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Replication Protein A (RPA70C) Negatively Regulates Ribonucleotide Reductase (RNR) in the model plant *Arabidopsis thaliana*

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Abstract

Replication protein A (RPA) is a heterotrimeric single-stranded DNA-binding protein that plays an important role in cellular responses to DNA damage. For example, RPA can activate the cell-cycle checkpoint protein ATR in the presence of persisting DNA damage. The model plant *Arabidopsis thaliana* has 5 functional homologous *RPA70* genes which may play specific roles in response to DNA damaging agents. One chemical that causes DNA damage in *Arabidopsis* is Hydroxyurea (HU), which blocks DNA replication by inhibiting activity in Ribonucleotide Reductase (RNR), an enzyme responsible for the production of free deoxyribonucleotides (dNTPs). In studies of *Arabidopsis* mutants, *atr* plants, but not *rpa70c,* were found to be hypersensitive to HU and had inhibited root and shoot growth and increased root hair formation. However, the double mutant *rpa70c.atr* was found to be less sensitive to HU and had an enhanced expression of RNR compared to *atr* single mutants. The double mutant's curious phenotype indicates that the double mutant suppresses the phenotype of the *atr* mutation and that the absence of the *RPA70C* gene partially reverses the effect of HU on *atr* mutant plants, probably due to the enhanced expression of RNR. These results indicate that *RPA70C* in conjunction with *ATR* plays a role in the regulation of DNA replication.

Introduction

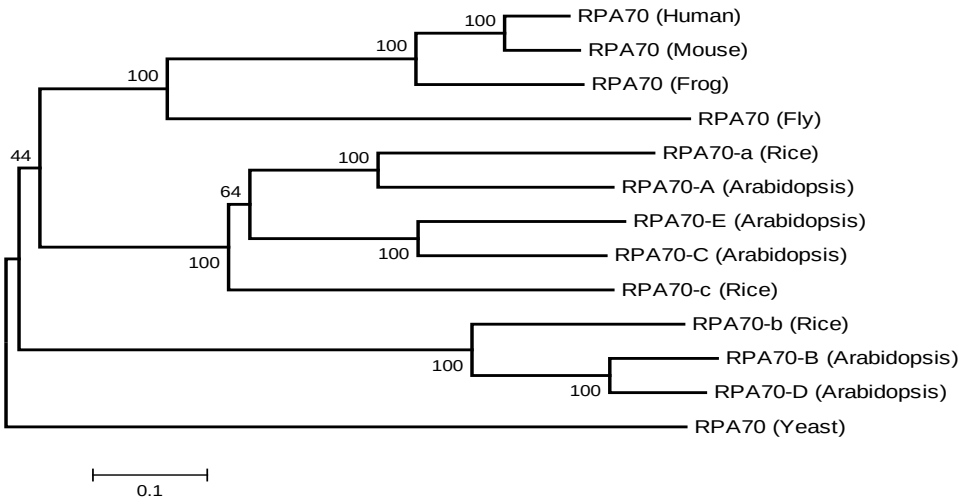
All living organisms are constantly exposed to DNA damage by means of a chemical or physical source. When damage occurs, eukaryotic organisms have a number of conserved mechanisms that can be used to recognize and respond to the damage. One protein that plays an important role in DNA damage response mechanisms is Replication Protein A (RPA), a heterotrimeric single-stranded DNA-binding protein that functions in DNA damage recognition, repair, and replication. In its normal function, RPA works in concert with other proteins such as Ataxia telangiectasia and Rad3 related protein (ATR), a universally conserved protein kinase, to sense persisting single-stranded DNA and to activate a cell cycle checkpoint (Culligan et al., 2004).

One of the many model organisms used to study RPA is the plant *Arabidopsis thaliana.* Most animals such as *Homo sapiens* have one gene for the Replication Protein A 70 subunit, RPA's largest subunit, and a mutation in the gene is fatal to the organism. However, *Arabidopsis* has five homologs of RPA70 (*RPA70A*-*RPA70E*). A mutation in any one of these genes is not lethal to *Arabidopsis,* therefore *Arabidopsis* was chosen as a model organism to genetically study its mutants in this study. *Arabidopsis* also has a short life cycle, produces many seeds, has a small size and genome and is applicable to vegetables which made it a favorable organism.

The phylogenetic tree in Figure A shows the evolutionary relationship of RPA70 across species. *RPA70 A, C* and *E* are closely related in one group and *RPA70 B* and *D* are closely related in another for *Arabidopsis*. Interestingly, in projects performed by other researchers, *RPA A, C* and *E* were found to be transcriptionally up-regulated in response to DNA double-

stranded breaks caused by ionizing radiation whereas *RPA70B* and *RPA70D* were not. These relationships indicate that each *Arabidopsis* homolog may have a distinct role in DNA damage recognition and repair and yet there may also be some redundancies in their functions. For this research, the *RPA70C* gene in particular was chosen to be the initial focus of the project in conjunction with *ATR*.

Figure A: **Phylogenetic Tree of RPA70 across Multiple Species**



Evolutionary relationship of the RPA70 homologs in *Arabidopsis thaliana.*

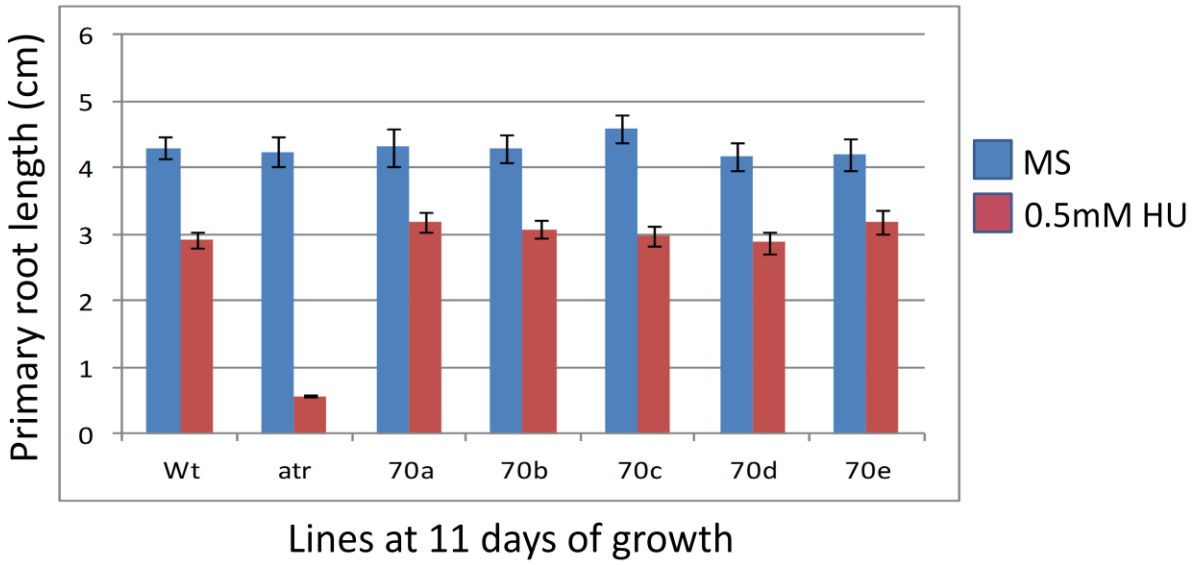
To induce DNA damage in the plants, the chemical hydroxyurea (HU) was used. HU is known to disrupt DNA replication by inhibiting the activity of Ribonucleotide reductase (RNR), a protein that synthesizes free deoxyribonucleotides (dNTPs). RNR has two components: the regulatory subunit RNR1 and the catalytic subunit RNR2 which has three homologs: RNR2A, RNR2B and RNR2C. It has been found that expansion of dNTP pools may help cells survive DNA damage by promoting the repair and/or the bypass of DNA lesions (Poli et al., 2012). Since RNR is the most important gene in the production of dNTPs and dNTP pools are rate limiting for DNA replication, its expression was also studied in relation to *RPA70C.*

Do the *Arabidopsis RPA70* genes have distinct roles in DNA damage response and DNA replication?

The *Arabidopsis thaliana RPA70* gene family has a diverse phylogenetic history, suggesting that individual RPA70 subunits may have distinct roles in DNA damage response pathways.

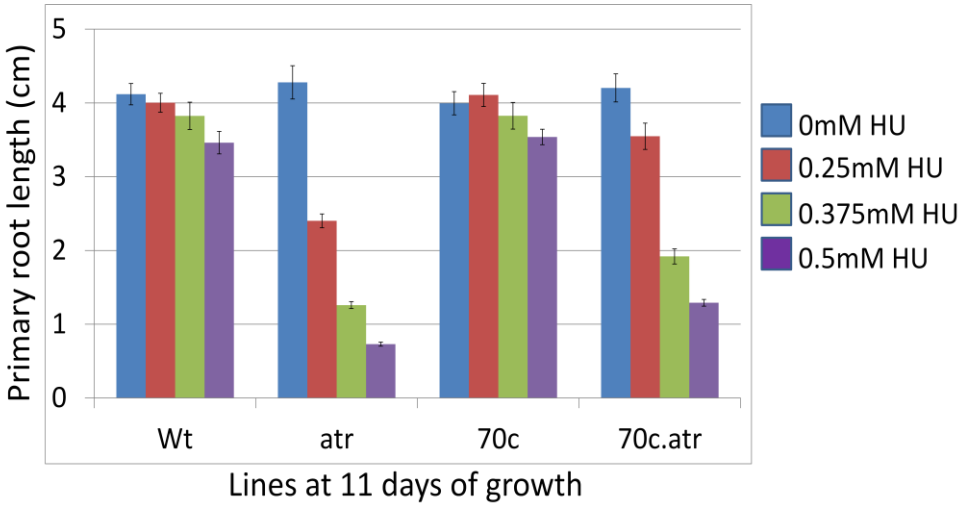
Results

Figure 1: The *rpa70* Single Mutants are Not Hypersensitive to HU



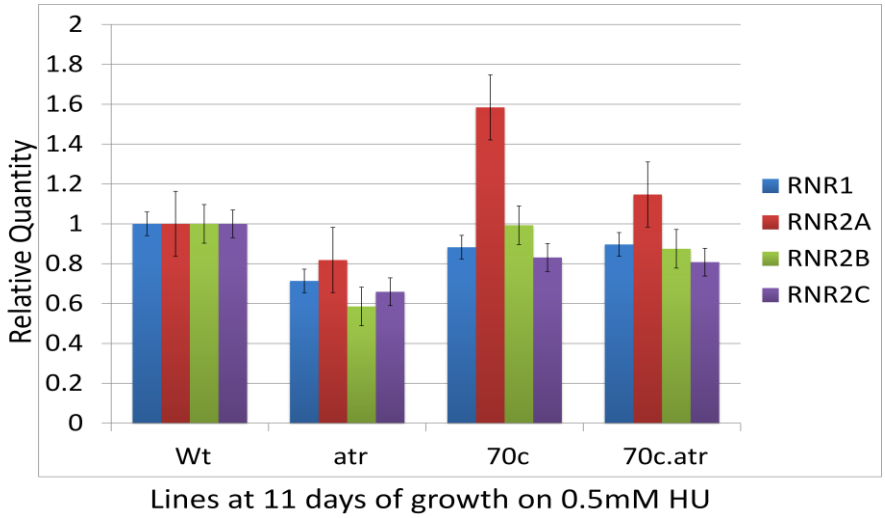
Single mutants of *rpa70* were grown on 1x MS with and without Hydroxyurea (0.5mM) treatment to see if any single mutant had sensitivity to HU. By measurement of root length as shown in Figure 1, all of the single mutants responded the same way to HU as the wildtype (Wt).

Figure 2: *rpa70c.atr* plants are Less Sensitive to HU than *atr* Plants



Double mutants of each *RPA70* gene were created with *atr* by crossing the single mutants with *atr* plants, growing up the heterozygotes, and then genotyping for double mutants in the following generation. Of the five combinations created, all were equally hypersensitive to HU like the *atr* single mutant except for *rpa70c.atr* which showed less sensitivity than the other mutants in all of the HU treatments (only data for *rpa70c.atr* is shown in Figure 2).

Figure 3: *rpa70c.atr* Displayed Enhanced Expression of RNR Compared to *atr*



For RNR study, two sets of *Wt, atr, rpa70c,* and *rpa70c.atr* lines were grown up concurrently for 11 days with one set on 1x MS and the other on 0.5mM HU. Tissue samples were then collected and crushed in liquid nitrogen and extracted for RNA. The RNA were then incubated with reverse transcriptase to make cDNA which were then used in quantitative real-time polymerase chain reaction (PCR) with four sets of primers to amplify the genes corresponding to the four components of RNR: RNR1, RNR2A, RNR2B, and RNR2C. Figure 3 shows the results of the quantitative real-time PCR showing the relative expression of each component of RNR.

Discussion

Part I: The *rpa70* Single Mutants are not Hypersensitive to HU

In Figure 1, of the single *rpa* mutants tested for sensitivity to HU, none were found to be hypersensitive to HU. The *atr* single mutant had already been established to be hypersensitive to HU so it was used as a positive control for that experiment (Culligan et al., 2004). When all of the single mutants were grown on HU, they had the same root length phenotype as the wild type and not *atr*, indicating that the five *rpa70* single mutants were not hypersensitive.

Part II: The *rpa70c* Mutation Suppresses the *atr* Phenotype in Response to HU

Double mutants of each of the *rpa70* subunits with *atr* were created to check for hypersensitivity to HU. Plants were grown on varying concentrations of HU in MS, and across all the treatments the result was that all the double mutants were positive for hypersensitivity except for *rpa70c.atr* which was not as sensitive. All of the mutants showed the same phenotype as *atr* on HU except for *rpa70c.atr* whose inhibited root growth was not as severe,

as indicated in Figure 2. This suggested that the *atr* phenotype was somehow being suppressed in the double mutant which allowed for the partial recovery from the effects of the HU. This may be due to the absence of *RPA70C* in the double mutant, but further studies in RNA expression needed to be done to determine why that is.

Part III: *RPA70C* Negatively Regulates RNR in Response to HU

Quantitative measurements of RNR gene expression using qRT-PCR as summarized in Figure 3 showed that the RNR regulatory subunit was down-regulated in *atr* mutants and expression of the RNR catalytic subunits was up-regulated in the absence of *RPA70C* and down-regulated in the absence of *ATR.* This was notable in that fact that RNR expression was lowest overall in *atr,* then increased in *rpa70c.atr,* and then increased further in *rpa70c.* Expression of the RNR2A regulatory subunit was notably increased in the absence of *RPA70C* and down-regulated in its presence, particularly in the *atr* single mutant. *RPA70C* thus has been found to negatively regulate RNR in response to HU and therefore has a distinct role in DNA damage response.

Since RNR is known to function in the replenishment of dNTPs and dNTP pools have been shown to push DNA repair and replication, the increased RNR expression in the absence of *RPA70C* in *atr* plants may be a contributing factor to the *rpa70c.atr* suppressor phenotype. The increased RNR expression may increase RNR activity and elongation to counter the effects of HU. However, this might not be the only contributing factor to *rpa70c.atr*'s milder sensitivity to HU compared to *atr*.

Part IV: *RPA70C* and RNR are not the only Contributing Factors to the *rpa70c.atr* Phenotype

HU indirectly stops DNA replication by means of inhibiting RNR, but *RPA70C* might also directly affect DNA replication to result in the *rpa70c.atr* phenotype. Also, the suppressor phenotype may be due to cell death in the roots or to permanent cell cycle arrest. To determine if there is death in the roots, Wt, *rpa70c*, *atr*, and *rpa70c.atr* lines are being tested for cell death in response to HU treatment using propidium iodide cell staining technique to study additional causes of the primary root growth inhibition. The accumulation of mutations in the four lines will also be observed to see if forced DNA damage repair by the increase in dNTP pools results in incorrect sequences and to what extent.

References

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Poli J, Tsaponina O, Crabbe L, Keszthelyi A, Pantesco V, Chabes A, Lengronne A, Pasero P. (2012). dNTP pools determine fork progression and origin usage under replication stress. EMBO J., 31(4), 883-94.