FINAL REPORT

Title: **Investigating the molecular mechanism of *TSO1* function in *Arabidopsis* cell division and meristem development**

Grant Number: **02-00ER20281** (07/01/97-06/30/03 with no cost extension to 06/30/04)

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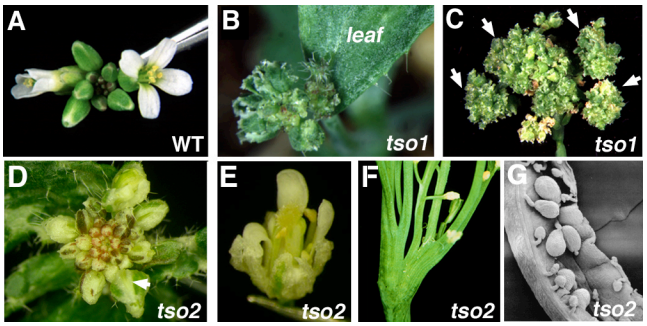
Arabidopsis tso1 and *tso2* mutations were both identified in the same EMS mutagenesis screen. They are both recessive and cause similarly abnormalities. *tso1* mutants develop callus-like flowers (Fig. 1B-C), and *tso2* mutants exhibit abnormal leaves and flowers with similar callus-like features (Fig. 1 D-E). However, *tso2* mutants are less severe than *tso1* and are fertile (Fig. 1G). They were both named *TSO*, meaning ugly in Chinese. The first DOE grant (97-00) led to the molecular cloning of *TSO1* (Song et al., 2000). The renewed application (00-03) was intended to further characterize the *TSO1* gene with only minor emphases on *TSO2*. However, the progress of *TSO1* has been limited by the departure of postdoctoral fellow Dr. J. Y. Song at the early stage of the project and by several failed attempts in replacing him. At the same time, the *TSO2* project was moving rapidly due to the persistent effort of a graduate student Chunxin Wang. Hence, we decided to deviate from the original aims to take advantage of the exciting research direction offered by studying *TSO2*. We have reported this change of direction in the 2002 progress report. Following summarize the major findings of *TSO2*.



1. *TSO2* encodes the small subunit of ribonucleotide reductase

Four *tso2* mutations were identified in two separate EMS mutagenesis screens. All four *tso2* alleles (*tso2-1, tso2-2, tso2-3*, and *tso2-4*) were recessive and gave similar phenotypes. Using a map-based approach, we cloned the *TSO2* gene and showed that it encodes the small subunit of the ribonucleotide reducatse (RNR). All four *tso2* alleles are missense mutations, with *tso2-1* and *tso2-4* mutating an identical amino acid (Fig. 2). *tso2-1* phenotype can be rescued by a cosmid clone harboring a RNR small subunit gene. RNR catalyzes the rate-limiting step in dNTP biosynthesis and hence is essential for DNA replication and DNA-damage repair. The enzymatic activity of RNR depends on the formation of a complex between two different subunits, R1 and R2. We measures dNTP level in *tso2* mutants and found a reduced level of all four dNTP in *tso2-1* floral tissues (Fig 3).

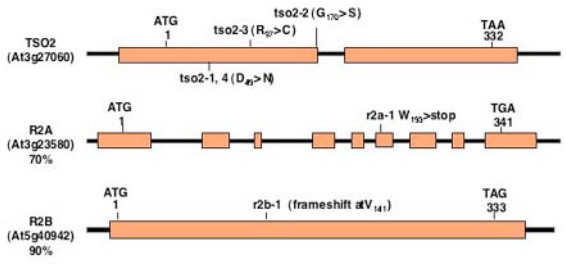


Figure 2. Schematic diagram of three *Arabidopsis* R2 genes. Rectangular boxes are exons. The start and stop codons and the position of each *r2* mutation are indicated. *tso2-1* and *tso2-4* are identical. Numbers indicate amino acid residues. % identity between *TSO2* and *R2A* or *R2B* are indicated.

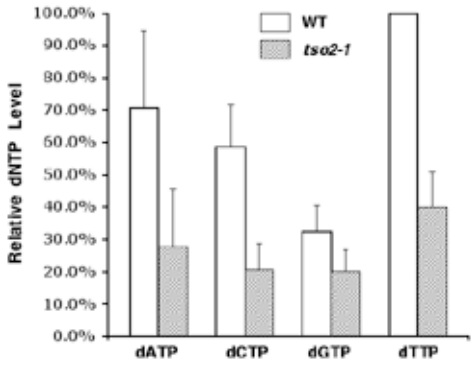


Figure 3. Relative dNTP levels in wild type (L*er*) and *tso2-1* floral tissues, averaging three different experiments. The dNTPs pool is measured by a polymerase-based assay

2. tso2-5 null mutants do not exhibit any phenotypes.

We subsequently identified a putative *tso2* null allele (*tso2-5*) by screening several T-DNA insertion databases. *tso2-5* is caused by a T-DNA insertion in the *TSO2* (WS ecotype). The T-DNA is inserted in exon I and causes a 10 bp deletion at the site of insertion, deleting 30th-34th residues of the protein. However, *tso2-5* does not exhibit any phenotype, suggesting that *tso2-1,2,3,4* missense alleles maybe recessive antimorphic alleles. We are in the process of transforming a *tso2-1* mutant gene into wild type and *tso2-5* plants to further verify the antimorphic nature of *tso2-1*.

3. *TSO2, R2A* and *R2B* are functionally redundant

We have identified two additional *R2* genes in the *Arabidopsis* genome named *R2A* (At3g23580) and *R2B* (At5g40942) (Fig. 2). *R2B* is 90% identical to *TSO2*, but is annotated as a pseudogene. The *R2B* from the Columbia (Col) ecotype, which was used for genome sequencing, possesses a 2 bp deletion resulting in a frameshift and a subsequent stop (Fig. 2). In contrast, we found that *R2B* from L*er* and WS ecotype does not harbor the 2 bp deletion and differ from Col in 13 additional nucleotides. *R2B* genomic DNA from L*er* (*R2B-L*) was fused to a strong and constitutive promoter 35S and this *35S::R2B-L* transgene was able to rescue all 36 *tso2-1* plants, indicating that L*er* or WS possesses a functional *R2B*.

R2A is 70% identical to *TSO2*. Using a reverse genetics method called TILLING (McCallum et al., 2000), we isolated an EMS-induced *r2a* mutation *(r2a-1)* in the Col accession. *r2a-1* is a nonsense mutation that deletes one third of the protein (Fig. 2). In the Col background, *r2a-1* is actually a *r2a-1 r2b-1* double mutant, and *r2a-1 r2b-1* plants are phenotypically wild type (Fig. 4A).

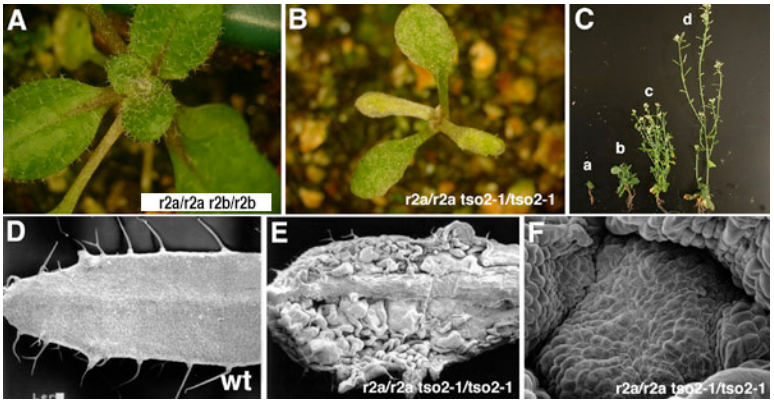


Figure 4. Genetic analyses of *r2* mutants (A) A young *r2a-1 r2b-1* seedling. (B) A *tso2-1 r2a-1* double mutant showing severe developmental arrest and abnormal leaf development. (C). A photograph of plants heterozygous for *r2a,* or *r2b,* or both *r2a* and *r2b* in *tso2-1* background. Specifically: (a) *tso2-1/tso2-1, r2a-1/+ r2b-1/+*; (b) *tso2-1/tso2-1, r2a-1/+;* (c) *tso2-1/tso2-1, r2b-1/+; (d) tso2-1* plants. (D) A wild-type (Ler) leaf. (E) A *tso2-1 r2a-1* double mutant leaf showing severely disrupted leaf surface. (F) A *tso2-1 r2a-1* double mutant SAM exhibiting developmental arrest.

We constructed *tso2-1 r2a-1* and *tso2-1 r2b-1* double mutants, which exhibited a much stronger phenotype than *tso2-1*. First, *tso2-1 r2a-1* double mutant seedlings did not develop beyond the 2-4 leaf stage with arrested shoot apical meristem (Fig. 4B,F). *tso2-1 r2a-1* seedlings have leaves with massively disorganized surfaces (Fig. 4E). Second, *tso2-1 r2b-1* double

mutants were embryo lethal. Finally, *tso2-1/tso2-1* plants heterozygous for *r2a-1, r2b-1,* or *r2a-1 r2b-1* were viable but exhibited a much stronger phenotype than *tso2-1/tso2-1* (Fig. 4C). Therefore, *R2A* and *R2B* are essential in *tso2-1* background.

4. The expression of *TSO2, R2A* and *R2B* during cell cycle and development

Are *TSO2, R2A* and *R2B* expressed differentially in different *Arabidopsis* tissues? *TSO2, R2A* and *R2B* transcripts were examined in different *Arabidopsis* tissues (Fig. 5A). *TSO2* transcripts were found in roots, rosette and cauline leaves, stems and flowers. While *R2A* transcripts were not detected in roots they were detected in rosette and cauline leaves, stems and flowers. In contrast, *R2B* transcripts were only detected by Southern blots of RT-PCR products and were present in all tissues tested (Fig. 5A in R2b.S panel). Thus, all three genes, in most cases, are expressed widely with *R2B* expression at a much lower level than *TSO2* and *R2A*.

To examine *TSO2* transcription during cell cycle, the β-glucuronidase (*GUS*) reporter gene was fused to the *TSO2* 1.2kb promoter. Transgenic seedlings harboring the *pTSO2::GUS* were treated with aphidicolin or colchicine, which arrest cells at S and M phase, respectively. *GUS* expression was detected in more cells and at higher levels when the seedlings were arrested at the S phase (Fig. 5C). In contrast, *GUS* expression was dramatically reduced when the seedlings were arrested at the M phase (Fig. 5D). Hence, *TSO2* transcription occurs predominantly in the S-phase. *TSO2* mRNA distribution during plant development was examined by *in situ* hybridization and by *pTSO2::GUS*. The sporadic rather than uniform pattern of *TSO2* mRNA in developing floral tissues (Fig. 5E, F) is characteristic of cell cycle phase-specific expression. In *pTSO2::GUS* transgenic plants, *GUS* expression is predominantly present in tissues and organs with active cell division activities, including young root tips, young leaves, and developing vasculatures (Fig. 5G, H). The *TSO2* expression pattern is consistent with its role in dNDP biosynthesis during DNA replication.

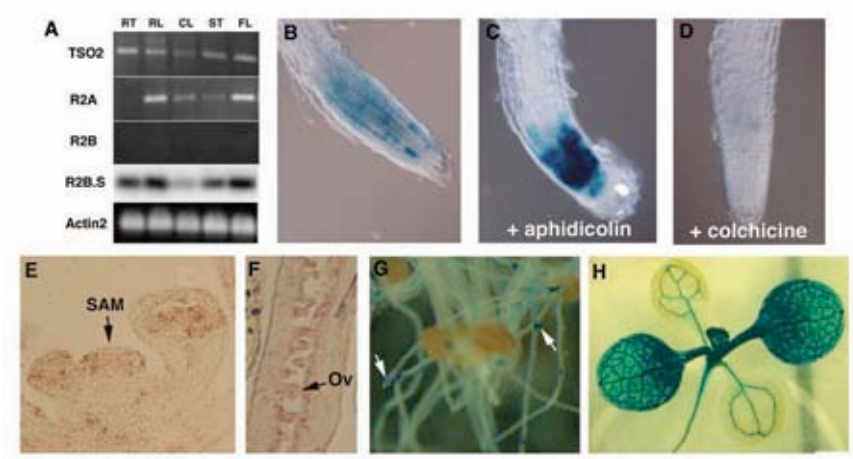


Figure 5. mRNA expression of *TSO2*, *R2A* and *R2B*. (A) Expression of *TSO2*, *R2A* and *R2B* mRNA measured by RT-PCR. RT: root; RL: rosette leaves; CL: cauline leaves; ST: stems; and FL: flowers. *Actin2* is the loading control. (B) *pTSO2::GUS* expression in a 3-day old wild type root. Note the sporadic GUS activity. (C) *pTSO2::GUS* expression in a 3-day old root treated with aphidicolin, revealing an increased number of *GUS*-expressing cells and an elevated level of *GUS* expression. (D) *pTSO2::GUS* expression in a 3-day old root treated with colchicine. Note the significantly reduced *GUS* activity. (E) *in situ* hybridization showing *TSO2* mRNA expression in wild-type SAM. Note the sporadic *TSO2* expression throughout the young floral primordia. (F) *in situ* hybridization showing sporadic *TSO2* expression in developing ovules (Ov). (G) p*TSO2:GUS* reporter expression in transgenic plants showing intense *GUS* activity in primary and lateral root meristems (arrows). (H) A 1-week old seedling showing *GUS* activity in shoot apex, young leaves, and vasculatures.

5. Cell cycle progression and resistance to UV-C is affected in tso2 mutants

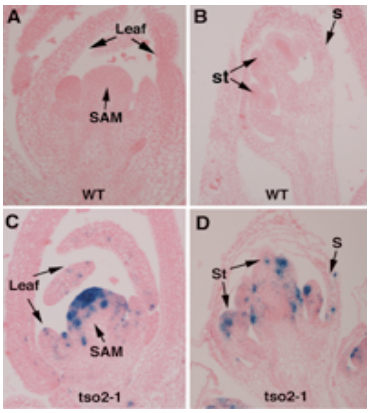


Figure 6. Cell cycle arrest in *tso2-1* mutants indicated by *pcycB1::GUS* expression. (A) *pcycB1::GUS* expression is not detectable in wild type SAM. (B) *pcycB1::GUS* expression is not detectable in wild type flowers showing sepals (s) and stamens (st). (C) *pcycB1:: GUS* is highly expressed in patches of cells in the *tso2-1* SAM and emerging leaf primordia, suggesting an increased number of cells at late G2 or early M phase. (D) *pcycB1::GUS* expression is found in cells of *tso2-1* floral organs including sepals (s), and stamens (st).

A *cyclin B1-GUS* chimeric protein driven by the *Cyclin B1;1* promoter (*pcycB1::GUS*) was previously developed as a reporter whose expression is detected only in the late G2/early M phases of the cell cycle in root tips (Colon-Carmona et al., 1999). We introduced this reporter into *tso2-1* mutants. While the *GUS* expression was normally not detectable in the above ground tissues of wild type plants (Fig. 6A,B), *GUS* was expressed strongly and in a sporadic fashion in *tso2-1* SAM and developing leaf and floral organs (Fig. 6C,D). The difference in *pcycB1::GUS* expression between wild type and *tso2-1 pcycB1::GUS* plants was reproducible, even when the wild type and *tso2-1* plants were siblings harboring identical *pcycB1::GUS* transgenes and when wild type and *tso2-1* sibling plants showed similar *GUS* expression in roots. Therefore, *tso2-1* above ground tissues may possess a significant number of cells arrested at the M/G2-phases of the cell cycle, which correlated with abnormal development in these tissues.

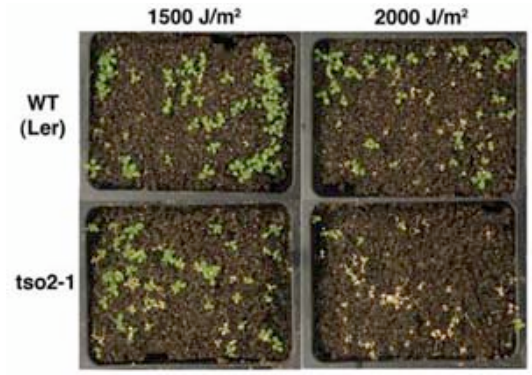


Figure 7. An increased sensitivity of *tso2-1* to UV-C as shown by a larger number of dead seedlings at both 1500 J/m^2 and 2000 J/m^2 doses.

We tested the sensitivity of wild type (L*er*), *tso2-1, r2b-1,* and *r2a-1 r2b-1* mutants to UV-C. *tso2-1* exhibited an increased sensitivity to UV-C (Fig. 7). *r2b-1* or *r2a-1 r2b-1* did not (data not shown). The similar level of UV-C sensitivity of *r2a-1 r2b-1* to wild type (L*er*) indicates that *TSO2* alone is sufficient to provide wild type level of protection against UV-C.

6. *tso2-1 r2a-1* accumulate DNA damage and undergo programmed cell death

Are reduced dNTP levels in *tso2-1* sufficient to impede DNA replication fork progression and induce DNA strand breaks? Using comet assay (Angelis et al., 1999), we measured DNA damage levels in the seedlings of *tso2* single and double mutants. While *tso2-1* or *r2a-1 r2b-1* double mutants did not exhibit increased DNA damage, *tso2-1 r2a-1* mutants exhibited significant increases in DNA damage (Fig. 8A). Consistent with the comet assay, only *tso2-1 r2a-1* seedlings were found to induce the expression of molecular markers associated with DNA strand breaks (Fig. 8B).

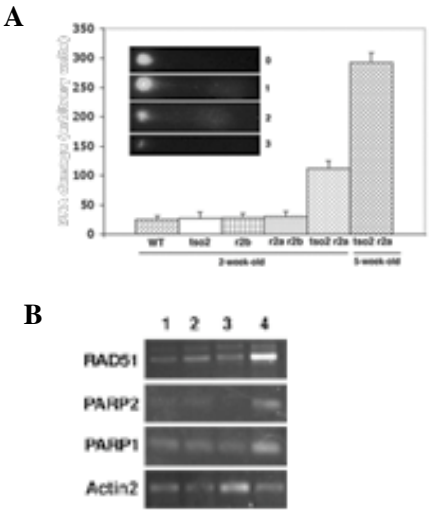


Figure 8. Increased DNA damage in *tso2-1 r2a-1* seedlings. (A) Results from the comet assay indicating the relative amount of DNA damage in different genotypes. While *tso2-1, r2b-1,* or *r2a-1 r2b-1* mutants exhibited similar levels of DNA damage to wild type (L*er*), *tso2-1 r2a-1* double mutants exhibited an increased level of DNA damage even at 2-week age. The DNA damage level is increased further in 5-week old *tso2-1 r2a-1* double mutants. The extent of DNA damage in each nucleus was indicated by 0, 1, 2, 3, or 4 units; examples of 0, 1, 2, and 3 are shown in the inset. The DNA damage units per genotype were derived by summing up the units from 100 nuclei. (B) RT-PCR was used to detect the induction of markers associated with DNA strand breaks. Lanes 1, 2, 3, and 4 correspond to WT (L*er*), *tso2-1, r2a-1 r2b-1,* and *tso2-1 r2a-1*, respectively.

In animals, genomic instability often induces programmed cell death (PCD) in a p53-dependent fashion. Using histochemical and molecular markers, we found PCD in *tso2-1 r2a-1* seedlings. Trypan Blue (TB) was used to stain dead cells. Large patches of TB-stained cells were observed in *tso2-1 r2a-1* double mutant leaves but not in wild type nor *tso2-1* (Fig. 8A). Further, a high level of H_2O_2 and a large number of callose depositions were detected in the leaves of *tso2-1 r2a-1* double mutants (Fig. 9A). Both H_2O_2 production and callose deposition are indicators of plant cells undergoing hypersensitive PCD during incompatible plant-pathogen interactions (Brodersen et al., 2002; Dietrich et al., 1994). Additionally, *tso2-1 r2a-1* seedlings expressed molecular markers associated with hypersensitive PCD in plants including a short chain alcohol dehydrogenase (*SAG13*), Peroxidase C (*PRXc*), *Glutathionine-S-Transferase* (*GST*), *Pathogenesis-Related 1* (*PR1*) and *EDS1* (Fig. 9B). However, *SAG12*, a cysteine protease specific to senescence-induced PCD, was not detected under the RT- PCR condition used (Fig. 9B). Overall, *tso2 r2a*-mediated PCD appears similar to hypersensitive PCD in plants. In plants, PCD has been investigated extensively in associated with hypersensitive response (HR) during incompatible pathogen-plant interaction and in association with senescence. Relatively little is known about how genomic instability and DNA-damage initiate PCD in plants. The *tso2 r2a* mutants provided us a unique opportunity to investigate PCD in the context of genome instability.

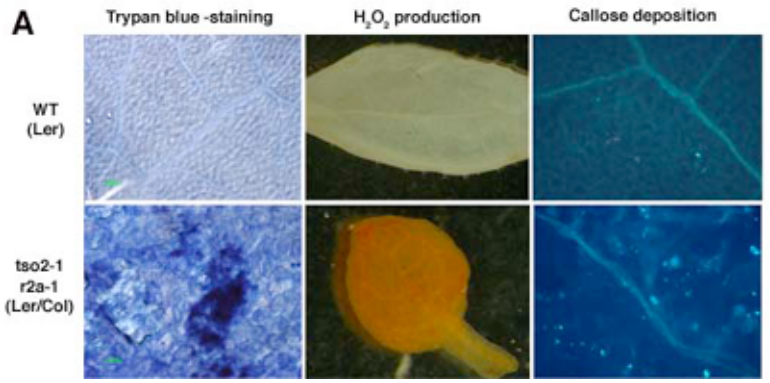


Figure 9. Expression of cell death markers in *tso2-1 r2a-1* mutants. (A) Leaves of wild type and *tso2-1 r2a-1* double mutants were examined for programmed cell death using three different histochemical markers: trypan blue staining, H_2O_2 production and callose deposition. Dark blue patches stained by trypan blue, reddish-brown deposits (reaction products between 3,3-diaminobenzidine and H_2O_2), and callose deposition revealed by aniline blue staining indicated programmed cell death in *tso2-1 r2a-1* mutants. (B) RT-PCR was used to detect different molecular markers associated with PCD.

7. Publications that acknowledge DOE funding

A. Song J, Leung T, Ehler LK, Wang C, and **Liu Z**. (2000) Regulation of meristem organization and cell division by *TSO1*, an Arabidopsis gene with cysteine-rich repeats. **Development** 127, 2207-2217.

B. Franks, R.G. and **Liu Z.** (2001) Floral Homeotic Gene Regulation. **Horticultural Reviews** vol. 27, 41-77.

C. **Z. Liu (**2003) Transcription Co-repressors in Flower Development. **Chinese Bulletin of Botany** 20 (4) :385-394

D. Chunxin Wang and Zhongchi Liu. Cell Death, Meristem Bifurcation and Synthetic Lethality Causeby Ribonucleatide Reductase Mutations in *Arabidopsis*. (Submitted).

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