ABSTRACT

Title of Dissertation: FUNCTIONAL ANALYSES OF *ARABIDOPSIS* RIBONUCLEOTIDE REDUCTASE SMALL SUBUNIT GENE FAMILY

Chunxin Wang, Doctor of Philosophy, 2004

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A fundamental question in plant development is how cell division events are coordinately regulated in the context of growth and development. To address this question, I chose to study a pleiotropic mutant, *tso2,* which exhibited developmental defects including callus-like floral organs and fasciated shoot meristem. I isolated the *TSO2* gene and showed it encodes the small subunit of ribonucleotide reductase (RNR). RNR catalyzes a rate-limiting step in the production of deoxyribonucleotides needed for DNA synthesis. Subsequently, I showed that *tso2* mutants reduce the dNTP levels.

To understand why *tso2* mutants, defective in this essential process, are still viable, I identified two homologs of *TSO2*, *R2A* and *R2B* in *Arabidopsis*. Mutations in *R2A* and *R2B* were isolated using a reverse genetic approach. While *r2a*, *r2b* single mutants or *r2a r2b* double mutants fail to display any visible phenotype, *r2a* and *r2b* mutations can enhance *tso2* genetically, resulting in seedling lethality and embryonic

lethality in *tso2 r2a* and *tso2 r2b*, respectively. Overexpression of either *R2A* or *R2B* can rescue the *tso2* mutants, suggesting that the three *R2* genes are functionally redundant.

In addition to the developmental defects, *tso2* mutants were more sensitive to HU (hydroxyurea) and UV-C, indicating that *TSO2* plays a major role in DNA repair. In *tso2 r2a* double mutant seedlings, increased DNA damage accumulates, leading to massive programmed cell death. In addition, release of transcriptional gene silencing was observed in *tso2 r2a* double mutants, suggesting that DNA damage can lead to epigenetic instability. To further identify regulators of *RNR* and novel components of plant DNA damage response pathways, 18 independent *tso2* suppressors were isolated in a genetic screen. These suppressors fall into at least four different complementation groups.

My genetic and molecular characterization of *TSO2* is the first functional study of RNR in plants. My results indicated that plants could initiate programmed cell death in response to DNA damage. The developmental defects in *tso2* mutants are caused by epigenetic instability and aberrant cell division. The isolation of potential *tso2* suppressors will be crucial to the understanding of plant DNA damage response pathway, an understudied area in plant biology.

FUNCTIONAL ANALYSES OF ARABIDOPSIS RIBONUCLEOTIDE REDUCTASE SMALL SUBUNIT GENE FAMILY

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

To my parents

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Abbreviation

4-NQO	4-nitroquinoline-1-oxide
APC	anaphase-protomoting complex
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia and Rad3-related
bru1	brushy1
CAK	CDK activating kinase
CAPS	cleavage amplified polymorphic sequence
cdc	cell-division-cycle
CDK	cyclin-dependent kinase
Cid	caffeine-induced death resistant
CKI	CDK inhibitor
CKS	CDK subunit
CLV	clavata
Col	Columbia
CRT	constitutive RNR3 transcription
CZ	central zone
dAK	deoxyadenosine kinase
dCAPS	degenerate cleavage amplified polymorphic sequence
dCK	deoxyguanosine kinase
DEL	DP-E2F-like
dGK	deoxycytidine kinase
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DNDP	deoxyribonucleoside diphosphate
DNMT	DNA methyltransferase
dNTP	deoxyribonucleoside triphosphate
DP	DRTF-1 polypeptide
DSB	double strand break
EMS	ethyl methanesulphonate
fas	fasciated
fis	fertilization-independent seed
FM	floral meristem
GST	glutathionine-S-Transferase
GUS	β-glucuronidase
HDAC	histone deacetylase
HMTase	histone methyltransferase
HR	hypersensitive response
HU	hydroxyurea
Ler	Landsberg erecta
LUC	luciferase
MAPK	mitogen-activating protein kinase

MDS	mitochondrial DNA-depletion syndromes
MMC	mitomycin C
MMS	methyl methanesulfonate
NDP	ribonucleoside diphosphate
ORF	open reading frame
OC	organizing center
PARP-2	poly (ADP-ribose) polymerase-2
PCD	programmed cell death
PCR	polymerase chain reaction
PR	pathogen-related
PRXc	peroxidase C
PZ	peripheral zone
RB	retinoblastoma
RBR	retinoblastoma-related
RNR	ribonucleotide reductase
ROS	reactive oxygen species
RZ	rib zone
SAG	senescence-associated-gene
SAM	shoot apical meristem
SCG	single cell gel electrophoresis
SEM	scanning electron microscopy
sin	short integuments
Sml	suppressor of mec1 lethality
Ssn	suppressor of snf1
sua	shuai
TB	trypan blue
TGS	transcriptional gene silencing
THF	5,10-methylenetetrahydrofolate
TILLING	targeting induced local lesions in genomes
TK	thymidine kinase
TS	thymidylate synthase
WS	Wassilewskija
wus	wuschel

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Chapter one

Literature Review

Overview

The longevity of plants is beyond the reach of animals. One of the unique features of plants is that plant growth continues throughout the lifespan and is modular. This powerful postembryonic growth originates from the special organization of the shoot apical meristem (SAM), the center for repetitive organ initiation. Due to the thick cell wall, the movement of plant cells is forbidden. This emphasizes the importance of control of cell division orientation and rate and cell expansion in plant development. How plant cell proliferation is regulated within the context of development has been a subject of debate for many years.

Another unique property of plants is their sedentary life style and their dependence on sunlight for photosynthesis. Therefore, plants are obliged to be constantly exposed to environmental mutagens, including UV irradiation and reactive oxygen species (ROS). The completed *Arabidopsis* genome revealed that plants have a large number of DNA repair genes (The *Arabidopsis* Genome Initiative, 2000). For example, *Arabidopsis* has six replication protein A (RPA1), four *Rad23* for nucleotide excision repair, sixteen DNA base glycosylases with roles in base excision repair, suggesting that plants have a higher repair capacity in response to constant exposure of DNA damaging agents. However, when DNA damage is beyond repair, apoptosis-like programmed cell death can occur in plants. In addition, plants have evolved some active defense

mechanisms to effectively protect themselves from pathogen attack, such as hypersensitive (HR)-mediated programmed cell death.

One key event in cell division and in DNA damage repair is DNA synthesis, in which DNA strands are duplicated with high fidelity. In all organisms, ribonucleotide reductase (RNR) provides the dNTPs for DNA synthesis by converting the ribonucleoside diphosphates (NDPs) into deoxyribonucleoside diphosphates (dNDPs). An ample and balanced dNTP pool is required for high fidelity DNA replication. Due to its vital role in DNA synthesis, RNR has been the target site for chemotherapy for cancer or infectious diseases.

This literature review aims to give relatively detailed background information for the Ph.D. research reported in this thesis. First, the organization of shoot apical meristem (SAM) and the maintenance mechanism underlying SAM will be described. Second, the progression of cell cycle in plants and DNA damage checkpoint will be described and discussed. Third, the regulation of cell division in the context of plant development will be discussed. Finally, the history, structure, regulation and application of RNR will be presented.

Organization of the shoot apical meristem (SAM)

Unlike animals whose body plan is already established in embryos, the elaboration of plant architecture occurs largely post-embryonically. All above-ground parts of flowering plants are derived from a group of cells positioned at the tip of the

stem, called shoot apical meristem (SAM). On one hand, the shoot apical meristem acts as a self-renewing source of undifferentiated, pluripotent stem cells. On the other hand, it is the reservoir of cells that will undergo differentiation and be utilized for organogenesis. As a consequence, continuous and repetitive formation of new structures and organs such as leaves and flowers results from the modular nature of the plant body plan. The amazing regenerative power of the SAM enables plants to live up to several thousand years as seen in some bristlecone pines.

How does the SAM maintain its regenerative ability for such a long time? Clonal analyses demonstrated that all of the postembryonic structures of the plants are derived from about three stem cells in every layer of the meristem (Stewart and Dermen, 1970). To reduce the chance of accumulating mutations during many rounds of cell divisions, stem cells in the SAM normally divide slowly. After cell division, some daughter cells will stay in the center and remain as new stem cells, other daughter cells are displaced to the periphery of the SAM to undergo differentiation into specific organs or secondary shoots. As shown in Fig. 1-1, the stem cells are positioned in the central zone (CZ) of the SAM. Surrounding the central zone is the peripheral zone (PZ), where cells divide quickly and will become incorporated into lateral organs. Underneath the central zone is the rib zone (RZ), where cell divisions give rise to the pith and the vascular system, contributing to the growth of the stem. Cells in these different regions were first distinguished by their cytohistological characteristics (Steves and Sussex, 1989), one of which is the different cell proliferation rates measured by their mitotic index or incorporation rates of radioactive thymidine. As noted earlier, cells divide more slowly in

the CZ than in the PZ and RZ. Obviously, in a growing meristem, a continuous flow of cells will transit from the CZ to the PZ or the RZ.

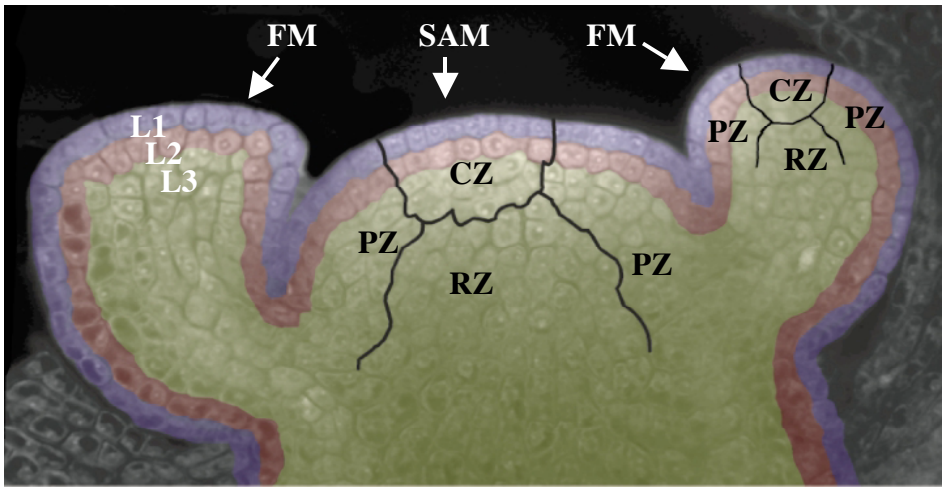


Figure 1-1. Schematic diagram of organization of shoot apcial meristem (SAM). The tunica corresponds to L1 and L2 layers and corpus corresponds to the internal L3 layer. The black outlines represent the approximate boundaries between the different meristematic zones. Although the cells of the PZ and the CZ are histologically distinguishable, there is no sharp boundary between each zone (from Carles and Fletcher, 2003).

The highly ordered structure of the SAM can also be divided into different layers. The outermost two layers, called L1 and L2, remain clonally separated from the underneath L3 cells as cells in L1 and L2 layers divide exclusively anticlinally (Fig. 1-1). Cells in L3 layer apparently divide in random orientations. The allocation of cells in the meristem into separate clonal cell layers suggests cell-lineage-dependent mechanism of development, which is, however, not the case. Studies with genetic mosaics showed that cell fate in plants is determined by the position of a cell, not by its clonal origin. These studies using genetic mosaics also showed that all three layers contribute to organ formation and growth of the stem, indicating coordinated cell proliferation and cell fate specification in different layers.

The intercellular communication could involve transfer of informational molecules through plasmodesmata, which establish a cytoplasmic continuity between neighboring cells. Therefore, the cells can act as a syncytium or symplasm. By tracking the movement of an inflorescent tracer dye, Rinne and van der Schoot (1998) found that the birch shoot meristem is compartmentalized into a central and a peripheral symplastic field, which could restrict the diffusion of small potential morphogens to the cells inside their boundaries. Gisel et al. (1999) reported that the symplastic connections between the phloem and shoot apex change over developmental time and the extent of the symplastic fields are dynamic.

Maintenance of shoot apical meristem

During the lifetime of a plant, the size of shoot apical meristem remains largely the same. However, the proliferation of the stem cells and the rate of organ formation vary in response to environmental stresses and developmental age. What is the underlying molecular mechanism to maintain a consistent number of stem cells? Mutations that cause a lack of shoot apical meristem or enlarged SAM led to the isolation of several regulators of SAM for initiation and maintenance. Mutations in three *clavata* loci (*clv1, clv2, clv3*) (Latin *clavatus*, meaning 'shaped like a club') cause the plants to produce a broader and distorted meristem, a phenomena called fasciation (Latin *fascia*, meaning 'bundle') (Fig.1-2B). *clv* mutants produce many extra floral meristems (FM) and many extra floral organs (Carles and Fletcher, 2003; Clark et al., 1993, 1995; Kayes and Clark, 1998). A simple explanation for this fasciation phenotype is that the three *CLV*

genes normally promote the transition of meristem cells from the central zone into the peripheral zone. Alternatively, *CLV* may inhibit the proliferation rate in the central zone. Since the mitotic index is not increased in the central zone in *clv* mutants, the increased size in SAMs and FMs is likely caused by the accumulation of cells that wait to be recruited by the developing organs in SAMs and FMs (Laufs et al., 1998).

Genetic and biochemical studies have demonstrated that the three *CLV* genes act in the same pathway by forming a complex. *CLV1* and *CLV2* encode a receptor-like kinase (RLK) and a receptor-like protein (RLP), respectively, whereas *CLV3* encodes a secreted polypeptide (Clark et al., 1997; Jeong et al., 1999; Fletcher et al., 1999). *CLV3* is mainly expressed in the L1 and L2 of the central zone while *CLV1* mRNA is mostly detected in the L3 of the central zone (Clark et al., 1997; Fletcher et al., 1999). The expression domain of the *CLV3* gene coincides with the location of stem cells and the *CLV3* gene has been used as a stem-cell marker. Consistent with its potential role as a ligand for *CLV1*/*CLV*2 receptors, immunological studies showed that *CLV3* is secreted from the overlying L1 and L2 cell layers and moves into the underlying L3 and lateral neighbors (Fig. 1-3). This is supported by genetic studies that the function of *CLV3* is dependent on both *CLV1* and *CLV*2.

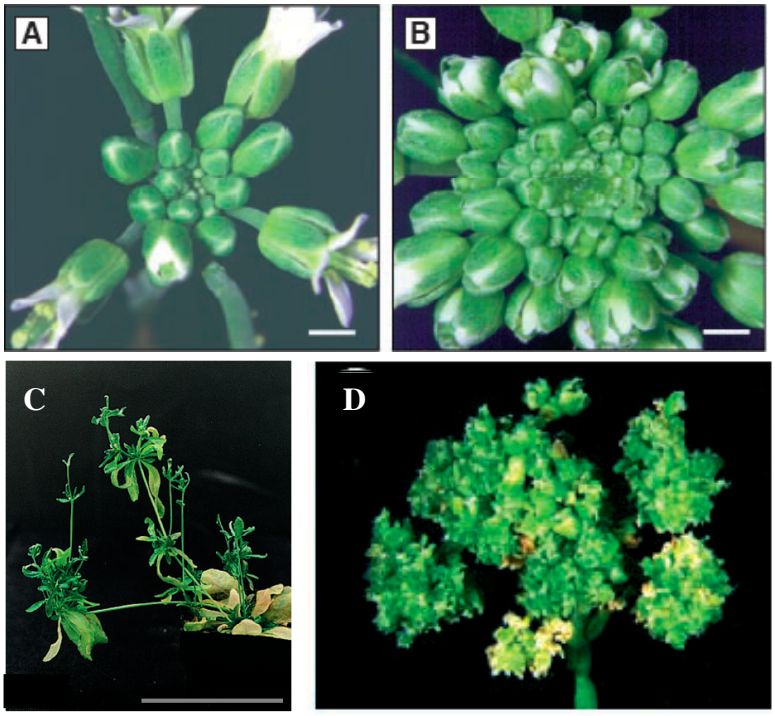


Figure 1-2. Mutants affecting shoot apical meristem development. (A) Wild type. (B) *clv3-2*. (C) a weak *wus-1* allele. (D) *tso1-1*.

wuschel (*wus*) mutants display an opposite phenotype from *clv*. In *wus* mutants, the shoot apical meristem is prematurely terminated, indicating that *WUS* gene is required for the maintenance of SAM (Laux et al., 1996). Weak *wus* mutants can undergo iterative processes of meristem initiation and premature arrest, leading to the production of disorganized groups of leaves and shoots, like 'tousled hair' (the German meaning of *wuschel*) (Fig. 1-2C). *WUS* encodes a homeobox protein and the mRNA is only found in a few cells, which constitute the so-called 'organizing center (OC)' in the L3 layer of the central zone, just beneath the *CLV3* expression domain (Mayer et al., 1998). Overexpression of *WUS* represses organ formation, induces *CLV3* expression and forms seedlings with an apex consisting entirely of undifferentiated meristematic cells (Schoof et al., 2000). This suggests that *WUS* mis-expression is necessary and sufficient to induce

CLV3 transcription and stem cell identity. Since *WUS* is not expressed within the stem cells but in the underlying organizing center, *WUS* activity in the OC was proposed to signal and specify the overlying cells as stem cells. Why are only the overlying cells induced to become stem cells? One possibility is that only these cells are competent to respond to the *WUS*-dependent signal. This is supported by observations that ubiquitously expressed *WUS* induces *CLV3* expression only in some cell types (Haecker and Laux, 2001). Alternatively, the *WUS*-mediated signal may be communicated via plasmodesmata in one direction only. The existence of symplasm or syncitum makes it possible that the movement or diffusion of small signal molecules is restricted (Rinne and van der Schoot, 1998).

wus mutants are epistatic to *clv* because *clv wus* double mutants display the *wus* mutant phenotype. In *clv3* mutants, the *WUS* expression domain expands laterally and into the L2 layer, suggesting that the *CLV* pathway normally restricts *WUS* transcription to the OC. Overexpression of *CLV3* throughout the SAM can mimic the *wus* loss-of-function phenotype, suggesting that *CLV3* activity is sufficient to repress *WUS* in SAM. Why is *WUS* only expressed in the OC in wild type plants? Perhaps, all *CLV3* protein produced in the apical stem cells is bound by *CLV1* in the third cell layer, preventing it from entering the OC cells underneath (Fig. 1-3). This is supported by the observation that 75% of the total amount of *CLV3* protein in cauliflower meristem extracts was found to be associated with *CLV1* (Trotochaud et al., 2000). *In vivo* evidence came from transgenic studies. Expression of *CLV3* from the epidermis using a L1-specific promoter *ATML1* could repress *WUS* in the OC non cell-autonomously and caused a *wus* mutant

phenotype. However, coexpressing *CLV3* and *CLV1* under the control of the *ATML1* promoter exhibited a wild type phenotype. In contrast, coexpressing *CLV3* and a mutant *CLV1* (*clv1-4*) under the control of the *ATML1* promoter still lead to a *wus*-like phenotype (Lenhard and Laux, 2003). Since *clv1-4* is a mutant form of *CLV1* that is predicted to bind less *CLV3* protein (Trotochaud et al., 1999), these results strongly suggest that *CLV1* can restrict the range of *CLV3* action via sequestration of the ligand. Therefore, *CLV1* fulfills a dual function: On one hand, it relays the *CLV3*-dependent signal into the receiving cells and ultimately causes repression of *WUS* transcription in apical stem cells. On the other hand, *CLV1* protects the underlying cells of the OC from *CLV3* by sequestering the ligand and thus, allows *WUS* expression in OC.

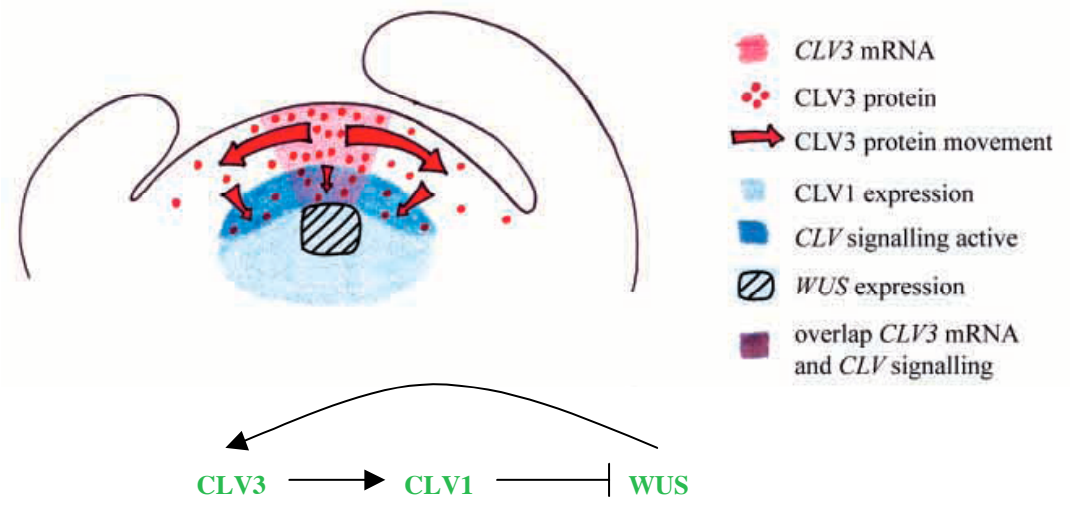


Figure 1-3. A model for CLV3 action as an intercellular signal in the SAM. (cited from Lenhard and Laux, 2003)

The feedback loop (Fig. 1-3) between *CLV3* and *WUS* thus, underlies the self-regulatory property of the SAM. If the *WUS*-dependent signal is too weak, *CLV3* expression will be decreased, leading to specification of fewer stem cells. When *CLV3*

level is low, the *CLV3*-dependent signal for *WUS* repression is also weakened. As a consequence, the *WUS* expression domain will expand and thus specify more stem cells. Conversely, if the *WUS*-dependent signal is too strong, *CLV3* expression will be increased and more stem cells will be specified. This, again, will lead to a strong repression of *WUS* and reduce *WUS* expression domain, leading to fewer stem cells. Thus, the size of the stem cell population in the SAM is continuously monitored and adjusted by the *WUS*-*CLV* feedback loop, leading to a constant size of the SAM.

The above model does not answer the question about how the organizing center is maintained, since new cells are continuously entering and leaving the *WUS* expression domain (the organizing center). Periclinal divisions of the overlying L3 stem cells result in a flow of cells through the OC: cells that enter the OC activate *WUS* expression, whereas cells leaving the OC towards the rib zone switch *WUS* off. One explanation is that the stem cells also send a positive signal inducing *WUS* expression in the OC. As the strength of the repressive signal drops sharply due to the sequestration of *CLV3* by *CLV1* at the outmost L3 layer, the activating signal can reach OC. Alternatively, the position of the OC is dependent on signals from underlying or lateral cells. It was shown that meristem maintenance requires the presence of young leaf primordia and is influenced by genes expressed in the leaves and vasculature (Moussian et al., 1998; Waites and Hudson, 1995).

CLV independent pathway

What happens if the SAM has more than one organizing center? What mechanisms prevent plants from forming additional OCs in their SAM? *Arabidopsis tso1* mutants seemed to be defective in this process. *tso1-1* also exhibited a fasciation phenotype resulted from extensive bifurcation or multiple splitting of the inflorescence meristems (Fig.1-2D). This is in contrast to the fasciaton in *clv* mutants, which is caused by a single enlarged SAM. *TSO1* encodes a putative DNA-binding protein with cysteine-rich repeats, bearing similarity with *Drosophila Enhancer of zeste* (Song et al., 2000). Song et al. proposed that one of the *TSO1* functions might be to limit central zone initiation either by repressing the expression of genes that normally activate central zone formation or by mediating communications between the central zone and its neighbors to prevent additional central zone initiation. Since cell division and cytokinesis defects were also observed in *tso1* mutants including partially formed cell walls and increased DNA ploidy (Liu and Meyerowitz, 1997), it is possible that the communications between the central zone and its neighbors are disrupted. It will be interesting to see if the *CLV-WUS* genes are ectopically expressed in those bifurcated meristems.

Recently, this type of fasciation was reported in a set of mutants that are defective in chromatin/DNA replication or DNA damage checkpoint. For instance, *AtCAP-E1* and *AtCAP-E2* are two condensing genes that are essential for mitotic chromosome condensation. While $E1^{-/-}E2^{-/-}$ plants are embryo lethal, $E1^{-/+}E2^{-/-}$ plants exhibited stem bifurcation associated with fasciation (Siddiqui et al., 2003). It is possible that the chromatin remains relatively decondensated, which could activate a checkpoint control to arrest the cell cycle. Actually, it has been shown that condensing is also involved in S-

phase checkpoint activation in other organisms (Aono et al., 2002). Since overexpression of cyclin D3 results in a variety of developmental defects including meristem disorganization (Riou-Khamlichi et al., 1999), this suggests that altered cell cycle can affect meristem development. Alternatively, condensins and other factors involved in DNA/chromatin dynamics can epigenetically alter gene expression programs. That is, in *E1*$^{-/+}$ *E2*$^{-/-}$ mutants, expression of certain genes like *WUS* might be altered.

Another example is *Arabidopsis MRE11* gene that encodes a component of the MRX complex (*Mre11*, Rad50, Xrs2/Nbs1) with roles in repairing double-strand breaks (DSB) and maintaining telomere length (Bundock and Hooykaas, 2002). It might be also involved in DNA damage checkpoint (D'Amours and Jackson, 2002). *mre11* mutants are hypersensitive to DNA damage and fail to grow directly in soil. The most severely affected seedlings grown on medium died after 8-weeks. Only the fittest *mre11* seedlings can survive in soil to form mature plants, which often showed bifurcation in the stems.

In *fasciated* (*fas1, fas2*) mutants, the clear histological distinction between the peripheral zone and central zone was lost, and the regular cell arrangement in L1 and L2 was also disrupted (Kaya et al., 2001). The expression domain of *WUS* expanded laterally but not uniformly in *fas1* mutants. Sometimes the *WUS* expression domain also shifted and/or expanded to outer cell layers, in some extreme cases, even to L1 cells. This is different from *clv* mutants, in which the *WUS* expression domain laterally expands and shifts one cell layer up but remains as two cell layers (Schoof et al., 2000). Genetic studies showed that *FAS* and *CLV* genes function in different pathways (Leyser and

Furner, 1992). *FAS1* and *FAS2* genes encode the two subunits of the chromosome assembly factor, which functions in assembling nucleosomes onto nascent DNA. It is likely that the role of *FAS* genes is to facilitate stable maintenance of gene expression states in the apical meristem. In *fas* mutants, the epigenetic repression of *WUS* might be lost, leading to ectopic expression of *WUS*.

The discovery of *bru1* mutants leads to a novel link between reponse to DNA damage and epigenetic gene silencing. *bru1-1* was isolated from the screen for mutants with elevated sensitivity to DNA damaging agents and *bru1-2* was obtained from the screen for mutations that release transcriptional gene silencing (TGS) at a transgenic luciferease marker gene (Takeda et al., 2004). In *bru1* mutants, the frequency of intra-chromosomal homologous recombinantion is elevated and genotoxic stress responses are constitutively activated, indicated by 2~3-fold induction of the poly (ADP-ribose) polymerase-2 (*PARP-2*) gene under normal growth conditions. The release of TGS in *bru1* is stochastic, indicated by the expression pattern of LUC and GUS reporter genes. It probably reflects random deregulation of silencing followed by mitotic transmission of the newly acquired active state (Takeda et al., 2004). *bru1* plants exhibited fasicated and often bifurcated shoot meristems. This is reminiscent of *fas*, *mre11* and *AtCAP-E1*$^{-/+}$*AtCAP-E2*$^{-/-}$ mutants that are defective in chromatin/DNA replication and S-phase DNA damage checkpoints of the cell cycle. Takeda et al. (2004) realized that there might be a link between chromatin/DNA replication, TGS and meristem maintenance. They found that *fas* mutants were hypersensitive to the DNA damaging agent MMS, and that *fas*, *mre11* and *AtCAP-E1*$^{-/+}$*AtCAP-E2*$^{-/-}$ mutants all abnormally express transcriptionally

silent information (TSI) (Steimer et al. 2000). TSI are pericentromeric repeats normally silent in wild type plants. In addition, *WUS* was also ectopically expressed in a stochastical manner in *bru1* mutants. These results suggest that defects in the S-phase DNA damage checkpoint or inaccuracies during chromatin/DNA replication cause instability of epigenetic states, which is often manifested by meristem fasciation due to mis-expression of meristem regulators such as *WUS*.

Cell division

Cell cycle

To better understand the role of cell division in plant development, we need to have good knowledge of cell cycle regulation in plants. At first glance, cell division is as simple as duplicating (S phase) and dividing the DNA (M phase). The problem is: all the genetic information of a cell has to be accurately copied and precisely segregated into two genetically identical daughter cells. In addition, cells have to grow and double the mass of proteins and organelles during the cell cycle. This is done in the so-called gap phases, G_1 and G_2. The gap phases also provide time for cells to check and ensure that the external and internal conditions are suitable for the next phase, and that the previous phase has been accurately and fully completed. In mammals, a cell can also enter a specialized phase called G_0 (G zero), in which cells permanently rest their division.

During G_1, cells must integrate external and internal signals before making the decision to initiate DNA replication, a commitment not only to the S phase but also to the completion of the cell cycle. Once committed, cells cannot revert back to G1. During

DNA synthesis, there is a mechanism to assure that the DNA duplication occurs only once. During G_2, a checkpoint is turned on to ensure that the onset of mitosis occurs only upon the completion of DNA replication and repair. During mitosis, the duplicated DNA first condenses into chromosomes, and mitotic spindles begin to form (prophase). When the nuclear envelope breaks down, the spindle microtubules are now able to interact with the chromosomes. Then, the duplicated chromosomes are aligned on the mitotic spindle, a characteristic of metaphase. During anaphase, the chromosomes are pulled to the opposite poles of the cell. In telophase, chromosomes are completely separated into two daughter cells. The nuclear envelope re-forms around the daughter nuclei. In animals, cytokinesis occurs by constricting the cell membrane between the two daughter nuclei until the final separation of the two cells. In plants, a structure called 'phragmoplast' forms at the site of the future new cell wall, which directs vesicles carrying cell wall materials to the center between the two daughter cells. Vesicle fusion results in new cell wall formation. Phragmoplast assembly starts centrally and expands toward the parental cell wall to divide the cell into two.

Progression of cell cycle

How is the cell cycle regulated at the molecular level? Extensive studies in yeast and mammals have shown that the activation of a special class of serine-threonine protein kinases, CDKs, is the core of cell cycle progression (Fig. 1-4). The activity of CDK (cyclin-dependent kinase) rises and falls as the cells progress through the cell cycle. CDKs are regulated at multiple levels, mainly by association and dissociation with different sets of cyclins during different cell cycle phases. The variation in CDK-activity

leads to changes in the phosphorylation state of proteins that initiate or regulate cell cycle events such as DNA replication, mitosis and cytokinesis. The CDKs are only active when bound to cyclins. Cyclins undergo a cycle of synthesis and degradation in each cell cycle. Consequently, the cyclic assembly and activation of the cyclin-Cdk complexes trigger different events in the cell cycle.

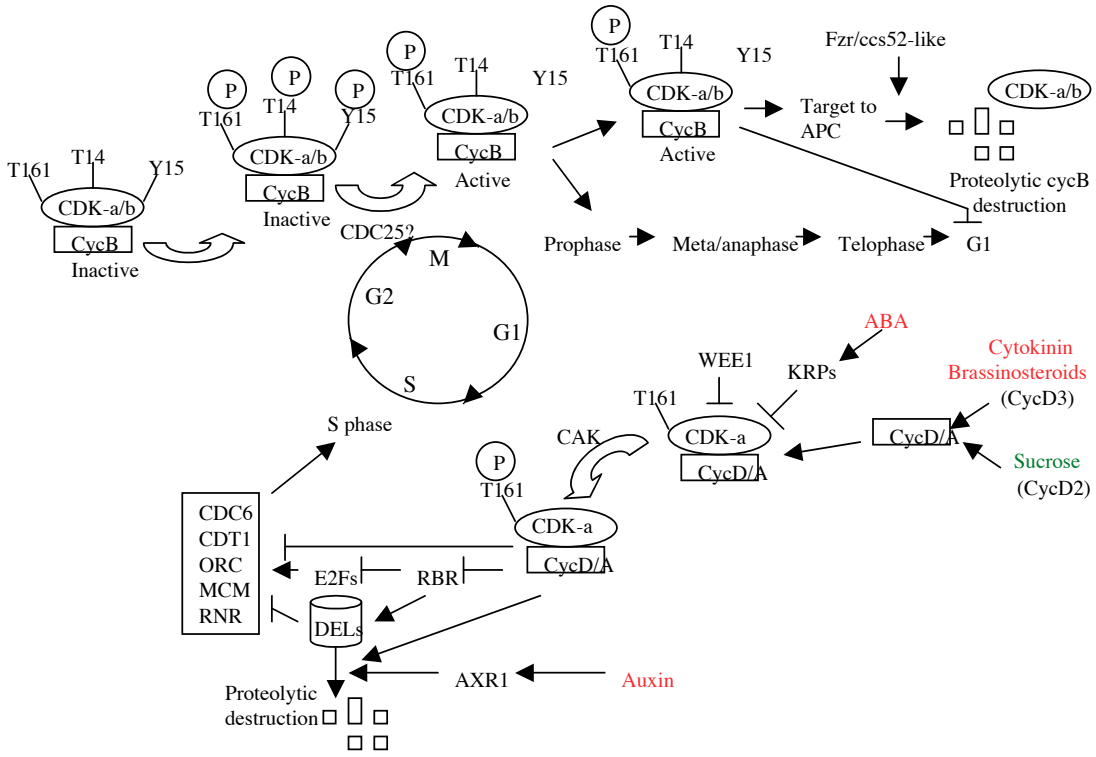


Figure 1-4. Schematic diagram of cell cycle progression in plants. CDK activities peak during G1→S and G2 →M transitions. External and intrinsic signals have different targets. This model is based on the mammalian cell cycle and adapted from den Boer and Murray (2000). Most factors have been identified in plants except the cdc25 phosphatase.

In plants, in response to cytokinin, brassinosteriods and sucrose, cyclin D3 and D2 are up-regulated, leading to activation of CDK-a. ABA can increase the level of inhibitor KPP to repress the CDK-a activity and thus, inhibit cell division. Rising levels of CycD—CDK-a kinase activity lead to phosphorylation of retinoblastoma-related

protein (RBR) and inactivate RBR. Once RBR is inactive, the E2F transcription factor is no longer repressed and S-phase genes are transcribed. DELs are E2F-like proteins but act as repressors (see later). Auxin can up-regulate AXR1 protein, which promotes the degradation of DEL protein. As a result, auxin promotes cell division. When cells approaching M-phase, CycB — CDK-a/b complex is inactive. The activation requires both CAK kinase and removal of inhibitory phosphates on T14 and Y15 by a CDC25-like phosphatase. The active complex triggers entry into prophase. A further control operating between telophase and G1 requires the destruction of mitotic CDK activity by anaphase-promoting complex (APC).

Retinoblastoma (RB) proteins

RB is the first tumor suppressor gene to be identified by its role in a rare pediatric cancer called retinoblastoma. The RB gene is mutated in a wide range of cancers in at least one third of all human tumors (Weinberg, 1992). RB is the main regulatory gene for the G1-S transition of the cell cycle. In early G1, RB is found in an underphosphorylated form. At a time close to the R (restriction) point, which demarcates the transition from a serum-dependent to serum-independent state (Pardee, 1989), the G1 cyclins bind to RB and direct their partner CDKs to phosphorylate and inactivate RB (Fig. 1-5b).

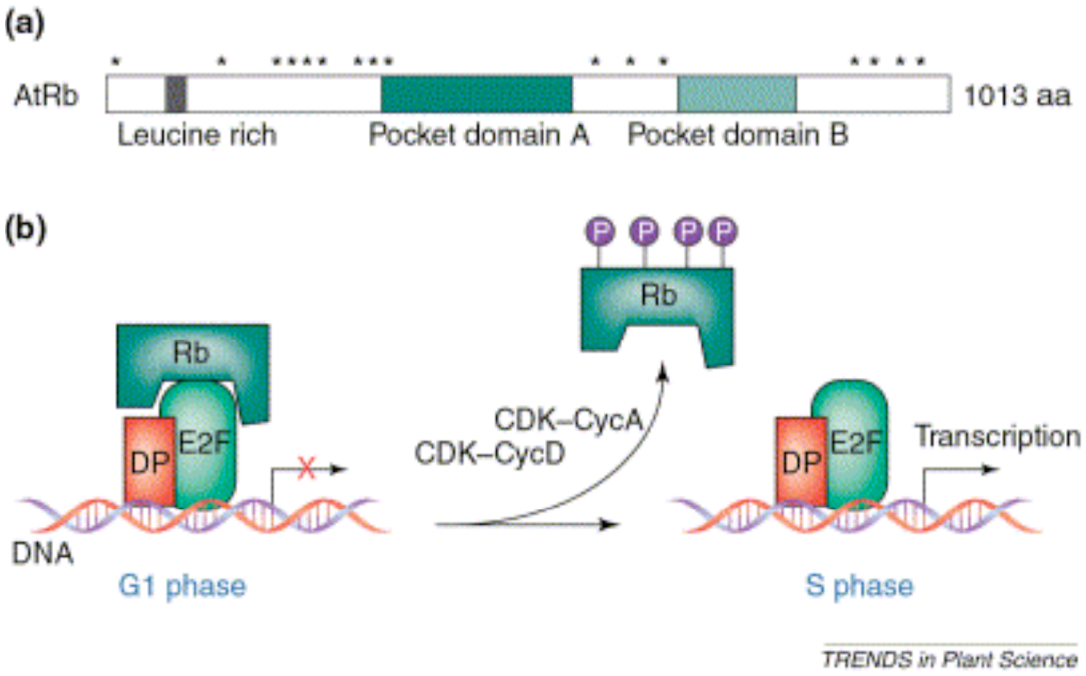


Figure 1-5. The model for activation of E2F-pRb pathway. (a) Structure of Arabidopsis retinoblastoma protein. Asterisks indicate the potential phosphorylation sites by CDK-cyclin complex. (b) During G0 or early G1 phase, hypophosphorylated Rb binds E2F/DP dimers and inactivates the E2F activity. During late G1 and early S phase, Rb is hyperphosphorylated, first by CDK-CycD and then by CDK-CycA, resulting in the dissociation of Rb from E2F. The released E2F/DP becomes active and promotes transcription of E2f-target genes in cell-cycle regulation, DNA synthesis and replication, and chromatin assembly(cited from Shen, 2002).

As shown in Figure 1-5a, RB harbors more than a dozen distinct sites of phosphorylation on either serine or threnonine residue. The A/B pocket domains physically interact with a set of protein factors with LXCXE motif including E2Fs and HDAC. The C-terminal region is responsible for binding to cyclins through R/KXL motif. In growth-arrested cells and during early G1, hypophosphorylated RB binds to E2F/DP dimmer and consequently repress the E2F/DP transcription activity by recruiting a set of chromatin-remodeling factors such as SWI-SNF (modification of nucleosome structures), HDAC (deacetylation of histones), HMTase (methylation of histones) and DNMT (methylation of DNA) to the promoters of E2F/DP target genes (Shen, 2002).

However, once hyperphosphorylated by the CDK/cyclin complex during late G1 and early S phase, RB is no longer able to bind E2F/DP dimers as phosphorylation causes RB to loose its grip on E2F, enabling E2F to activate a cohort of client genes (Chellappan et al., 1991). Since E2F regulates the expression of numerous genes needed for cell cycle entry and DNA synthesis, loss-of-function of RB will result in constitutive activation of E2F, thus leading to loss of control of cell proliferation.

E2F transcription factors

As mentioned above, the key target of RB gene is E2F transcription factor. E2F was a critical cellular factor in the E1A-mediated activation of the Adenovirus E2 promoter, thus, the name E2F (Nevins, 1992). It was observed that E1A caused a cellular protein (later shown to be the RB) to dissociate from E2F and activate E2F by sequestering RB. E2F is identical to the differentiation-regulated transcription factor DRTF. In a variety of organisms, E2F is found to exist as a gene family. So far, there are seven E2Fs reported in human. In addition, a distantly related protein, called DP (first identified as DRTF-1 polypeptide) (Girling et al., 1993), always forms a heterodimer with E2F and binds to DNA. Therefore, people very often combine the two, and use E2F/DP to describe their function in literature.

The most intriguing aspect of the E2F gene family is the existence of a 'sibling rivalry' among them. E2F1, E2F2 and E2F3 are activators whereas E2F4, E2F5, E2F6 and E2F7 are repressors. While activating E2Fs are specifically regulated by their association with RB in normal cells, the repressive E2Fs are mainly associated with the

related pocket proteins (p107 or p130) or lack the binding domain for any of the pocket proteins (see below) (Trimarchi and Lees, 2002). Nonetheless, they all recognize the same consensus site TTTCCCGC. Hence, different transcription responses invoked by the individual E2F/DP species depend on the identity of the E2F moiety as well as proteins associated with them. Overexpression of each activating E2F is sufficient to irreversibly commit cells to re-enter the cell cycle, while the combined mutations of E2F1, E2F2 and E2F3 are sufficient to completely block cellular proliferation (Lukas et al., 1996;Wu et al., 2001). In addition, de-regulated E2F activity can trigger apoptosis through either p53-dependent or –independent mechanisms when the total pool of transcriptional activity contributed by the activating E2Fs exceeds a higher threshold (Trimarchi and Lees, 2001). Although the three activating E2Fs share redundant functions, they might play different roles in normal development shown by the tissue-specific defects of corresponding E2F-difficiency mice (Trimarchi and Lees, 2001).

Core cell cycle genes in *Arabidopsis*

Although cell cycle has been extensively studied in yeast and animals, little is known about the regulation of cell cycle in plants. The high level of sequence similarity shared between the plant and animal cell cycle regulators such as E2F/DP, RB proteins, and other core cell cycle regulators, suggests the conservation of cell cycle mechanism. Recent high-quality, homology-based annotation of the *Arabidopsis* genome revealed that there are 61 core cell cycle genes, belonging to seven selected families (Vandepoele et al, 2002) (Table 1-1). In yeast, one CDK is sufficient to drive cells through all cell cycle phases whereas in multicellular organisms, a family of related CDKs with specific functions are involved. The *Arabidopsis* genome has eight CDKs with one A-type, four

B-type, two C-type, one E-type and four CDK-activating kinases (CAK) (three D-type and one F-type) (Vandepoele et al, 2002). A-type CDKs have been shown to regulate both the G1-to-S and G2-to-M transitions whereas B-type CDKs is only involved in controlling G2-to-M checkpoint (Porceddu et al., 2001). The large number of CDK genes suggests the complexity of cell cycle regulation in plants.

Table 1-1. Core cell cycle genes in *Arabidopsis*

Major players	Subtypes	No. of genes	Note
CDK	CDK-A	1	G1-S and G2-M
	CDK-B	4	G2-M
	CDK-C	2	?
	CDK-E	1	?
CAK	CAK-D	3	CDK activation kinase
	CAK-F	1	
CYCA	CYCA1	2	mitotic cyclins
	CYCA2	4	
	CYCA3	4	
CYCB	CYCB1	4	mitotic cyclins
	CYCB2	4	
	CYCB3	1	
CYCD	CYCD1	1	G1 cyclins
	CYCD2	1	
	CYCD3	3	
	CYCD4	2	
	CYCD5	1	
	CYCD6	1	
	CYCD7	1	
CYCH	no	1	regulate CAK activity
CKS	no	2	docking factros for CDK
KRP	no	7	CDK inhibitor
WEE1	no	1	negative regulator of CDK/cyclin
RBR	no	1	tumor suppressor
E2F	E2Fa	1	transcription factor (G1-S transition)
	E2Fb	1	
	E2Fc	1	
DP	DPa	1	binding partners for E2F
	DPb	1	
DEL	DEL1	1	DP-E2F-like, repressor
	DEL2	1	
	DEL3	1	

The timing of CDK activation is determined mainly by a large number of cyclins, whose levels fluctuate in the cell cycle. There are two major groups of cyclins: mitotic cyclins (A- and B-type cyclins) and G1-specific cyclins (D-type cyclins). H-type cyclins regulate the CAK activity. *Arabidopsis* has ten A-type cyclins, nine B-type cyclins, ten D-type (seven subclasses: one D1-type, one D2-type, three D3-type, two D4-type and one D5, D6, D7-type). There is only one H-type cyclin in *Arabidopsis* genome.

There are two CKS (CDK subunit) proteins (CKS1 and CKS2) in *Arabidopsis*, which act as docking factors to mediate the interaction between CDKs and their substrates or regulatory proteins. CDK inhibitor (CKI) proteins can bind to specific CDKs and inactivate CDKs. In *Arabidopsis*, all seven CKI genes belong to the group of Kip/Cip CKIs and hence, are designated KRP1 to KRP7 (De Veylder et al., 2001). CDK/cyclin activity is negatively regulated by WEE1 kinase, which phosphorylates CDK subunit. This inhibitory effect can be removed by CDC25 phosphatase. There is one WEE1 homolog gene but no CDC25 homolog in *Arabidopsis*.

RB (retinoblastoma) and the E2F/DP proteins are key regulators that control the start of DNA replication. While only one Rb gene exists in the *Arabidopsis* genome, eight E2F/DP genes are found, which can be divided into three groups. The first group comprises E2Fa, E2Fb, and E2Fc, which are most similar to the mammalian E2F factors (46% overall similarity). The second group consists of two DP genes (DPa and DPb). The third group contains three genes with 59% similarity among themselves, 21% and 18% similarity to E2F and DP genes, respectively, which is named DP-E2F-like (DEL1,

DEL2, DEL3). They were previously named E2Fd, E2Fe and E2Ff, respectively. Unlike the E2F and DP genes that harbor DNA binding, dimerization, Marked and Rb binding domains, DEL genes only contain two duplicated DNA binding domains that are highly similar to the E2F DNA binding domain (Fig. 1-6). The DNA binding domain of E2F/DP/DEL recognize the same consensus motif (TTTSSCGSS, S being C or G).

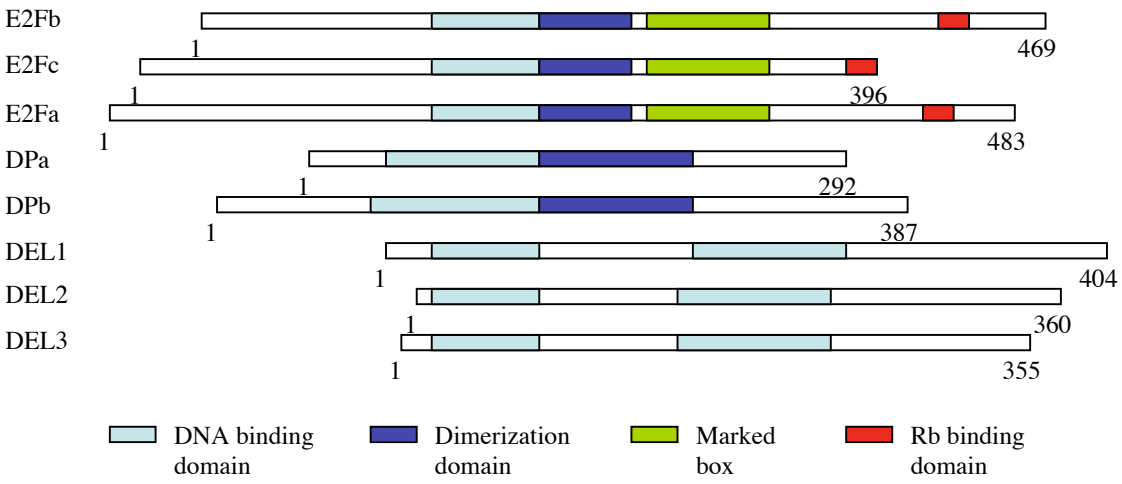


Figure 1-6. Structures of E2F gene family. E2Fa, E2Fb and E2Fc are in the same class while DPa and DPb belong to the second class. DEL1, DEL2 and DEL3 are unique in that they lack a dimerization domain and act more likely as repressors (cited from Vandepoele et al, 2002).

Based on the DNA binding site, Ramirez-Parra et al (2003) conducted a genome-wide analysis of E2F-regulated genes in *Arabidopsis*. In addition to a large number of genes belonging to cell cycle and DNA replication (31.3%), and transcription (21.7%), a variety of putative E2F target genes are found to be involved in other processes such as defense, signal transduction, and metabolism. This is consistent with the observation in animals that E2F/DP transcription factors regulate gene expression not only in proliferating cells but also during differentiation, development and apoptotic response (Muller et al., 2001; Ren et al., 2002; Wells et al., 2002). Further evidence came from a

genome-wide microarray analysis of E2Fa-DPa overexpression in plants (Vlieghe et al., 2003), in which a number of up-regulated genes are involved in cell wall biosynthesis and nitrate assimilation.

Cell division and development

A fundamental question in plant development is how cell division events are coordinated and integrated with cell expansion, leading to the normal growth patterns. The basic cell cycle machinery in plants is controlled by a variety of internal and external signals (Hemerly et al, 1999; Mironov et al, 1999). Upstream controls can act at distinct levels such as modulating CDK level and activation, cell cycle timing and cell division pattern and frequency to generate the final plant architecture. Previous studies showed that plant morphogenesis is not strictly dependent on rates and precise planes of cell division (Smith et al. 1996; Reynolds et al. 1998; Mironov et al. 1999). For example, the maize *tangled* mutant has misaligned cell division orientations in developing leaves, but its overall leaf shape is normal (Smith et al., 1996). In *Arabidopsis short integuments 2* (*sin2*) mutants, cell number in the outer integuments of ovules is reduced 5-10-fold, but the overall morphology of the *sin2* is relatively normal (Broadhvest et al., 2000). It was suggested that when the basic cell cycle machinery is somewhat perturbed, it can be locally sensed via cell-cell communication and finally balanced with cell expansion to maintain normal growth patterns (Meyerowitz 1996; Scheres and Heidstra 1999). Obviously, this kind of tolerance of altered cell number or size has its limit.

When specifically expressed in embryos under the promoter of *2S2* albumin gene, the dominant-negative *cdc2aAt* mutant causes a distorted apical-basal pattern, suggesting that cell division events are essential for the elaboration of the apical-basal pattern and morphogenesis of embryos (Hemerly et al., 2000). Constitutive expression of E2F/DP in tobacco altered plant morphology with curled leaves, round petals and shortened pistils. Mature leaves of the transgenic plants contained increased numbers of small cells (Kosugi and Ohashi, 2003).

Overexpression of E2Fa/DPa in *Arabidopsis* also results in uncontrolled cell division and delayed differentiation. Transgenic plants arrest growth early with several curled leaves (De Veylder et al., 2002). This is in contrast to ectopical expression of cyclin B1 and cyclin D2, which promote plant growth (Doerner et al., 1996; Cockcroft et al., 2000). It suggests that overexpression of E2Fa/DPa overrides signals that regulate cell differentiation and that the balance between division and differentiation is vital for normal plant development. *Arabidopsis* cyclin D3 is transcriptionally regulated by sucrose and cytokinins (Soni et al., 1995), suggesting that cyclin D3 could relay developmental signals to the cell cycle machinery. Overexpression of cyclin D3 results in developmental aberrations such as extensive leaf curling, disorganized shoot meristem and delayed senescence (Riou-Khamlichi et al., 1999).

However, most of these studies are limited to ovexpression of cell cycle genes and these gain-of-function studies might not reveal the normal function of each of the cell cycle genes in plant development. On one hand, extreme redundancies among the cell

cycle genes in plants prevent single loss-of-function mutants from exhibiting any developmental defects. On the other hand, some cell cycle genes play such essential roles that they are indispensable for viability. For instance, the anaphase-promoting complex (APC) regulates mitotic progression and exit by controlling the stability of cell cycle regulatory proteins such as mitotic cyclins. *Arabidopsis apc2* is female gametophytic lethal and arrested mainly at one or two-nucleus stage of megagametogenesis (Capron et al., 2003). In *hobbit* mutants, the organization of cell layers in shoot apical meristem is disrupted due to unordered cell division patterns. Cell division ceases after an initial proliferation phase, leading to seedling lethality (Billou et al., 2002). *HOBBIT* encodes a homolog of the CDC27 subunit of APC. The deregulation of auxin reporter genes and repressors AXR3/IAA17 in *hobbit* mutants suggests that *HOBBIT/CDC27B* controls auxin-mediated cell division and differentiation responses.

Mutations in plant retinoblastoma protein (RB) homolog also cause a gametophytic lethality (Ebel et al., 2004). *Arabidopsis* is currently the only plant known to contain a single retinoblastoma-related gene (RBR1). In *rbr1* mutants, the mature unfertilized megagametophyte fails to arrest mitosis and undergoes excessive nuclear proliferation in the embryo sac, which is in consistent with its negative role in cell cycle progression. In addition, the central cell nucleus in *rbr1* mutants can initiate autonomous endosperm development reminiscent of *fertilization-independent seed* (*fis*) mutants (Ebel et al., 2004), suggesting that correctly exiting the cell cycle is required for normal gametogenesis and repression of autonomous endosperm development.

DNA damage checkpoint

Progression of the cell cycle is not always smooth as cells are facing constant physical and chemical assaults including reactive metabolic products, which can induce heritable mutations or DNA strand breaks. A surveillance mechanism has evolved so that the integrity of DNA is monitored during the cell cycle. The DNA damage checkpoint will slow or arrest the cell cycle and lead to induction of genes participating in DNA repair. Increasing evidence suggests that these checkpoints are not specific points during the cell cycle at which genomic integrity is assessed, instead they continuously monitor the integrity of the genome and control cell cycle progression accordingly (Nasmyth, 1996). The same proteins involved in regulating the orderly progression through the cell cycle are also involved in the checkpoint responses. Conversely, DNA damage checkpoint proteins like ATM and ATR, previously thought to be only involved in DNA damage responses, have been shown to regulate normal DNA replication in unperturbed cells (Shechter et al., 2004).

The DNA damage checkpoint has three components; sensor, signal transducer, and effectors (Sancar et al., 2004). The sensors monitor DNA for structural abnormalities and then initiate the checkpoint signal. Transducers further transmit and amplify this signal. Effectors execute the action leading to the biological consequences. However, there is no absolute demarcation between the various components of the checkpoint. For instance, the damage sensor ATM also acts as a signal transducer (Sancar et al., 2004). A fourth class of checkpoint proteins, called mediators, is conceptually placed between sensors and signal transducers. In addition, many components of the DNA damage checkpoint pathways are shared with G1/S, intra-S, and G2/M checkpoint pathways.

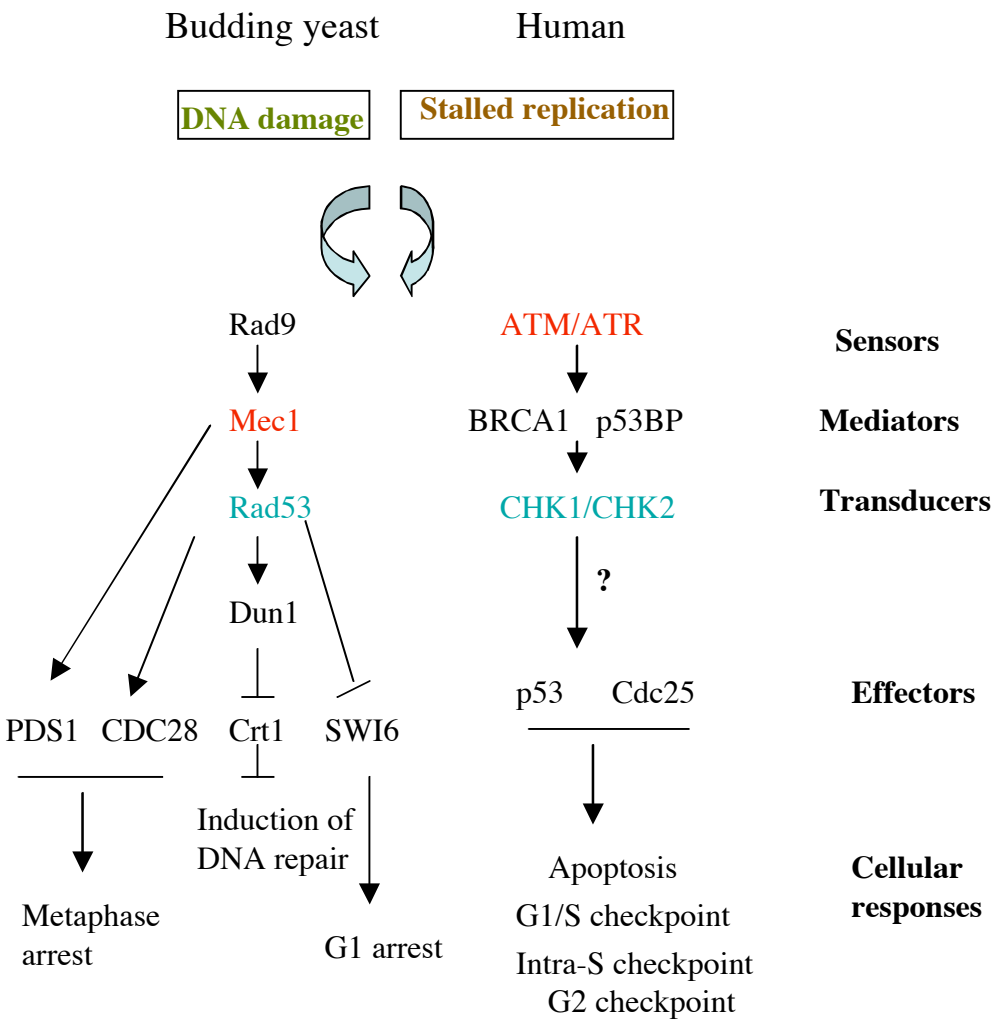


Figure 1-7. Components of the DNA damage checkpoints in yeast and human cells. The damage is detected by sensors. With the aid of mediators, sensors transduce the signal to transducers, which, in turn, activate or inactivate other proteins. These effectors directly participate in various cellular responses including cell cycle arrest and damage repair. Proteins shown in same color are conserved from yeast to human.

Checkpoint proteins are well conserved from yeast to human, indicating that the basic organization of these pathways has been preserved throughout evolution. For example, yeast Mec1 and human ATM/ATR are members of the evolutionarily conserved subfamily of phosphatidylinositol 3-kinase (PI3-kinase) (Elledge, 1996). In yeast, the

Rad24/Rad17 subclass of genes and Rad9 are the two major proteins involved in sensing DNA damage (Lowndes and Murguia, 2000). Although Mec1, Rad53 and Dun1 are regarded as the signal transducer, Mec1 is actually required for phosphorylation of Rad9 (Vialard et al., 1998). Rad53 can recognize and specifically bind to hyperphosphorylated Rad9 (Durocher, et al., 1999). Since Rad9 remains bound to chromation after DNA damage, this mechanism would recruit Rad53 to lesions, allowing localized amplification of the checkpoint signal (Lowndes and Murguia, 2000).

In mammalian cells, the tumor suppressor p53 protein is the core of the DNA damage checkpoint pathway. As a transcription factor once activated by DNA damage, p53 can turn on p21 transcription, which in turn inactivates CDK4. As a consequence, cell cycle is arrested at G1/S phase. P53 also regulates a large number of target genes including those involved in DNA repair. In addition, p53 can monitor the extent of DNA damage and trigger apoptosis to remove damaged cells. Cells undergoing apoptosis shrink, develop bubble-like blebs on their surface, and degrade their chromatin. The mitochondria of apoptotic cells eventually break down into small, membrane-wrapped fragments and are engulfed by phagocytic cells (Lam et al., 2001). The pattern of events is so orderly that the process is often called programmed cell death (PCD).

Programmed cell death

Programmed cell death (PCD) is needed not only to destroy cells that represent a threat to the integrity of the organism, such as those damaged cells, but also intrinsic to the cell for proper development. For instance, apoptosis occurs during the resorption of

the tadpole tail at the time of its metamorphosis. The formation of the fingers and toes of the fetus requires removal of the tissues between them, which is carried out by apoptosis. In plants, PCD is needed for terminal differentiation of xylem vessels. Senescence is an active programmed cell death process to recycle the energy and resources to other parts. Hypersensitive response (HR)-mediated PCD is an important mechanism in defense against pathogens.

Several morphological and biochemical similarities were found between animal cells undergoing apoptosis and plant cells undergoing PCD, such as condensation and shrinkage of the cytoplasm and nucleus, caspase-like proteolytic activity, cytochrome c release from mitochondria, the formation of DNA-containing (apoptotic-like) bodies and genomic DNA degradation or DNA laddering (Pennell and Lamb, 1997; Danon et al., 2000). However, plant cells do not engulf their dead neighbors due to the presence of cell wall. Instead, in xylogenesis, the dead plant cells become part of the very architecture of the plant performing crucial functions such as water conducting and mechanical support (Greenberg, 1996).

Ribonucleotide reductase (RNR)

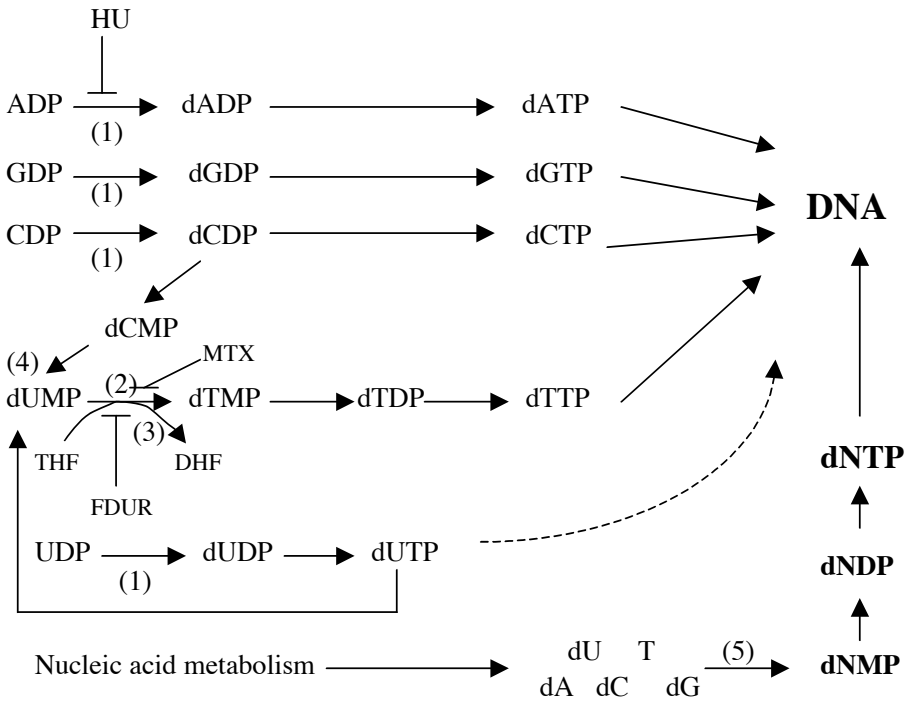
Biosynthesis and metabolism of deoxyribonucleotide

Deoxyribonucleotides are of paramount importance for DNA replication and repair. Two processes — the conversion of ribose to deoxyribose and the conversion of uracil to thymine, are essential to *de novo* deoxyribonucleotide biosynthesis. Not

surprisingly, both processes are target sites for chemotherapy for cancer or infectious diseases. Thus, enzymes catalyzing both steps are under intensive study.

Ribonucleotide reductase (RNR) catalyzes the reduction of all four common ribonucleotides to the corresponding 2'- deoxyribonucleotides in all organisms. RNR acts upon ribonucleoside diphosphate (NDPs) substrates. Once reduced, dADP, dGDP and dCDP are converted directly to the corresponding triphosphate by nucleoside diphosphate kinase. Biosynthesis of dTTP is completed partly from dUDP and partly from dCDP (Fig. 1-8). First, dCDP is dephosphorylated to dCMP, which then undergoes deamination by dCMP deaminase to become dUMP. Second, dUDP is phosphorylated to dUTP, which is then cleaved by highly active dUTPase, to produce dUMP. This step is very important to exclude uracil from DNA. dUMP can arise in DNA either by spontaneous deamination of dCMP, which will lead to mutations, or by dUTP incorporation. Since the cell cannot tell whether a dUMP arises from the deamination of dCMP or from natural incorporation, a surveillance system has evolved to excise dUMP no matter how it arises.

After dUMP is formed, it is converted to dTMP by thymidylate synthase (TS). 5,10-methylenetetrahydrofolate (THF) serves as a redox cofactor to give rise to dihydrofolate (DHF) as a byproduct of the reaction (Fig. 1-8). In some species, such as plants, dihydrofolate reductase (DHFR) and thymidylate synthase (TS) are encoded by a single gene, called DHFR-TS. dTMP, once formed, is converted to dTTP by two successive phosphorylations.



De Novo synthesis
(1) Ribonucleotide reductase
(2) Thymidylate synthase
(3) Dihydrofolate reductase
(4) DCMP deaminase

(5) Salvage pathway
Thymidine kinase
Deoxyadenosine kinase
Deoxycytidine kinase

Figure 1-8. Diagram of *de novo* and salvage pathways for biosynthesis of dNTPs. The salvage pathway allows the reutilization of material obtained from degradation of DNA, regulates the flux of deoxyribonucleotides in and out of cells and together with the *de novo* pathway, contributes to the setting of the intracellular levels of dNTPs.

Salvage Pathway

Conversion of NDPs into dNDPs is not the only pathway for dNTP synthesis. Free deoxypurine nucleosides and deoxypyrimidine nucleosides can also be utilized for dNTPs production, which is called the salvage pathway (Fig. 1-8). This is accomplished by the corresponding deoxyribonucleoside kinases including thymidine kinase (TK), deoxyadenosine kinase (dAK), deoxyguanosine kinase (dGK) and deoxycytidine kinase

(dCK). Salvage pathways vary from species to species, partly in their contents of deoxyribonucleoside kinases. For example, human cells contain only four deoxyribonucleoside kinases including a cytosolic dCK, a mitochondria-localized dGK and two isoforms of thymidine kinase: one localized in cytosol (TK1) and one in mitochondria (TK2). The dCK can act on deoxyadenosine and deoxyguanosine as well as deoxycytidine. The TK2 can also act upon deoxycytidine and deoxyuridine. Of the four deoxyribonucleoside kinases, only TK1 is cell cycle-regulated with peak expression at S-phase, resembling enzymes of the de novo deoxyribonucleotide synthesis. Since mitochondrial DNA is constantly replicating even in quiescent cells, the constant supply of dNTPs is vital to the maintenance of the mitochondrial genome. Hence, the main supply of dNTPs for mtDNA synthesis comes from the salvage pathway initiated by dGK and TK2, which are constitutively expressed.

The importance of salvage pathway for dNTP synthesis is best illustrated by an autosomal recessive disorder called mitochondrial DNA-depletion syndromes (MDS). MDS patients show progressive muscle weakness or liver failure, and the majority of affected individuals die during the first year of life (Mandel et al. 2001). The mtDNA/nuclear DNA ratio could be reduced to 8-39% of the control mean in MDS patients, mainly in liver and brain tissues. MDS is caused by defective dGK (Mandel et al. 2001). Saada and his colleagues (2001) showed that mutations in TK2 are responsible for the mitochondrial DNA depletion myopathy.

History of ribonucleotide reductase

The first ribonucleotide reductase (RNR) was cloned in *E.coli* in 1961, a big shock to the field since back then, organic chemists were not aware of reactions in which a carbon-bound OH-group could be replaced directly by hydrogen (Jordan and Reichard, 1998). RNR was the first protein free radical to be discovered: an organic free radical, identified as tyrosyl-122, forms part of the polypeptide structure of the small subunits of the enzyme and is essential for its activity (Ehrenberg and Reichard, 1972; Sjoberg et al., 1978). The radical is so stable that it survived the two-week period taken to purify the enzyme.

The *E.coli* RNR became the prototype of class I enzymes to which all eukaryotic reductases belong. Later, class I reductases were subdivided into two subgroups. One class (Ia) has the original E.coli enzyme as the prototype, which also includes the eukaryotic enzymes. The second class (Ib) is present exclusively in bacteria. RNR in *Lactobacillus leichmannii* was found to require adenosylcobalamin, leading researchers to believe that the reductases were B_{12} enzymes. This is because pernicious anemia, a disease caused by defective DNA synthesis due to malfunctional RNR, can be cured by administration of vitamin B_{12} (Blakley and Barker, 1964). The requirement of adenosylcobalamin is an exclusive feature of class II reductases (see Table 1-2). Since the generation of the tyrosyl radical of class I enzymes requires oxygen for its formation, the discovery of a second ribonucleotide reductase in anaerobically growing E.coli came as no surprise (Fontecave et al., 1989). The anaerobic enzyme, the prototype of class III reductases, contains an iron-sulfur cluster and uses this cluster and S-adenosylmethionine to generate the glycyl radical (Ollagnier et al., 1996).

The three RNR classes share a common tertiary structure in spite of large differences in the primary sequences. The known three-dimensional structures of class I and class III show overwhelming structural similarity. Sequence comparison between class I and class II enzymes shows that they contain identical, strategically placed amino acids involved in catalysis and allosteric regulation. With the elucidation of an increasing number of primary sequences of class II and class III enzymes, there is now almost no doubt that the R1 large subunits of the three classes share a common ancestor.

Table 1-2. Overview of the characteristics of the ribonucleotide reductase classes

	Class Ia	Class Ib	Class II	Class III
Oxygen dependence	Aerobic	Aerobic	Aerobic/anaerobic	Anaerobic
Structure	a2b2	a2b2	a(a2)	a2b2
Genes	*nrdAB*	*nrdEF*		*nrdDG*
Radical	Tyr…Cys	Tyr…Cys	AdB12…Cys	AdoMet…Gly…Cys?
Metal site	Fe-O-Fe	Fe-O-Fe Mn-O-Mn	Co	Fe-S
Substrate	NDP	NDP	NDP/NTP	NTP
Reductant	Thioredoxin Glutaredoxin	NrdH-redoxin Glutaredoxin	Thioredoxin	Formate
Allosteric sites/ polypeptide chain	2	1	1	2
dATP inhibition	Yes	No	No	Yes
Occurrence	Eukaryotes Eubacteria Bacteriophages Viruses	Eubacteria	Archaebacteria Eubacteria Bacterophages	Archaebacteria Eubacteria Bacterophages
Prototype	*E.Coli* Mouse	*S.typhimurium* *C.ammoniagenes*	*L.leishmannii*	*E.Coli*

As shown in Table1-2 (adapted from Jordan and Reichard, 1998), all classes but class II enzymes consist of two homodimeric proteins, R1 (α_2) and R2 (β_2). The large R1 subunit of class I enzyme harbors the catalytic site and binding sites for allosteric effectors whereas the small R2 subunit of class I enzymes contains an oxygen-linked diferric center and a stable tyrosyl free radical. In contrast, a stable oxygen-sensitive

glycyl radical is located on the large subunit of class III enzymes whereas the small subunit contains an iron-sulfur cluster that together with S-adenosylmethionine generate the glycyl radical. Since the glycyl radical of class III is rapidly destroyed by oxygen (King and Reichard, 1995), class III enzymes are strictly anaerobic. However, radical formation from adnosylcobalamin of class II enzymes does not require oxygen nor is the radical sensitive to oxygen. Therefore, class II enzymes function in both aerobic and anaerobic organisms.

Structure of ribonucleotide reductase

Class I RNR is a tetramer protein complex (Fig. 1-9 and Fig.1-10), consisting of two nonidentical homodimers: the large R1 subunit and the small R2 subunit. The large homodimer mediates both catalysis and allosteric interactions and the small homodimer provide the tyrosine free radical for catalysis. The large subunit has three binding sites. The first site binds ATP/dATP to control the overall activity. The negative feedback regulation of RNR by dATP is a mechanism to make sure that cells will not use up NTPs, which are the backbones for RNA synthesis. The second site binds ATP, dATP, dGTP and dTTP to control the specificity. This level of regulation leads to the production of a balanced dNTP pool (illustrated in Fig. 1-9). The third site binds the substrates (NDPs) as a catalytic site. In addition, there is a redox site where thioreddoxin or glutaredoxin reduces the disulfide bonds generated during catalysis. The ultimate reducing power comes from NADPH.

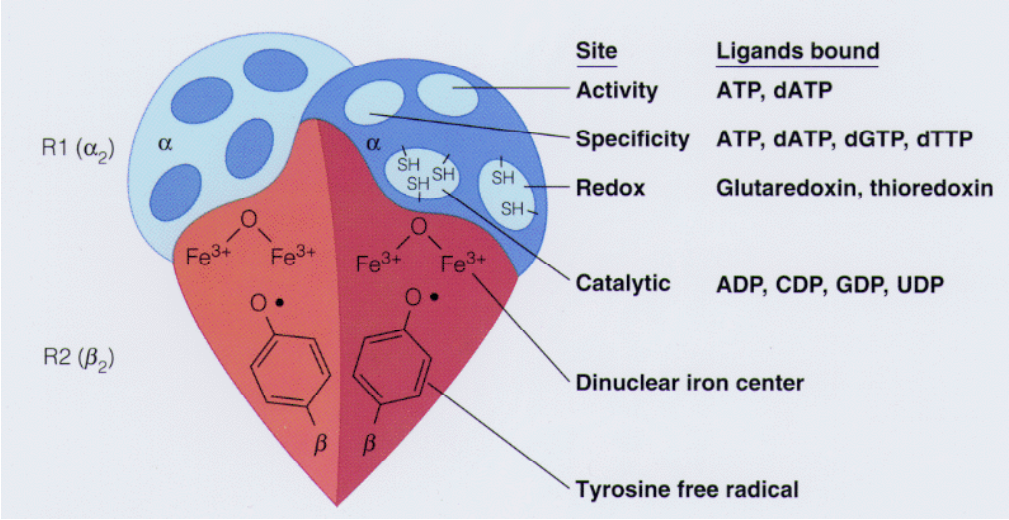


Figure 1-9. Schematic diagram of RNR structure. R1 dimer are shown as blue spheres while R1 dimer in red. The diferric iron center is linked to oxygen and is essential for radical generation on the neighboring tyrosine. (cited from Mathews, van Holde and Ahern (2000) Biochemistry, 3rd edition p810)

How does one enzyme catalyze the reduction of four different ribonucleotides and how does it manage to produce a balanced dNTP pool? The three binding sites for activity, specificity and catalysis in the R1 large subunit provide an intricate regulation of the order in which ribonucleotides are bound and reduced (Fig. 1-10). The rationale is that cell division will not occur unless the cell's energy state is high. When the cell's energy state is high, the concentration of ATP is high but the dNTP level is low. So, ATP will bind to the activity site of R1, turning on the RNR activity. ATP will also binds to the substrate specificity site, leading to the production of dUDP and dCDP. As noted earlier, both dUDP and dCDP are precursors for dTTP. When the concentrations of dUDP and dCDP go up, the concentration of dTTP also rises. Then, dTTP is able to compete with ATP for the substrate specificity site. When dTTP is bound, then GDP becomes the substrate and dGDP is made. dGDP is rapidly converted into dGTP, which,

upon reaching a certain concentration, will compete with dTTP for the specificity site. When dGTP is bound to the specificity site of R1, ADP becomes the substrate and dADP is produced. dADP is again converted into dATP, which, upon reaching a certain concentration, will compete with ATP for the activity site. When dATP is bound the RNR is turned off.

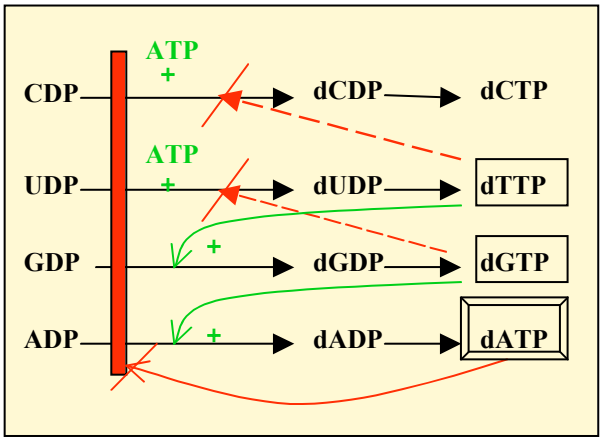


Figure 1-10. Schematic diagram of regulation of RNR activity. The red rectangle bar designates the activity site, which is activated by ATP and inhibited by dATP. The red dashed line indicates inhibition and green line indicates activation.

As shown in Fig. 1-11, the R1 dimer (on the top) is an unstable structure as both subunits and domains can move with respect to each other. This may form a structural basis for the allosteric regulation and catalysis. The binding of R2 can stabilize the R1 dimer. The shape of the R1 dimer is complementary to the upper part of the heart-shaped structure of the R2 dimer (on the bottom), allowing the R1 dimer to sit like a saddle on the top of the R2 dimer. The upper part of R2 fits neatly into the cavities of R1, in agreement with the location of conserved residues in this part of the R2. There is still space for the substrate to pass into the active site, but only nucleoside diphosphates are allowed to bind as substrates as the ribose moiety of a nucleoside triphosphate would not be able to bind close to the active site (shown as yellow spheres in Fig. 1-11).

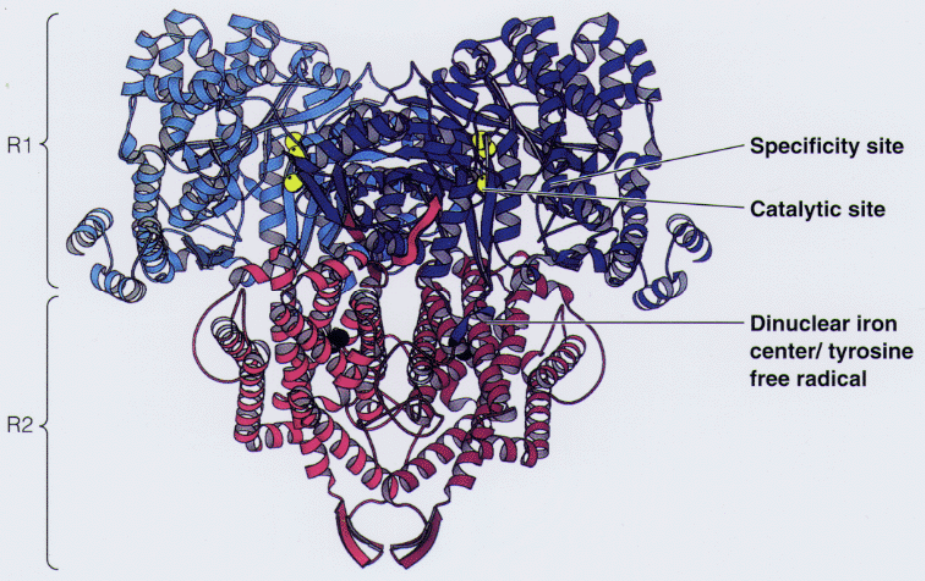


Figure 1-11. Three-dimensional structure of RNR. The R1 dimer sits like a saddle on the top of R2 dimer, which is heart-shaped. (cited from Uhlin and Eklund, 1994)

The C-terminus of R2 protein (at most 30 residues) accounts for all of its interactions with R1 in *E.coli*. Like the N-terminus, this carboxyl domain is not conserved between species, reflecting the species-specific interaction between R1 and R2. Surprisingly, the last two residues of R2 are always Asp and Phe in most species. As short as 7 polypeptides, corresponding to the C-terminus of R2, can strongly compete with the full-length R2 (375 aa) for binding of R1. Substitution of one amino acid in the C-terminus of R2, such as E350A or Y356A can still bind to R1 tightly. However, E350A exhibits a low activity (240 times less active than the wild type), and Y356A is completely inactive (Climent et al, 1992), suggesting that the conformation of the holoenzyme complex might be changed.

Unusual organization of RNR genes in yeast

The large subunit and the small subunit are encoded by two genes in yeast, *RNR1* and *RNR3* for R1, and *RNR2* and *RNR4* for R2 (see Table 1-3). Unlike other yeast *RNR*

genes, expression of RNR3 is undetectable under normal growth conditions but can be induced up to 100-fold by DNA damage (Elledge and Davis, 1990), suggesting that *RNR3* is mainly involved in the DNA repair pathway. Nevertheless, the *RNR3* mutant has no obvious phenotype, even under DNA damage conditions. Domkin et al (2002) found that the *in vitro* activity of RNR3 is less than 1% of the RNR1 activity even though the two proteins share 80% identity. This is partly because RNR3 does not readily dimerize. *In vivo,* the protein levels of RNR3 after DNA damage never reach more than 10% of the RNR1 levels. However, a strong synergism between the RNR3 and RNR1 was observed. A catalytically inactive RNR1-C428A mutant can increase the endogenous activity of RNR3 by at least 10-fold. This, in combination with the fact that RNR3 lacks a functional allosteric activity site, may provide a selective advantage for yeast growing in natural ecological niches under permanent DNA-damaging conditions when the levels of RNR1 are limiting for ribonucleotide reduction.

RNR4 reveals another intriguing story. RNR4 and RNR2 are only 54% identical. In addition, RNR2 contains a 51-amino-acid N-terminal peptide that is absent in RNR4. Most strikingly, six of 16 residues that are absolutely conserved in all R2 proteins from *E.coli* to mammals are changed in RNR4, including the two histidines and one glutamate that are directly involved in the coordination of the iron complex (Huang and Elledge, 1997). This suggests that RNR4 is very likely catalytically inactive, or at least its function is not dependent on the tyrosyl radical. Since Tyr131 is an invariant residue that is responsible for generating the free radical in the R2 subunit, Wang et al (1997) replaced the Tyr131 in RNR4 with Phe and found that *RNR4-Y131F* was still able to complement

the *RNR4-Δ1* deletion allele. However, like RNR2, RNR4 is also essential for mitotic viability as *rnr4* null mutants are lethal. To make things more complicated, overexpression of RNR2 cannot rescue the *rnr4* mutant phenotype and vice verse, indicating that the two small subunit genes are not functionally redundant. Huang and Elledge (1997) speculated that RNR4 might play a structural or a regulatory nonenzymatic role in RNR activity.

Table 1-3. Overview of RNR genes in eukaryotes

Species	Gene	Subunit	Cell cycle regulation	DNA damage inducibility	Structure	Subcellular localization
S.cerevisiae	Rnr1	Large	10-fold	5-fold	$\alpha_2\beta\beta'$	majority in cytoplasm
	Rnr2	Small	2-fold	25-fold	$\alpha\alpha'\beta\beta'$	majority in cytoplasm
	Rnr3	Large	?	100-fold		
	Rnr4	Small	No	yes		majority in nucleus
Human	RRM1	Large	4-fold	9-fold	$\alpha_2\beta_2$	cytoplasm
	RRM2	Small	4-fold	13-fold	$\alpha_2\beta'_2$	cytoplasm
	p53R2	Small		yes		nucleus
Tobacco	RNR1-1	Large				
	RNR1-2	Large	3.3-fold			
	RNR2	Small	6.6-fold			
Arabidopsis	R1	Large			$\alpha_2\beta\beta'$?	
	TSO2	Small	yes		$\alpha_2\beta\beta$?	
					$\alpha_2\beta'\beta'$?	
					$\alpha_2\beta\beta''$?	
	R2a	Small				
	R2b	Small				

Chabes et al (2000) examined in detail the structure and biochemical properties of the two yeast small subunits. They demonstrated that the crucial role of RNR4 is to fold correctly and stabilize the radical-storing RNR2 by forming a stable 1:1 RNR2/RNR4 complex. No RNR activity was observed with RNR2 alone or with RNR4 alone in the presence of RNR1, suggesting that RNR2/RNR4 heterodimer is the only active form.

Regulation of RNR genes

Considering the essential role of RNR in DNA synthesis, the multileveled and complicated regulation of RNR expression and activity is not surprising. First, the RNR activity is cell cycle-regulated. This could be executed at transcriptional (mRNA induction), posttranscriptional (mRNA stability), translational (protein induction) and/or posttranslational (protein degradation or subcellular localization) levels. Either one of the two subunits or both subunits are under stringent regulation in different species. Second, in addition to its role in DNA replication, RNR also provides dNTPs for DNA damage repair. Therefore, RNR genes are regulated by DNA damage checkpoints.

Controlled degradation of RNR protein

In mammalian cells, transcription of the *R1* and *R2* genes is cell cycle-regulated with maximal levels during S-phase. However, because of its long half-life (20 h), the R1 protein is in excess and is present constantly during all phases of the cell cycle (Kolberg et al. 2004). In contrast, the R2 protein and its enzyme activity are present at high levels during S-phase and at lower levels in G0 and G1. Although neither DNA damage nor replication blocks promote the *R2* gene transcription, they could delay the degradation of the R2 protein and hence, increase the overall RNR activity. Interestingly, mammalian cells evolved an additional R2 gene, called p53R2, which seems to be specifically employed in response to DNA damage (see later).

What is the mechanism underlying the R2 protein degradation in mitosis? Chabes and her colleagues (2003) found that mouse R2 protein contains a conserved KEN box at its N-terminus and demonstrated that it is a new target for anaphase-promoting complex

(APC)-Cdh1-mediated proteolysis. R2 protein with mutated KEN box or without the KEN box is not degraded in late mitosis. Since the p53R2 protein lacks the N-terminal 33-aa residues containing KEN box in R2 protein, it easily escapes the degradation mediated by the APC-Cdh1 complex. Why is it so important for the cell to degrade the R2 protein in late mitosis? As the R1 protein is present in all phases of the cell cycle, the undegraded R2 protein in mitosis will keep RNR activity high, which may cause unscheduled DNA replication. In addition, increasing evidence suggests that a deregulated R2 protein might be a tumor progressor determinant (see later). Thus unwanted R2 protein must be removed from the cells.

Specified isoform of R2 for DNA repair

In yeast, all four *RNR* genes can be induced by DNA damage outside S-phase. Mammalian cells use a different strategy. As mentioned earlier, a special *R2* gene (*p53R2*) has evolved. Another distinction is that the p53R2 protein is nuclear localized while all other R2s are cytoplasmic. This *p53R2* is a direct target of p53 and can be induced by p53 in response to various genotoxic stresses. Tanaka and his colleagues (2000) speculated that there might be two independent pathways for supply of dNTPs, one involving normal maintenance of dNTPs for DNA replication during S phase and the other involving supplying dNTPs for urgent repair of DNA damage. They found that after DNA damage, the level of p53R2 protein is increased whereas the level of R2 is decreased. The RNR activity after DNA damage is exclusively dependent on p53R2 as RNR activity can be abolished by *p53R2* antisense interference (Yamaguchi et al. 2001). Moreover, a point mutation in *p53R2* was found in a cancer cell line HCT116, which

causes a substitution of a highly conserved valine into leucine. This mutation not only abolished the RNR activity after DNA damage, but also caused remarkable apoptosis after γ-irradiation. p53AIP1, a pivotal mediator of p53-dependent apoptosis, is induced in this p53R2 mutant cell line. These results suggest that inactivation of p53R2 may lead to accumulation of too much DNA damags that can not be repaired, which eventually triggers the p53-dependent apoptotic pathway to eliminate those damaged cells.

Is the RNR activity still induced in p53 mutants where p53R2 is no longer active? The answer is yes. While both p53R2 protein level and the p53R2 binding to hRRM1 (human R1 protein) is down-regulated in cells with mutant p53, hRRM2 (human R2) protein level and its binding to hRRM1 was up-regulated. In addition, RNR activity is still increased after UV treatment in cells with mutant p53, suggesting that hRRM2 complements p53R2 to form RNR holoenzyme and maintain RNR activity after UV treatment when p53 is inactive (Zhou et al., 2003). Consistently, expression of antisense hRRM2 in cells with mutant p53 led to decreased hRRM2 level and resulted in greater sensitivity to UV than observed in cells with active p53. There might be a mechanism that can only switch on hRRM2 expression when p53 is inactive. Lin et al. (2004) also found that hRRM2 can be employed to supply dNTPs for repair of DNA damage in HCT-116 cells (p53-/-) with impaired p53-dependent induction of p53R2.

Translocalization of RNR proteins in response to DNA damage

In yeast, RNR1 and RNR3 are predominantly localized to the cytoplasm whereas RNR2 and RNR4 are predominantly present in the nucleus during the normal cell cycle.

However, under DNA damage and replication stress, RNR2 and RNR4 are redistributed to the cytoplasm in a checkpoint Mec1/Rad53/Dun1-dependent manner (Yao et al, 2003). In fission yeast, a small protein, Spd1, acts to anchor the R2 to the nucleus (Liu et al. 2003). In the presence of DNA damage, the checkpoint pathway inactivates Spd1 via the COP9/signalosome. The removal of Spd1 resulted in the R2 staying in cytoplasm, allowing the formation of holoenzyme with the R1 subunit. In *spd1* mutants, the R2 protein will be always present in cytoplasm and remain active. In *csn1* or *csn2* mutants (*Csn1* and *Csn2* genes encode two subunits of COP9/signalosome), Spd1 will not be degraded, locking the R2 protein in nucleus. Since R1 is predominantly present in cytoplasm, the compartmentalization of R2 to the nucleus results in the absence of or less RNR holoenzyme activity. The fact that *csn1*mutants are sensitive to DNA damage and *spd1* mutants can suppress the DNA damage-sensitivity of *csn1* suggests that the CSN-dependent as well as the checkpoint-dependent relocalization of R2 is required for an efficient DNA damage response.

This relocalization mechanism displays an opposite direction in different species. Tobacco RNR1a can be relocated from cytoplasm into nuclei upon UV-C treatment (Lincker et al., 2004). In mammalian cells, all three RNR subunits (hRRM1, hRRM2 and p53R2) are located in cytoplasm. However, both p53R2 and hRRM2 physically bind to p53 and hence, cannot bind to hRRM1. Upon UV-C treatment, the binding affinity between p53 and the two small RNR subunits decreases. All three RNR subunits translocate to the nucleus and the active tetrameric RNR complex forms (Fig. 1-12).

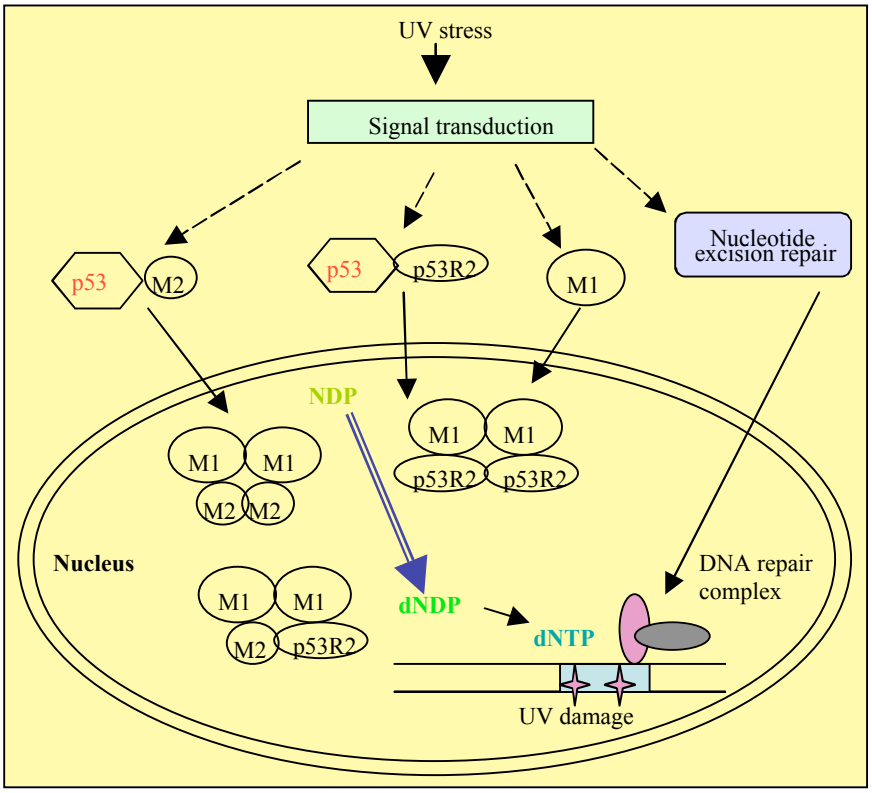


Figure 1-12. Translocation of human RNR subunits upon DNA damage. hRRM1, the large subunit, is shown as M1 and hRRM2, the small subunit as M2. Once DNA damage occurs, the signal transduction pathway leads to the dissociation of p53 from hRRM2 and p53R2. Meanwhile, genes in nucleotide excision repair are up-regulated and the repair proteins move into the nucleus, recognize and bind to the damage sites. All three RNR subunits translocate into the nucleus and form active holoenzyme, producing dNTPs for repair.

Control of RNR mRNA stability

Saitoh et al (2002) found that Cid13 encodes a cytoplasmic Poly(A) polymerase regulating RNR mRNA in fission yeast. *Cid13* shares a high level of sequence similarity to Trf4/5 family of DNA polymeras. However, Cid13 is a cytoplasmic protein and lacks detectable DNA polymerase activity. Overexpression of *Cid13* rescues hydroxyurea (HU) sensitivity of many checkpoint mutants (Saitoh et al., 2002). In addition, Δ*cid13* cells are HU sensitive and have reduced dNTP pools, suggesting a role of Cid13 in controlling RNR activity. The *RNR2* gene in fission yeast, *suc22* mRNA poly(A) tail was 10

nucleotides shorter in Δ*cid13* cells than that in wild type. Cid13 associates with poly(A) binding proteins *in vivo* and can polyadenylate mRNA *in vitro*. The onset of DNA replication marks a point of rapid consumption of dNTPs. DNA damage or heat shock may also strain the capacity to synthesize dNTPs. In these circumstances, *suc22* mRNA polyadenylation by *Cid13* may provide a mechanism of rapidly enhancing dNTP synthesis, bypassing steps of promoter activation, synthesis and processing of mRNAs, and mRNA nuclear export. Such a capacity may be crucial for survival during times of stress or to ensure optimal dNTP availability during DNA replication.

Protein inhibitor of RNR

Added to the already complex regulation of RNR is the discovery of a novel small protein (104 aa), *Sml1*, as a negative regulator of dNTP pools by binding directly to yeast RNR1. *Sml1* was isolated as a suppressor of two essential checkpoint genes MEC1 and RAD53. *sml1* mutants exhibited increased levels of dNTPs (about 2.5-fold) without altering RNR genes transcription. Chabes et al. (1999) reported that *Sml1* binds to RNR1 but not to RNR2, in a 1:1 ratio. Zhao et al. (2000) further dissected the Sml1 protein by randomly mutagenizing the *Sml1* ORF. They identified seven mutations that did not affect protein expression and the mutant protein could still suppress lethality caused by *mec1* and *rad53*. All seven mutations abolished the interaction between Sml1 and RNR1 or human R1. This demonstrates that the binding mechanism between *Sml1* and the RNR large subunit is conserved from yeast to human and the Sml1/R1 interaction is required for Sml1 to inhibit R1. However, no homolog of *Sml1* in other species so far has been identified based on the sequence similarity.

In the model proposed by Zhao et al. (1998), *Sml1* binds RNR1 to inhibit RNR activity when DNA synthesis is not required. During S phase or after DNA damage, the checkpoint pathway inactivates the *Sml1* to allow RNR activation. Consistent with this model, *Sml1* protein levels fluctuate during the cell cycle, being lowest during the S phase. The decrease of *Sml1* levels in S phase and after DNA damage and HU treatment is dependent on Mec1/Rad53 and other checkpoint genes. Later, it was found that Dun1, a downstream kinase of Mec1/Rad53, genetically and physically interacts with Sml1 *in vivo* and can phosphorylate Sml1 *in vitro*. Such posttranslational regulation of RNR can be achieved in the absence of protein synthesis, resulting in a more rapid change in RNR activity than that gained by transcriptional regulation.

Repression of RNR gene expression

During the screen for mutations that cause constitutive expression of *RNR3* under normal growth conditions, two large groups of CRT (constitutive RNR transcription) genes were isolated (Zhou and Elledge, 1992). The first group includes CRT3/RNR4, CRT5/POL1/CDC17, CRT6/RNR2, CRT7/RNR1, and CRT9/TMP1/CDC21. Mutations in genes of this group activate *RNR3* transcription by generating an endogenous DNA damage signal. The second group includes CRT1, CRT4/TUP1, and CRT8/SSN6. SSN6 and TUP1 are two global repressors recruited to particular promoters by interacting with target-specific DNA-binding proteins.

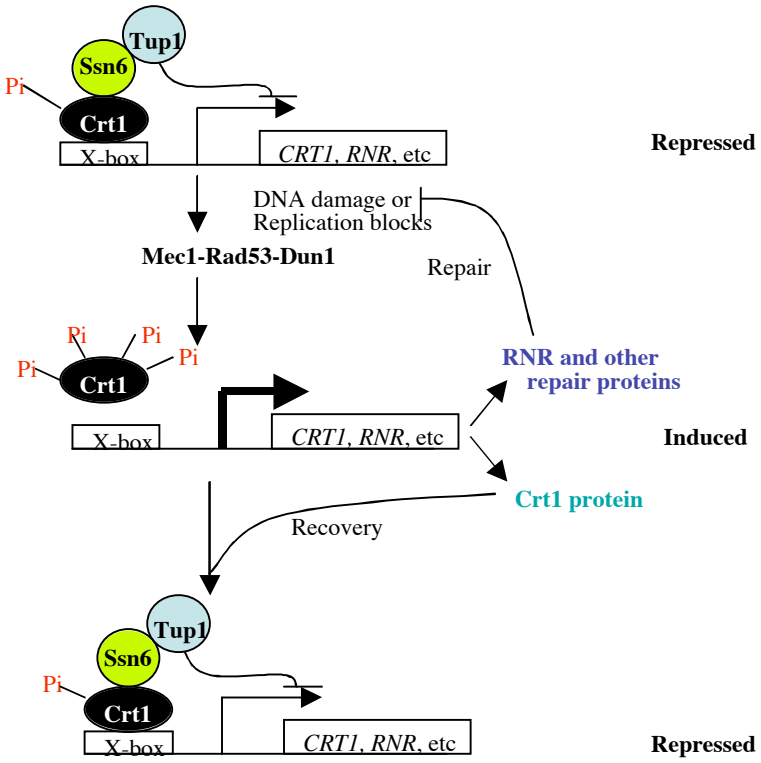


Figure 1-13. A model illustrating CRT1 mediated repression of RNR genes.(cited from Huang et al., 1998)

CRT1 encodes a homolog of the mammalian RFX family of DNA-binding proteins. It recognizes a conserved DNA sequence of 13 nucleotides, called the X-box. More than one X-box (with different strengths) was found in the promoters of RNR2, RNR3 and RNR4 as well as CRT1 itself, which is required for the CRT1-mediated repression *in vivo*. Among the different *crt* mutants, *crt1, tup1* and *ssn6* mutants exhibit the strongest derepression of both *RNR3* and *RNR2* (from 50 to 200-fold) (Zhao and Elledge, 1992). CTR1, TUP1 and SSN6 form a complex to mediate the repression of RNR2 (Huang et al., 1998), which was supported by a pull-down assay. In the model proposed by Huang et al. (1998) (Fig. 1-13), under normal conditions, CRT1 binds to the X-boxes and recruits SSN6 and TUP1 co-repressors to the promoter of the target genes to repress their transcription. In response to replication blocks or DNA damage, CRT1

becomes phosphorylated by Mec1-Rad53-Dun1 kinase cascade and loses its ability to bind X-boxes. This leads to transcriptional induction of its target genes including RNR as well as CRT1 itself. Accordingly, overexpressiong of CRT1 inhibits RNR induction. As DNA damage is repaired or replication blocks are removed, the kinase cascade becomes inactive. The increasing level of CRT1 will bind to X-boxes again, resulting in rapid restoration of the repressed state.

dNTP pool as a signal

In yeast, Mec1 and Rad53 are responsible for the transcriptional and cell cycle response to both DNA damage and replication blocks, thus either *mec1* or *rad53* null mutants are lethal. To one's surprise, overexpression of *RNR1* or *RNR3* (but not *RNR2* or *RNR4*) can suppress Δ*rad53* and Δ*mec1* lethality. Desany et al. (1998) found that even a twofold increase in *RNR1* gene dosage is sufficient for suppression. As overexpression of *RNR1* has no effect on the timing of S-phase completion or the overall rate of DNA synthesis, it suggests that the essential function of Rad53 and Mec1 is to maintain an adequate dNTPs supply. The fact that low amounts of exogenously supplied RNR1 can efficiently suppress lethality suggests that the defect responsible for lethality is just below the threshold for survival.

A nucleotide sensor must be activated to prevent replication in response to the low dNTP pools. Inhibition of replication fork progression by low dNTP pools leads to prolonged S phase associated with increased chromosome breakage (Windle et al., 1991; Di Leonardo et al. 1993), which may be the initial lesions required for gene

amplification, a form of genetic instability (Ma et al. 1993; Kuo et al. 1994). It was reported that synthesis of DNA from limiting levels of dNTPs could lead to accumulation of short DNA fragments and chromosome breakages, and cell death after a few divisions (Reichard, 1988). However, the nature of the nucleotide sensor remains unknown.

Although limiting levels of dNTPs could be error-prone for DNA synthesis, overproduced dNTPs are also detrimental to DNA synthesis. Chabes et al. (2003) reported that an increased dNTP concentration leads to a higher mutation rate in yeast but, at the same time, helps yeast cells to survive DNA damage. They speculate that excessive dNTP concentrations allow more efficient bypass of DNA lesions by DNA polymerases, which is beneficial to individual cells whose survival needs surpass the risk of higher mutation rate. However, in multi-cellular higher eukaryotes, accumulation of mutations will eventually lead to disasters. This survival mechanism in yeast may find no place in higher eukaryotes.

Chemotherapy

RNR has long been the target site for chemotherapy for cancer due to its essential role in DNA synthesis. Hydroxyurea is a well-known therapeutic agent produced by Bristol-Myers Squibb as Hydrea and by Roxane as Hydroxyurea Capsule, which is a specific but reversible inhibitor of the small subunit R2. In addition, the species-specific interaction between the large subunit and the small subunit also provides a good strategy to develop a peptidomimetic drug that competes specifically with the binding of the viral R2 to R1.

Recently, several studies provided renewed interest in targeting RNR in the development of anticancer therapeutics. It was found that RNR activity, and the level of R2, are markedly altered in malignant cells exposed to the growth factor TGF-β in rodent and human tumor cells. These observations suggest that RNR, and particularly the rate-limiting R2 subunit, may be critically involved in mechanisms controlling malignant progression. Fan et al (1996) reported that expression of R2 in benign BALB/c 3T3 and NIH 3T3 cells leads to a greatly increased frequency of focus formation in cooperation with H-ras transformation. Moreover, stable expression of R2 leads to significant increases in the membrane-associated Raf-1 protein and mitogen-activating protein kinase-2 (MAPK-2) activity. Since deregulated R2 expression causes no obvious differences in growth rates or cell cycle-phase distributions, their results demonstrated that the R2 protein can participate in other critical cellular functions in addition to ribonucleotide reduction and that deregulated R2 is a novel tumor progressor determinant.

What about deregulation of R1, the large subunit? Surprisingly, stable expression of R1, with or without R2 expression, led to suppression of tumorgenic and/or metastatic potential (Fan et al, 1997), suggesting that R1 and R2 play opposite roles in determining malignancy and a balance in the levels of R1 and R2 might be a very important control point.

As a new strategy, Lee et al. (2003) screened and identified a 20-mer oligonucleotide complementary to the coding region of R2, GTI-2040. They showed that

GTI-2040 specifically inhibits expression of R2 mRNA in a number of cell lines. The antisense oligo also displays sequence-specific and dose-dependent antitumor activity *in vivo* with superior efficacy compared with known RNR-based therapeutic agents, such as 5-fluorouracil (FU), gemcitabine and vinblastine.

Eklund et al. (2001) discussed the possibility of designing specific inhibitors to normal RNR, especially the R2, based on the fact that human cells have an additional p53R2 for DNA repair. Since cancers often have mutations in the p53 pathway, they are presumably unable to make p53R2. Therefore, cancer cells would die if the normal R2 were inhibited, whereas normal cells with p53 could still survive on the dNTPs supplied by p53R2. The discovery that p53R2 is 158-fold more susceptible to the iron chelator deferoxamine mesylate than the human R2 and 2.5-fold less sensitive to hyroxyurea than R2 (Shao et al. 2004) makes this strategy very attractive. Unfortunately, the real picture is always more complicated as an additional p53-independent induction of p53R2 might exist. Kolber et al. (2004) found that in some breast cancer cell lines with mutations in p53, p53R2 is present throughout the cell cycle in relatively high amounts.

Chapter two

Functional Analyses of *Arabidopsis* Ribonucleotide Reductase Small Subunit Gene Family

Abstract

Ribonucleotide reductase (RNR), comprising two large subunits (R1) and two small subunits (R2), catalyzes a rate-limiting step in the production of deoxyribonucleotides needed for DNA synthesis. Previous studies in yeast and mammals indicated that defective RNR often led to cell cycle arrest, growth retardation and p53-dependent apoptosis. Given that plants are constantly exposed to environmental mutagens and plant cells are totipotent, an understanding of RNR function in plants is of great importance. We isolated and characterized mutations in all three *R2* genes (*TSO2, R2A* and *R2B*) in *Arabidopsis*. We showed that while neither *r2a*, *r2b* nor *r2a r2b* double mutants display any visible phenotype, *tso2* mutants, with reduced dNTP levels, exhibited developmental defects including callus-like floral organs and fasciated shoot apical meristem. The *TSO2* gene is cell cycle-regulated and can be induced by HU and DNA damaging agents such as MMS. *tso2* mutants were more sensitive to HU and UV-C and *tso2 r2a* seedlings exhibited increased DNA damage and massive programmed cell death. The release of transcriptional gene silencing observed in *tso2 r2a* mutants supports a link between defective DNA replication and epigenetic stability. Our data suggests that plants can initiate programmed cell death upon DNA damage despite the absence of p53 in its genome.

Introduction

Fundamental to all life forms is the ability to replicate and repair DNA. Ribonucleotide reductase (RNR), comprising two large subunits (R1) and two small subunits (R2), catalyzes a rate-limiting step in the production of dNDP needed for DNA synthesis (Elledge et al., 1992). The R1 subunit binds the nucleoside diphosphate substrates and allosteric effectors and the R2 subunit contains the di-iron tyrosyl radical cofactor essential for the reduction of NDP to dNDP (Elledge et al., 1992). RNR enzymatic activity can be efficiently inhibited by hydroxyurea (HU), a chemical used to block DNA synthesis and cell cycle progression (Reichard, 1988). The enzyme activity and the mRNA expression of the *RNR* genes are cell cycle-regulated in different species with maximal values in the S phase (Chaboute *et al.*, 1998; Elledge *et al.*, 1992; Reichard, 1988). In addition to regulation by the cell cycle, RNR genes are induced by DNA damage when DNA repair is required outside S-phase. It has been shown that aberrant expression of R2 directly alters the malignant potential of tumor cells (Amara et al., 1996). A failure to control the size of dNTP pools and /or their relative amounts leads to cell death or genetic abnormalities (Reichard, 1988).

Genetic analyses of *rnr* mutants in yeast and mammals indicated that defective RNR often led to cell cycle arrest, growth retardation and p53-depedent apoptosis (Elledge et al., 1992; Kimura et al., 2003). Defective feedback regulation of RNR led to elevate dNTP levels resulting in increased mutation rates but enhanced resistance to mutagens (Chabes et al., 2003). These genetic studies demonstrated that RNR plays a key role in maintaining genomic stability by providing proper dNTP pools.

RNR is one of the best-studied transcriptional targets of the DNA damage checkpoint pathway in yeast and mammals. In mammals, p53R2, a RNR small subunit, is a direct target of the tumor suppressor protein p53 (Tanaka et al., 2000). In yeast, which does not have p53, the checkpoint pathway induces RNR via several mechanisms including alleviation of the Crt1-mediated transcriptional repression (Huang et al., 1998), phosphorylation-mediated removal of an inhibitory protein Sml1, (Zhao et al., 2001; Zhao et al., 1998), and redistribution of RNR2 and RNR4 from nucleus to cytoplasm (Yao et al., 2003). The existence of multiple mechanisms regulating RNR activities underscores the importance of proper dNTP pool for the fitness and survival of an organism.

In contrast to yeast and mammals, our knowledge of RNR function in plants is limited. *R1* and *R2* genes have been previously isolated from tobacco and *Arabidopsis* (Chaboute et al., 1998; Philipps et al., 1995) and their S-phase specific expression was shown to depend on the E2F-like motifs in their promoters (Chaboute et al., 2002; Chaboute et al., 2000). Transient translocation of a GFP-R1 protein from cytoplasm to nucleus in response to UV irradiation was recently reported (Lincker et al., 2004). However, no mutation in any of the plant *RNR* genes has been reported. The function of RNR in maintaining plant genomic stability and genetic variability remains unknown.

Here we report the first functional dissection of RNR in higher plants. *Arabidopsis TSO1* and *TSO2* genes were previously identified in the same genetic screen by mutations that cause the formation of callus-like floral organs. These two loci were

named *TSO*, meaning ugly in Chinese (Liu et al., 1997). *TSO1* was shown to encode a novel nuclear protein with two cysteine-rich repeats and *tso1* mutants exhibited defects in cell division (Liu et al., 1997; Song et al., 2000). The molecular isolation of *TSO2* reported here revealed a direct role of *TSO2* in the cell division as *TSO2* encodes one of the three *R2* genes in *Arabidopsis*. Subsequent identification and characterization of mutations in all three *R2* genes in the *Arabidopsis* genome provided the first insights into RNR function for plant growth and response to environmental mutagens.

Results

tso2-1 **is a pleiotropic mutant**

Four *tso2* mutants (*tso2-1, 2, 3, 4*) were isolated from EMS mutagenesis and they all are recessive and phenotypically similar. Therefore, *tso2-1* was chosen for further analysis. In *tso2-1* mutants, the morphology of roots and the first four rosette leaves are normal. Subsequently, *tso2* mutants display abnormal leaf and floral morphology (Fig. 2-1A,B), including white sectors in green organs (Fig. 2-1C, D), uneven thickness, rough surface, and irregular margins of leaves or floral organs. Very often, the sepals are wrinkled and have irregular margins (Fig. 2-1E, F). The siliques of *tso2* mutants are shorter, wrinkled and have white patches or sectors (Fig. 2-7J). In addition, embryo development inside the siliques exhibits a high frequency of abortion (Fig. 2-1J-M). In the wild type, normal embryos turn green (usually five days after pollination). However, in *tso2-1* mutants, white aborted embryos (Fig. 2-1K) or brown wrinkled, abnormal embryos (Fig. 2-1M) are often observed. We counted forty siliques (at late stage) from

both wild type and *tso2-1* mutants. While none of the wild type siliques contains white embryos, all *tso2* siliques contain white aborted embryos ranging from 5% to 60%.

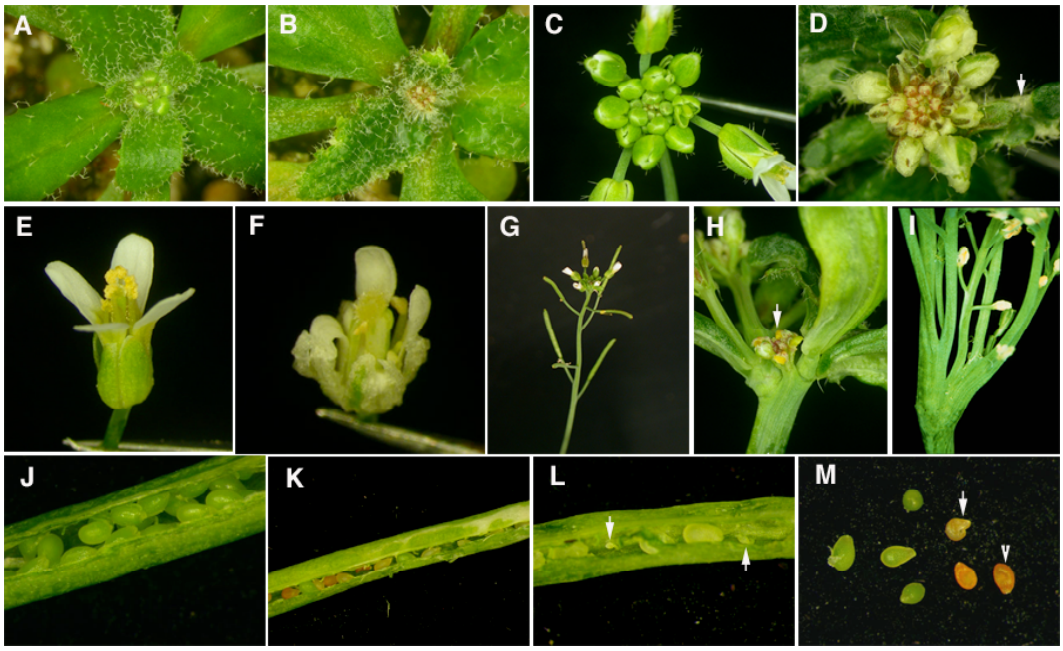


Figure 2-1. Phenotype of *tso2* mutants. (A) A wild type (ler) plant. (B) *tso2-1* mutant. (C) A wild type inflorescence. (D) *tso2-1* inflorescence. Note the white/green leaves start to exhibit abnormal morphology such as the irregular marginal white tissues (arrow). (E) A wild type flower. (F) A *tso2-1* flower. Note the wrinkled sepals and sepals with white margins. (G) A wild type shoot. (H) A *tso2-1* shoot showing arrested shoot apex (indicated by an arrow) and bifurcation. (I) A fasciated *tso2-1* plant, showing the multiple bifurcation. (J) A wild type silique at late developmental stage. Note the mature seeds turning green. (K) A *tso2-1* silique. Note the seeds at different stages. (L) A *tso2-1* silique. Two seeds are arrested at very early stage (indicated by arrows). (M) seeds removed from one *tso2-1* silique. Note the brown defective seeds (indicated by an arrow).

Occasionally, *tso2-1* mutant stems broaden and flatten and split into multiple shoots. This fasciation occurs both in reproductive stage and vegetative development stages (Fig. 2-1H and I). Sometimes, the main inflorescence remains arrested, even when the cauline leaves are fully developed. Occasionally the main inflorescence meristem is arrested but the secondary shoots develop normally (Fig. 2-1H, indicated by arrow).

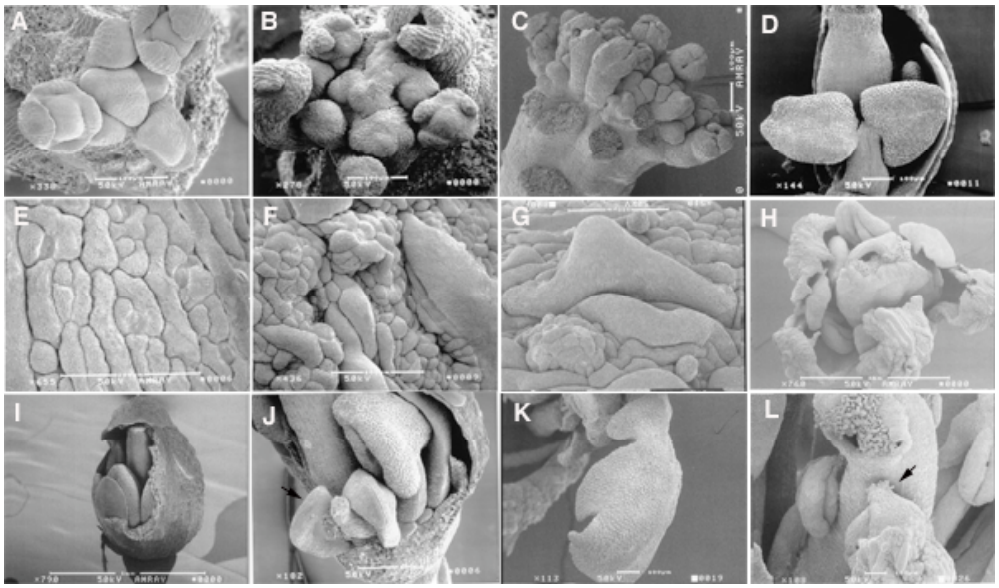


Figure 2-2. Scanning electronic microscopic analysis of *tso2* mutants. (A) wild type (Ler), showing the inflorescence apex. (B, C) *tso2-1* mutants showing the fasciated shoot apex. (D) A flower of *tso2* mutants. Two sepals and petals are removed to show a twin anther. (E) Wild type sepal epidermis. Immature stomata (arrow) are interspersed with long cells. (F) *tso2-1* sepal epidermis. Clusters of epidermal cells are projected above the surface. (G) A close-up of sepal epidermal cells showing enlarged cells. (H) A *tso2-1* flower, showing the unfused capels. (I) A wild type flower. (J) A *tso2-1* flower. Three petals are at different developmental stages. (K) A serrated petal in *tso2* flowers. (L) Partial homeotic transformation in *tso2-1* flowers. Note the stigmatic-like tissues (indicated by an arrow) on top of an anther.

The wild type shoot apical meristem (SAM) is a dome-like structure with floral primordia on the periphery (Fig. 2-2A). In *tso2-1* mutants, occasionally the SAM splits into multiple apical meristems (Fig. 2-2B). Sometimes the fasciated shoots are flattened (Fig. 2-2C). In wild type, the sepal epidermal cells are well-organized in the same plane while in *tso2-1* mutants, sepal epidermal cells are disorganized with protruding cell clusters (Fig. 2-2F, G). In addition, the sepals very often display irregularly serrated margins (Fig. 2-2H). Occasionally, petals are also serrated (Fig. 2-2K). Sometimes, we observed asynchronized development for petals (Fig. 2-2J) and unfused or twisted carpels

(Fig. 2-2H, L). Partial homeotic transformation of stamens into carpels is also observed: on the top of the anther, stigmatic-like tissues are formed (Fig. 2-2L).

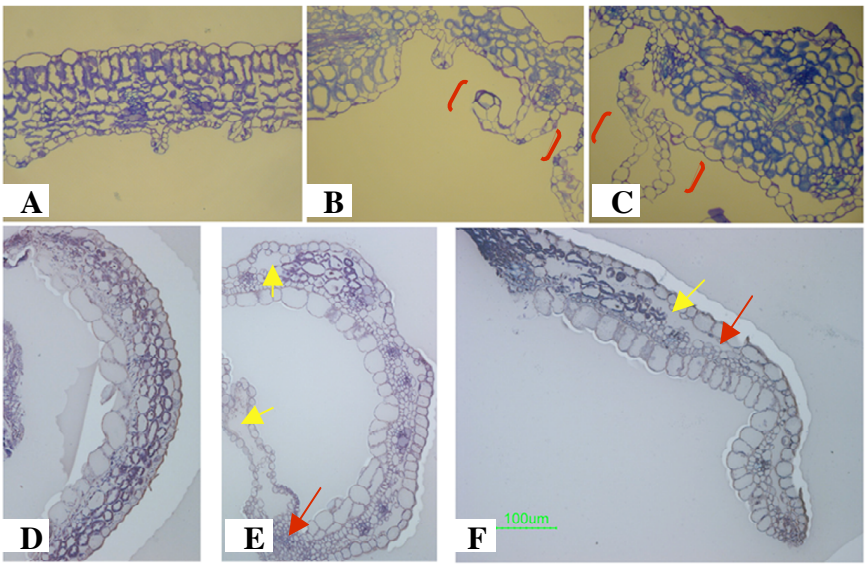


Figure 2-3. 2 μm cross section of wild type and *tso2-1* mutant leaves (top panel) and siliques (bottom panel). (A) wild type. Mesophyll cells are well organized and contain chloroplasts (stained dark purple). (B, C) *tso2-1*. Note the unusual large intracellular air space and disorganized mesophyll cell layers indicated by the red bracket. (D) wild type silique. (E, F) *tso2-1* silique. Subepidermal cells in the white sector region (shown in Figure 2-7K) contain fewer or no chloroplasts. The white sector region also has larger intracellular air space (indicated by yellow arrows). Note some extremely smaller cells (indicated by red arrows).

Chloroplast development is defective in *tso2-1*

The appearance of white sectors in green organs could be due to a lack of chloroplasts in mesophyll cells or due to a lack of mesophyll cells. Histological analysis of leaf and silique tissues was performed in wild type and *tso2-1*. Occasionally, one valve of the silique is completely white in *tso2-1*, which occurred more often in *tso2-1 r2b*/+ double mutants (see later, Fig.2-7K). The siliques were cut in half and the white and green sectors were fixed separately. In wild type and in the green sectors of *tso2-1*

siliques, the subepidermal cells contain a large number of chloroplasts outlining the cell wall, which are stained in dark purple. In contrast, the majority of the subepidermal cells in *tso2-1* white siliques do not contain chloroplasts. Therefore, the white sector is caused partly by a lack of chloroplasts in mesophyll cells (Fig.2-3E) and partly by abnormal airspaces, indicating a lack of mesophyll cells (Fig. 2-3F). In addition, a large number of small cells are present and unevenly distributed (Fig.2-3E and F). The uneven thickness and rough surface of the leaves, floral organs and siliques are probably caused by unevenly distributed cell layers and unusually large intracellular air space.

Molecular cloning of the *TSO2* gene

A mapping population of 358 *tso2* plants selected from F2 generation of a cross between *tso2-1* (in Ler background) and *sup-5* (in Col background) was used to map the *TSO2* gene. *TSO2* was gradually mapped to a 20-kb region (from 32-53kb) located within one BAC clone, MOJ10 (Fig.2-4). A cosmid library was constructed from this BAC and overlapping cosmids spanning this 20-kb region were identified. Two overlapping cosmids (D and G) were transformed into *tso2* mutants. Cosmid D rescued the *tso2* mutants while cosmid G failed to rescue the mutant.

Cosmid D covers the 39-62 kb region of MOJ10 (Fig.2-4). Two recombinants at 53-kb region indicated that *tso2* is within this 39-53 kb region of MOJ10, where four genes reside. All four genes have been sequenced in *tso2* alleles. Only the RNR (ribonucleotide reductase) small subunit gene (at3g27060) was found to have a mutation in all four *tso2* alleles. The invariant aspartic acid (D) at residue 49, which is involved in

binding to the large subunit of ribonucleotide reductase (Philipps, *et al.*, 1995), is changed to asparagine (N) in both *tso2-1* and *tso2-4* alleles. In *tso2-2*, Glycine 170 is substituted by serine while arginine 97 is replaced with cysteine in *tso2-3* (Fig.2-5).

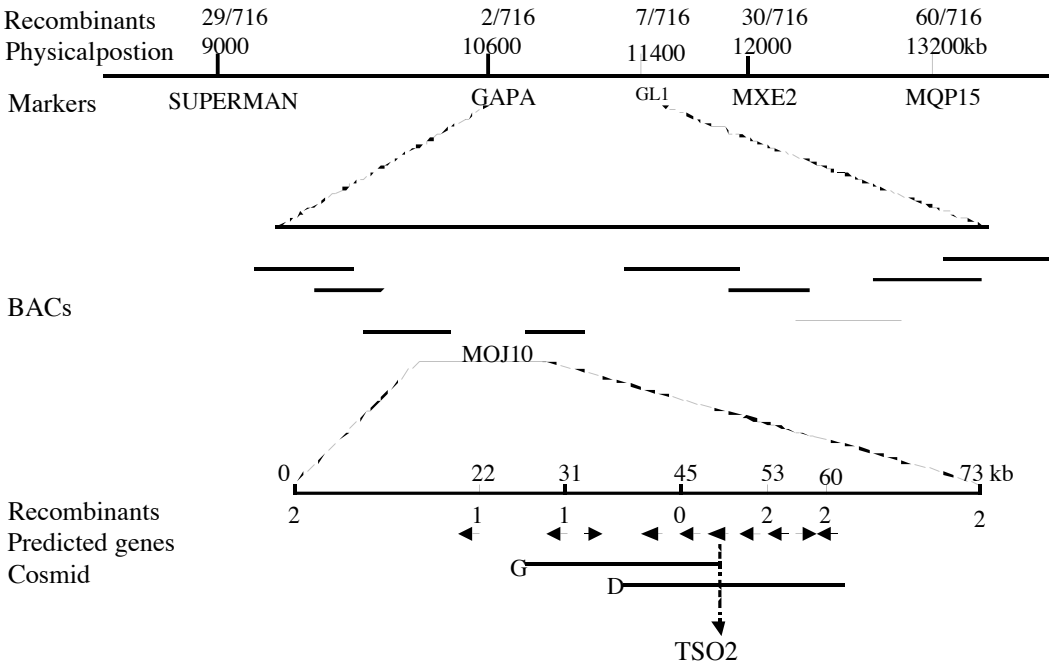


Figure 2-4. Schematic diagram of map-based cloning of the *TSO2* gene.

<u>*Arabidopsis* genome has three *R2* genes</u>

Since *tso2* mutants are all viable and fertile, other redundant genes in the *Arabidopsis* genome may compensate for *tso2*. A close examination of the *Arabidopsis* genome revealed two additional *R2* genes named *R2A* (At3g23580) and *R2B* (At5g40942) (Fig. 2-5). *R2A* was previously isolated by Philipps *et al.* (1995), while *R2B* was annotated first as a pseudogene and recently as a truncated gene (at5g40942) by the *Arabidopsis* genome sequence project (http://www.*Arabidopsis*.org). The *R2B* from the Columbia (Col) accession, which was used for genome sequencing, possesses a 2 bp

deletion resulting in a frameshift and a subsequent stop (Fig. 2-5). In contrast, *R2B* from L*er* and Wassilewskija (WS) accessions do not harbor the 2 bp deletion and differ from Col in 13 additional nucleotides, five of which change amino acids (Table 2-1).

Table 2-1. Polymorphisms in the *R2B* gene between Col and Ler

Nucleotide change (Ler-Col)[a]	Position[b]	Nature of the mutation
A-T	393	silent
T-C	411	silent
TG-deletion in Col	418-419	frameshift
G-A	438	silent
C-A	573	silent
C-A	639	silent
C-T	661	Leu-Phe
C-T	726	silent
C-T	736	Pro-Ser
C-G	824	Ala-Gly
G-C	842	Ser-Thr
A-G	879	silent
A-G	931	Ile-Val

[a]No polymorphism is found between Ler and WS
[b]The start codon ATG of the Ler *R2B* gene is designated +1.

As shown in Fig.2-5, the gene structure of *R2B* is similar to *TSO2*. While *TSO2* has one intron, *R2B* has no introns. Since *R2B* and *TSO2* share 82% identity to each other at the nucleotide level, it suggests that they might be derived from gene duplication. In contrast, *R2A* only shares 70% identity to *TSO2* at amino acid level and almost no similarity at nucleotide level. *R2A* has a distinct gene structure with eight introns, indicating that *R2A* has a different origin from *TSO2* and *R2B*.

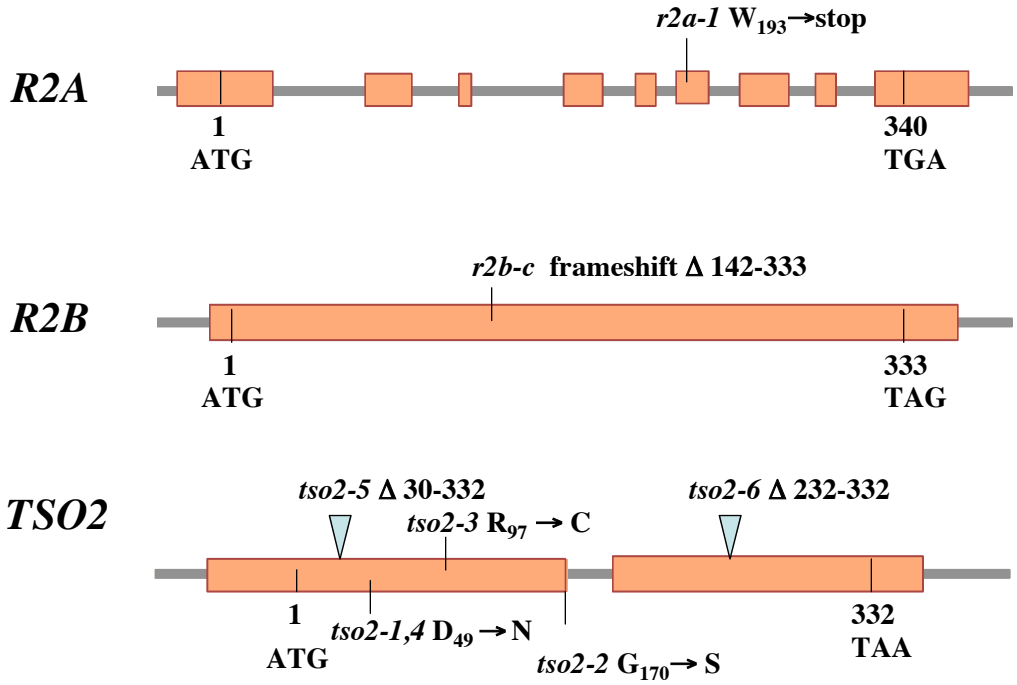


Figure 2-5. Gene structures of the three *R2* genes in *Arabidopsis*. Exons are shown as rectangles. The lines indicate introns and UTRs.Triangles indicate the positions of T-DNA insertion.

All RNR proteins are localized in cytoplasm with exception of human p53R2. None of the three *Arabidopsis* R2 proteins has a nuclear localization signal. Intriguingly, TSO2 and R2B proteins have a potential PEST sequence (MPSMPEEPLLTPTPD) at the N-terminus, which acts as a protein degradation signal. In addition, three E2F binding sites (consensus sequence TTTG/CG/CCGC) are found in the promoter of the *TSO2* gene (Table 2-2). There is one E2F binding site in *R2B* promoter but not in *R2A*. E2Fs are transcription factors that are essential for G1/S transition (see Chapter one), regulating a large number of genes such as cyclin A, cyclin E, pRb, $p34^{cdc2}$, DNA polymerase α, ORC1, PCNA, DHFR, TK, RNR etc (Chaboute et al., 2000, 2002; Lavia and Jansen-Durr, 1999). Interestingly, all the E2F binding sites shown in Table 2-2 are located within –700 in the promoters of *RNR* genes, consistent with a genome-wide survey that the

majority of E2F binding sites are located within 1 kb promoter region starting from the ATG (Ramirez-Parra et al., 2003)

Table 2-2. E2F binding sites in the promoters of various RNR genes

RNR gene	Position (ATG as +1)	Binding site
TSO2	-307	CCGCCAAA
	-329	CCGGCAAA
	-469	TTTGGCGC
R2B	-666	TTTGGCGC
R1	-228	CCGGGAAA
	-232	TTTCCCGG
	-251	GCGGGAAA
Rice *R2A*	-197	GCGCGAAA
	-262	TTTCCCGG
	-314	CCGCGAAA
Rice *R2B*	-168	TTTGGCGG
Tobacco *RNR1b*	-177	GCGGGAAA
Tobacco *R2*	-294	GCGGCAAA
	-355	TTTCCCGC

Note: cannonical binding sites are shown in red.

Genetic interaction among three *R2* genes

In order to further dissect the functions of the three *R2* genes, we isolated an EMS-induced *r2a* mutation *(r2a-1)* in the Col accession using a reverse genetics method known as TILLING (McCallum et al., 2000). *r2a-1* is a nonsense mutation that deletes one third of the protein (Fig. 2-5). 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. 2-6A).

Aided by PCR-based markers specific to each mutant allele, the F2 progeny of a cross between *tso2-1* (L*er*) and *r2a-1 r2b-1* (Col) was analyzed. *tso2-1 r2a-1* and *tso2-1 r2b-1* double mutants were identified, which exhibited a much stronger phenotype than either parent. First, *tso2-1 r2a-1* double mutant seedlings did not develop beyond the two

to four-leaf stage (Fig. 2-6C). Their SAM appeared arrested (Fig. 2-6D), and their leaves exhibited massively disorganized surfaces (Fig. 2-6E, F). Second, *tso2-1 r2b-1* double mutants were embryo lethal. PCR-based genotyping detected *tso2-1 r2b-1* double mutants only among aborted seeds, but not among viable plants from *tso2-1/tso2-1; r2b-1/+* parents (Table 2-3). 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* as shown by their smaller statures, more severely reduced fertility, more frequent stem fasciation, and larger white sectors (Fig. 2-6G-I). These results indicate that *R2A* and *R2B* become essential in the *tso2-1* background.

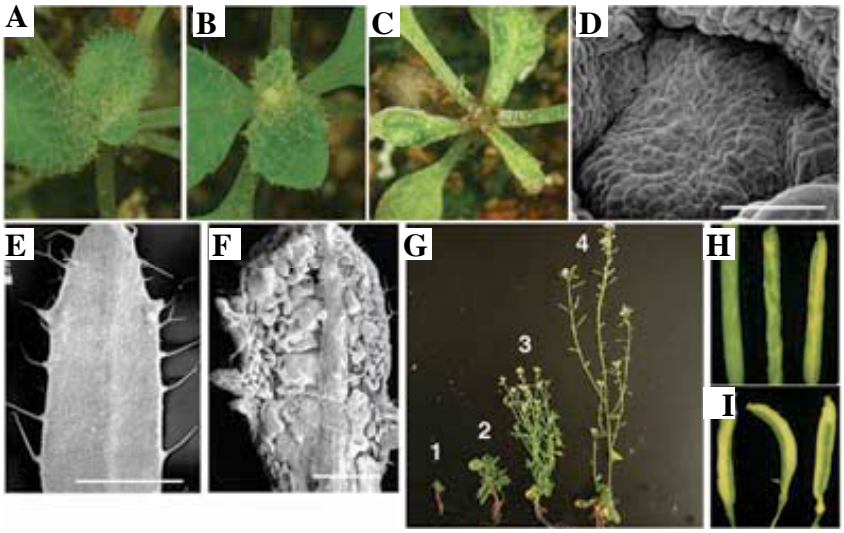


Figure 2-6. Genetic interaction among the three R2 genes. (a) *r2a-1 r2b-1* seedling. (B) A *tso2-1* seedling. White tissues are forming in the emerging leaves. (C) A typical *tso2-1 r2a-1* double mutant seedling, which will not develop further. (D) The SAM of a *tso2-1 r2a-1* seedling that is flat and appears arrested. (E) A wild-type leaf. (F) A *tso2-1 r2a-1* leaf showing severely disrupted leaf surface. (G) 6-week old *tso2-1/tso2-1* plants singly or doubly heterozygous for *r2a-1* or/and *r2b-1*. (1) *tso2-1/tso2-1; r2a-1/+; r2b-1/+*. (2) *tso2-1/tso2-1; r2a-1/+*. (3) *tso2-1/tso2-1; r2b-1/+*. (4) *tso2-1/tso2-1*. (H) *tso2-1* siliques showing small white sectors in the green carpel valve. (I) *tso2-1/tso2-1; r2b-1/+* siliques showing larger white sectors and smaller siliques. Bar: 100 μm in D; 1mm in E, F.

Table 2-3. Genotyping among the progeny of *tso2-1/tso2-1 r2b-1/+* parent[a]

	r2b genotype	*r2b-1/r2b-1*	*r2b-1 /+*	+/+	Total
No. of Plants[b]	Expected	9	18	9	36
	Observed	0	22	14	36
No. of seeds[c]	Green	0	13	5	18
	White	2	33	3	38

[a]: Only the genotype at R2B is shown as all plants are *tso2-1/tso2-1* .
[b]: 36 *tso2-1/tso2-1* plants were PCR-genotyped for the *r2b* locus.
[c]: 18 normal (green) and 38 abnormal (white) seeds were genotyped.

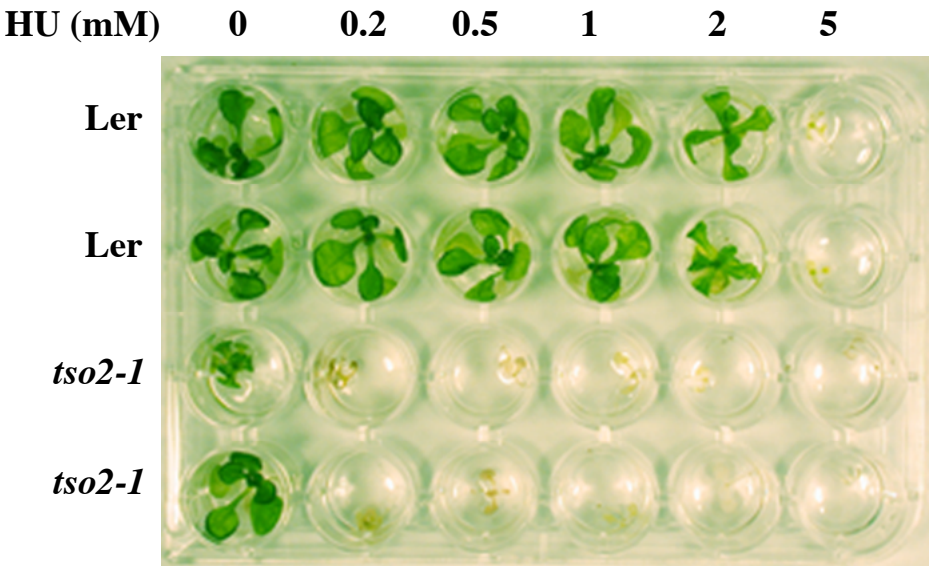


Figure 2-7. *tso2-1* is hypersensitive to HU treatment. Wild type (Ler) and *tso2-1* plants were grown in duplicate in the same plate and the experiment was repeated three times with similar results.

tso2-1 is sensitive to hydroxyurea (HU)

Hydorxyurea (HU) has been shown to be the specific inhibitor of the R2 subunit (acting as the radical scavenger) and yeast *rnr* mutants are hypersensitive to HU treatment. Does it hold true for *r2* mutants in *Arabidopsis*? To this end, 5-day-old

seedlings of *r2* mutants (*tso2-1*, *r2a r2b*, *r2b*) and wild type (Ler) were exposed to different concentrations of HU and the sensitivity was assayed one week later. As shown in Fig. 2-7, *tso2-1* is sensitive to HU treatment at a concentration 10-fold lower than the concentration wild type is sensitive to. Wild type seedlings are still viable in the presence of 2 mM HU while *tso2-1* seedlings cannot survive under 0.2 mM HU. However, neither *r2b* single mutant (Col) nor *r2a r2b* double mutants showed increased sensitivity to HU (data not shown), suggesting that RNR activity in these mutants is similar to wild type and TSO2 alone is sufficient to confer wild type-like resistance to HU.

dNTP pool size is reduced in *tso2-1* mutants

Although the *TSO2* gene encodes the small subunit of RNR, it is based on its sequence homolog from other species. Are the developmental defects indeed caused by a defective R2? Could the other *R2* genes (*R2A* and *R2B*) maintain the RNR activity to near wild type levels? Two established methods are currently used to measure RNR activity: one utilizes radioactively labeled substrate (normally CDP) and monitors product (dCDP) formation. The other monitors NADPH consumption. We measured the dNTP pool sizes directly in different *r2* mutants using DNA polymerase assay with inflorescence tissues since impaired RNR activity will lead to reduction of dNTP levels.

Whereas wild type (Ler), *r2b* and *r2a r2b* double mutants showed similar dNTP levels (data not shown), *tso2-1* mutants contain significantly reduced dNTP pools (Fig. 2-8). In all eukaryotic cells, dTTP is always the most abundant, and dGTP the least. The relative levels of dATP and dCTP vary in different species (Mathews and Ji, 1992). Since

dCTP is reduced to only 35% of wild type, and dGTP drops to 60% of wild type, this different degree of reduction could lead to an imbalanced dNTP pool in *tso2-1*. The reduction of dNTP pool sizes in *tso2-1* mutants, but not in *r2b* and *r2a r2b* mutants, is consistent with the observation that only *tso2-1* is hypersensitive to HU treatment and exhibits developmental defects.

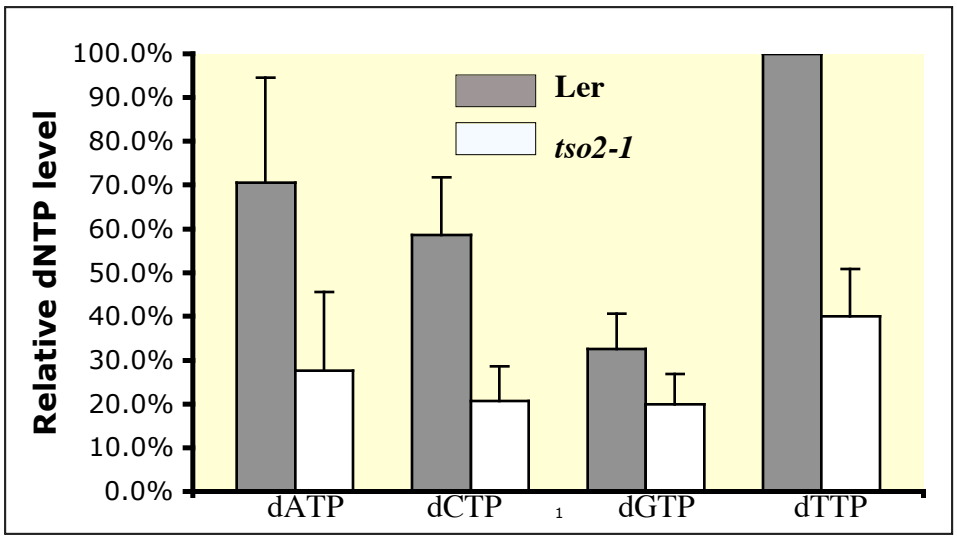


Figure 2-8. Relative dNTP level in Ler and *tso2-1* mutants. The level of dTTP (which is the most abundant in eukaryotic cells) in Ler is designated as 100%, and the level of other three dNTPs was adjusted accordingly. The measurement was repeated three times with different batches of tissues.The level was normalized to an internal control, ADH activity (see Methods and Materials)

The mRNA expression patterns of *TSO2*, *R2A* and *R2B*

As mentioned earlier, the first four rosette leaves of *tso2-1* do not exhibit any visible defects. Could this be due to the tissue specific or developmental stage-specific expression of *TSO2* or other R2 genes? *TSO2, R2A* and *R2B* transcripts were examined in different *Arabidopsis* tissues (Fig. 2-9A). *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. 2-9A 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*.

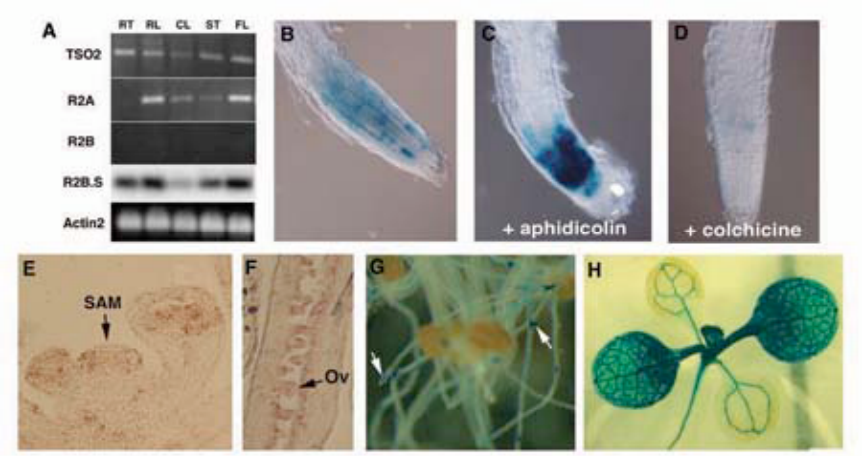


Figure 2-9. mRNA expression of *TSO2*, *R2A* and *R2B*. (A) Expression of *TSO2*, *R2A* and *R2B* mRNA. RT: root; RL: rosette leaves; CL: cauline leaves; ST: stems; and FL: flowers. The RT-PCR products of *R2B* were only detected by Southern hybridization with a *R2B*-specific probe shown in lane *R2B*.S. *Actin2* is the loading control. (B) *pTSO2::GUS* expression in a 3-day old wild type root. (C) *pTSO2::GUS* expression in a 3-day old root treated with aphidicolin, (D) *pTSO2::GUS* expression in a 3-day old root treated with colchicine. (E) *in situ* hybridization showing *TSO2* mRNA expression in wild-type SAM. (F) *in situ* hybridization showing sporadic *TSO2* expression in developing ovules (Ov). (G) *pTSO2:GUS* reporter gene 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.

RNR genes are cell cycle-regulated at the transcriptional level in most species. To examine *TSO2* transcription during the 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. 2-9C). In contrast, *GUS* expression

was dramatically reduced when the seedlings were arrested at the M phase (Fig. 2-9D). 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. 2-9E, 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. 2-9G, H). The *TSO2* expression pattern is consistent with its role in dNTP biosynthesis during DNA replication.

DNA damage induction of the RNR genes

Yeast RNR genes can be induced by various DNA damage agents. In contrast, mammalian RNR genes are specifically induced only by UV-C but not HU or methyl methanesulfonate (MMS). Do plant *RNR* genes show a similar response to DNA damage induction to animals or yeast? We treated Col cell suspensions with HU, MMS and 4-nitroquinoline-1-oxide (4-NQO) for 2, 12 and 24 hrs. Total RNA was extracted at each time spot and RT-PCR was performed with a small number of cycles to avoid saturation. PCR products were Southern blotted and hybridized with gene-specific primers (see Materials and Methods). As shown in Fig. 2-10, all four RNR genes can be induced by HU. *TSO2* is induced about 20-fold, *R2B* 13-fold, *R1* 5-fold and *R2A* 2-fold. However, both *R2A* and *R1* genes are not induced by MMS and 4-NQO whereas *TSO2* and *R2b* were slightly induced (about 2-fold).

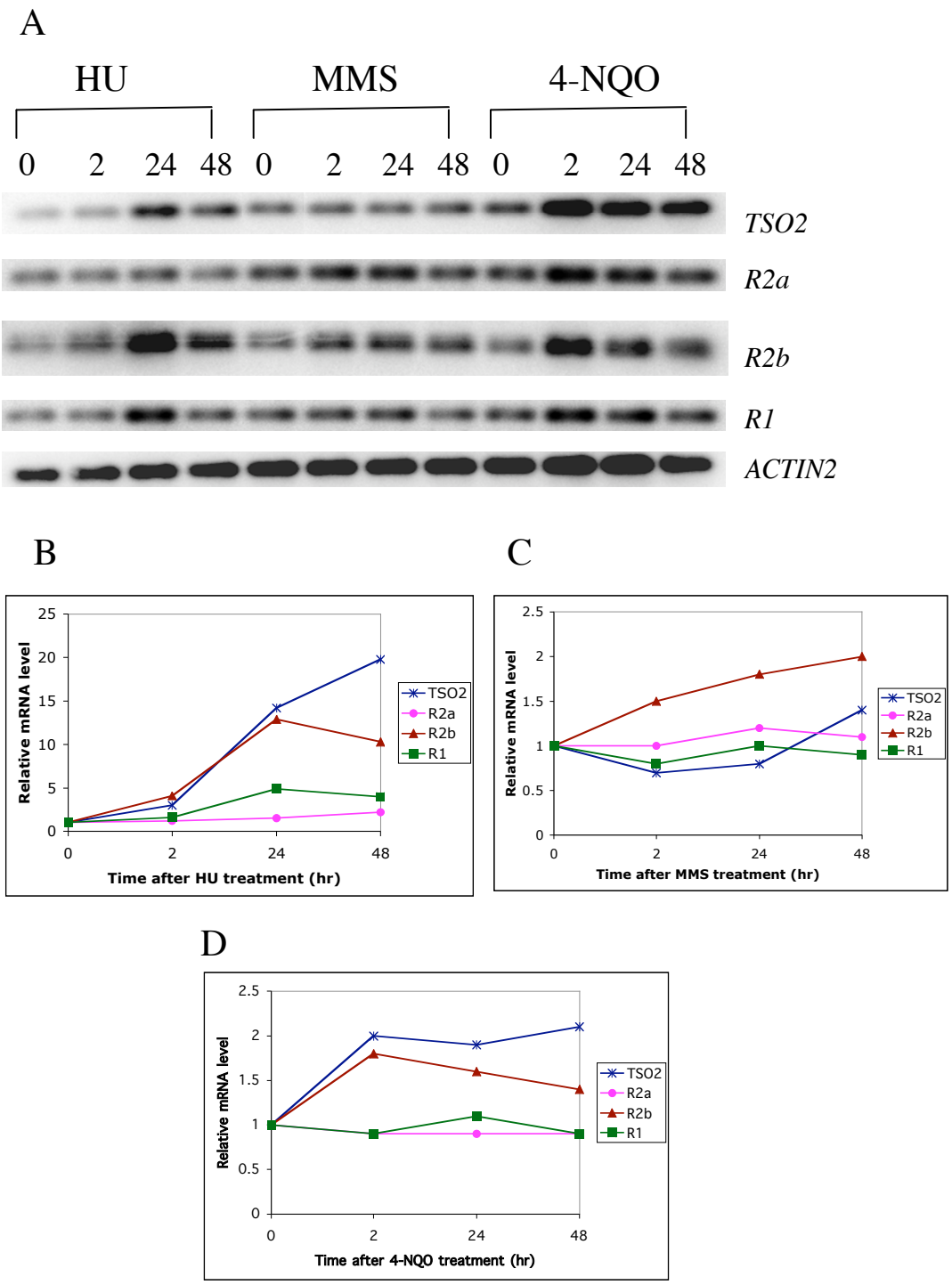


Figure 2-10. DNA damage induction of RNR genes. (A) semi-quantitative RT-PCR. RT-PCR was performed with 12-20 cycles. The PCR products were Southern blotted and hybridized with gene-specific probes. (B-D) Normalization of mRNA expression level under HU treatment (B), MMS treatment (C) and 4-NQO treatment (D). The mRNA levels of each RNR gene at different time points were compared with the mRNA level without treatment, which is defined as 1.

***tso2-1* exhibits defects in cell cycle progression**

Since yeast *rnr* mutants display a *cdc* phenotype, we tested if cell cycle progression is affected in *tso2-1* mutants. A *cyclin B1-GUS* chimeric protein driven by the *Cyclin B1;1* promoter (*pcycB1::GUS*) was introduced into *tso2-1* mutants. The reporter expression is detected only in the late G2/early M phases of the cell cycle in root tips (Colon-Carmona et al., 1999). While the *GUS* expression was normally not detectable in the above ground tissues of wild type plants (Fig. 2-11A, B), *GUS* was

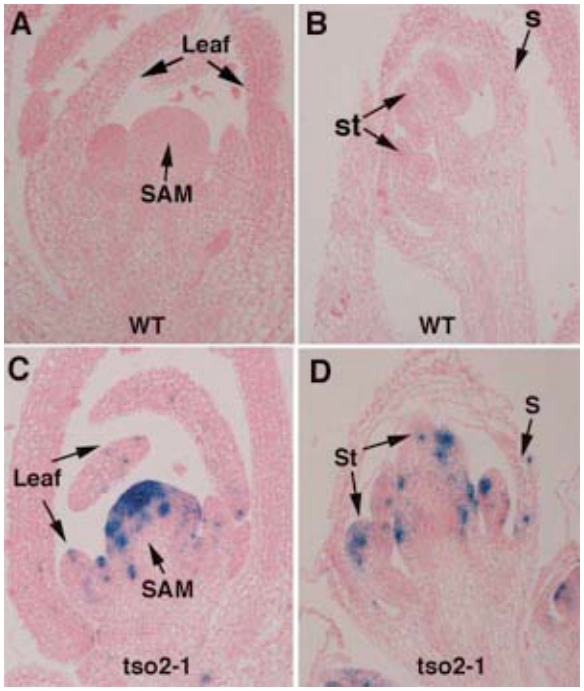


Figure 2-11. Cell cycle arrest in *tso2-1* mutants indicated by *pcycB1::GUS* expression. (A) *pcycB1::GUS* expression is not detectable in wild type SAM. (B) *pcycB1::GUS* expression is not detectable in wild type flowers showing sepals (s) and stamens (st). (C) *pcycB1:: GUS* is highly expressed in 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).

expressed strongly and in a sporadic fashion in *tso2-1* SAM and developing leaf and floral organs (Fig. 2-11C, 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.

***tso2-1* seedlings show increased sensitivity to UV-C**

Since RNR also provides dNTPs for DNA damage repair, the reduced dNTP pool sizes in *tso2-1* might lead to hypersensitivity to DNA damage treatment, which is the case for yeast *rnr* mutants. Therefore, we tested the sensitivity of *tso2-1* to different DNA damaging agents including MMS (alkalating agent), Mitomycin C (MMC, cross-linking agent) and 4-NQO (UV-mimetic agent). With the same assay used for HU sensitivity (Fig. 2-7), we did not observe any difference in the sensitivity to MMS, MMC and 4-NQO among Ler , Col, *r2a r2b* and *tso2-1* (data not shown). Filatov et al. (1996) found that mammalian *RNR* genes could be induced by UV-C but not by HU or MMS. They argued that mammalian RNR might be induced only by DNA damage requiring nucleotide excision repair. Although 4-NQO is a UV-mimetic agent, published studies have shown that different repair pathways might be used for DNA damage caused by 4-NQO and UV-C. Therefore, 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. 2-12A, B) while *r2b-1* or *r2a-1 r2b-1* did not (data not shown). The similar level of UV-C sensitivity of *r2a-1 r2b-1* double mutants to wild type (L*er*) indicates that *TSO2* alone is sufficient to provide wild type level of protection against UV-C. The embryonic and seedling lethality prevented us from testing *tso2-1 r2b-1* or *tso2-1 r2a-1* double mutants, which may show a more significant sensitivity to UV-C than *tso2-1* single mutants.

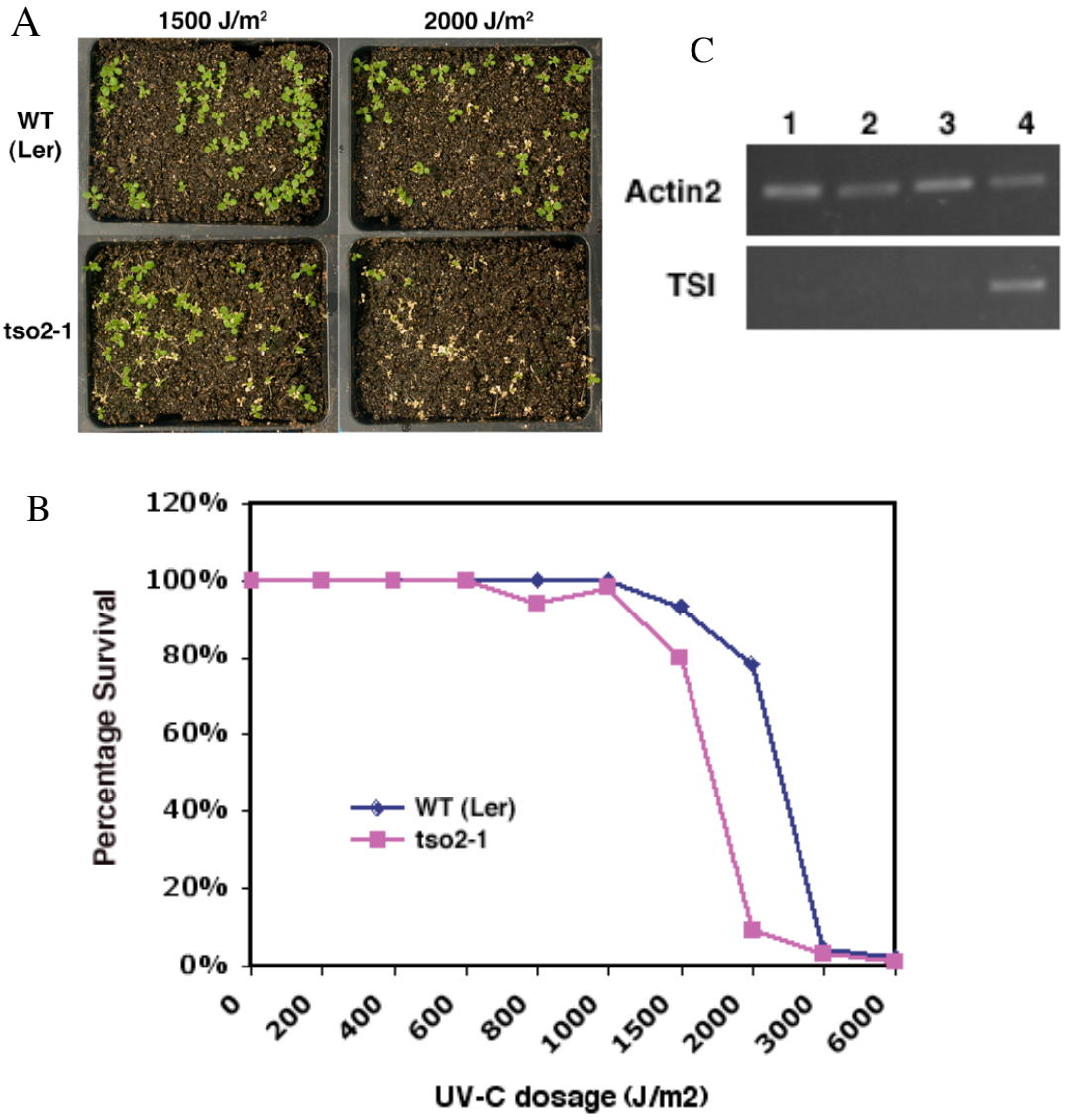


Figure 2-12. Increased sensitivity to UV-C and epigenetic misregulation in *r2* mutants. (A) An increased sensitivity of *tso2-1* to UV-C as shown by a larger number of dead seedlings at both 1500 J/m^2 and 2000 J/m^2 doses. (B) A graph illustrating the survival rates of wild type (L*er*) and *tso2-1* seedlings under increasing UV-C dosages. (C) 25 cycles of RT-PCR were used to detect *TSI* transcripts. Lanes 1, 2, 3, and 4 correspond to WT (L*er*), *tso2-1*, *r2a-1 r2b-1*, and *tso2-1 r2a-1,* respectively.

***tso2-1 r2a-1* double mutants exhibit defects in epigenetic regulation, accumulate DNA damage, and undergo programmed cell death (PCD)**

The phenotype of *tso2-1* associated with meristem fasciation and increased sensitivity to DNA-damaging agents resembles several *Arabidopsis* mutants defective in DNA/chromatin replication and assembly, including mutants of *BRU1, FAS1, FAS2, AtCAP-E1, AtCAP-E2,* and *AtMRE11* (Bundock and Hooykaas, 2002; Kaya et al., 2001; Siddiqui et al., 2003; Takeda et al., 2004). All these mutants were found to release transcriptional gene silencing (TGS) (Takeda et al., 2004) at the pericentromeric repeats called *Transcriptional Silent Information* (*TSI*) (Steimer et al., 2000). We found that *tso2-1 r2a-1* double mutant seedlings also released silencing at *TSI* but *tso2-1* single mutant seedlings did not (Fig. 2-12C). We also tested *tso2-1* single mutants at later developmental stages. There is no release of TSI silencing in *tso2-1* inflorescence tissues (data not shown).

Are reduced dNTP levels in *tso2-1* sufficient to impede DNA replication fork progression and induce DNA strand breaks? Using comet assay (single cell gel (SCG) electrophoresis) (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 significantly increased DNA damage (Fig. 2-13A). 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. 2-13B). These markers included Poly (ADP-ribose) polymerase-1 *(AtPARP1), AtPARP2,* and *AtRAD51* (Doucet-Chabeaud et al., 2001; Doutriaux et al., 1998).

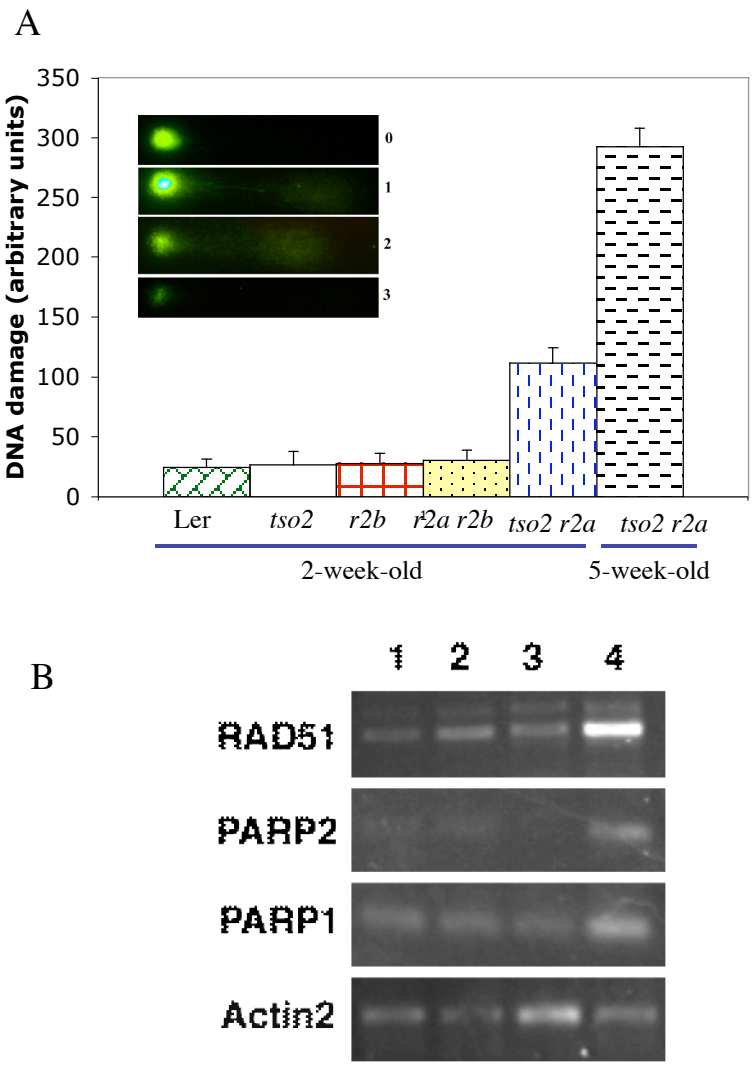


Figure 2-13. Increased DNA damage in *tso2-1 r2a-1* seedlings. (A) Results from the comet assay indicating the relative amount of DNA damage in different genotypes. While *tso2-1, r2b-1,* or *r2a-1 r2b-1* mutants exhibited similar levels of DNA damage to wild type (L*er*), *tso2-1 r2a-1* double mutants 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) 27-30 cycles of RT-PCR were used to detect the induction of *AtPARP1, AtPARP2* and *AtRAD51*. Lanes 1, 2, 3, and 4 correspond to WT (L*er*), *tso2-1, r2a-1 r2b-1,* and *tso2-1 r2a-1*, respectively.

The *tso2 r2a* double mutants do not survive beyond the 2 ~ 4-leaf stage. Very often we found that the leaves of the double mutants turn yellowish, show symptoms of chlorosis and eventually die. These symptoms resemble senescence and hypersensitive response (HR)-mediated programmed cell death. In animals, genomic instability often induces programmed cell death (PCD) in a p53-dependent fashion (Chernova Olga et al., 1995; Vogelstein et al., 2000). We tested if the accumulating DNA damage in *tso2-1 r2a-1* seedlings observed above could lead to PCD using histochemical and molecular markers. Trypan blue (TB) was used to stain dead cells (Rate et al., 1999). 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. 2-14A). 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. 2-14A). 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 (Brodersen et al., 2002) including *SAG13*, Peroxidase C (*PRXc*), *Glutathionine-S-Transferase* (*GST*), *Pathogenesis-Related 1* (*PR1*) and *EDS1* (Fig. 2-14B). However, *SAG12*, a cysteine protease specific to senescence-induced PCD (Pontier et al., 1999), was not detected under the RT- PCR condition used (Fig. 2-14B). Overall, *tso2 r2a*-mediated PCD appears similar to hypersensitive PCD in plants.

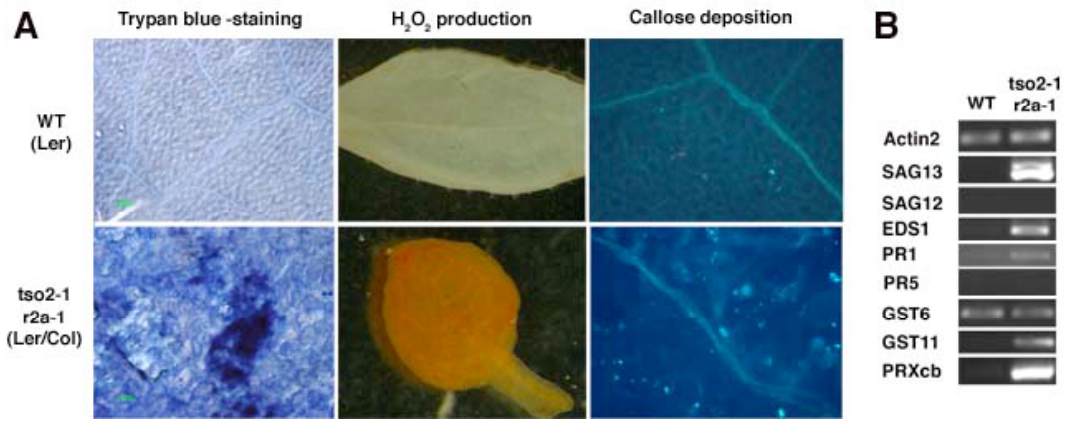


Figure 2-14. Expression of cell death markers in *tso2-1 r2a-1* mutants. (A) Leaves of wild type and *tso2-1 r2a-1* double mutants were examined for programmed cell death using three different histochemical markers: trypan blue staining, H_2O_2 production and callose deposition. Dark blue patches stained 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) 25 cycles of RT-PCR were used to detect different molecular markers associated with PCD. *SAG13, EDS1, PR1, GST11,* and *PRXcb* were induced to various levels in *tso2-1 r2a-1* mutants. *GST6* remained the same in both wild type and *tso2-1 r2a-1*. *SAG12* and *PR5* were not induced. However, *SAG 12* could be detected using 30 cycles of PCR (data not shown).

***tso2-1* is an recessive antimorphic allele**

Since *tso2-1,2,3,* and *4* alleles are all missense mutations, it is not known if they are loss-of-function alleles or antimorphic. A null mutation in *TSO2* will help distinguish the above alternatives. By sequence-indexed database search, I identified a T-DNA insertion in *TSO2*. This allele was named *tso2-5*, in which the T-DNA is inserted 80 bp downstream of the start codon ATG and causes a 10-bp deletion at the insertion site (Fig. 2-5), resulting in a frame-shift and truncation. Therefore, even though *TSO2* mRNA level is only reduced 2-fold (data not shown) in *tso2-5* mutants, *tso2-5* is very likely a null allele. Surprisingly, *tso2-5* does not exhibit any visible phenotype (Fig. 2-15A).

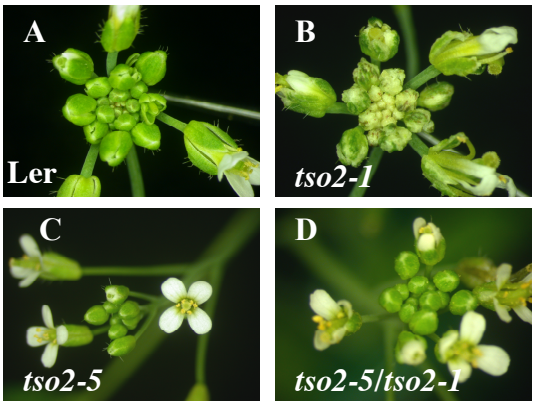


Figure 2-15. *tso2-5* null allele display a wild type phenotype. (A) wild type (Ler). (B) *tso2-1*. (C) *tso2-5*. (D) *tso2-1*/*tso2-5*.

Since *tso2-5* putative null allele displays no phenotype, the missense *tso2* alleles are likely antimorphic by either titrating out R1 or by heterodimerizing with and inactivating R2A or R2B. Since *tso2-1* is recessive, it suggests that *tso2-1* dosage may be important to cause a phenotype. To confirm that, we first crossed *tso2-1* into *tso2-5* to generate a *tso2-1/tso2-5* transheterozgyote. *tso2-1/tso2-5* showed wild type phenotype (Fig. 2-15D). We crossed *tso2-5* with *r2a r2b* double mutants to generate triple mutants. *tso2-5 r2a r2b* triple mutants are embryonic lethal because this triple mutant could not be isolated from the progeny of wild type-like *tso2-5/+; r2a/r2a; r2b/r2b* plants. *tso2-5 r2b* double mutants still display no phenotype. However, *tso2-5/tso2-5; r2a/+; r2b/+* and *tso2-5/tso2-5; r2a/+* both are *tso2-1*-like. This indicates that two copies of the poisonous *tso2-1* have a similar effect as removing 100% TSO2 as well as 50 % of R2A and R2B. These genetic data suggest that *tso2-1* is an antimorphic allele.

Another surprising observation is that *tso2*-5/tso2-5; *r2a-1/r2a-1* is more severe than *tso2-1* single mutants and is similar to *tso2-1/tso2-1; r2a-1/r2a-1*, which are seedling lethal (Fig. 2-16). In some extreme cases, *tso2*-5 *r2a* double mutants only

develop one fully expanded cotyledon, and the development of the other cotyledon is retarded (Fig. 2-16E). If *tso2-1* interferes with both R2A and R2B, *tso2-1 r2a* double mutants should display a more severe phenotype than *tso2*-5 *r2a*. This suggests that the poisonous *tso2-1* protein may only interfere with R2A, but not R2B.

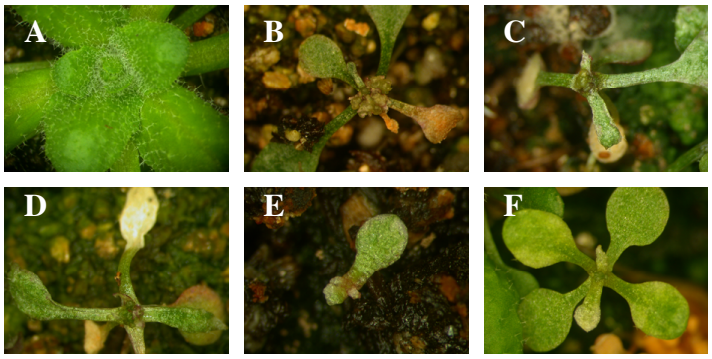


Figure 2-16. *tso2-1 r2a* and *tso2-5 r2a* exhibit similar phenotype. (A) *r2a*. (B-C) *tso2-1 r2a*. (D-F) *tso2-5 r2a*.

Discussion

Our molecular genetic studies of *tso2* led to the identification of mutations in all three *R2* genes in the *Arabidopsis* genome. The analyses of single, double and triple *r2* mutants demonstrated for the first time that normal dNTP pool and RNR function is critical for the plant response to mutagens and for proper plant development. Our data is supported by a recent transcriptome profiling study showing a 10-fold induction of *TSO2* transcripts upon treatment with bleomycin plus mitomycin C (Chen et al., 2003). Interestingly, while all four *tso2* missense alleles exhibited morphological defects, single *r2a-1, r2b-1* and double *r2a-1 r2b-1* mutants did not. We propose several alternative but nonexclusive explanations. First, *TSO2* may play a more prominent role than *R2A* and *R2B*. Second, *R2A* and *R2B* may function only under certain environmental or

physiological conditions. Third, the four *tso2* missense alleles may be recessive antimorphic alleles. Recessive antimorphism is possible if the mutant *TSO2* proteins interact with R1 or other R2 subunits, inactivating the RNR enzyme complex altogether, leading to a more severe phenotype than the single knock-out mutants. This antimorphic nature of *tso2-1* missense mutation is supported by the identification of *tso2-5* and genetic analysis between *tso2-1* and *tso2-5*.

Differential regulation of three *R2* genes

Although all three *R2* genes are expressed in almost all tested tissues with different abundance (Fig. 2-9A), there might be differential regulation for each of them. First, no or very low levels of *R2A* transcript are observed in roots. The expression level of *R2B* in cauline leaves is much lower than that in other tissues (Fig. 2-9A). Second, the GUS staining pattern and *in situ* hybridization indicated that the *TSO2* gene is cell cycle-regulated, with a maximal expression levels in S-phase. Whether *R2A* and *R2B* are cell cycle-regulated remains to be tested. Finally, the existence of E2F binding sites in the promoters of the *TSO2* and *R2B* but not *R2A* genes suggests that they are regulated by different mechanisms. The consensus E2F binding site is TTT(C/G)(C/G)CGC in both human and plants. The *TSO2* gene contains one canonical (TTTGGCGC) and two atypcial E2F binding sites (TTTGGCGG and TTTGCCGG in opposite direction) whereas the *R2B* gene has only one canonical E2F binding site (TTTGGCGC). The only potential E2F binding site (TTTCGCCG) in the *R2A* gene has CC at position 6 and 7, the two positions that are intolerant for any change for E2F binding (Ouellette et al., 1992, Ramirez-Parra et al., 2003). Thus, R2A is unlikely to be regulated by E2F.

Chamovitz and his colleagues found that *Arabidopsis* R2A but not TSO2, physically interacts with CSN7/FUS5, one subunit of the COP9 signalosome (CSN) complex (Levanon et al., 2003). This suggests that R2A might be regulated at the protein level, rather than at mRNA level, resembling the mammalian R2 protein (Chabes et al., 2003). TSO2 and R2A also differ in their ability to rescue yeast *rnr* mutants (Levanon et al., 2003).

Intriguingly, cell cycle regulation of the human *R1* gene is controlled by the YY-1 transcription factor (Johansson et al., 1998). In contrast, the human *R2* gene contains a CCAAT box (recognized by NF-Y) and an adjacent E2F4 binding site (TCTCCCGC) for its cell cycle-regulation. It is noteworthy that E2F4 acts as a repressor (Chabes et al., 2003). Transcriptional regulation of human *R1* and *R2* genes by different transcription factors might allow the cells to fine-tune its RNR activity in response to different signals. It is possible that other transcription factors in addition to E2F/DP are involved in regulating plant *RNR* genes.

Induction of RNR genes by DNA damage

In yeast, the control of dNTP pool sizes has evolved as an important survival mechanism (Zhao et al., 2003). Not surprisingly, RNR genes are the targets of the surveillance machinery in response to different stresses. As a consequence, *rnr* mutants are hypersensitive to various DNA damaging agents including MMS, 4-NQO, and UV. In contrast, mammalian *RNR* genes seem to respond only to specific DNA damages. The expression of mouse *R2* gene cannot be induced by MMS or HU. However, the mouse *R1*

gene can be induced up to 3-fold, and the *R2* gene 10-fold, by UV in a dose-dependent manner (Filatove et al., 1996). They argued that MMS causes base damage by alkylation, which is thought to be repaired by a short patch repair mechanism or dealkylation that requires very little if any dNTPs (Filatove et al., 1996). Only upon UV irradiation, which produces bulky lesions in DNA, are *RNR* genes induced to provide dNTPs needed for the nucleotide excision repair pathway.

Our data on the induction of *Arabidopsis RNR* genes by DNA damage suggest that plants might respond to DNA damage in a similar manner as animals, at least in terms of induction of RNR genes. Like mammalian RNR genes, *Arabidopsis R1* and *R2A* genes are not responsive to MMS and 4-NQO induction and the *TSO2* and *R2B* genes are only slightly induced. Consistent with this, neither *r2a r2b* nor *tso2-1* plants are hypersensitive to MMS or 4-NQO treatments. However, unlike animals, *Arabidopsis RNR* genes can be induced by HU with the *TSO2* gene increased by 20-fold but *R2A* by only 2-fold. We have not tested the induction of *RNR* genes by UV treatment. We showed that *tso2-1* mutants are hypersensitive to UV-C (Fig. 2-12), suggesting that RNR activity in *tso2-1* is suboptimal for repairing bulky lesions caused by UV-C. Culligan et al. (2004) reported that the *Arabidopsis R1* gene is not induced by either HU or UV-B treatment. The discrepancy could be due to different tissues used (they used whole seedlings and we used cell suspensions) or different methods (they used Northern blot and we used a more sensitive semi-quantitative RT-PCR). In addition, they used 1 mM HU while we used 20 mM HU.

Using comet assay, Menke et al. (2001) found that over a wide range of concentrations and treatment times, various DNA damaging agents including MMS and the radiomimetic bleomycin, could cause DNA damage. However, combinations of bleomycin and mitomycin C exert a strong induction of DNA damage-responsive genes, such as *Rad17*, *Rad51*, and poly (ADP-ribose) polymerase (*PARP-2)* (Chen et al., 2003). Under their conditions (1-1.5 μg/ml bleomycin and 50-75 μM mitomycin C), *TSO2* was induced 10-fold. This suggests that plant *RNR* genes might respond only to certain types of DNA damage. In the case of *tso2-1* mutants, the presence of *R2A* and *R2B* may be able to provide enough dNTPs for small DNA damage caused by MMS and 4-NQO.

The induction of *RNR* genes by HU suggests that, unlike in animals, there might be a feedback regulation of *RNR* genes in response to depletion of the dNTP pools in plants. This is because HU, the specific inhibitor of the small subunit of RNR, will abolish RNR activity and hence deplete the dNTP pools. In the yeast *rnr2-314* mutant, the damage response pathway is partially constitutive, as indicated by a 10-fold increase in the RNR2 promoter activity compared with the wild type (Elledge and Davis, 1989). This is consistent with our data that DNA damage accumulates and DNA repair genes are up-regulated in *tso2 r2a* double mutants (Fig. 2-13).

Decreased dNTP levels affect organelle replication

One unique feature of *tso2* mutants is the formation of white sectors on green organs. We observed a lack of chloroplasts in the photosynthetic mesophyll cells, unusual air spaces beneath the epidermis, and abnormally small mesophyll cells in *tso2* mutant tissues (Fig. 2-3E and F). In yeast, lower RNR activity or reduced dNTP level was

shown to increase the formation of mitochondrial DNA-deficient cells (petite cells) (Zhao et al., 2001; Zhao et al., 1998), suggesting that organelle DNA replication is highly sensitive to reduced dNTP levels. Therefore, stochastic depletions of chloroplasts and/or mitochondria may underlie the white sectors and small cells in *tso2* mutants.

In animals, the salvage pathway is involved in providing the dNTPs for mitochondrial genome replication (see Chapter one). Several human disorders such as mitochondrial DNA-depletion syndromes (MDS) are caused by mutations in the dNTP salvage pathway. The white sectors and small cells in *tso2* strongly suggest that the *de novo* biosynthesis of dNTP is required for both chloroplast and mitochondrial DNA replication. The development of white sectors occurring in the fifth leaf and later-arising organs of *tso2* could result from a gradual dilution of dNTP, which may be highly enriched in embryos/seedlings via salvage pathways (Reichard, 1988; Saada et al., 2001). Interestingly, *Arabidopsis* thymidine kinase 1 (TK1) seems to be a cytoplasmic protein whereas thymidine kinase 2 (TK2) is localized in mitochondria. This is similar to human where TK2 is also a mitochondrial protein. Deoxyguanisine kinase (dGK), however, is a chloroplast protein in *Arabidopsis*. It seems that both *de novo* and salvage pathways are involved in the replication of all three genomes in plants.

Fasciation is an indirect consequence of epigenetic mis-regulation in *tso2* mutants

Fasciation is manifested by distorted phyllotaxy, broadening and flattening and, in extreme cases, bifurcation of the stem. Several mutants that have an extreme fasciation phenotype, such as *clv1*, *clv2* and *clv3* mutants (Clark et al., 1993; Kayes and Clark,

1998; Fletcher et al., 1999), have been well studied in *Arabidopsis*. In these mutants, the fasciation is manifested as enlarged shoot apical meristem, sometimes 1000-times larger than wild type (Clark et al., 1997). Another class of fasciation mutants exhibits bifurcation of the stem. In *tso1-1* mutants, fasciation results from extensive bifurcation or multiple splits of the inflorescence meristem (Song et al, 2001). Like *tso1*, *tso2* mutants belong to this second class of fasciation mutants. In *tso2 r2a/+* and *tso2 r2a/+ r2b/+* plants, meristem bifurcation is more frequently observed than in *tso2-1* single mutants. Mutants that are defective in either DNA/chromatin replication or assembly also exhibit this second class of fasication. These mutants include *bru1, fas1, fas2, AtCAP-E1, AtCAP-E2, top1α, top1β* and *mre11* (see Chapter one; Bundock and Hooykaas, 2002; Kaya et al., 2001; Siddiqui et al., 2003; Takeda et al., 2004). *TOP1α* and *TOP1β* encode a type I DNA toposiomerase, promoting the relaxation of supercoiled DNA by introducing a transient double-strand breaks (DSB) and acting in a number of different DNA metabolisms such as DNA replication, transcription, repair, and chromatin compaction (Takahashi et al., 2002). *FAS1* and *FAS2* encode the two subunits of chromatin assembly factor with a function in assembling nucleosomes onto nascent DNA (Kaya et al., 2000). The *MRE11* gene encodes a component of the MRX complex with roles in repairing DSB and maintaing telomere length (Bundock and Hooykass, 2002). The *BRU1* gene encodes a novel nuclear protein (Takeda et al., 2004).

Takeda et al. (2004) found that *bru1, fas1, fas2, mre11, AtCAP-E1-/-* and *AtCAPE2-/-* are hypersensitive to DNA damage and can release transcriptional gene silencing (TGS). This prompted them to propose that defects in the S-phase DNA-

damage checkpoint, or chromatin/DNA replication, can cause epigenetic instability of newly replicated chromatin. As a result, release of gene silencing at crucial regulatory gene loci such as *WUS* will lead to fasciation (Mayer et al., 1998; Takeda et al., 2004). We observed the release of silencing at the pericentromeric repeats in *tso2 r2a* double mutants (Fig. 2-13C), suggesting that epigenetic mis-regulation of *WUSCHEL* and *AGAMOUS* may also underlie the fasciated SAM and homeotic transformation of floral organs observed in *tso2-1* mutants.

In *Drosophila*, a tesmin/TSO1 homolog, Mip120 is a member of Myb-containing complex involved in site-specific DNA replication (Beall et al., 2002). It was shown that, Mip120 and other Myb-interacting proteins are members of a repressive complex to ensure a silenced transcriptional and/or replicative state. Mutations of each of the members will lead to unscheduled DNA replication (Beall et al., 2004). The increased DNA ploidy in *tso1* meristem cells suggests that *TSO1* might function in a similar way. Therefore, it is very likely that the fasciation in *tso1* mutants is also caused by defective DNA replication. The link between chromatin/DNA replication and maintenance of gene silencing might also exist in other organisms. Cell polarity is disrupted in *C. elegans* in *div* mutants that are defective in DNA polymerases (Encalada et al, 2000), which is likely caused by ectopic expression of certain developmental regulator genes.

Since *rnr* mutants of yeast or mammals have not been reported to have epigenetic defects, it remains to be seen if release of TGS could underlie growth abnormalities in *rnr* mutants of fungi or animals. Alternatively, defects in cytokinesis (shown in *tso1*) and cell

division (shown in *tso2*) may interfere with the *CLAVATA/WUSCHEL* signaling pathway so that multiple stem cell organizing centers (specified by *WUSCHEL*) are initiated.

Cell division and development

Could the phenotype of tso2 be attributed solely to reduced dNTP pool sizes and defective DNA repair? Alternatively, could RNR function in some unknown capacity? Downes et al (2000) reported that mammalian S-phase checkpoint integrity depends on transformation status and purine dexoyribonucleosides. They found that purine deoxyribonucleosides could prevent the caffeine-induced activation of mitotic $p34^{cdc2}$ kinase. They speculated that normal high S-phase intracellular levels of purine deoxyribonucleotides could act as an intracellular signal for S-phase, and could retard progression towards mitosis. This explains why RNR is involved in oncogenic transformation more so than RNR being a simple metabolic enzyme.

***tso2-1* is a recessive antimorphic allele**

According to Muller's definition (1932), if a mutant allele behaves identically to a deficiency or deletion, it is a null. If an allele leads to more severe phenotypes than a deficiency, it is an antimorph. A very common example of antimorph is the dominant-negative effect. A dominant-negative allele normally produces a truncated or inactive protein that interferes with its own wild type protein. The interference could be carried out by: (1) competing with the wild type copy for the substrate or binding partner; (2) in the case of homodimer or multimer formation, titrating out the wild type copy; therefore, leading to a dominant effect.

Several recessive antimorphic alleles were reported in *Drosophila* (Marenda et al. 2003). However, the underlying molecular mechanism remains unclear. As RNR is a tetrameric complex and *R2* consists of a small gene family in *Arabidopsis*, it is not surprising that the null or loss-of-function alleles of all three *R2* genes exhibited no phenotype due to functional redundancy, and the missense mutation *tso2-1* exhibits developmental defects due to its antimorphic nature. The mutant *tso2-1,2,3,4* proteins can be catalytically inactive but retain the ability to bind to other R2 or R1 subunits. Alternatively, the mutant proteins lose their ability to bind one subunit but retain the ability to bind other subunit. In either case, the mutant protein can compete the R1 or R2 subunits with other functional RNR subunits.

tso2-5 is a putative null allele as the T-DNA is inserted in the very beginning of the *TSO2* gene and causes a 10-bp deletion at the insertion site. The resulting protein could be either truncated (containing the first 30 aa) or frame-shifted. *r2a*-1 is caused by a premature stop codon, but retains two-thirds of the protein. The *R2A* mRNA level is reduced in *r2a-1* due to nonsense-mediated decay (data not shown), suggesting that *r2a-1* might be a null allele. The *R2B* pseudogene in Col ecotype will make a truncated protein (four-tenth of the wild type), indicating that *r2b* is likely a null allele.

The ultimate proof of *tso2-1* as an antimorphic allele is to introduce the *tso2-1* mutant transgene into wild type (Ler) or *tso2*-5 plants to see if the transgene can confer mutant phenotype in a wild type background, and to see if it is dosage-dependent (to

explain why *tso2-1* is recessive). The *tso2-1* gene could be expressed under a strong constitutive 35S promoter. *p35S::TSO2-1* could cause a *tso2-1*-like phenotype in both Ler and *tso2-1* plants. In addition, further experiments need to be done in confirm that *tso2-5* is a null. Since *TSO2* transcripts are only reduced 2-fold in *tso2-5*, it might suggest that the T-DNA could be spliced out or a cryptic promoter in the T-DNA sequence can still drive the transcription of *TSO2* from downstream of the T-DNA insertion in *tso2-5*. First, Northern analysis could be performed to reveal the real size of the *TSO2* transcript in *tso2-5*. Second, 5' RACE could be conducted with a primer downstream of the T-DNA insertion site to unveil the sequence of the *TSO2* transcript, which can determine if frame-shift does occur in *tso2-5*. The last and indisputable evidence could be harvested from Western analysis to detect if any TSO2 protein is made in *tso2-5*. It is note worthy that although *tso2-5* alone displays no visible phenotype, *tso2-5/tso2-5 r2a/+* exhibits a *tso2-1* phenotype. In addition, *tso2-5 r2a* and *tso2-1 r2a* show a similar phenotype. Moreover, while *tso2-5 r2b* still fail to show any phenotype, *tso2-1 r2b* is embryonic lethal. These genetic results are strongly against that *tso2-5* might be a weak and *tso2-1* a strong loss-of-function mutant.

***tso2*-mediated PCD**

Senescence and the hypersensitive reponse (HR)-mediated cell death are the two major types of programmed cell death (PCD) in plants. As a defense mechanism, cells undergo PCD to limit the spread of the invaders (HR) or to recycle the energy and resources (senescence). In animals, genomic instability often leads to p53 dependent or p53-independent apoptosis (Chernova et al., 1995; Vogelstein et al., 2000), so that cells

with extensive DNA damage and mutations are eliminated, in order to avoid the passage of defective genetic information to the next generation.

While genomic instability-caused apoptosis has been well studied in animals, it is rarely reported in plants and the mechanism remains unknown. Since plants do not have a p53 homolog, it is likely that plants evolved a different signal transduction pathway in response to genomic instability. Both PCD and DNA damage are not observed in *tso2-1* single mutants, but both were observed in *tso2 r2a* double mutants, suggesting a causal correlation between DNA damage and PCD.

Several histochemical and molecular markers were used to detect PCD in *tso2 r2a* double mutants. SAG13, a widely recognized PCD marker, is induced in *tso2 r2a* double mutants. *EDS* and *PR-1*, which are up-regulated in HR-mediated PCD, also accumulate in the double mutants (Fig. 2-15B). SAG12 is a senescence-specific marker, the expression of which is strictly dependent on age (Noh and Amasino, 1999). We detected a low level of SAG12 in *tso2 r2a* (with longer cycles of PCR), suggesting that a senescence process might also be turned on. It is possible that PCDs, triggered by different signals in plants, share common downstream effectors. By examining if PCD occurs in other plant mutants defective in genome integrity, one may pinpoint the DNA/cellular lesions required to switch on PCD. Although there is no p53 homolog in plants, our data suggest that plants also possess a mechanism to induce cell cycle arrest and eliminate damaged cells via PCD. The viable *tso2* mutants will facilitate genetic screens for mutations in p53-like genes in higher plants.

Chapter three

Isolation of Suppressors of *tso2-1*

Abstract

To further understand the regulation of RNR genes and to identify novel components of plant checkpoint pathways, a genetic screen of suppressors of *tso2-1* was performed. A preliminary screen from only 4,000 M2 seeds led to the isolation of 18 independent suppressor mutations that can partially or completely suppress the phenotype of *tso2-1*. Two of these 18 suppressors display a novel phenotype and are dominant. Complementation tests showed that the 18 suppressors fall into at least four different complementation groups. The potential pathways that these suppressor mutations may define are discussed.

Introduction

It is widely known that due to genetic redundancy, knockout mutations in a gene may not lead to any visible or detectable phenotype. *Arabidopsis* is a superb model organism for genetics, as the large number of T-DNA insertion and transposon-tagged lines makes it possible to knockout almost every gene in the genome. If a gene belongs to a small gene family, each individual gene can be knocked-out or knocked-down and double or triple mutants can be constructed to reveal their functions. For instance, the *Arabidopsis* genome has three *SEPPLATA* (*SEP*) genes. Single mutants of *sep1*, *sep2* or *sep3* or double mutants of any two combinations do not exhibit any visible phenotype. The triple mutant *sep1 sep2 sep3*, however, produces flowers consisting of all sepals,

revealing the functions of these three *SEP* genes in petal, stamen and carpel development (Pelaz et al. 2000).

Modifier screens have been very powerful to isolate genes whose functions are hidden or masked by their redundant genes. This is based on the principal that functions of a gene become more crucial in certain genetic backgrounds, where the functions of its redundant members are reduced. A good example is the isolation of several C-class floral genes in *Arabidopsis*. For a long time, *AGAMOUS* (*AG*) was the only C-class gene identified. Additional C–class genes, *HUA1* and *HUA2*, were only isolated much later in a modifier screen in the background harboring a weak *ag-4* allele. Single mutants of *hua1* or *hua2* and even the *hua1 hua2* double mutants do not display any floral defects on their own but can dramatically enhance the weak *ag-4* phenotype (Chen and Meyerowitz, 1999). Such "modifier" screens are widely practiced in other genetic organisms such as *Drosophila* and *C.elegans*.

Ribonucleotide reductase (RNR) is an essential enzyme that catalyzes the rate-limiting step for the production of the deoxyribonucleotides required for DNA synthesis. Therefore, the *RNR* genes have been tightly regulated at different levels. For example, *RNR* genes can be transcriptionally regulated by Crt1 repressors, post-transcriptionally by Cird13, post-translationally regulated by Sml1 inhibition protein or by cytoplasm-to-nucleus translocation (For detail, see Chapter one). Although a large amount of information is available on the regulatory mechanisms of *RNR* in yeast and animals, little is known about how *RNR* expression and activity are regulated in plants. As shown in

Chapter two, unlike yeast, expression of Arabidopsis *RNR* genes are not induced, or slightly induced, by certain types of DNA damaging agents such as MMS and 4-NQO (at least under the conditions we used). Since mammalian *RNR* genes cannot be induced by MMS, it suggests that similar mechanisms of regulation of RNR in response to DNA damage might be conserved between animals and plants. However, the *TSO2* gene can be induced up to 10-fold by a combination of bleomycin and mitomycin C whereas human *RNR* genes can be induced 3~10-fold by UV treatment. The expression level of *R2B* is very low compared to *TSO2* and *R2A* (Fig. 2-10A). Is *R2B* normally repressed by general co-repressors? Is there a Sml1 type inhibitory protein of RNR in *Arabidopsis*? Mutations that inactivate negative regulators of RNR may suppress *tso2-1* phenotypes. Taking advantage of the fertile phenotype of *tso2-1* and the fact that the dNTP level is below the threshold for normal development in *tso2-1*, creating a sensitive genetic background, a modifier screen will be highly informative.

In yeast, a genetic screen aimed at identifying genes that regulate *RNR3* turned out to be a big success. Several important DNA damage and replication checkpoint genes were isolated and novel regulation mechanisms were revealed (Zhou and Elledge, 1992). In yeast, the mRNA level of *RNR3* is almost undetectable under normal growth conditions but can be induced up to 100-fold by DNA damaging agents. Taking advantage of this feature, Zhou and Elledge designed a genetic screen to isolate mutants that can express high levels of *RNR3* in the absence of DNA damaging agents. More than 202 independent *crt* (constitutive *RNR3* transcription) mutants were obtained and fell into 9 complementation groups. Five of the *CRT* genes have been identified as previously

cloned genes: *TUP1*, *SSN6*, *RNR1*, *RNR2*, and *POL1/CDC17*. *crt4*, which encodes the *TUP1*, has 58 alleles and *crt1*, which encodes a DNA binding protein, has 131 alleles. CRT1 acts by recruiting the general repressors *TUP1* and *SSN6* to the promoters of damage-inducible genes including RNR. The discovery that *POL1/CDC17* is one of the *CRT* genes illustrated for the first time that directly blocking DNA replication can provide a signal to induce the DNA damage response (Zhou and Elledge, 1992).

Due to the essential role of RNR, *rnr* null alleles are embryonic lethal in *Drosophila* and mice, preventing us from understanding the functions of RNR in later developmental stages in higher eukaryotes. *tso2-1* is the first viable *rnr* mutant that exhibits developmental defects in higher eukaryotes. Genetic screen for suppressors or enhancers of *tso2-1* will provide a tremendous opportunity to uncover genes involved in the regulation of RNR or DNA replication. There are at least eight different mechanisms by which a mutated gene could suppress the *tso2-1* phenotype. First, mutations in genes encoding transcriptional regulators of the *TSO2* gene can be identified. Mutations in this type of regulatory genes will reduce or abolish *tso2-1* transcription, leading to a reduction of the antimorphic *tso2-1* product. As described in Chapter two, the *tso2-5* null allele and the *tso2-1/tso2-5* transheterozygote both show a wild type phenotype. Hence, reduced *tso2-1* may be equal to *tso2-1*/*tso2*-5 or *tso2*-5/*tso2*-5. Second, a nonsense mutation in the *TSO2* ORF can act as the intragenic suppressor. Such suppression will eliminate the antimorphic *tso2-1* protein product. Third, mutations in negative regulatory genes of R1, like the budding yeast *SML1* (see Chapter one), may release the inhibitory effect on the R1 subunit and enhance the overall RNR activity. Fourth, gain of function mutations in

genes involved in the RNR salvage pathway, or recessive mutations in genes that negatively regulate the salvage pathway, may result in more dNTP production, thus compensating for the *tso2-1* effect. Fifth, gain-of-function mutations in *R1* or other *R2* genes (*R2a* and *R2b*) may result in higher RNR activity so that the *tso2-1* effect will be compensated or compromised. In both yeast and human, mutations in the allosteric activity site of R1 that eliminate the dATP feedback inhibition results in two times higher dNTP production (Chabes et al., 2003). Sixth, mutations in genes involved in posttranslational regulation of RNR may either stabilize the R1 or other R2 subunit, or promote the degradation of the *TSO2* protein. As a consequence, the effect of *tso2-1* can be reduced, eliminated or compromised. Seventh, mutations in genes involved in DNA replication, such as DNA polymerase may somehow more efficiently utilize the low dNTPs in *tso2-1* to reach normal DNA replication, thus compromising the effect of *tso2-1*. Although unlikely, it was found that overexpression of R1 could rescue a mutant phenotype caused by defective mitochondrial DNA polymerase MIP1 gene in yeast (Lecrenier and Foury, 1995). Eighth, mutations in genes involved in DNA replication checkpoints or a sensor of dNTPs, might trigger a feedback pathway that more effectively induces the expression of other *RNR* genes. Mutations in any one of these eight pathways alone might not display a visible phenotype but may be recovered in *tso2-1* background.

This chapter describes the isolation of suppressors of *tso2-1*. Eighteen suppressors of *tso2-1*, named *shuai* (*sua*) (means handsome in Chinese), were screened from only 4,000 M2 seeds. Complementation tests indicate that they fall into at least four complementation groups. Cloning of these *sua* genes in the future will not only enhance

our understanding of the regulation of RNR genes in plants, but also shed light on how the size of dNTPs pool is maintained and how cell division is integrated in the context of development in plants.

Results

The suppressor screen was designed to isolate mutations that could suppress the phenotype of *tso2-1*, either partially or completely. If the suppression is complete, then wild type-like plants are expected to emerge in the M2 generation. Caution has been exercised in the screen as *tso2-1* plants exhibit a certain degree of variation in phenotypic severity from batches to batches and under different growth conditions. To this end, all the potential suppressors were grown to the M3 generation and the phenotype of each line was confirmed. For those lines that continue to show the suppressor phenotype, PCR-based genotyping was conducted to make sure they have *tso2-1* homozygous mutation in the background in order to eliminate wild type contaminants. The majority of the lines were also sequenced in the TSO2 ORF to look for any potential intragenic suppressors. The sequencing results were always consistent with the PCR-based genotyping using the dCAPS marker, i.e. detecting original *tso2-1*. None of them were intragenic suppressors. Since the suppressors can suppress the *tso2* phenotype, we name them *shuai* (*sua*) (means handsome in Chinese).

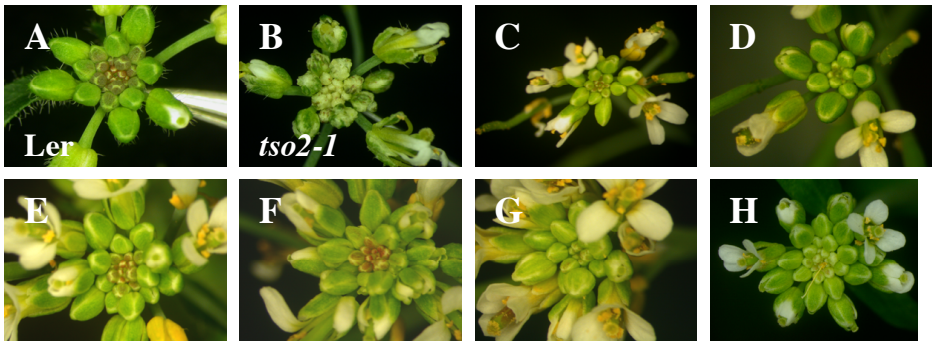


Figure 3-1. Representative suppressor lines of *tso2-1*. (A) wild type (Ler). (B) tso2-1.(C-H) suppressor lines DS2, DS8, DS11, DS12, DS15 and DS6. Note that the center of the inflorescence is whitish in DS6, indicating it partially suppresses *tso2-1*.

Forty-one wild type-like plants, or plants displaying very subtle *tso2-1* phenotypes, were initially isolated from screening about 4, 000 M2 plants (from 50 M1 families). Seeds were collected from these 41 lines and grown alongside *tso2-1* plants for phenotypic comparison. Three lines exhibited a *tso2-1* phenotype and were eliminated. Thirteen lines segregated in a 3:1 ratio of wild type and *tso2* phenotypes in the M3, and genotyping/sequencing analyses showed that none of the wild type-plants were *tso2-1* homozygous, suggesting that they were likely derived from cross-pollination between *tso2-1* and wild type. Seven lines are wild type contaminants as they were wild type at the *TSO2* locus. The remaining18 suppressors were kept for further analysis. These 18 lines all showed a wild type phenotype but were homozygous for *tso2-1* mutation (Fig. 3-1). Since they were isolated from different M1 families, they were very likely independent lines. Later on, one of the 18 lines, DS6 consistently exhibited a very subtle *tso2-1* phenotype (shown in Fig. 3-1H) where only the center of the inflorescence became whitish in later developmental stages, suggesting that DS6 only partially suppresses *tso2-1*.

Table 3-1. Complementation test of 9 suppressor lines

	DS12	DS15	DS17	DS19	DS21
DS2	+	+	+	+	+
DS6	+	+	+	+	+
DS8	+	+	X	+	X
DS11	+	+	+	+	+

Note: "+" complementated; "X" not complementated.

In order to determine whether these 18 lines were allelic to each other, crosses were made among these lines. To reduce the workload, the suppressor lines were randomly divided into four groups. Lines in group A were crossed to lines in Group B and Lines in group C were crossed to lines in Group D. Results from the complementation tests are shown in Table 3-1 and Table 3-2.

Table 3-2. Complementation test of 7 suppressor lines

	DS38	DS41	DS53
DS31	X	N/A	+
DS33	+	partial	X
DS35	+	partial	X
DS42	+	partial	X

Note: "+" complementated; "X" not complementated
"N/A" no data.

Assuming each line is recessive, if the two lines complement each other (shown as "+" in the table), the F1 generation will display a *tso2-1* phenotype as they can no longer suppress the *tso2-1* phenotype. Thus, they belong to different complementation groups. If the two lines do not complement each other (shown as "X" in the table), they belong to the same complementation group and the F1 generation will still exhibit a wild

type phenotype because the *tso2-1* phenotype is still suppressed. As shown in Table 3-1, DS8, DS17 and DS21 belong to the same complementation group and they are allelic to each other. While DS 2, DS 6 and DS11 can complement with each of DS12, DS15 and DS19, these 9 lines fall into at least two other complementation groups. Crosses among DS2, DS6 and DS1as well as among DS12, DS15 and DS19 are in progress.

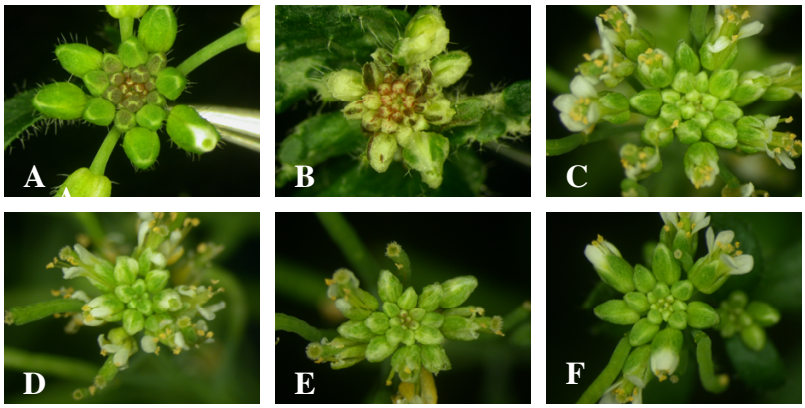


Figure 3-2. DS41 might be semi-dominant. (A) Wild type. (B) *tso2-1*. (C) F1 plants from the cross between DS41 and DS33. (D) F1 plants from the cross between DS41 and DS35. (E-F) F1 plants from the cross between DS41 and DS42.

As shown in Table 3-2, DS33, DS42 and DS53 cannot complement each other and hence, they are allelic to each other. DS31 and DS38 belong to the same complementation group. Interestingly, the F1 plants from the crosses between DS41 and DS33, DS41 and DS35, or DS41 and DS42 exhibit an intermediate phenotype (Fig. 3-2 C-F), that is, the phenotype is much weaker than the *tso2-1* plants grown side by side. Some of the floral organs still have white sectors but the leaves are normal. The intermediate phenotype could be due to the environmental variations or due to DS41 being semi-dominant.

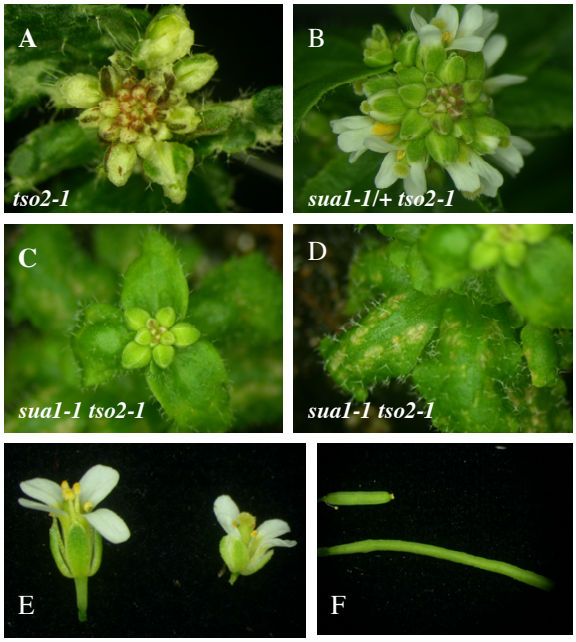


Figure 3-3. Phenotype of *sua1-1* suppressor (DS101) in *tso2-1* background. (A) *tso2-1*; (B) *sua1-1/+ tso2-1*. Note that the down-dripping inflorescence; (C) *sua1-1 tso2-1*. The plant is dwarf and the inflorescence is smaller consisting of a few number of flowers; (D) *sua1-1 tso2-1*. Note leaves with chlorotic lesions characteristic of cell death. (E) A single flower of wild type (Ler) (left) and *sua1-1/+ tso2-1* (right). (F) A silique of wild type (bottom) and *sua1-1/+ tso2-1* (top).

To segregate the suppressor mutations away from *tso2-1*, each suppressor line was crossed into wild type (Ler) and each suppressor mutation will be recovered in the F2. It will be difficult to identify suppressor lines that do not display any visible phenotype in the absence of *tso2-1*. In this case, the suppressors will always be kept in *tso2-1* background and the double mutants (suppressors in *tso2-1* background) will be used for map-based cloning.

Although the majority of the suppressor lines exhibited a wild type phenotype, two suppressors showed a novel phenotype in addition to their ability to suppress the *tso2-1* phenotype. In the progeny of the self-pollinated DS101 line (which we named

sua1-1), one quarter of the plants showed a *tso2-1* phenotype. One quarter of the progeny (presumably *sua1/sua1 tso2-1/tso2-1*) were dwarf and completely sterile with compact inflorescences (Fig. 3-3C). The rosette leaves of this dwarf plant displayed chlorotic lesions, a characteristic of cell death (Fig. 3-3D). The other half of the progeny (*sua1/+ tso2-1/tso2-1*) showed a compact inflorescence and the flowers were smaller, with downward pointing siliques, only one-third the length of the wild type siliques (Fig. 3-3B, E, F). The segregation pattern indicated that DS101 is a dominant mutant. Another suppressor, DS102, behaves similarly to DS101 (data not shown) and is likely allelic to DS101. Complementation tests between them are in progress. In the F1 progeny of a cross between *sua1-1/+ tso2-1* and wild type, none of the plants exhibited this novel phenotype. That is, *sua1-1/+ tso2-1/+* plants are wild type in phenotype, suggesting that *sua1-1* in a wild type background is recessive.

Table 3-3. Potential complementation groups of the 18 suppressors

	Alleles in each group	Recessive or dominant
Group 1	DS8, DS17, DS21	recesive
Group 2[a]	DS2, DS6, DS11	recesive
Group 3[a]	DS12, DS15, DS19	recesive
Group 4	DS41	semi-domiant?
Group 5	DS31, DS38	recesive
Group 6	DS33, DS35, DS42, DS53	recesive
Group 7[a]	DS101, DS102	dominant

[a]: Allelism has not been verified by complementation test among the group members

In summary, 15 recessive, one semi-dominant, and two dominant suppressors of *tso2-1* were isolated from a screen of 4,000 M2 seeds. 18 suppressors fall into 3-10 complementation groups. We preliminarily assigned them into six complementation groups (Table 3-3). Crosses between lines in Group 1, 2, 3 and lines in Group 4, 5, 6 are

in progress. Crosses among the lines within Group 2, Group 3, and Group 7 are also in progress.

As mentioned in Chapter two, *tso2-1* is likely an antimorphic allele. A null allele, *tso2*-5 can suppress the *tso2-1* phenotype as *tso2-1*/*tso2*-5 exhibited a wild type phenotype. This suggests that a cis-mutation, either in the promoter of *tso2-1* that abolishs the expression of *tso2-1,* or in the *tso2-1* ORF that produces a truncated tso2-1 protein, which removes the poisonous effect of *tso2-1,* will suppress *tso2-1*. Obviously, these intragenic suppressors will be dominant. Nevertheless, we sequenced the *tso2-1* ORF in all 15 recessive suppressors and one dominant suppressor, and found no cis-mutations in any of these suppressor lines.

Discussion

At first glance, the number of suppressors is unusually high from the preliminary screen of about 4,000 M2 seeds even after the number of real suppressors is reduced from 41 to 18. Considering that 202 *crt* mutants were isolated from the small genome of yeast, it is likely that the number of *Arabidopsis* genes involved in the regulation of *RNR* is large, suggesting that the regulation of *RNR* in plants is highly complex.

It is surprising that none of the 18 suppressor lines is an intragenic suppressor. Based on the sequencing results, no nonsense mutations were found in the *TSO2* ORF in any of the 18 suppressor lines. There are two possible explanations. First, mutations in many genes in at least eight different pathways can suppress the *tso2-1* phenotype and

nonsense mutations in the *TSO2* gene are not among the most common ways to suppress *tso2-1*. Again, since the intragenic suppressors are expected to be dominant, such dominant suppressors might have been eliminated at the M1 generation because of their wild type phenotype, which could be mistaken for contaminants. A more focused screen in the M1 generation might be necessary to identify such mutants. Alternatively, *tso2-1* might not be an antimorphic allele. This is not supported,however, by the fact that the *tso2-5* null allele and *tso2-5/tso2-1* transheterozygotes are wild type. In addition, even if *tso2-1* were not an antimorphic allele, *tso2-5* like mutations could still suppress the *tso2-1* phenotype and; hence, act as intragenic suppressors.

Although backcrosses between the suppressors and *tso2-1* were not conducted, the complementation tests suggest that 16 of the 18 suppressors are recessive. Since all 16 suppressor lines were grown to the M3 generation and none segregated a *tso2-1* phenotype in the M3, they are all homozygous for the suppressor mutations. If any one of the suppressor lines is dominant, then the F1 progeny of the cross between the dominant homozygous suppressor and a recessive suppressor should all show the suppressed phenotype and no *tso2-1* phenotypes. In contrast, if all the F1 progeny of the cross between two suppressors showed a *tso2-1* phenotype, then both suppressor lines are recessive. A detailed genetic analysis of the complementation test is shown in Fig. 3-4.

The mutations in the suppressor lines could be quickly divided into two classes. One class is a cis-mutation. They are either nonsense mutations in the *TSO2* ORF, producing a truncated protein, or point mutations or deletions in the *TSO2* promoter,

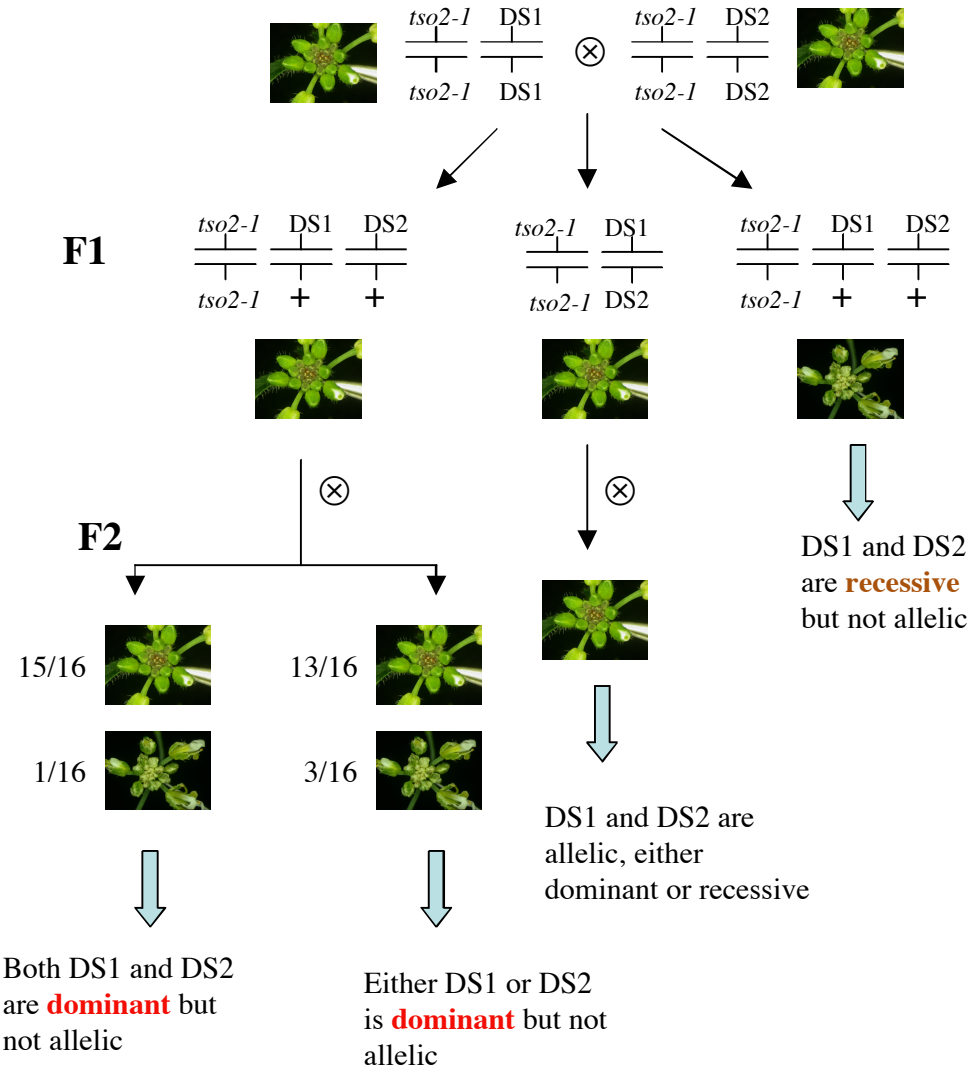


Figure 3-4. Diagram of complementation tesst of suppressor lines. If the two lines can complement each other, the F1 plants will exhibit *tso2-1* phenotype, suggesting the two lines are recessive and not allelic to each other.

reducing or abolishing *TSO2* transcription. In both cases, the poisonous effect of *tso2-1* is removed. As a result, *tso2-1*$^{sup\ (null)}$/*tso2-1* is wild type in phenotype and should be dominant over *tso2-1*. The two dominant suppressor lines (DS101 and DS102) are unlikely cis-mutations as they exhibited novel phenotypes such as downward pointing siliques, compact inflorescences, and dwarfism. It is possible that these dominant suppressors encode transcription factors that control the expression of *TSO2* as well as

other target genes responsible for the novel phenotypes. Alternatively, the dominant suppressors (DS101 and DS102) could be involved in protein degradation pathways; mutations of which may increase the levels of R1, compensating for *tso2-1* poisonous effect. This may also increase the stability of other proteins, leading to novel phenotypes.

Trans-mutation is the second class of suppressors. The majority of this class should be recessive. This type of mutation could define global repressors (like yeast Tup1 and Ssn6), RNR specific repressors (like Crt1 in yeast) or RNR inhibitors (like yeast Sml1). They could also define genes in RNR mRNA stability (like Cid13 in yeast), genes in DNA replication checkpoints, DNA polymerase, or the salvage pathway of dNTP biosynthesis. The first thing we can do before mapping and cloning of these suppressor genes is to determine the mRNA levels of RNR in the suppressors by RT-PCR. It will help us to distinguish among these different pathways in which the suppressors might be involved.

Chapter four

Concluding Remarks

Cell division and development

Although the basic events of the cell cycle have been well studied in yeast and animals, how cell division is regulated in the context of development is not clear. Two opposing views have been proposed to address the function of cell division in plant growth, which are probably also applicable to animals (Kaplan, 1992). The 'organismal theory' regards cells as merely compartments of developmental space that is defined at a higher level by genes specifying the morphology. Therefore, the final body plan could be altered by mechanisms that do not directly affect cell-cycle parameters. This theory also implies that the stereotyped cell division patterns become insignificant. The opposite 'cell theory' considers cells as fundamental building blocks of the organism, and thus, the spatial and temporal control of cell proliferation is essential for the pattern formation.

While supports for each theory are accumulating (see Chapter one), there are increasing observations suggesting that the two theories are not mutually exclusive, and development could be controlled at both levels. For example, constitutive expression of the cell cycle inhibitory protein KRP2 in *Arabidopsis* reduces cell division rates and forms leaves with fewer, but larger cells (De Veylder et al., 2001; Wang et al., 2000), indicating an overall organismal control. However, the leaves of KRP2-overexpressing plants also become narrow and serrated, and grow at much lower rates, indicating a cellular mechanism.

Overexpressing E2Fa induces extra cell divisions, leading to enlarged cotyledons. However, when its dimerization partner DPa is also overproduced, massive over-proliferation occurs in most organs, resulting in distorted growth (De Veylder et al., 2002). These data suggest that cell cycle regulation is not just a simple speeding up or slowing down of cell production, which translates in faster or slower growth. Growth and development is governed by a variety of signaling (including light, temperature, internal positional and developmental cues) and growth substances (such as hormones and nutrients), which can create an interaction between cellular and whole-organ behavior and allow cells to compensate for impaired cell division by increasing cell expansion and vice versa.

Mutants with aberrant (or incomplete) cell division tend to show stronger developmental defects than those with reduced or increased cell division rates. It is probably because these kinds of mutants also disrupt cell-to-cell communication, through which a general machinery can manage cell division locally. As mentioned in Chapter one, *tso1* mutants cause defects in both cell division and cytokinesis. *tso1* exhibits enlarged and fasicated inflorescence meristems and callus-like flowers consisting of misshapen sepals only. Recent discovery of *Drosophila* p120, a *TSO1* homolog, being a member of Myb-containing repressive complex to ensure a silenced transcriptional and/or replicative state (Beall et al., 2002), suggests a similar role of *TSO1* in DNA replication.

The similar fasication phenotype in *tso2* and other mutants such as *fas1*, *bru1* and *mre11*, indicates a link between DNA/chromatin replication or DNA damage checkpoint and epigenetic stability. That is, mis-regulation of the shoot apical meristem regulatory gene *WUS* likely underlies the fasication phenotype shared by these mutants. However, unlike *fas1*, *bru1* and *mre11* mutants, in which morphological defects are largely limited to meristem fasciation, both *tso1* and *tso2* exhibit additional developmental defects that could not be explained by ectopic expression of certain developmental genes. For example, *tso1-1* flowers could not develop any petals, stamens and carples. The sepals are in irregular shape and very often the number of sepals is increased from 4 to 6 (Liu et al., 1997). In *tso2-1* mutants, sepal epidermal cells are disorganized and often outgrown to form protrusions from the flat surface. One explanation is that the delayed or aberrant cell division could disrupt the local cell-to-cell communication and hence, the positional information is changed. An upstream machinery that controls cell division and cell growth in plant development by assessing planes and rates of cell division and directing the division of neighboring cells accordingly, may fail to adjust to this change, leading to improper development.

Compensatory proliferation

The outgrowth of cells in *tso2-1* sepal epidermis and the uneven thickness of rosette leaves could also be due to over-proliferation in certain regions. This over-proliferation is more obvious in the leaves of *tso2 r2a* double mutants. Then, how to explain that, a lower dNTP pool caused by impaired RNR activity in *tso2-1* could lead to over proliferation? The striking similarity of *tso2 r2a* double mutants and *Drosophila*

hippo mutants suggests that there might also be a compensatory proliferation induced by certain mechanisms in plants (Wu et al., 2003).

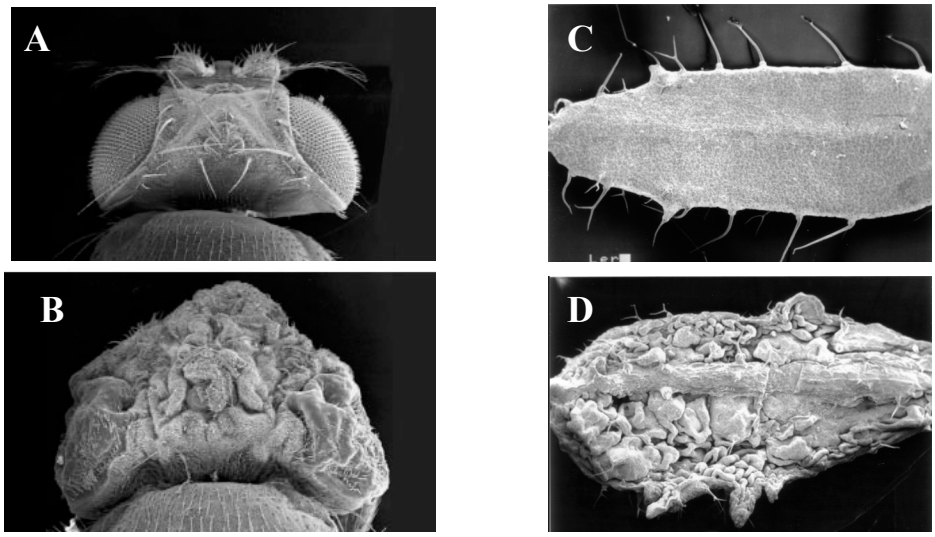


Figure 4-1. Eyes of *Drosophila* (left panel) and leaves of *Arabidopsis* (right panel). (A)The head of a normal fruitfly, showing its perfectly organized eyes; (B) The head of *hippo* mutant, showing vastly overgrown tissues resulted from increased cell proliferation and decreased cell death; (C) A leaf of wild type, showing flat smooth surface; (D) A leaf of *tso2 r2a* double mutants, showing disorganized and overgrown cells. (A-B) Pictures are reproduced from Wu et al., 2003.

Genetic and molecular analyses showed that *Hippo* is important for transcriptional repression of cyclin E and DIAP1, an inhibitor of apoptosis. Therefore, in *hippo* mutants, cell division is promoted whereas cell death is inhibited, leading to over proliferation in eyes (Wu et al., 2003). The phenotype is in contrast to overexpression of growth stimulating genes Myc or E2F in *Drosophila*, which results in extra cell growth or division, but has little or no effect on body size (Neufeld et al., 1998). Normally, cell division and cell death are coupled so that excessive cell growth and proliferation could

be compensated for by extra apoptosis. Conversely, cell death could also lead to compensatory proliferation (Milan et al., 1997).

Recently, Huh et al (2004) reported that stimulation of cell death signaling in the wing disc, even in the absence of cell death itself, resulted in increased proliferation. They proposed that the *Dronc* gene, as an apoptosis inducer, plays two distinct roles. In its primary role, *Dronc* transduces death signals in the cells in which it is activated. Meanwhile, it promotes these cells to produce a signal that drives the proliferation of nearby cells. Similarly in *tso2 r2a* double mutants, a signal might be produced in those cells defective in DNA replication or delayed in cell division, to inform surrounding cells that a particular developmental event has not been completed and/or provides instructions on what to do next. It is also possible that differentiation of surrounding cells is delayed and hence, their cell fates might be changed due to altered positional information.

The shoot meristem of *tso2 r2a* double mutants often enlarged and developed a tumor-like structure. Surprisingly, our preliminary *in situ* data showed that expression of both *STM* and *ANT* genes are largely reduced in the shoot meristem of *tso2 r2a* double mutants (data not shown). *STM* is a shoot meristem regulator gene that is specifically expressed in the shoot meristem whereas *ANT* is an organogenesis marker that is only expressed in developing organ primordia. This result may suggest that cells in the enlarged and disorganized shoot meristem of *tso2 r2a* double mutants fail to gain their differentiation status or their cell fates are unspecified. It is also possible that these cells

are dying, and hence the transcription machinery is shut down so that neither *STM* nor *ANT* can be expressed.

In *hippo* mutants, it was suggested that the mutant cells still retain the ability to differentiate, but become incompetent at recognizing or responding to signals that would normally promote differentiation. The resembling phenotype shown in *tso2* suggests the existence of a similar mechanism in plants. Further studies of *tso2* and *tso1*, and the isolation of both *tso2* and *tso1* suppressors will provide important insights into the question of how cells acquire, integrate, and utilize positional information on the status of their environment to control growth and differentiation.

Chapter five

Materials and Methods

Plant growth, mutant strains, and materials

Plants were grown under 16 hour light/8 hour dark cycle at 20^{o}C. The *tso2-1, -2, -3,* and *-4* alleles were isolated in two separate EMS mutagenesis screens (Liu and Meyerowitz 1995; Levin, Fletcher et al. 1998), and all four alleles were generated in the L*er* background. . *tso2-5* was a T-DNA insertion line (217A06) in WS background purchased from INRA-Versailles (http://flagdb-genoplante-info.infobiogen.fr/projects/fst/DocsIntro/introCollection.html). *tso2-6* was a T-DNA insertion line (Salk_140203) in Col background obtained from *Arabidopsis* Biological Resource Center (ABRC). The method of TILLING (McCallum, Comai et al. 2000) was used to isolate *r2a-1*. Specifically, a primer pair 5' TTTGCTGTGAGGCTGGTCGCTT TT 3' and 5' CTTCCAGATTCGATGGCGGATTCA3' was used to amplify ~ 1 kb *R2A* genomic DNA from EMS-mutagenized Col M2 plants. HPLC-based detection of DNA heteroduplex led to the identification of *r2a-1*. A primer pair, 5' CGATTAAATCTTCTT CACCGGA 3' and 5' GGCTCCAATCCTTTTTGGAT 3' was used to PCR-amplify the entire *R2B* genomic DNA from three different *Arabidopsis* accessions (Col, Ler, and WS) and 15 polymorphisms were found between Col and Ler including a 2-bp insertion that gives rises to a functional *R2B* gene in Ler and WS. *pcycB1:GUS* plant seeds were provided by Dr. Peter Doerner (Colon-Carmona et al., 1999). *TSO2* and *R2A* cDNAs, RZL13g10F (AV546418) and SQ022c10F (AV555679), were provided by the Kazusa DNA Research Institute.

Genetic analyses

tso2-1 (L*er*) was crossed with *r2a-1 r2b-1* (Col). The F1 heterozygous progeny for all three mutations are wild type. dCAPS and CAPS markers (see Appendix Table A1) were used to screen *tso2*-like F2 plants at *R2A* and *R2B* loci to identify plants of various genotypes. PCR-based genotyping was performed for individual seeds from *tso2-1/tso2-1; r2b-1/+* parents. *tso2-1 r2b-1* double mutants were only detected among aborted seeds (Table 2-3). The *r2a-1/r2a-1; tso2-1/+* plants, which are phenotypically normal, were identified from the same cross described above by PCR-based genotyping of normal-looking plants in the F2. *tso2-1 r2a-1* double mutants were obtained from the self progeny of *tso2-1/+*; *r2a-1/r2a-1*plants. *tso2-5* was also crossed with *r2a r2b* double mutants. The F1 heterozygous progeny for all three mutations is wild type. *tso2-5 r2b* was identified from the F2 generation. *tso2-5/tso2-5 r2a/+* plants display a *tso2-1*-like phenotype. *tso2-5 r2a* double mutants were identified from the self progeny of *tso2-5/tso2-5 r2a/+* plants.

Microscopic and histological analysis

For scanning electron microscopy (SEM), samples were fixed, coated, dissected and photographed as described previously (Bowman et al, 1989). Images were directly captured with the semicaps software and the AMRAY 1000A scanning electron microscope. Whole mount floral photomicrographs were taken through a Zeiss Stemi SV6 dissecting microscope. Slides containing longitudinal sections of inflorescences from *in situ* hybridization experiments were examined and photographed under a NIKON

ECL1PSE E600W microscope with Nomarski optics equipped with a digital still camera DXM1200.

For thin tissue sections, leaves, inflorescences and siliques were fixed for 3 hr at 4 °C in 4% glutaraldehyde, 50 mM NaPO4, pH 7.0, then rinsed in 50 mM NaPO4, pH 7.0 overnight. Some siliques were cut on both sides of the septum so that individual green or white valves were fixed separately. After through ethanol series, the tissues were embedded in JB-4 according to manufacturer's instruction (Electron Microscopy Sciences, catalog 14270-000). Two μm sections were stained in 0.1% Toluidine Blue in 0.1% sodium borate.

Map-based cloning of *TSO2*

tso2-1 (in L*er* background) was crossed to *sup-2* (in Col background). A total of 358 F_2 *tso2* plants were assayed individually for linkage to various CAPS and dCAPS markers (Appendix Table A1), which mapped *TSO2* to a 20-kb region within the P1 clone, MOJ10. A cosmid library was constructed from MOJ10 using the binary vector pCLD04541. The procedure is essentially the same as in Bent et al. (1994). Two overlapping cosmids, D and G, were transformed into *tso2-1* mutants using the floral dip method. All eight transgenic plants harboring Cosmid D and none of the five transgenic plants harboring cosmid G are wild-type in phenotype, suggesting that cosmid D, not G contains the *TSO2* gene. Cosmid D showed 100% co-segregation with the rescued phenotype in T2 transgenic plants. Sequencing of the four candidate genes *At3g27040, At3g27050, At3g27060* and *At3g27070* in cosmid D only identified a mutation in *At3g27060* in *tso2-1,* suggesting that *At3g27060* is *TSO2*.

Molecular analyses of *TSO2*, *R2A* and *R2B*

p35S::R2B was constructed by PCR-amplifying the ORF using Ler genomic DNA as a template. The PCR fragment was subsequently inserted into the pBI121 vector (Clonetech). The R2B primer pairs are 5' GCTCTAGACGATTAAATCTTCTTCACCG GA 3' and 5' CGGGATCCGGCTCCAATCCTTTTTGGAT 3'. Xba I and BamH I sites were introduced into 5' and 3' primers respectively. For the pTSO2::GUS reporter construct, a 2.1 kb BamH I/EcoR I fragment containing the GUS ORF and a 3' Nos was excised from pBI121 and inserted to the BamH I/EcoR I site of pBIN20 (Hennegan and Danna, 2000) to create the pBIN20GUS vector. PCR-amplification of 1.2 kb promoter sequence of TSO2 was conducted with following forward primer pair: 5' GCTCTAGAA TAAGGCCCTGTTCGTTTCC3' and 5'CGGGATCCGAATCTGTCTG GGGTTGGT G 3'. PCR products were digested with BamH I/Xba I and inserted into BamH I/Xba I-digested pBIN20GUS vector. All PCR was performed with high fidelity DNA Polymerase Pwo or Tgo (Roche Molecular Biochemicals). GUS staining was according to Parcy et al., (1998). GUS stained tissues were embedded in paraplast and sectioned according to Parcy et al.

For semi-quantitative RT-PCR, total RNA was isolated with Tri-Reagent (Sigma) from one-week old roots and seedlings, two-week-old rosette leaves, four-week-old cauline leaves, stems and inflorescences. Two μg of total RNAs were used to synthesize cDNA with oligo (dT) and Superscript II reverse transcriptase (Invitrogen). PCR was conducted with 1 μl of diluted RT reaction at 94°C, 20 sec; 57 °C, 20 sec; 72 °C, 40 sec

for 20-25 cycles. The primers for TSO2, R2A and R2B were listed in the Appendix, Table A2. The PCR products were Southern blotted and hybridized with a R2B specific probe.

To assay the induction of cell death-related markers, DNA damage-inducible genes and transcriptional silence information (TSI), similar RNA isolation, RT-reaction, 20-25 cycles of PCR were used. Primers for each of the genes are listed in the Appendix, Table A2.

In situ hybridization was essentially the same as previously described (Liu et al., 2000) except that the RNA probes were not hydrolyzed. The *TSO2* EST clone (RZL13g10F) was linearized with Hind III and served as a template for the *TSO2* antisense probe using a T7 promoter.

DNA damage induction of RNR expression

Cell line T87 was a gift from Dr. Rebecca Stevens (Mariconti et al., 2002) and was subcultured weekly in B5 Gamborg's medium (Sigma), pH 5.8, supplemented with 3% sucrose and 1 μM NAA at 25°C in darkness. Four-day-old cell suspensions were treated with 20 mM HU, 0.01% MMS and 2 μg/ml 4-NQO, respectively. At different time points, cells were harvested and quickly frozen in liquid nitrogen before isolation of total RNA. Total RNA was isolated with Tri-Reagent (Sigma) and 2 μg total RNA was used for cDNA synthesis primed by oligo $(dT)_{18}$. RT-PCR was performed with 11 cycles for *TSO2*, 13 cycles for *R2A*, *R1* and *Actin2*, 17 cycles for *R2B*. PCR products were

Southern blotted and hybridized with gene-specific probes (developed from 3'UTR region).

Measurement of dNTP pool levels

dNTPs pool was measured by a polymerase-based assay (Roy, Beuneu et al. 1999). Inflorescences with unopened flower buds were harvested in liquid nitrogen, ground to fine powder, weighed, and extracted with 60% cold methanol by vigorous vortex. The extracts were heated at 95^{o}C for 5 minutes and centrifuged at 17,000g for 15 minutes. The supernatants were dried in a Speedvac, resuspended in sterile distilled water, and stored at –20^{o}C. 1 μl of each sample was used for the polymerase assay. Commercial dNTPs were tested in parallel to establish a linear calibration curve. As the internal control, a portion of the ground tissues was assayed for alcohol dehydrogenase (ADH) activity (Russell, Wong et al. 1990). The ADH activity per gram sample was then used to normalize the dNTP levels for each gram of ground tissues.

Comet assay

A cometAssay kit from Trevigen (Gaithersburg, MD) was used with minor modifications. One to two week-old seedlings were chopped with a razor in a Petri dish that was kept on ice and contained 500 μl 1 × PBS plus 20 mM EDTA. The resulting mixture was filtered through a 60 μm nylon mesh. 40 μl nuclei were mixed with 400 μl 1% low-melting agarose (pre-warmed at 37°C) and placed onto Trevigen pre-coated slides. After incubating in Lysis Solution (2.5 M NaCl, 100 mM EDTA pH 10, 10 mM Tris, 1% Sodium Lauryl Sarcosinate and 1% Triton X-100) for 1 hour at 4°C, the nuclei

on slides were unwound in Alkaline Solution (0.3 N NaOH, 5 mM EDTA) for 40 minutes and neutralized 2-3 times in 1 × TBE for 5 minutes. The slides were run at 1 V/cM for 10 minutes in 1 × TBE and then dipped in 70% ethanol for 5 minutes. After air dry, the slides were stained with a 1:10,000 dilution of SYBR Green. Anti-fade solution (1% p-Phenylenediamine dihydrochloride in 0.1 × PBS and 90% glycerol) was added to slides that were examined by epifluorescence microscopy. The percentage of DNA in each comet tail (T DNA%) was evaluated by the Comet Score software (http://www.autocomet.com) and assigned with a number (0, 1, 2, 3, or 4) with a higher number corresponding to a higher T DNA% (Collins, Dusinska et al. 1997). DNA damage units for each genotype were derived by averaging the data from four slides. Onr hundred comets were scored per slide.

UV-C sensitivity assay

Ten-day-old seedlings were irradiated by UV-C at a distance of 5 centimeter with different dosages using a Stratalinker 1800 (Stratagene). Seedlings were then grown under F40GO gold fluorescent lights for 5 days to avoid photoreactivation. Plants were returned to normal lighting for 1-2 weeks before scoring survival rate and photography.

DNA damage sensitivity assay

Assays of hydroxyurea (HU), mitomycin C (MMC), and methyl methanesulfonate (MMS) sensitivity are according to Masson et al (1997). Briefly, 5-day-old seedlings were transferred into 24-well plates, each well containing 1 ml of 1 × MS medium (Sigma) with various concentrations of HU, MMC or MMS. Plates were placed under normal growth conditions for one to two-weeks before photographs were taken.

Cell death assays

For trypan blue assay (Rate, Cuenca et al. 1999), 10-day old rosette leaves were boiled in lactophenol containing 10 mg of trypan blue for 1 minute, cleared in alcoholic lactophneol (95% ethanol:lactophenol at 2:1) for 2 minutes, washed in 50% ethanol and stored in water. For aniline blue staining of callose (Rate, Cuenca et al. 1999), 10-day old rosette leaves were boiled for 2 minutes in alcoholic lactophenol, rinsed in 50% ethanol and then in water. Cleared and rinsed leaves were stained for 1 hour at room teperature in a solution of 0.05% aniline blue in 0.15 M K_2HPO4. Stained leaves were examined under ultraviolet epifluorescence. DAB-uptake method (Thordal-Christensen, Zhang et al. 1997) was used to detect H_2O_2. Seedlings were placed in 1 mg/ml 3,3'-diaminobenzidine (DAB) in 10 mM ascorbic acid for 2 hours. The seedlings were then boiled in 96% ethanol for 10 minutes and stored in 96% ethanol. H_2O_2 production is visualized as a reddish-brown coloration.

Mutagenesis of *tso2-1* seeds

About 5,000 tso2-1 seeds were treated with 0.1% Tween-20 for 15 min and then with 0.3% EMS over night. After the EMS was removed and seeds were rinsed with water for 4 hrs, the seeds were resuspended in 50 ml 0.1% agar and dispersed into 50 planting pots. They were cold treated at 4°C for 3 days and then placed under 16 hr light at 20°C. Seeds were pooled from M1 plants of each pot so that 50 M1 families were collected. About 80 seeds from each of the M1 families were sown and screened for suppressors of *tso2-1*. Those plants that displayed a wild type, or a very subtle *tso2-1* phenotype, were scored as potential suppressors and seeds were collected from individual lines.

Complementation test

The M3 potential suppressor lines were grown to flowering to confirm that they consistently showed a wild type phenotype. After the phenotype was confirmed, their genotype was verified with the dCAPS marker for *tso2-1*. During the first batch of screening, 9 suppressor lines were isolated and randomly divided into 2 groups. Lines in Group 1 were crossed with lines in Group 2 and each line was also backcrossed with wild type (Ler). During the second round of screening, 9 more suppressor lines were obtained. Two of them are dominant with novel phenotypes. The rest of 7 lines were again randomly divided into two groups and crosses were made between these two groups. The phenotypes of F1 plants from those crosses were recorded. If all the F1 plants displayed a *tso2-1* phenotype, then the two lines used for that cross were scored as " complemented", that is, they belong to different complementation groups. If all the F1 plants exhibited a wild type phenotype, then the two lines used for that cross were scored as "not complemented", that is, they belong to the same complementation group and they are allelic to each other.

Appendix

Table A1. CAPS and dCAPS markers used in this study

Marker name	Restriction enzyme	Restriction fragments for Ler (bp)	Restriction fragments for Col (bp)	Oligonucleotide sequence
MQP15	Hind III	1128	871, 257	Left: ATCTCCAATCTCAAATCTCC
				Right: ATGTAGCACTAAGAAACCAGCC
MXE2	Hinf I	551, 322, 63	614, 322	Left: CGATAGCATAGATGTTTCAAGC
				Righte: AAGGAAATACAACGCTTAGTCG
MMG15	Sac I	705,613,134	839, 613	Left: CGGCAGTGGTGTTGATTTCACG
				Right: TACAACAAAACACAAAGCCC
MOJ10-0K	BstU I	1488	1244, 244	Left: AGTAAGCCAGCGAAAAAGAAGG
				Right: CACGACCGTTGTTCTTGAATGG
MOJ10-31K		99	113	Left: GGTTTAGCTTTAGACTCGGTTT
				Right: AGGTCTAAGTTTTGAAATTTTATCA
MOJ10-45K	EcoR I	611, 565, 111	676, 611	Left: CTGTGAAATCAGCCCCTCAT
				Right: CGTTCGATTGGATGGAACTT
MOJ10-53K	Taq I	774, 473, 51	774, 524	Left: TCAGACCTCGGCACAGATAA
				Right: CTGGTCTTGGTTCCATTCTAAA
MOJ10-73K	Dde I	652[a], 567, 151	612, 567, 151	Left: GAGGCAACTTCACGACGAATGG
				Right: TGGTGAGTCCATAACACAATCC

Marker name	Restriction enzyme	Restriciton fragments for Ler (bp)	Restriciton fragments for mutant (bp)	Oligonucleotide sequence
tso2-1	Mbo II	62	90	Left: AAGAAGTAGATCTATCAGAA
				Right: AGAAAGCGAGGACGTGTTTG
tso2-2	Kpn I	599, 521	1120	Left: AGCATCAAAACAAACGCTCT
				Right: AATCCCTTCAAAGATGCCTTC
tso2-3	Hha I[b]	532, 300, 145	852, 145	Left: same as above
				Right: AACCTTAGAGAAGCATC
r2a-1	PflM I	785, 154	939	Left: TTTGCTGTGAGGCTGGTCGCTTTT
				Right: CTTCCAGATTCGATGGCGGATTCA
r2b-C	Hae III	600, 600	1200	Left: CGATTAAATCTTCTTCACCGGA
				Right: GGCTCCAATCCTTTTTGGAT

[a]There is about 40-bp insertion in Ler sequence.

[b]Hha I produces three more smaller fragments (71,63,10) in both Ler and *tso2-3*.

Table A2. Gene-specific rimers used for RT-PCR

Genes	Forward primer	Reverse primer
TSO2	CATCTCTTTCGTGCCATTGA	TCGCAGACGATTGATTTCAC
R2A	ACTGATGTGCAACAGTGGGA	TCCTTCTCCTTCGAGTCCTTT
R2B	CGTTTGAAAGCGATTGTTTG	TGATCATCATTAAACGCAGC
R1	AGGAACTCCAAGGCCTCAAT	CCCTGTGTTCTTCCTTTCCA
Actin2	GTTGGGATGAACCAGAAGGA	CTTACAATTTCCCGCTCTTC
EDS1	GGAGCAGTCGTAATCTTCGC	CCAAATGTCACACAACGAGG
GST6	TCAAGCTCGTCTTCCCTTGT	GGTTGCCTTGACTTTCTTGC
SAG12	CCAAACTAAAATGTCGCCGT	TTGACTCAGTTGTCAAGCCG
PR-5	TCGTCTTCATCACAAGTGGC	TCGGATCCATGATCCTAAGC
PRXcb	AGTTAAGGTCGGACCCTCGT	CTCTCCTTCCCAAAGGAACC
SAG13	CTCTCGTGACCAACGAGTGA	TCATTTGCTTCTCCAACACG
GST11	GCAGGAATCAAAGTTTTCGG	GGAGCCAAGGGAGACAAGTT
PR-1	GTGCTCTTGTTCTTCCCTCG	ACTTTGGCACATCCGAGTCT
TSI	GAACTCATGGATACCCTAAAATAC	CTCTACCCTTTGCATTCATGAATC
Rad51	GGCCATGTACATTGATGCTG	CCAGCAAAAAGAGCTGAACC
PARP-1	CAAATTCAAGAGCAGGCACA	CATGGCATCGGTAAAGTCCT
PARP-2	AACGAGGCTTGAGGAACTGA	CGTATATCCCGAATGCGTCT

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