The protein kinase TOUSLED is required for maintenance of transcriptional gene silencing in *Arabidopsis*

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TOUSLED-like kinases (TLKs) are highly conserved in plants and animals, but direct evidence linking TLKs and transcriptional gene silencing is lacking. We isolated two new alleles of *TOUSLED* (*TSL*). Mutations of *TSL* in *ros1* reactivate the transcriptionally silent *35S-NPTII* transgene and the transcriptionally silent endogenous loci *TSI* (*TRANSCRIPTIONAL SILENCING INFORMATION*). Chromatin immunoprecipitation (ChIP) analysis shows that histone H3Lys9 dimethylation is decreased in the reactivated transgene and endogenous *TSI* loci in the *tsl ros1* mutant. However, there is no change in DNA methylation in the affected loci. Western blot and ChIP assay suggest that *TSL* might not be responsible for histone H3Ser10 phosphorylation. The *tsl* seedlings were more sensitive to DNA damage reagent methyl methanesulphonate and UV-B light. Our results provide direct evidence for a crucial role of the TOUSLED protein kinase in the maintenance of transcriptional gene silencing in some genomic regions in a DNA-methylation-independent manner in *Arabidopsis*.

Keywords: TOUSLED protein kinase; ROS1; transcriptional gene silencing

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INTRODUCTION

TOUSLED (*TSL*), which encodes a nuclear Ser/Thr protein kinase, was first cloned from *Arabidopsis* (Roe *et al*, 1993). Mutations in *TSL* impaired the development of flowers and leaves (Roe *et al*, 1993). TOUSLED-like kinases (TLKs) in animals are cell-cycle

regulated and sensitive to DNA-damaging agents and inhibitors of DNA replication (Sillje *et al*, 1999). Strong TLK kinase activities are linked to active DNA replication during the S-phase, when it directly phosphorylates ANTI-SILENCING FUNCTION 1 (ASF1; Sillje & Nigg, 2001; Carrera *et al*, 2003; Groth *et al*, 2003). *TSL* in *Arabidopsis* also interacts with and phosphorylates ASF1b *in vitro* (Ehsan *et al*, 2004). ASF1 recruits H3 and H4 histones to form REPLICATION-COUPLED ASSEMBLY FACTOR together with CHROMATIN ASSEMBLY FACTOR 1 (CAF1) to assist the assembly of nucleosomes onto newly synthesized DNA during replication (Mello & Almouzni, 2001; Tyler, 2002). Mutations in *Arabidopsis* FAS1 and FAS2 (subunits of CAF1) lead to developmental defects and impair transcriptional gene silencing (TGS; Kaya *et al*, 2001; Takeda *et al*, 2004; Ono *et al*, 2006). *fas1* and *fas2* mutants are hypersensitive to DNA-damaging agents, suggesting that they function in DNA repair (Takeda *et al*, 2004). However, there is no direct evidence to show whether TLKs are linked to TGS.

Previous studies showed that two linked transgenes, *RD29A-LUC* and *35S-NPTII*, become silenced due to a mutation in a putative DNA glycosylase/lyase REPRESSOR OF SILENCING 1 (ROS1; Gong *et al*, 2002). A mutant screen for suppressors of *ros1* mutant (*ror*) has identified a gene encoding REPLICATION PROTEIN A2 (RPA2A/ROR1; Kapoor *et al*, 2005a; Xia *et al*, 2006). In this study, we characterized another two *ros1* suppressors. These were found to be new alleles of the *tsl* mutant. We provide evidence that *TSL* is important in chromatin regulation and in maintaining TGS in some genomic regions of *Arabidopsis*.

RESULTS

tsl mutations reactivate the silenced *35S-NPTII*

Here, we used the *Arabidopsis* accession C24 carrying a T-DNA locus with *35S-NPTII* and *RD29A-LUC* genes as starting C24 wild-type plants (Ishitani *et al*, 1997). From a screening for suppressors of the *ros1-1* mutant (Gong *et al*, 2002), we obtained two allelic

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Fig 1 | *tsl* mutations impair the transcriptional gene silencing of 35S-NPTII, but have no effect on the expression of RD29A-LUC or endogenous RD29A gene in the *ros1* mutant. In this study, we used *Arabidopsis* accession C24 carrying a T-DNA locus with RD29A-LUC and 35S-NPTII genes as C24 wild type. (A) C24 wild-type (WT), *ros1*, *tsl4 ros1* and *tsl5 ros1* seedlings grown on MS medium. (B) Comparison of the kanamycin sensitivity of C24 WT, *ros1*, *tsl4 ros1* and *tsl5 ros1* seedlings grown on MS medium containing 50 mg/l kanamycin (Kan). (C,D) Comparison of WT, *ros1* and *tsl4 ros1* seedlings grown on 150 mg/l kanamycin (C) or 200 mg/l kanamycin (D). (E) The expression of RD29A-LUC monitored by luminescence imaging of wild-type (WT), *ros1*, *tsl4 ros1* and *tsl5 ros1* seedlings (A) after ABA treatment for 5 h. (F) The comparison of endogenous RD29A and COR47 gene expression after ABA treatment for 5 h. TUBULIN was used as a loading control. (G) Northern blot analysis of the expression of the NPTII gene. Ribosomal RNA was used as a control. ABA, abscisic acid; COR47, COLD-REGULATED; MS, Murashige-Skoog medium; *ros1*, repressor of silencing 1; *tsl*, tousled.

and recessive mutants *tsl4 ros1* and *tsl5 ros1*. Map-based cloning identified the mutations in the *TSL* (*At5g20930*) gene. The mutations in *tsl4 ros1* and *tsl5 ros1* were found in positions 3904 (changed 3' splicing site from AG to AA) and 4423 (from TCA for Ser to TTA for Leu), respectively, counting from the first ATG initiation codon. Both mutants show similar growth phenotypes, as described previously (Roe et al, 1993). Genetics analysis indicated that the growth defect phenotypes are independent of *ros1* mutation. Previous studies have shown that the activity of TSL is regulated by the cell cycle (Ehsan et al, 2004). However, because most *ros1* plants do not show any growth phenotypes as compared with wild type (Gong et al, 2002), it is thought that ROS1 might not be involved in cell-cycle regulation. We also obtained two T-DNA insertion mutants (SALK_047307 as *tsl6*, SALK_082965 as *tsl7*) from the Ohio *Arabidopsis* Stock Center.

As shown in Fig 1B–D, *tsl4 ros1/tsl5 ros1* mutants were more tolerant to kanamycin than *ros1*, but were more sensitive to kanamycin than wild type. *tsl* mutations did not reactivate the

expression of silenced RD29A-LUC and endogenous RD29A when analysed by bioluminescence imaging and northern blot after treatment with 100 μ M abscisic acid (Fig 1E,F), but partly reactivated the silenced 35S-NPTII in the *ros1* mutant as compared with wild type (Fig 1G).

tsl mutations partly reactivate the silenced *TSIs*

It is known that the transcriptional silencing of *TSIs* can be released by some mutations (Xia et al, 2006). As shown in Fig 2A, *TSIs* were expressed at higher levels in *tsl4 ros1* than in *ros1* or wild type. *tsl* mutation reactivates the expression of *TSIs* independent of *ros1*, as shown by a T-DNA insertion line *tsl6* without *ros1* mutation (Fig 2A). In comparison, *TSIs* were expressed at higher levels in the two DNA hypomethylation mutants *hog1 ros1* (*hog1* for homology-dependent gene silencing 1; Xia et al, 2006) and *ddm1* than in *tsl* (Fig 2A). However, *tsl* mutations did not reactivate the silenced transposon gene *At2g12240*, but *hog1* and *ddm1* mutations reactivated (Fig 2A).

Fig 2 | *tsl* mutation does not affect DNA methylation but increases the expression of *TSI*. (A) Northern blot analysis of *TSI* expression. Col, Columbia wild type; *tsl6*, a T-DNA line in Columbia accession. The *At2g12240* was a silenced transposon gene in wild type (WT). *TUBULIN* was used as a loading reference. (B) DNA methylation at the endogenous *RD29A* promoter (left) digested with *Bst*UI, and transgene *RD29A-LUC* promoter (right) digested with *Mlu*I. (C,D) Bisulphite sequencing analyses of DNA methylation for total *RD29A* promoter (including both transgene and endogenous *RD29A* promoter) (C) and endogenous *RD29A* promoter (D). (E) DNA methylation at the 35S promoter in WT, *ros1* and *tsl4* *ros1* analysed by bisulphite sequencing. (F) Comparison of DNA methylation in WT, *ros1* and *tsl4* *ros1* at centromeric DNA (*cen*-DNA; left), rDNA (middle) and *Ta3* (right) digested with *Hpa*II or *Msp*I.

tsl mutations do not affect DNA methylation

Both the transgene and endogenous *RD29A* promoters are hypermethylated in the *ros1* mutant compared with wild type (Gong et al, 2002). As previously described (Gong et al, 2002), we compared the DNA methylation levels by using both DNA-methylation-sensitive enzymes and bisulphite sequencing, and found no DNA methylation difference in both the transgene *RD29A* promoter and endogenous *RD29A* promoter between *tsl4* *ros1* and *ros1* (Fig 2B–D). Bisulphite sequencing also confirmed that, as in *ros1* and wild type, no DNA methylation was found at the 35S promoter in *tsl4* *ros1* (Fig 2E). In addition, no clear DNA methylation differences were found in the regions of centromeric DNA, 180-bp rDNA and *Ta3* transposon (Fig 2F). These results indicate that *tsl* mutations reactivate certain silenced genes in a DNA-methylation-independent manner.

Reduced histone H3Lys9me2 in *tsl4* *ros1* mutants

We carried out chromatin immunoprecipitation (ChIP) experiments to examine whether *tsl* mutations cause changes in histone modifications in the *RD29A* promoter, 35S promoter, partial *LUC* (from 64 to 382 of luciferase open reading frame) and *TSI* regions. As previously described (Xia et al, 2006), we used the transcriptionally active *ACTIN* promoter as a control for the dimethylated H3Lys4 and the silenced *Ta3* retrotransposon (Konieczny et al, 1991) as another control for the H3Lys9 dimethylation (Fig 3A). When no antibodies were added, no PCR products were amplified. Consistent with previous results, the *ros1* mutation increased H3Lys9 dimethylation in the 35S promoter compared with the wild type (Kapoor et al, 2005a; Xia et al, 2006). However, H3Lys9 dimethylation was reduced and the H3Lys4 dimethylation increased at the 35S promoter in *tsl4* *ros1*

Fig 3 | *tsl* mutations reactivate expression of the silenced *35S-NPTII* and *TSI* by reducing histone H3Lys9 dimethylation in the *ros1* mutant, but do not affect histone H3Ser10 phosphorylation. (A) Chromatin immunoprecipitation (ChIP) analysis was carried out on *35S* promoter, *RD29A* promoter, partial *LUC* and *TSI* regions with specific antibodies against dimethylated H3Lys9 (anti-H3Lys9), dimethylated H3Lys4 (anti-H3Lys4) and phosphorylated H3Ser10. PCR amplifications of *ACTIN* and *Ta3* were used as controls for anti-H3Lys4 and anti-H3Lys9, respectively. No antibody precipitation was used as a negative control. WT, wild type. (B) Western blot analyses of histone H3 modifications at H3Lys4, H3Lys9 and H3Ser10 by using the above antibodies in wild type, *ros1* and *tsl4 ros1*. Western blot of histone H3 was used as a loading control.

to a level similar to that in the wild type (Fig 3A). By contrast, both H3Lys4 and H3Lys9 dimethylation levels of *RD29A* promoter and partial *LUC* (from 64 to 382 of the coding region) in *tsl4 ros1* were similar to that in *ros1*, which is apparently reduced or increased compared with that in wild type.

For *TSI*, the high H3Lys9 dimethylation in *ros1* was reduced in the *tsl4 ros1* mutant to a level similar to that in wild type. However, we did not observe any change in H3Lys4 methylation among *ros1*, *tsl4 ros1* and the wild type for *TSI* (Fig 3A). These results indicate that *tsl* mutations cause changes in H3Lys9 dimethylation in the reactivated loci.

tsl mutations and H3Ser10 phosphorylation

We used ChIP assay to determine whether the phosphorylation of H3Ser10 is related to the reactivation of *35S-NPTII* with a polyclonal antibody specific for this modification. PCR analysis of the precipitated DNA in the regions of *RD29A* promoter, *35S* promoter, partial *LUC*, *Ta3*, *ACTIN* and *TSI* did not produce a difference between 2-week-old seedlings of *ros1*, *tsl4 ros1* and wild type. Western blot analysis using total purified histone proteins isolated from 2-week-old seedlings further confirmed that H3Ser10 phosphorylation level did not change in the absence of TSL kinase (Fig 3B). At the same time, we compared the histone H3Lys4 and H3Lys9 dimethylation levels among wild type, *ros1* and *tsl4 ros1* by western blot, and found no difference (Fig 3B). Histone H3 was used as a loading control in these experiments. These results indicate that TSL did not affect the overall H3Lys4 and H3Lys9 dimethylation, and might not participate directly in histone H3Ser10 phosphorylation.

tsl mutants are sensitive to UV-B light and MMS

Previous studies in mammals indicated that TLKs are targets of the DNA-damage checkpoint and are important in DNA repair (Groth et al, 2003). *tsl* mutant plants growing under normal light conditions frequently showed local necrosis on leaves, which is

seldom found in *ros1* or wild-type plants (Fig 4A). This leaf damage in *tsl* mutants might be due to hypersensitivity to irradiation. Furthermore, UV-treated 5-day-old seedlings of *tsl4 ros1* became yellow and their growth was greatly inhibited as compared with *ros1* or wild-type seedlings. Methyl methane-sulphonate (MMS) is an alkylating agent that causes double-strand DNA breaks. The growth of *tsl4 ros1* or single *tsl4* mutant seedlings treated with 0.0075% or 0.01% MMS for 2 days was greatly inhibited as compared with *ros1* or wild type, but no clear leaf necrosis was observed (Fig 4C, 2 days). After 4-day MMS treatment, the seedlings were first transferred to Murashige-Skoog (MS) medium and grown for one more day so that the leaf necrosis phenotype could be more easily observed. With 0.0075% or 0.01% MMS treatment, all *tsl4 ros1* or *tsl4* seedlings were more critically damaged than *ros1* and wild-type seedlings. In all of these experiments, the *ros1* seedlings were more sensitive to MMS than wild type, which is consistent with our previous results (Gong et al, 2002; Xia et al, 2006). These observations suggest that *tsl* mutants are defective in repairing DNA damage induced by both UV-B and MMS.

DISCUSSION

The formation of heterochromatin depends on DNA methylation and/or histone modifications in plants (Soppe et al, 2002; Tariq et al, 2003). Mutations in some *Arabidopsis* genes alter DNA methylation, thereby releasing TGS, whereas mutations in other genes release TGS independent of DNA methylation (Xia et al, 2006). The latter group of genes includes MORPHEUS' MOLECULE 1 (MOM1; Amedeo et al, 2000), CAF1 subunits FAS1 and FAS2 (Tyler et al, 1999; Takeda et al, 2004; Ono et al, 2006), MRE11 condensin complexes (Takeda et al, 2004), BRU1 (a novel protein with the overlapping functions of CAF1; Takeda et al, 2004) and ROR1/RPA2A (REPLICATION PROTEIN A2; Elmayan et al, 2005; Kapoor et al, 2005a; Xia et al, 2006). Interestingly, except for *mom1*, all the other mutants are also sensitive to

Fig 4 | *tsl* mutants are sensitive to UV-B light and the double-strand DNA-damaging reagent methyl methanesulphonate. (A) Comparison of the growth phenotypes among wild type (WT), *ros1* and *tsl4 ros1* growing under normal conditions. (a) *tsl4 ros1* mutants; (b) leaf necrosis phenotypes in WT, *ros1* and *tsl4 ros1* mutants; (c) WT; (d) *ros1* mutants. (B) Phenotypic comparison of WT, *ros1* and *tsl4 ros1* mutants after treatment with UV-B light. (C) Sensitive comparison of C24 WT, *ros1*, *tsl4 ros1* and *tsl4* seedlings grown on medium containing 0.0075% and 0.01% methyl methanesulphonate (MMS). Five-day-old seedlings were transferred to MS liquid medium containing different concentrations of MMS and grown for another 2 or 4 days. For 2-day treatment, photos were taken immediately (2 days); for 4-day treatment, seedlings were first transferred to MS solid medium and further cultured for 1 day, and then photos were taken (4 + 1 days). Seedlings grown on MS medium were used as growth control. Scale bars, 1 cm.

DNA-damaging agents and show pleiotropic developmental defects. The *tsl* mutants show similar pleiotropic developmental defects as the above-mentioned mutants (Roe *et al*, 1993) and are sensitive to UV-B irradiation and MMS. *tsl* mutations release TGS independent of DNA methylation. Combined with previous results in animals and plants, our results indicate that TLKs together with other histone chaperones regulate chromatin assembly, chromatin remodelling and heterochromatin independently of DNA methylation in some genomic loci.

Both *TSL* and *ROR1* were isolated as suppressors of the *ros1-1* mutation. *ROS1* works as a DNA demethylation enzyme to erase DNA methylation, guided possibly by short interfering RNAs from the *RD29A* promoter (Gong *et al*, 2002; Kapoor *et al*, 2005b; Agius *et al*, 2006). The *ros1* mutations lead to hypermethylation and TGS of the *RD29A* promoter, but do not affect DNA methylation in the *35S* promoter. TGS of the *35S* promoter in *ros1* might result from spreading of the heterochromatin, which originates from the *RD29A* promoter (Kapoor *et al*, 2005a; Xia *et al*, 2006). Our ChIP analysis indicated that histone H3Lys4 and H3Lys9 dimethylation levels in the *RD29A* promoter and partial *LUC* are not affected by *tsl* mutations, which is consistent with the silenced *RD29A-LUC* in *tsl* mutant. However, *TSL* mutations lead to an increase of H3Lys4 dimethylation and a reduction of H3Lys9

dimethylation in the *35S* promoter region, and reactivate the expression of *35S-NPTII*. In *Arabidopsis*, KRYPTONITE is found to be responsible for H3Lys9 methylation, which in turn regulates CpNpG DNA methylation (Johnson *et al*, 2002). As *tsl* mutations affect H3Lys9 but not DNA methylation, the mechanism by which *TSL* affects KRYPTONITE or other proteins to regulate histone H3Lys9 methylation remains unclear. Our results indicate that establishment and spreading of TGS in *RD29A-LUC* and *35S-NPTII* are regulated by distinct epigenetic pathways. *TSL* and *ROR1* are required for heterochromatin spreading, which might originate from the *RD29A* promoter and extend to *35S-NPTII*, and result in silencing of *35S-NPTII*, but not for maintaining the TGS of *RD29A-LUC* in the T-DNA locus (Xia *et al*, 2006).

Recently, Zhang *et al* (2006) reported that a loss-of-function mutation in *Drosophila Jil1* encoding a histone H3Ser10 kinase results in the ectopic spreading of heterochromatin markers dimethyl H3Lys9 and heterochromatin protein 1 (HP1), suggesting that *Jil1* kinase activity antagonizes Su(var)3-9-mediated heterochromatinization for maintaining euchromatin. Depletion of the mitotic kinase Aurora B, which phosphorylates H3Ser10 in mammals, also suggests that H3Ser10 phosphorylation is necessary for antagonizing the interaction of HP1 with H3Lys9 trimethylation (Fischle *et al*, 2005). H3Ser10 is also

phosphorylated by TLKs in *Drosophila* and mammals, but not in *Caenorhabditis elegans* (Li et al, 2001; Carrera et al, 2003). In animals, phosphorylation of H3 has been linked to gene activation and chromatin condensation during mitotic/meiotic events (Nowak & Corces, 2004). However, in maize, phosphorylation of histone H3Ser10 is necessary for chromatid cohesion during meiosis (Kaszas & Cande, 2000). Previous studies indicated that TSL does phosphorylate histone H3 *in vitro*, but H3Ser10 phosphorylation could not be detected *in vivo* in flower buds (Ehsan et al, 2004). In this study, we used histone proteins from 2-week-old seedlings to check the levels, and found no clear H3Ser10 phosphorylation difference among *ros1*, *tsl4* *ros1* and wild type by western blot. ChIP assays for different genomic regions including *RD29A* and *35S* promoter further suggest that TSL might not be involved in H3Ser10 phosphorylation. Whether *Arabidopsis* TSL targets other phosphorylation sites of H3 or other histones needs to be explored in future work.

METHODS

The methods including plant growth, mutant screening, Southern blot for DNA methylation analysis, northern blot and genomic bisulphite sequencing are described previously (Xia et al, 2006).

Genetic analysis and positional cloning of the TSL gene. We identified two allelic mutants, named *tsl4* *ros1* and *tsl5* *ros1*, which can grow on MS medium containing 50 mg/l kanamycin with similar developmental defects. Genetics analysis indicated that two mutants are allelic and recessive. The *tsl4* *ros1* mutant was backcrossed to the *ros1* mutant three times. The homozygous *tsl4* *ros1* mutant in the C24 accession with a T-DNA locus was crossed to the wild-type Columbia. The mutants of F₂ plants were picked up on the basis of developmental defect phenotypes. Mapping-based cloning was carried out by using simple sequence-length polymorphism markers (primer sequences will be provided on request). The candidate gene *TOUSLED* from wild-type and *tsl4* *ros1* or *tsl5* *ros1* mutant plants was sequenced to find the mutation.

ChIP assay and western blot analysis. ChIP was carried out as described previously (Xia et al, 2006) by using the anti-dimethyl-histone H3 (Lys9; Upstate, Charlottesville, VA, USA; 07-441), anti-dimethyl-histone H3 (Lys4; Upstate; 07-030) and anti-phospho-histone H3 (Ser10) antibodies. DNA was analysed by PCR using the following primer pairs: Actin, Ta3, 35S promoter, TSL, *RD29A* promoter and LUC (primer sequences will be provided on request). All PCR reactions were carried out in 25 µl, starting with 5 min at 95 °C, followed by 24–35 cycles.

Western blot was carried out as described previously (Xia et al, 2006) by using the antibodies used for the ChIP assay described above.

UV and methyl methanesulphonate treatment. UV and MMS treatment were carried out as described previously (Revenkova et al, 1999). Briefly, 5-day-old seedlings grown on vertical MS plates were irradiated with UV (254 nm) at 5 KJ/m². After irradiation, seedlings were kept in the dark for 24 h and then transferred to standard growth conditions. Photos were taken after 1 week. For MMS treatment, 5-day-old seedlings grown on 1% agar solid MS medium were transferred to MS liquid containing different concentrations of MMS. Photos were taken after 2-day treatment. For 4-day treatment, the seedlings were first transferred from MMS medium to MMS-free medium and grown for another 1 day and then photos were taken.

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