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Characterization of *Arabidopsis thaliana* lines with T-DNA insertions in the mitochondrial ribosomal protein genes *Rps14* and *Rps19*

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Abstract

In *Arabidopsis*, most of the genes encoding mitochondrial ribosomal proteins are located in the nucleus and only seven are present in the mitochondrial genome. Assembly of a functional ribosome requires coordinated expression of ribosomal protein encoding genes located in both these organelles. Genes and promoters of nuclear encoded mitochondrial ribosomal protein coding genes of plants have not been well characterized so far. In the present study we have characterized *Arabidopsis thaliana* SALK mutant lines with T-DNA insertion in *Rps14* or *Rps19* gene. The location of T-DNA insertion in the mutant lines was confirmed and plants homozygous and hemizygous for T-DNA insertion were identified for both *Rps14* and *Rps19* genes. In homozygous T-DNA mutant lines of both *Rps14* and *Rps19* genes, the expression was estimated using RT-PCR. *Rps14* and *Rps19* transcripts similar to wild type were present in homozygous mutant plants of *Rps14* and *Rps19* which indicated that T-DNA insertion has not affected their expression.

Key words: T-DNA, mitochondria, ribosomal protein genes, *Arabidopsis thaliana*

Introduction

Mitochondria are DNA-containing, semi-autonomous organelles of the eukaryotic cell and are bounded by a double membrane. They are the sites of synthesis of ATP through oxidative phosphorylation and are therefore called the 'power houses' of the cell. According to endosymbiotic theory of origin of mitochondria (Margulis 1970), the progenitor eukaryotic cell engulfed an α-proteobacterium, and during the establishment of endosymbiotic relationship, most of the genetic material of the bacterium was transferred to the nucleus of the eukaryotic cell (Gray et al. 2001). Mitochondrial genome size varies widely among plants (200-2400 kb) and is 10 to 100 times larger than their animal counterparts (Palmer 1990, 1992). However, the gene content of mitochondrial genome of plants is modest (around 60 protein coding genes) and is less variable among species. For example, *Arabidopsis thaliana* mitochondrial genome is 366 kb and has 57 protein coding genes (Unseld et al. 1997) whereas the 1685 kb cucumber mitochondrial genome has only 37 protein coding genes (Alverson et al. 2011).

Each cell contains several (100 to 1000) mitochondria and mitochondrial genome replication occurs independently of the nuclear genome. Further, mitochondria have an active protein synthesis machinery and synthesize proteins encoded by the mitochondrial genome. Mitochondria assemble their own ribosomes for translation of mRNAs encoded by the mitochondrial genome. Mitochondrial ribosomes are made up of rRNA and ribosomal proteins. All rRNA genes are encoded by mitochondrial genome but most of the ribosomal protein genes are nuclear encoded and transported back to the mitochondria. Due to various events of gene transfer from mitochondria to the nucleus, the number of ribosomal proteins encoded by the mitochondrial genome varies among different species. For example mitochondrial genomes of *A. thaliana, Beta vulgaris* and *Oryza sativa* have 7, 6 and 11 genes coding for mitochondrial ribosomal proteins (Kubo et al. 2000; Unseld et al. 1997; Notsu et al. 2002). To constitute a functional ribosome, all ribosomal proteins are perhaps required in equimolar quantity. This calls for coordinated expression of mitochondrial and nuclear encoded ribosomal protein coding genes. In the nucleus, most of the mitochondrial genes are single copy while some are present as multi-

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copy. Thus, mitochondrial ribosomal protein coding genes offer unique opportunity to study mitochondrial – nuclear genome interaction.

Mitochondria are maternally inherited in angiosperms and hence there is very limited intraspecific diversity for the mitochondrial genome. Mitochondria are essential for cell function and mitochondrial gene mutations affecting mitochondrial function are generally lethal. Further, polyploid condition of the mitochondrial genome hinders selection/manifestation of spontaneous or induced mutations in the mitochondrial genome. Thus, genetic studies of mitochondrial genomes are severely limited. Cytoplasmic male sterility (CMS) is the only trait of agronomic significance in angiosperms conditioned by the mitochondrial genome. This, however, does not imply that mitochondrial genome has no role to play in plant development. In fact, majority of mitochondrial-genes code for proteins of the respiratory pathway or components protein synthesis machinery and therefore render it difficult to assign specific functions to individual genes.

T-DNA insertion lines are a good source for functional characterization of genes and promoters. In *Arabidopsis* for almost every gene multiple T-DNA insertion lines are available. In this study, we chose to concentrate on *Rps14* and *Rps19* genes. These are single-copy nuclear genes in *Arabidopsis*. We procured SALK lines for *Rps14* and *Rps19* genes having insertion in the intergenic region and 3' UTR region, respectively, from ABRC. SALK lines having T-DNA insertion in the coding region of *Rps14* and *Rps19* genes were not available. Besides the role of *Rps14* and *Rps19* gene mutants on plant morphology, we have examined promoter of *Rps19* and found that the intergenic region and the 5' UTR, served as minimal promoter for *Rps19* gene, whereas the 447 bp spanning the 3' UTR and last exon of the *Rps19* upstream gene *AT5g47310* is required for *uidA* expression in vegetative and reproductive tissues at high levels (Lata et al. 2017). The aim of present study is to examine SALK lines (SALK_141650C and SALK_142827.55.50.X) for *Rps14* and *Rps19*, respectively.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and the SALK lines (SALK_141650C and SALK_142827. 55.50.X) (Table 1) obtained from ABRC were used in the present study. Seeds of wild type (WT) and SALK lines were surface sterilized with 70% ethanol and 0.1% SDS-HgCl2 solution, placed on plates containing Murashige and Skoog medium (1962) and incubated in culture room 22±1°C with 16 h light/8 h dark photoperiod.The seedlings (~15-day-old) were shifted to pots containing Soilrite (Keltech Energies Ltd., Bengaluru, India) and grown in glass house at 22±1°C, 16 h light/8 h dark photoperiod.

Table 1. Detail of Salk line with T-DNA insertion in the *Rps14* and *Rps19* genes

Gene name	Stock name	Polymorphism (insertion) site	Stock centre
Rps 14	SALK_141650C	Promoter	ABRC
Rps 19	SALK_142827.55.50.x	3'UTR	ABRC

Isolation of A. thaliana genomic DNA

Total DNA was isolated by CTAB method according to the modified protocol of Doyle and Doyle (1990). Young rosette leaves (~1g) were collected from individual plants of (*Rps14/Rps19* SALK lines) and ground to fine powder using liquid nitrogen. Ground leaf powder was transferred to a 50 ml polypropylene centrifuge tube containing 15 ml of pre-warmed CTAB DNA extraction buffer. 0.2% of â-mercaptoethenol was added to the DNA extraction buffer and mixed thoroughly by inverting. Samples were incubated at 65°C for 1 h and mixed 2-3 times by gently inverting the tube. 15 ml of chloroform-isoamyl alcohol (24:1) was added and mixed by inversion many times to emulsify. Tubes containing the mixture were centrifuged at 10,000 rpm for 10 min at room temperature. Upper aqueous phase was removed with a wide-bore pipette and transferred to a clean centrifuge tube and to it 2/3rd volume of pre-chilled isopropanol was added and mixed quickly by inverting the tube. DNA was pelleted by centrifugation at 10,000 rpm for 15-20 min at 4°C and supernatant was removed. DNA pellet was washed using 70% ethanol and centrifuged at 10,000 rpm for 5-10 min. Pellet was air dried and dissolved in 200 µl autoclaved double distilled H_2O and kept at 37°C for 1 h. RNase (50 µg/ml) was added to DNA sample and incubated at 37°C for 1 h to remove RNA.

Designing of primers for SALK line verification, PCR reaction mixture and PCR conditions

Rps14 and *Rps19* bulk DNA isolated from pooled leaf

samples from individual plant of SALK line were checked for presence of *nptII* gene using *nptII*gene specific primers (Table 2). T-DNA iSect Primer Design Tool (http://signal.salk.edu/tdnaprimers) was used to design primers for confirmation of T-DNA insertion. P1 and P2 primers were designed for both *Rps14* and *Rps19* genes. LB serves as a common primer for SALK lines. Two separate reactions were carried out for screening by combining P1+P2 and LB+ P2 primers. P1+P2 reaction gives a band in wild type and heterozygous line while LB+P2 reaction gives a band in homozygous and heterozygous line. Sequences of primers are given in the table 2. PCR reactions were carried out using Taq DNA polymerase (Bangalore, Genei) under standard conditions. 50-100 ng of genomic DNA was taken as template, 1 unit of Taq DNA polymerase was added in the 25 µl PCR reaction. PCR cycle programme involved initial Denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, Primer annealing at 60°C for 30 sec and extension at 72°C for 45 sec.

Isolation of total RNA from Arabidopsis and synthesis of cDNA

Total RNA was isolated from rosette leaves of WT and identified homozygous *Rps14* and *Rps19* T-DNA insertion plants, About 1 g of tissue was ground to fine powder in liquid nitrogen and Tri-reagent (Sigma-Aldrich) was added @1 ml per 50-100 mg of tissue. It was allowed to thaw and mixed properly using a pipette. Insoluble material was removed from the homogenate by centrifugation at 12,000 rpm for 10 min at 4^{o}C. The aqueous phase was transferred to a fresh tube and chloroform was added @0.2 ml per 1 ml Tri-reagent. The tubes were shaken vigorously by hand for 15 seconds and incubated at 15-30^{o}C for 5 min. The samples were centrifuged at 12,000 rpm for 10 min at 4^{o}C. Upper aqueous phase was taken in a fresh tube and RNA was precipitated by adding isopropanol @0.5 ml per 1 ml of Tri-reagent used for initial homogenization. Samples were incubated at 15-30^{o}C for 10 min and centrifuged at 12,000 rpm for 10 min at 4^{o}C. Supernatant was discarded and RNA pellet was washed with 70% ethanol, briefly air-dried for 5-10 min and dissolved in DEPC-treated sterile double distilled water and kept at –70^{o}C. To remove any DNA contamination RNA samples were subjected to DNaseI (Sigma, USA) treatment. First strand of cDNA was synthesized using SuperscriptIII Reverse Transcriptase kit (Invitrogen). The reaction mixture was set with RNA 2 µl (1 µg), oligo (dT) primers 0.5 µl, dNTP mix 1 µl, RT enzyme mix 10 µl and DEPC water 6.5 µl to make final volume 20 µl. The contents were mixed gently and incubated first at 25°C for 10 min, then at 42°C for 60 min. Enzyme was inactivated by incubating at 85°C for 5 min and then reaction mix was chilled on ice. To this 1 µl of *E. coli* RNase H was added to remove RNA strand and incubated at 37°C for 20 min. The final ss-cDNA was stored at –20°C till further use. For downstream analysis ss-cDNA was used as the template. This was quantified using Nanodrop spectrophotometer and concentration was adjusted to 50 ng/µl in all samples.

RT-PCR and qRT PCR analysis

RT-PCR and qRT PCR was done to analyze the expression levels of *Rps19* and *Rps14* genes in Salk lines using *Rps14/Rps19* gene specific forward and reverse primers (Table 2). The reaction was set up with single strand cDNA (template) 2 µL (100 ng), Buffer (10X) 5 µL, dNTP (10mM) 1.5 µL, *Rps19* Forward primer 1 µL (20 pmol), *Rps19* Reverse primer 1 µL (20 pmol), Taq polymerase 1 µL (3U), Sterile double distilled water 34.5 µL to make total reaction volume 50 µL. The amplification was performed in a Thermal cycler C1000^{TM} Thermal cycler (BIO-RAD), with the amplification conditions, one cycle of 94°C for for 3 min followed by 35 cycles of 94°C for 1 min, 58°C for 0.5 min and 72°C for 0.5 min. Final extension at 72°C for 10 min. qRT-PCR was performed with StepOnePlus^{TM} Real-time PCR machine (Life Technologies) and Power SYBR® Green PCR Master Mix (Life Technologies). qRT-PCR was done with three biological replicates and three technical replicates. *Actin2 (At3g18780)* was used as to normalize samples for transcript variation and relative expression was calculated using $-\Delta\Delta CT$ method (Livak and Schmittgen (2001). The primers used for qRT-PCR of *Rps19* and *Actin2* are given in Table 2.

Results and discussion

Evolution and structural organization of Rps14, Rps19 genes and location of T-DNA insertion in SALK lines

Mitochondrial ribosomal protein (MRP) coding genes of plants represent a unique case; the majority of MRP genes are present in the nucleus while a few are retained in the mitochondrial-genome (Table 3). Further, some of the MRP genes are present in one species but absent in others (Table 3). Yet others are present as single copy nuclear genes in one species whereas in other species they are present in mitochondria (Table 3). Thus, it is not clear how many and which of the

Table 2. List of primes used for SALK line verification and PCR study

Primer name	Primer sequence (5'-3')
*npt*II Forward	ATGATTGAACAAGATGGATTGCACGC
*npt*II Reverse	TCAGAAGAACTCGTCAAGAAGGCG
Rps14 (P1)	CTGTTCCTGATTCTCGCAAAG
Rps14 (P2)	TCAAATTGGTCTACAATGGCC
Rps19 (P1)	CGGGAGGTCTAGAGGTTATGG
Rps19 (P2)	AAATCCCTTCAAGCCTAGACG
LB	ATTTTGCCGATTTCGGAAC
Rps19 RT Forward	GAGGATTCTGCCAACTCTGC
Rps19 RT Reverse	ACCGCCGAATCAACATACTC
Nos Reverse	TAACATAGATGACACCGCGC
Actin2 qRT Forward	GACCTTTAACTCTCCCGCTATG
Actin2 qRT Reverse	GAGACACACCATCACCAGAAT
Rps14 qRT Forward	GCGAAATGCGAGACAAGAATC
Rps14 qRT Reverse	GATCGACCAGTGAACACACA

respect to their function in plant development.

As per TAIR annotation, *Rps14* (*At2g34520*) is a gene with single exon of 495 bp and has a 46 bp 5' UTR. An intron of 90 bp interrupts the 3' UTR of 434 bp. It is separated from its upstream gene (*At2g34530*) of unknown function by 203 bp of intergenic region (Fig. 1a). In the SALK line, T-DNA is inserted in the intergenic region (Fig. 1c). Similarly, the *Rps19* (*AT5g47320*) gene has 639 bp coding sequence (CDS), the CDS has four exons and three introns. *Rps19* gene has 5' and 3' UTR of 98 bp and 321 bp, respectively, and a 552 bp intron (+90/+641) interrupts the 5' UTR. A *PPPDE* (putative thiol peptidase family protein) (*AT5g47310*) gene is present upstream of *Rps19* gene. A 229 bp intergenic region lies between *Rps19* and *PPPDE* (Fig. 1b). In the SALK line the T-DNA insertion lies in the 3' UTR of *Rps19* (Fig. 1d).

Isolation and confirmation of SALK lines for T-DNA insertion in the Rps14 and Rps19 genes and isolation of homozygous mutant plants

The particulars of Salk lines obtained from Arabidopsis Biological Resource Centre (ABRC) are given in the Table 1. *npt*II gene is used as the selectable marker in SALK lines. Seeds of SALK lines were plated on MS medium having 50 mg/l kanamycin. *Rps14* SALK line seeds germinated on MS medium and gave green and albino seedlings on kanamycin containing plates.

Table 3. *Rps14* and *Rps19* gene status in various plant species in nuclear and mitochondrial genome

Gene	Plant	Nucleus	Mitochondria	Reference
Rps14	*Arabidopsis*	+	Ψ	Aubert et al. 1992; Brandt et al. 1993; Unseld et al. 1997
	Brassica		+	Ye et al. 1993; Handa 2003
	Hordeum	+	Ψ	Ong and Palmer 2006
	Zea mays	+	×	Figueroa et al. 1999
	Solanum tuberosum	+	Ψ	Quiñones et al. 1996
	Oryza sativa	+	Ψ	Notsu et al. 2002; Kubo et al. 1999
	Sorghum	+	×	Ong and Palmer 2006
	Triticum aestivum	+	Ψ	Sandoval et al. 2004
Rps19	*Arabidopsis*	+	Ψ	Sanchez et al. 1996
	Beta vulgaris	+	×	Matsunaga et al. 2013
	Zea mays	+	×	Adams et al. 2002
	Oryza sativa	Ψ	+	Ueda et al. 2008
	Glycine max	+	+	Adams et al. 2002

Symbol '+' represents intact gene, 'Ψ' indicates gene exits as pseudo gene in mitochondrial genome and '×' shows gene is absent from mitochondrial genome

MRP genes are essential and how their expression is coordinated. Therefore, in this study we chose to investigate *Rps14* and *Rps19* genes of *A. thaliana* with

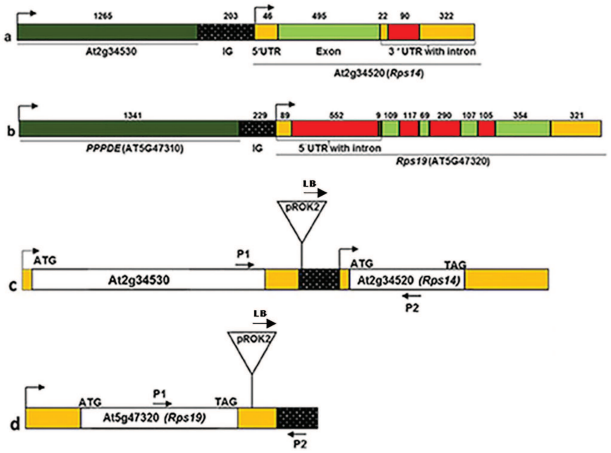

Fig. 1. Schematic diagram showing *Rps14* and *Rps19* gene organization and location of T–DNA insertion in the SALK lines. a. Representation showing various components of *Rps14* gene. b. Representation of various components of *Rps19* gene. c. Diagram showing T–DNA insertion in the intergenic region of the *Rps14* gene in the SALK_141650C line. d. Representation of T–DNA insertion in the 3' UTR of the *Rps19* gene in the SALK_142827.55.50.x line. IG- intergenic region, numbers indicate size (bp) of the fragment, red and green color represents intron and exon respectively, black and orange color indicates intergenic region and UTR respectively

However, no green seedlings were recovered on plates containing *Rps19* SALK line seeds. Therefore, *Rps19* seeds were sown on plain MS medium and green seedlings obtained were used to test the presence of T-DNA. DNA was isolated from a pool of 3-5 seedlings of *Rps14* and *Rps19* lines and used in PCR with *npt*II specific primers (Table 2). An expected 550 bp amplicon was obtained in two replicate samples of each line which indicated the presence of T-DNA (Fig. 2a).

To validate the T-DNA insertion site primers were designed from the T-DNA region and the flanking plant genomic region as per the TAIR guidelines. For *Rps14*, the primer *Rps14* P1 was designed from the *Rps14* upstream gene (*At2g34520*) sequences flanking the left side of T-DNA and primer *Rps14* P2 (Table 2) was designed corresponding to the *Rps14* sequences flanking the right side of T-DNA (Fig. 1c). Likewise for *Rps19, Rps19* P1 and *Rps19* P2 (Table 2) were designed from *Rps19* and the intergenic region flanking the left and right side of T-DNA region, respectively (Fig. 1d). Primer LB was designed from the left border of T-DNA region (Table 2). In PCR with *Rps14* P1+ *Rps14* P2 primers an expected 950 bp amplicon was obtained with WT DNA as template but no amplification was obtained in WT with the primers *Rps14* P2+ LB. On the other hand, in *Rps14* seedlings, some plants gave two amplicons of 950 bp and 650 bp whereas others gave a single 650 bp amplicon (Fig. 2b). This indicated that 650 bp amplicon was derived from T-DNA and flanking plant DNA and the plants showing only 650 bp amplicons are homozygous for T-DNA insertion whereas those showing 650 and 950 bp amplicons are hemizygous. In case of *Rps19,* PCR with WT DNA gave a 900 bp amplicon with *Rps19* P1+ *Rps19* P2 primers whereas no amplification was obtained in WT with the *Rps19* P2 + LB primers. In

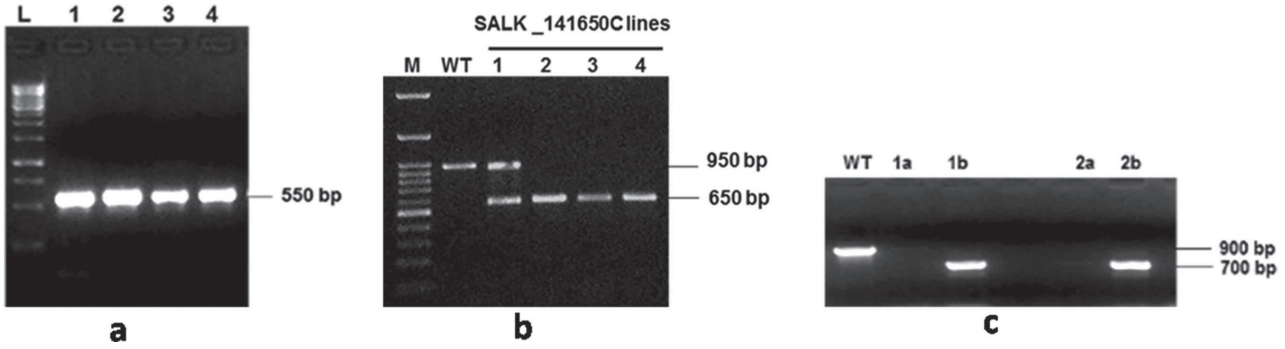

Fig. 2. a. Verification of T-DNA insertion in SALK lines. Gel picture showing amplification of 550 bp *npt*II specific fragment in PCR of SALK lines. L: 1 kb DNA ladder, Lane-1, 2-*Rps14,* Lane-3, 4-*Rps19;* b. Identification of hemizygous and homozygous T-DNA insertion plants among *Rps14* SALK progenies. Gel picture of PCR amplicons obtained in WT and *Rps14* progeny plants. Presence of two amplicons with primers *Rps14* P1, *Rps14* P2 and LB in progeny 1 indicates hemizygous condition of T-DNA. L: 100 bp DNA ladder and c. Establishment of zygosity status of T-DNA in progenies of *Rps19* SALK line. Gel picture of PCR amplicons obtained in WT and *Rps19* progenies. Occurrence of a single but distinct amplicon in WT and *Rps19* SALK progenies indicated both the *Rps19* plants are homozygous for T-DNA

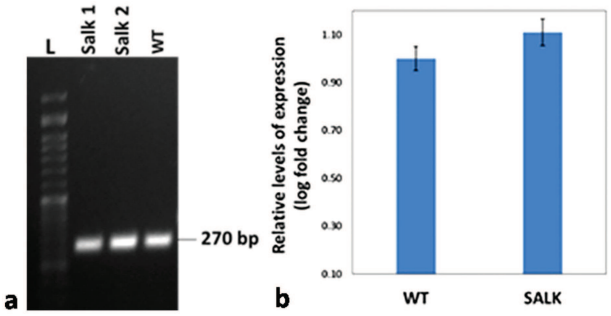

Fig. 3. a. RT PCR analysis to verify the expression of *Rps19* gene in the SALK line. Gel picture showing 270 bp obtained in WT and homozygous T-DNA insertion plants in RT-PCR. L: 100 bp DNA ladder and b. qRT-PCR estimation of *Rps14* transcript: Schematic bar chart showing relative abundance of *Rps14* transcripts in rosette leaves of WT and homozygous *Rps14* SALK progenies

contrast, in individual plants of *Rps19* SALK line, a single amplicon of 700 bp was observed with primers *Rps19* P2 + LB whereas no amplicon was obtained with the *Rps19* P1+ *Rps19* P2 (Fig. 2c). This clearly indicated that *Rps19* seedlings were homozygous for T-DNA insertion.

Estimation of presence of Rps19 and Rps14 transcript in Rps19 and Rps14 plants respectively homozygous for T-DNA insertion and phenotyping

RT PCR with *Rps19* specific primers gave the expected amplicon of 270 bp in both WT and homozygous T-DNA insertion plants (Fig. 3a). The intensity of bands was also comparable among all plants tested. This clearly indicated that T-DNA insertion did not affect the expression of *Rps19* gene in the SALK line SALK_142827.55.50.X. qRT-PCR results of *Rps14* are presented in Fig. 3b. *Rps14* transcripts were found in both WT and homozygous mutant plants (SALK_141650C). In fact, SALK mutant plants showed a slightly higher level of *Rps14* transcripts as compared with WT. Thus T-DNA insertion in *Rps14* was also found not to disrupt gene transcription. Absence of SALK lines in the coding region of these genes indicated that these genes might be essential for plant development. Presence of *Rps14* and *Rps19* transcripts comparable to WT in homozygous mutant line of *Rps14* and *Rps19* indicated that T-DNA insertion has not affected their expression. Homozygous plants of *Rps14* and *Rps19* were raised in phytotron till maturity. Plants were examined throughout for any deviation in morphology. No significant morphological differences were observed between SALK homozygotes and WT plants at any stage of development. These are in agreement with the RT-PCR results where transcripts of *Rps14* and *Rps19* were not altered in the mutant lines.

Authors' contribution

Conceptualization of research (SRB, SL); Designing of the experiments (SRB, SL); Contribution of experimental materials (SRB); Execution of field/lab experiments and data collection (SL, AW); Analysis of data and interpretation (SL, AW); Preparation of manuscript (SL).

Declaration

The authors declare no conflict of interest.

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