Project Title:	*Influence of Translation Initiation on Organellar Protein Targeting in Arabidopsis*
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A primary focus of the Mackenzie laboratory is the elucidation of processes and machinery for mitochondrial genome maintenance and transmission in higher plants. Leading to the previous grant was the discovery that numerous organellar DNA maintenance components in plants appeared to be dual targeted to mitochondria and plastids (Elo et al. 2003; Mackenzie 2005). Of particular interest was the observation that some twin (tandemly arrayed) dual targeting presequences appeared to utilize non-AUG alternative translation initiation, allowing for multiple translation starts at a single gene (Christensen et al. 2005). Two aspects of this phenomenon were of particular interest: (1) Alternative translation initiation might provide a mechanism to regulate protein targeting temporally and spatially, a possibility that had not been demonstrated previously, and (2) alternative translation initiation might occur in genes involved in nuclear-controlled mitochondrial genome recombination, thought to be exclusively mitochondrial in their function. We pursued three aims, with an emphasis on two specific genes of interest: *POLγ2*, an organellar DNA polymerase, and *MSH1*, a *MutS* homolog thought to participate in mitochondrial, but not plastid, genome recombination surveillance. Our aims were to:

- *Aim 1*. Identify additional genes within Arabidopsis and other genomes that employ non-AUG alternative translation initiation.
- *Aim 2*. Locate sequences upstream to the annotated AUG that confer alternative non-AUG translation initiation activity.
- *Aim 3*. Identify *cis* and *trans* factors that influence start site selection in genes with non-AUG starts.

Our studies, described in Wamboldt et al. (2009), identified over 200 Arabidopsis genes thought to employ alternative translation initiation at a CTG initiator, with five confirmed in our study, and over 1000 genes with potential for other initiator non-AUG codons. Included within this list was the flowering time locus *FCA*, where protein data derived independently by another group are consistent with an alternative CTG upstream initiator (Macknight et al. 2002). Features for alternative translation initiation in the *Polγ2* gene were conserved across five different plant species.

Experiments utilizing an *in vitro* transcription/translation system showed that alternative translation initiation activity occurs via leaky ribosome scanning mechanisms consistent with Kozak models (Kozak, 2002). In the case of *POLγ2*, an ATG contained in weak Kozak context resides 7 codons downstream to a CTG in strong Kozak context (purines at -1, -2, -3 and +4 positions). Initiation at the ATG encodes a plastid targeting protein, initiation at CTG a mitochondrial. Within root tissues, mitochondrial targeting is observed, while in leaf tissues, dual targeting is evident. An initiation factor, eIF4a1, was shown to bind preferentially to the alternative CTG site.

Investigation of the organellar polymerase *POLγ2* provided compelling evidence for alternative translation initiation in the regulation of protein targeting. However, studies of *MSH1* were inconclusive. Transgenic analysis of *MSH1* protein targeting with presequence:GFP fusions produced evidence of dual targeting in experiments involving transient expression (protoplast

transfection, leaf infiltrations, particle bombardment), but consistently produced evidence of mitochondrial targeting in stable transformants. Mitochondrial targeting was also observed when gene presequences were tested from *MSH1* homologs in other plant species (Abdelnoor et al. 2006). We were interested to learn whether MSH1 might be dual targeted under particular cellular conditions, and strictly mitochondrially targeted in others. Alternatively, our results might represent experimental artifact of transgenic protein over-expression in the cell.

Because our laboratory is keenly interested to understand the function of *MSH1*, its role in maintaining mitochondrial genome stability, and its influence on plant development, we directed our experimental approach to these questions. Recently we have shown that disruption of *MSH1* in Arabidopsis results in the simultaneous activation of recombination at over 33 (now shown to be 36) repeat pairs in the mitochondrial genome, producing massive rearrangement of the genome (Arrieta-Montiel et al. 2009). Previous studies of the Arabidopsis mitochondrial genome by others have suggested considerable variation in structure and sequence content between ecotypes Col-0, L*er* and C24 (Forner et al. 2005; 2008). However, disruption of *MSH1* in the three ecotypes reveals that each contains identical mitochondrial sequence information in identical context; the variation observed is apparently the consequence of differing patterns of recombination within each genome (Arrieta-Montiel et al. 2009).

Mitochondrial recombination is similarly influenced in five other plant species (tomato, soybean, tobacco, millet, sorghum) with RNAi-mediated suppression of their *MSH1* homologs (Sandhu et al. 2007; Sandhu and Mackenzie, unpublished). In all cases of *MSH1* disruption, mitochondrial genome rearrangements were accompanied by dramatic changes in plant growth and development. Among these are the induction of cytoplasmic male sterility, a GA-responsive reduced growth rate, non-flowering phenotype, and leaf variegation (Figure 1). The variegation phenotype, involving altered plastid development, might be interpreted as an effect of *MSH1* directly on the plastid, if the protein were dual targeted.

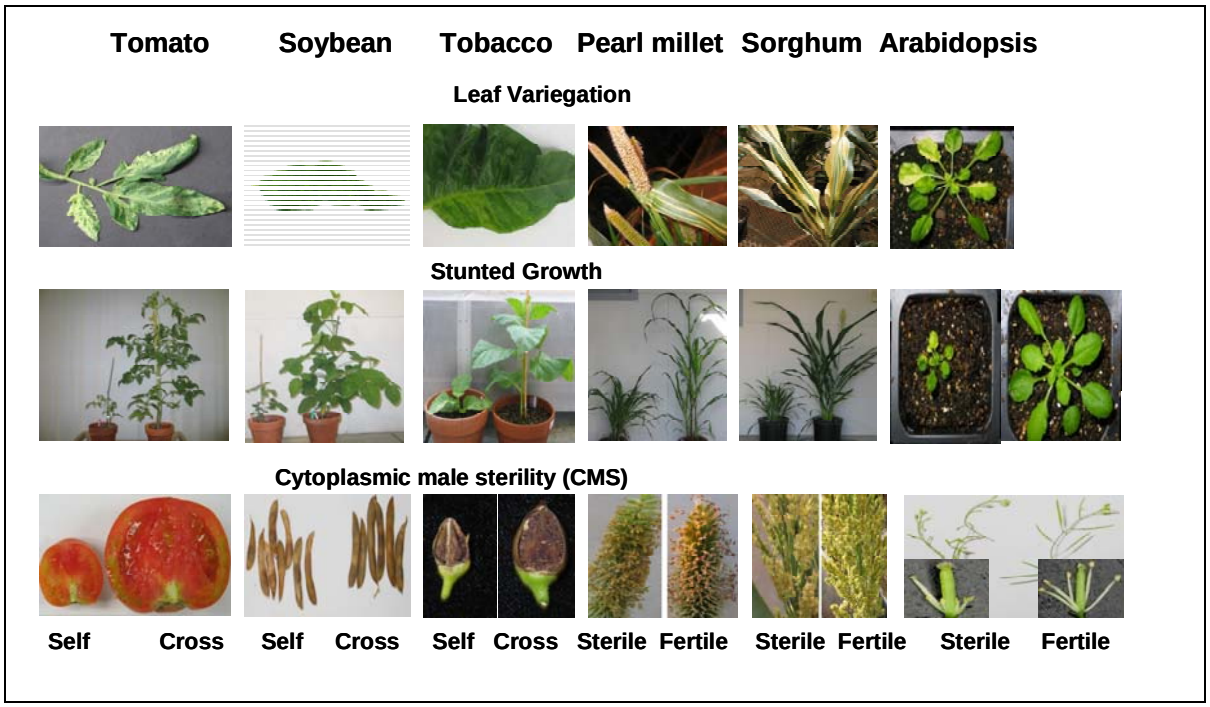


Figure 1. *MSH1* suppression influences distinct developmental pathways in six different species. Three common phenotypes are shown for RNAi suppression lines and a T-DNA insertion mutant (Arabidopsis).

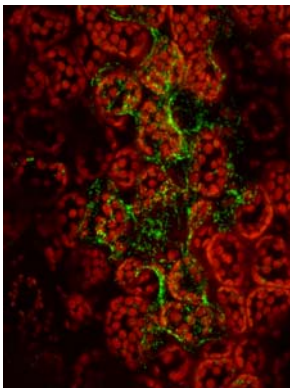
The observations made from these cross-species comparisons, together with transcript profiling results, imply that changes within the mitochondrial genotype influence plant developmental pathways reproducibly. ROS is a potent signaling molecule that can be produced by mitochondrial perturbation, but the elicitation of distinct developmental phenotypes in *msh1* lines implies reprogramming more complex than ROS modulation alone. More extensive rearrangement of the plant mitochondrial genome can be induced in an *msh1 recA3* double mutant, involving disruption of two distinct nuclear genes that influence mitochondrial recombination properties (Shedge et al. 2007). The *msh1 recA3* double mutant is slow growing, delayed in flowering, and strikingly enhanced in stress response, evidenced by transcript profiling and heat stress response experiments (Shedge et al. 2010). Following heat treatment at 37^{o}C for 24 hours, the double mutant recovers to set seed, while 100% of wildtype and single mutants die.

Disruption of *msh1* also influences plastid behavior. Whether this influence is direct, by dual targeting of *MSH1* protein to mitochondria and plastids, or indirect, via retrograde regulation from mitochondrion to nucleus to influence plastid development, is under investigation. It appears that MSH1 may be dual targeted, functioning distinctly within the plastid. However, several pieces of evidence appear to contradict this assumption: (1) complete sequencing of the plastid genome in the *msh1* mutant by our group has revealed no evidence of recombination or sequence variation (data not shown); (2) transcript profiling of the *msh1* mutant shows an altered pattern of expression of nuclear genes controlling assembly of the photosynthetic apparatus (Table 1), evidence of altered mitochondrial gene expression (Shedge et al. 2010), but no clear evidence of altered plastid gene expression; and (3) the *MSH1* presequence targets GFP reporter protein to mitochondria in Arabidopsis stable transformants (Figure 2). Nevertheless, we have been unable to exclude the possibility that plastid targeting might also occur. To address this question, gene constructions were made that combine the *MSH1* native promoter (750 nt) to the full length *MSH1* gene sequence with and without a COOH-fused GFP reporter. These constructions have been used to test the feasibility of functional complementation of *MSH1* by its introduction to Arabidopsis *MSH1/msh1* heterozygous plants and demonstration of mitochondrial genome stability in the resulting *msh1/msh1* transgenic segregants. This *MSH1* gene construction has also been modified to substitute a mitochondrial (*RecA3*) or plastid (*POL*γ*2*) targeting sequence for the *MSH1* presequence in order to test whether a strictly mitochondrial- or plastid-targeted form of *MSH1* is sufficient to fully complement. These experiments are ongoing and will be completed well before the end of the present grant period (8/2010).

Table 1. Programmed Nuclear Gene Response: Altered expression of nuclear genes encoding photosynthesis components

AGI No.	Gene	*msh1*	*var2*	*im*	Soybean
AT5G66570	PSBO-1	-1.3	NS	-1.4	-1.4
AT3G50820	PSBO-2	-1.4	NS	-1.2	-1.2
AT1G61520	LHCA3	-1.2	-1.5	-1.5	-1.3
AT3G27690	LHCB2:4	-1.7	-5.7	-3.1	NS
AT3G08940	LHCB4.2	-1.2	-2.2	-2.3	-1.2
AT3G54890	LHCA1	-1.3	-1.4	-1.7	NS
AT1G15820	LHCB6	-1.3	-1.4	-2.3	-1.6
AT4G02770	PSAD-1	-1.6	-1.6	-1.6	NA
AT2G30790	PSBP-2	-2.0	-2.9	-1.8	NA
AT2G20570	GLK1	-1.9	-5.6	-1.4	-1.6
AT5G44190	GLK2	-1.3	-2.6	-1.5	NA

Figure 2. MSH1 shows evidence of mitochondrial targeting. An Arabidopsis stable transformant with a gene construction fusing the *MSH1* pre-sequence to GFP under the control of CaMV 35S promoter provides evidence of mitochondrial targeting by confocal laser scanning microscopy. The plastids autofluoresce red. 60X



Publications during present funding period (one additional in preparation):

Arrieta-Montiel MP, Shedge V, Davila J, Christensen AC, Mackenzie SA. 2009. Diversity of the Arabidopsis mitochondrial genome occurs via nuclear-controlled recombination activity. Genetics. Oct 12. Epub ahead of print. (while this publication was funded predominantly from an NSF grant to S.M., information from the study directly relates to the current proposal).

Shedge V, Davila J, Arrieta-Montiel MP, Mohammed S, Mackenzie SA. 2010. Extensive rearrangement of the Arabidopsis mitochondrial genome elicits cellular conditions for thermotolerance. Manuscript submitted.

Wamboldt Y, Mohammed S, Elowsky C, Wittgren C, de Paula WB, Mackenzie SA. 2009. Participation of leaky ribosome scanning in protein dual targeting by alternative translation initiation in higher plants. Plant Cell 21:157-67.

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