site and 0.2 kb at the right border of T-DNA. The sequencing and BLAST search revealed that the insert was integrated into chromosome 1 and tagged gene coding for PPR containing protein At1g53330 in the region behind the nucleotide 1054 (19900749 bp on AGI map) downstream of the start codon (Fig. 3). The T-DNA integration site is 976 kb distant from marker nga 280 (on AGI map) which showed genetic linkage in previously described mutant gene mapping.

Complementation of the CB-1265 mutant

Due to lower fertility and viability of mutant plants we tried to transform not only fertile mutants with clear phenotypic changes (recessive homozygotes) but also heterozygotes identified by the embryo test. (Plants with no defective embryo were scored as wt homozygotes and plants with defective embryos were scored as heterozygotes.) The results confirmed very low efficiency when transforming mutant plants. Only two transgenic seedlings (both with pLV-76 T-DNA) were detected among approximately 24,000 seeds (480 mg) resulting from the transformation of 12 plants (efficiency 0.008%). The phenotype was clearly identical in comparison with Col-0 control plants. After the transformation of heterozygots, the efficiency was quite higher. Forty-six transformants were obtained by transformation with construct pLV-76 (the efficiency was 0.128%) and 29 in the case of transformation with construct pLV-77 (the efficiency was 0.115%). All detected transformants were resistant simultaneously to hygromycin and kanamycin. The presence of both marker genes was checked by PCR. To compare phenotype of double transgenic plants with wt Col-0 plants after hetrozygots transformation, we have taken into consideration the fact that just about one fourth of the plants were expected to represent recessive homozygots for At1g53330 gene mutation. We have scored morphological traits of transformed plants from seed germination to seed production with special attention to embryo microscopic observations. All plants transformed with pLV-76 T-DNA (At1g53330 cDNA driven by 35S promoter) had complemented phenotype comparable to Col-0 controls. The seed germination of transformed plants was not delayed significantly, as was observed for CB_1265 mutants. Also an embryo test confirmed the presence of no defective embryos. During the screening of T_1 plants after transformation of heterozygotes with pLV-77 T-DNA, five plants with defects similar to that observed in CB-1265 line were

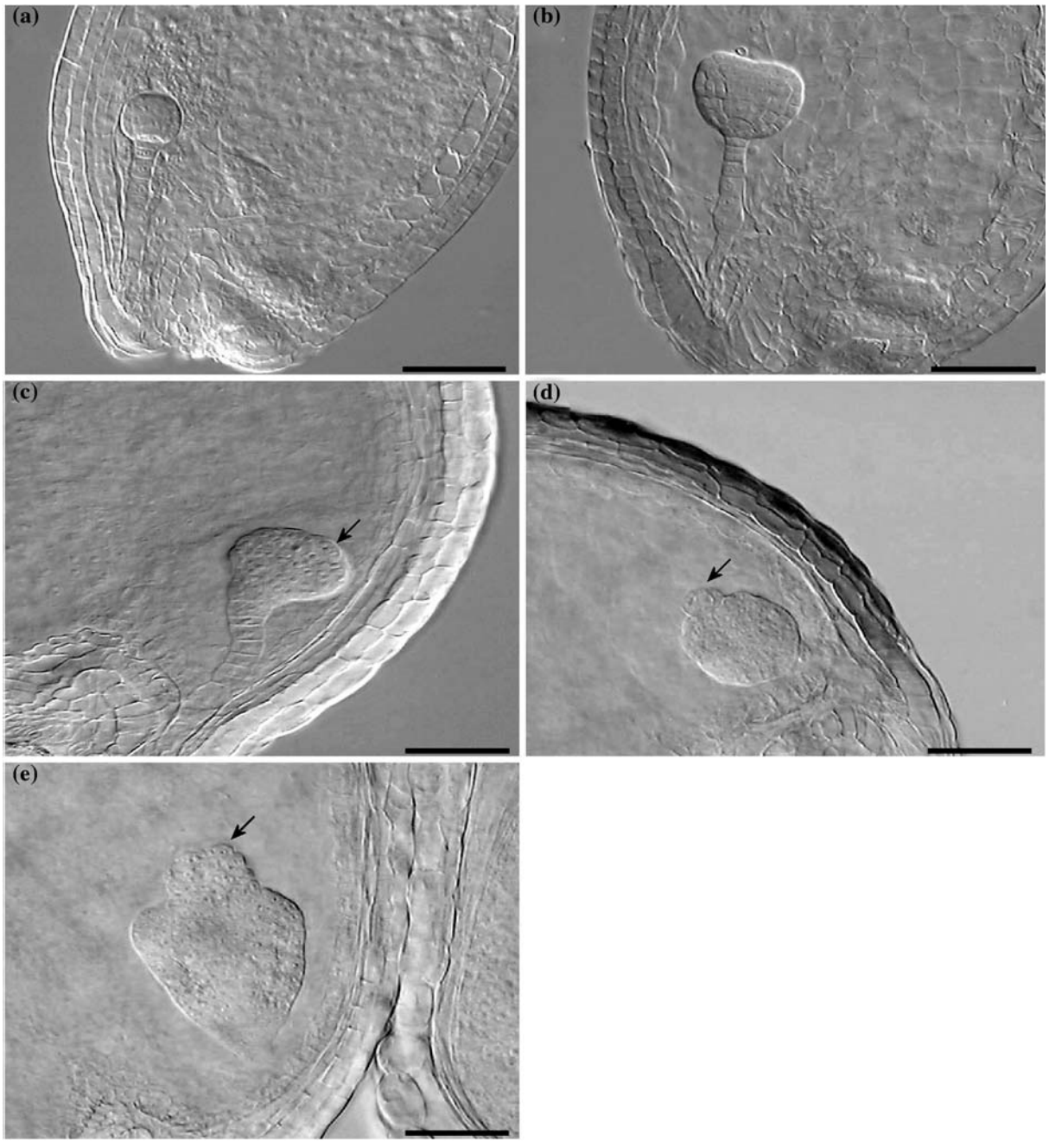

Fig. 2 Embryos of *Arabidopsis thaliana* seeds dissected from immature siliques observed with DIC microscopy after clearing treatment. wt embryo of ecotype Columbia (Col-0) at globular **a** and globular to heart transition stage **b**; CB_1265 mutant embryos at globular **c**, **d** and globular to heart transition stage **e**. The arrow shows the observed defects caused by T-DNA insertion. Bar=100 μm

found. They grew slower in comparison to wt Col-0 control, developed prolonged cotyledons with broad petioles and produced few siliques on many secondary stems. Also embryonal defects were observed comparable to that in the CB_1265 line.

RT-PCR verified that there was no At1g53330 mRNA production in the homozygous mutant plants—in all forms described above. Expression in wt plants was detected in flower and seedling tissue, whereas in leaf tissue only a very slight RT-PCR product was found. In contrast to plants with pLV-77 T-DNA, pLV-76 transformed plants had clear RT-PCR products in all tissues. Northern analysis confirmed the results from RT-PCR, except wt plants that showed merely a very slight At1g53330 gene expression (Fig. 4).

Table 2 Results of embryo-test performed on heterozygous CB_1265 plants[a]

	T_3	F_1 CB_1265 × Col-0	F_1 Col-0 × CB_1265	F_2 CB_1265 × Col-0	F_2 Col-0 × CB-1265
wt	486	610	577	1109	848
Mutant	456	190	179	343	274
Ratio tested	1:1	3:1	3:1	3:1	3:1
χ^2	0.96	0.67	0.71	1.47	0.20

Individual immature seeds were evaluated

[a] Plants with no defective embryo were scored as wt homozygotes and plants with defective embryos were scored as heterozygotes

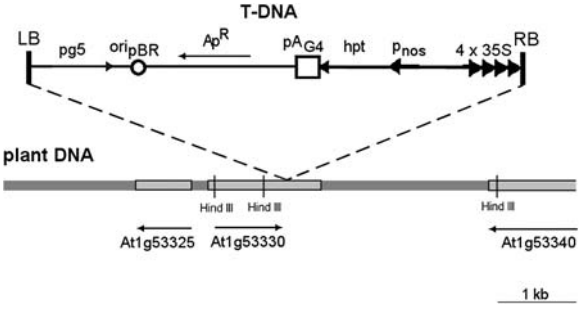

Fig. 3 A schematic map of T-DNA integrated in the *Arabidopsis* At1g53330 gene. The pPCVRN4 T-DNA contains following sequences (Koncz et al. 1994): pg5—promoter of T-DNA gene 5; ori_{pBR} - pBR322 replication origin; Ap^R—ampicillin resistance gene; pA_{g4}—polyadenylation sequence of T-DNA gene 4; hpt—hygromycin phosphotransferase gene; pnos—promoter of the nopaline synthase gene; 4×35S en—tetramer of enhancer of 35S promoter from CaMV virus. To isolate the T-DNA arms in conjunction with plant DNA sequences by inverse PCR (iPCR), the plant DNA was digested with *Hind*III enzyme and self-ligated. The amplified iPCR product was isolated from the agarose gel. Plant DNA segments linked to the T-DNA arms were sequenced using primers lb2/lb4 at the left T-DNA border (LB), and RB1/RB2 primers at the right T-DNA border (RB)

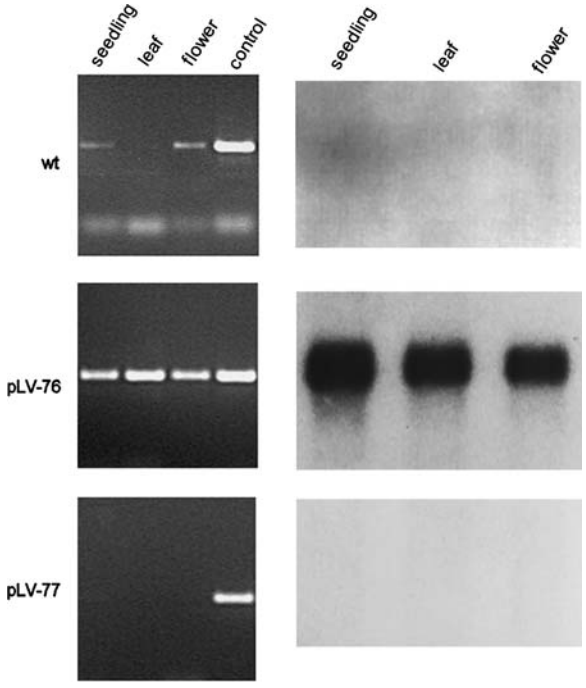

Fig. 4 RT-PCR (left) and Northern (right) analysis of the At1g53330 gene in wt and plants which were transformed by pLV-76 and pLV-77 constructs. The size of RT-PCR products was 1054 bp. As a positive control PCR products from a DNA template amplified by the same primers as used for RT-PCR were loaded

Bioinformatic analysis of the tagged PPR At1g53330 gene

The At1g53330 gene is 1416 bp long and is composed of eight repeats of a classical PPR motif (P) together with one short PPR motif (S) classifying it to a subfamily P. The constitution of the PPR repeats together with their alignment is shown on Fig. 5. The gene is located on chromosome 1 on the shorter arm in the region with infrequent occurrence of other PPR genes. The PPR protein At1g53330.1 was predicted to be targeted to mitochondria by TargetP program, whereas Predotar did not find any targeting signal peptides. The score of the targeting prediction of these two programs is shown in Table 3. MitoProt software (Claros and Vincens 1996) confirmed targeting of At1g53330.1 PPR protein to mitochondria with probability 0.9048.

Discussion

Mutant isolation and genetic analysis of the mutant

Although the vector plasmid pPCVRN4 was constructed for the activation tagging approach (Koncz et al. 1994), a recessive mutant has been isolated in our work. Gain-of-function mutations remain still rare, but this type of mutation could be very useful for elucidating the function of unknown genes that have redundant partners in the genome. However, getting a dominant mutation depends strongly on the site of integration (Weigel et al. 2000). In any case, the occurrence of recessive mutants is more obvious. A similar spectrum of mutants with more than 70% recessive knock-out mutations was also described in

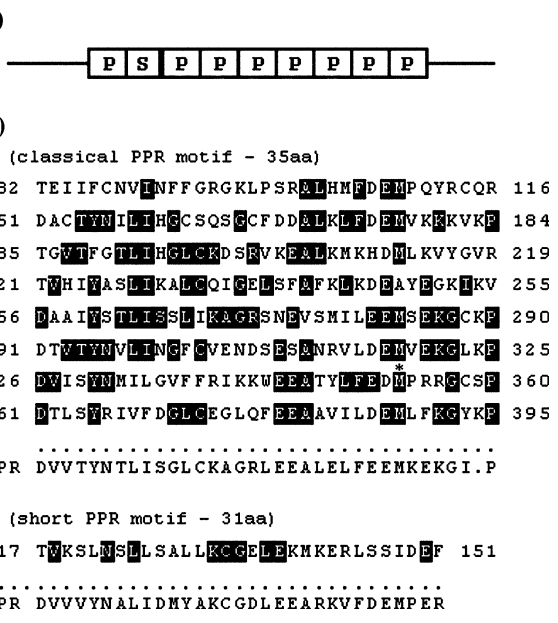

Fig. 5 Schematic structure of the At1g53330 gene showing the order of classical (P) and short PPR motifs (S) **a** and alignment of all identified PPR motifs **b**. Residues identical to the consensus motif (shown at bottom under dotted lines) are shaded in black. The site of the T-DNA insertion in CB_1265 mutant is marked by * above the amino acid symbol

Table 3 The scores for the presence of the targeting signal peptides of the At1g53330 PPR gene

	Predotar v. 1.03	TargetP v. 1.01
mTP[a]	0.18	0.525
cTP[b]	0.00	0.482
SP[c]	0.01	0.066
Other[d]	0.82	0.037
Prediction	None	M[e]
RC[f]	ND[g]	5

[a] Mitochondrial targeting peptide prediction score

[b] Chloroplast transit peptide prediction score

[c] Signal peptide prediction score

[d] Prediction score for the occurrence of signal peptide outside organelles

[e] Target of the peptide M=mitochondria

[f] Reliability class; a measure of the size of the difference (diff) between the highest and the second highest output scores. The lower value on the RC, the safer the prediction on that particular sequence. There are five reliability classes (RC 1 is for diff > 0.800; RC2: 0.800 > diff > 0.600; RC3: 0.600 > diff > 0.400; RC4: 0.400 > diff > 0.200; RC 5 is for diff < 0.200)

[g] Not included in the Predotar v. 1.03 output

another activation tagging collections (Ogarkova et al. 2001).

Co-segregation analysis revealed that the altered phenotype of our mutant was due to a T-DNA insertion. Usually, a fair proportion of the mutations observed are not linked to a functional T-DNA insert (Feldmann 1991). This is probably due to an erroneous, uncompleted genetic repair process supporting T-DNA integration (Koncz et al. 1992).

The inverse PCR (iPCR) method was applied for cloning the sequences flanking both border repeats of T-DNA. We have cleaved the genomic DNA of mutant plants separately by two enzymes. One of them (*Hind*III) does not cleave within the T-DNA insert and the other (*Xba*I) has restriction sites within T-DNA. This strategy was chosen because not all fragments are usually successfully sequenced using the iPCR approach. It is probably due to irregular integration of T-DNA that can involve nicking the left border (Tinland et al. 1994). The occurrence of only one specific iPCR product as well as Southern blot analysis confirmed the presence of one insertion site of T-DNA in the plant genome.

All sequenced iPCR products indicated that the T-DNA insert was integrated into the At1g53330 gene on chromosome 1 which encodes for a protein containing tandem arrays of PPRs.

Characterization of the tagged PPR gene

The identified gene encoding PPR protein At1g53330.1 belongs to a large family along with 441 members as revealed computational analysis of the *Arabidopsis* genome (Small and Peeters 2000). Lurin et al. (2004) have provided a detailed bioinformatic analysis of all *Arabidopsis* genes for PPR proteins together with expression studies. Although PPR genes are relatively evenly distributed throughout all 10 chromosome arms, obvious clusters are located in some regions. The densest grouping of PPR genes lies on chromosome 1 (around 23 Mb; 19 genes and several probable pseudogenes lie within little more than 1 Mb). Assignment of the At1g53330 gene on the chromosome map shows that this gene is not a member of such a cluster. The prediction of organelle targeting of PPR protein At1g53330.1 was rather ambiguous. The protein is targeted to mitochondria according to TargetP (value 0.525; available at http://www.cbs.dtu.dk/services/TargetP) and MitoProt (value 0.905; available at http://ihg.gsf.de/ihg/mitoprot.html) programs, whereas Predotar (http://genoplante-info.infobiogen.fr/predotar) did not find any targeting signal peptides (Table 3). In any case, the possibility of non-targeting of the At1g53330.1 protein is very low. Just a fractional proportion of PPR proteins is assumed to be localized outside the organelles of plant cells as described in Oguchi et al. (2004). According to Lurin et al. (2004) the level of expression of the At1g53330 gene is relatively low in leaves and also in flowers in comparison to other PPR genes.

Phenotype analysis of the PPR mutant

The function of PPR proteins is not known well, so studying individual mutants can contribute to finding its role in plants. In general, it is assumed that PPR proteins play constitutive, often essential roles in mitochondria and chloroplasts, possibly via binding to organellar transcripts (Small and Peeters 2000).

Existing functional analyses of mutations of PPR genes result mainly from studies of individual mutants (Bentolila et al. 2002; Hashimoto et al. 2003; Koizuka et al. 2003; Meierhoff et al. 2003; Williams and Barkan 2003; Komori et al. 2004; Oguchi et al. 2004; Yamazaki et al. 2004) induced by forward genetics approaches. Such mutants regardless of the plant object were usually characterized from its biochemical or physiological properties. Morphological abnormalities were observed in just a few cases. In non-*Arabidopsis* species, Williams and Barkan (2003) described the phenotype of maize *PPR2* mutant with defects in plastid ribosome accumulation. Lack of plastid rRNA and translation products resulted in albino seedlings and also ''ivory'' phenotype of leaves. Concerning *Arabidopsis* PPR mutants, a majority of them do not have any obvious macroscopic phenotype. Meierhoff et al. (2003) observed phenotypic changes under specific conditions on *hcf152* mutants. The seedlings were lethal under autotrophic growth conditions on soil, but on a sucrose-containing medium they developed similarly as wt plants. Similarly to maize *PPR2* mutant (Williams and Barkan 2003), the phenotype of *hcf152* plants arose from the loss of parts of the photosynthetic apparatus (cytochrome b_6f complex). Furthermore, some other PPR mutants were identified during direct genetic screens for embryo-lethal mutations in the frame of the *Arabidopsis* Seed Genes project (Tzafrir et al. 2003). These results indicated that many PPR genes have an essential function that leads to the abortion of the embryos if the genes are affected. Reverse genetics screens (Henikoff et al. 2004) seem to be more likely to compare the phenotypic effects on PPR mutants. Sixteen embryonal and also one pigment defect for mutants of PPR genes are noticed in ''The Arabidopsis Information Resource'' (TAIR) database (http://www.arabidopsis.org). There is no connection between embryonal defect occurrence and protein target. The first attempt to perform a systematic functional reverse screen of PPR mutants was done by Lurin et al. (2004). They characterized 25 insertion mutations corresponding to 21 members of PPR family genes (the At1g53330 gene was not included). Among them six mutants were lethal in the early stages of embryo development. For the 19 remaining PPR mutants, homozygous plants were found, but a clear visible phenotype was observed just in five mutant lines: three of them present short siliques and one exhibited a ''slow growth'' phenotype.

In our case, the segregation analysis in T_2 and following generations as well as the segregation after the back-cross with wt plant revealed that the mutant gene is required for the embryo development at the transition from globular to heart stage. A crucial mechanism of embryo development is suppressed at this stage that blocks its further development. This is probably connected with shoot apical meristem initiation and maintenance during embryogenesis (Jürgens 1995). However, the gene knock-out does not affect all the embryos. Some plants are able to regenerate if they pass the heart stage and develop cotyledons (hence the term semi-lethal). We could find approximately an equal proportion of embryo-lethal plants as well as fertile plants either with many pleiotropic effects or just with slightly changed morphology. However, most of the mutants started to grow with a distinctive delay in comparison to wt. The plants, which were able to develop cotyledons, continued in their growth and produced fertile siliques.

Complementation of the CB-1265 mutant

The question as to whether the disrupted At1g53330 gene is indeed responsible for the observed phenotype or if the morphological changes are the result of at least two mutational events had to be answered after the phenotypic analysis. Two approaches are usually offered: complementation of the mutant phenotype or a comparison with another insertion line with the T-DNA tag in the same gene.

We have prepared two T-DNA constructs with the At1g53330 gene: (i) CDS driven by a 35S promoter (pLV-76); (ii) CDS with native promoter sequences (pLV-77). Because the tagged mutant plants already contained the hpt gene for selection on hygromycin, we have included nptII gene in our complementation constructs. Due to uncertain expectations of successful transformation of recessive homozygots we have transformed also heterozygotes identified by the embryo test. Luckily, we have identified two T_1 double transgenic plants after direct transformation of recessive homozygotes, both with pLV-76 T-DNA. The phenotype in T_1 as well as in T_2 generation was clearly comparable to wt plants. pLV-77 transformants were selected only after transformation of heterozygots. Out of 29 plants growing on a medium supplemented with antibiotics, 24 were of wt phenotype but five of them showed similar morphological changes as did recessive homozygots in CB_1265 line. This proportion is in agreement with the expected 3:1 ratio for when complementation does not occur after transformation of heterozygotes (χ^2=0.93). It seems that the sequences, which we have amplified as a

native promoter were not sufficient to make At1g53330 gene again fully functional. The gap between the neighboring At1g53325 gene and At1g53330 translational start codon is 394 bp long and we have cloned a region of 282 bp for pLV-77 T-DNA construction. Probably also other sequences are necessary for the right At1g53330 gene expression. TSSP-TCM software suitable for plant promoters searching (Shahmuradov et al. 2005) did not show us any results for the At1g53330 gene.

Lurin et al. (2004) found that expression of *Arabidopsis* PPR genes is usually at low levels. They detected a much higher proportion of transcripts from the PPR subfamily than from P L-S subfamily genes. Evidence of expression of the vast majority of PPR genes can be obtained but the results are dependent on the technique used. We have detected clear RT-PCR products only in samples from pLV-76 complemented plants. Wt plants gave clear RT-PCR products only for RNA isolated from flower tissue. Northern analysis confirmed At1g53330 gene expression also only in pLV-76 complemented plants, the signal in wt plants was too weak. We compared data also from expression analysis performed by Ian Small's lab (personal communication) for the other 15 PPR genes in which mutations caused embryonal defects (according to a phenotypic analysis published by Lurin et al. (2004)). It showed that levels of their expression are very differential. Only four of them had an expression detectable by RT-PCR in leaves and for seven genes no RT-PCR product was obtained from any tissue.

Nevertheless, the main question still remains why the expression of the phenotype is so complex? Several explanations are possible. One of them could be the presence of the enhancer of the 35S promoter at the right border of the T-DNA insert. It was found that genes located up to 8.2 kb away from the enhancer sequence can be activated (Ichikawa et al. 2003). Regarding the At1g53330 gene we could consider an influence on the At1g53310 gene coding phosphoenolpyruvate carboxylase (EC4.1.1.31), At1g53320 gene coding for phosphodiesterase, At1g53325 gene coding for a protein interaction domain, and an At1g53345 hypothetical protein. More likely, a strong maternal effect on embryo development occurs in our mutant line. It seems that the mutant phenotype correlates in some way with the parental phenotype. When assuming that the At153330.1 PPR protein is targeted to the mitochondria, the sorting and selection of mitochondrial genomes (Budar et al. 2003), that are more compatible with the mutation, should be taken into account. Maternal programming of embryonic morphogenesis was thought to be exclusive for animal eggs, but recently also *Arabidopsis* mutants with a pronounced maternal effect on embryo development were described (Ray et al. 1996; Grossniklaus et al. 1998). The phenotypic variability could also be due to more trivial reasons such as chromosomal rearrangements (Nacry et al. 1998) or epigenetic effects (Madlung et al. 2002). To distinguish among different hypotheses, we need to continue with backcrosses to examine the inheritability of the phenotypic variants and preferably find another mutant alleles for comparison. Four lines with insertion in the At1g53330 gene were searched out at the Salk Institute Genomic Analysis Laboratory (SIGnAL) using the ''T-DNA Express *Arabidopsis* Gene Mapping Tool'' (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al. 2003), but only one line is currently available through the ''*Arabidopsis* Biological Resource Center'' (ABRC) or the ''Nottingham *Arabidopsis* Stock Center'' (NASC). This line marked SAIL 553_B_08 (TAIR accession 1006534498) shows a phenotype without any obvious morphological changes. The reason could be the fact that the T-DNA is integrated very closely but not directly within the At153330 gene. Thus, some gene interference between T-DNA and the plant gene may be supposed but a direct gene knock-out has not occurred.

An investigation of the role of PPR genes can bring new insight into functional genomics. An extensive search for *Arabidiopsis* mutants affected in the expression of particular PPR proteins as well as a more detailed look at protein action, the affinity to RNA or other protein molecules, will be needed to elucidate the exact functions of one of the largest and least understood protein families in plants.

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