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# MITOCHONDRIAL GENOME STRUCTURE AND DOUBLE STRAND BREAK REPAIR IN *ARABIDOPSIS THALIANA*

by

Sterling Ericsson

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MITOCHONDRIAL GENOME STRUCTURE AND DOUBLE STRAND BREAK REPAIR IN *ARABIDOPSIS THALIANA*

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University of Nebraska, 2021

Advisor: Alan C. Christensen

The mitochondrial genomes of plants are known for their variability in size and arrangement, yet low mutation rates in coding sequences. These opposing characteristics have been suggested to be due to the stability of their DNA maintenance processes and the usage of a double strand break repair (DSBR) system. The components of this repair complex remain poorly understood and only a few of the involved enzymes have been identified, making direct analysis difficult. The flowering plant species *Arabidopsis thaliana* has been used as a model organism for genome modification and subsequent phenotypic and genotypic impacts. The aims of this research were to determine the effectiveness of restriction enzymes inserted into a plant cell to cause mitochondrial double strand breaks (DSBs), to see how these breaks impact recombination at repeat sequences undergoing DSBR, and to determine the influence of stress responses on the functionality of the DSBR system. To this end, a wild type Arabidopsis was inoculated with the horizontal gene transfer bacteria *Agrobacterium tumefaciens* containing a designed gene cassette. This cassette included the sequence for the *Pvu*II restriction enzyme to induce DSBs, an ethanol inducible promoter for the *Pvu*II sequence, and a mitochondrial-targeting peptide (from the AOX1A gene) to ensure transport into the

mitochondria. The transgenic *Pvu*II plants were subjected to various stress conditions, including reduced light and cold stress, and the effect of these stresses on recombination rates following ethanol induction of the transgenic restriction enzyme were compared. Recombination was investigated using the L repeat sequence in the Arabidopsis mitochondrial genome and qPCR analysis of the L1 and L2 repeat locations. The experimental groups for reduced light and cold stress, with and without ethanol induction, showed increased recombination after induction of *Pvu*II, and altered recombination accumulation after stress was also applied. This shows that DSBs can be successfully induced in plant mitochondria through inserted restriction enzymes and that this results in higher recombinant sequence accumulation during DSBR, along with stresses changing the functionality of this repair.

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# CHAPTER 1: BACKGROUND

The mitochondrial genomes of the different kingdoms of eukaryotic life can be highly conserved and similar in design, such as in the animal kingdom, and then quite different in structure, organization, and size in other sections of the tree of life. With animals, the size is generally small from 14 to 20 kilobase pairs (kbp) and the genome has a key set of genes organized into a circular structure that contains little non-coding DNA (Altman and Katz 1976; Kolesnikov and Gerasimov 2012). Plant mitochondrial genomes are significantly different in structure and organization, featuring sizes that can be several megabases (mb) long and lacking a circular organizational system and instead employing predominantly linear fragments that overlap and generally produce circular maps and assemblies (Ward et al 1981; Oldenburg and Bendich 1996) A large number of internal repeat sequences in the plant mitochondrial genome, which are variable across species, are involved in genome rearrangement through recombination between the repeats (Unseld et al. 1997). It has been observed that plant mitochondrial protein-coding genes nevertheless have a slow evolutionary rate as compared to animal mitochondria while also showing a relatively high rearrangement rate (Palmer and Herbon 1988). A hypothesis to explain the discrepancy between low mutation rates and high rates of genome expansion in non-coding regions is that double strand break repair (DSBR) pathways, including break-induced replication (BIR) and gene conversion, are responsible (Christensen 2013, 2014, 2018). A greater understanding of the mitochondrial DNA repair system can give insight into plant aging and fertility characteristics, along

with the source of a highly valued agricultural trait called cytoplasmic male sterility (CMS) that would allow for direct control over plant breeding and hybridization (Touzet and Meyer 2014; Kazama 2019).

## 1.1 Plant mitochondrial genome structure

Variation in size, shape, and appearance is the theme when it comes to plants and that is no different for their mitochondrial genome structures. The majority of research and investigation of plant mitochondrial genomes has been done in the angiosperms, the flowering plants, for their usage in agriculture, industrial production, and for ornamental purposes. Even among angiosperms, complex differences in their mitochondrial genome are evident. Their massive nucleotide size difference, ranging from 200 kilobases (kb) to several megabases, makes structural analysis and species comparisons difficult (Palmer and Herbon 1988). Coding sequences in plant mitochondrial genomes have been highly conserved, with the majority of alterations being due to expansive rearrangements and duplications of non-coding regions (Ward et al. 1981; Palmer and Herbon 1988). The mitochondrial genome of *Arabidopsis thaliana* (Figure 1) encodes 32 proteins and approximately 22 components of translation including tRNAs and rRNAs, showing little difference from animal or fungal mitochondria. However, the majority of the non-coding regions are derived from an unknown origin, with only a minimal amount being able to be confirmed as nuclear or viral in origin (Palmer and Herbon 1988; Unseld et al. 1997).

The number of mitochondria within a plant cell often outnumbers the real copy number of the mitochondrial genome though this has only been observed in leaf tissues (Arimura 2018). This results in an individual mitochondrion lacking a full copy of the genome in most circumstances, with many having none of the genome at all. In order to maintain and utilize the genes within the mitochondrial genome for upkeep, the mitochondria go through a process of fusion and fission to utilize needed gene transcription and protein factors. The uneven distribution of mitochondria throughout the plant cell has major ramifications for DNA synthesis and the speed of plant development and regeneration (Arimura 2018). Strangely, thus far, only several fission genes have been identified in the mitochondrial genome, with no genes directly responsible for the fusion process having yet been found when studying the model plant *A. thaliana*, including as potential genes in the nuclear genome targeted to the mitochondria (Arimura et al. 2004). There are, however, prospective regulatory genes involved in the fusion process and the intermitochondrial association and clustering required for fusion to occur, including the nuclear genome originating protein FRIENDLY whose function was identified in 2014 (Zawily et al. 2014).

The mitochondrial genome itself has been observed through physical analyses to be made up of primarily linearly stranded molecules that can be circularly mapped, along with a small number of sub-genomic circular components (Bendich 2007). Fusion of the mitochondria, as previously described, allows for rolling circle replication to be performed and is hypothesized to be initiated from the process of mitochondrial fusion

itself (Shedge et al. 2007). The replication procedure allows for further recombination and rearrangement of the genome, especially at sites containing repeated sequence (Arrieta-Montiel et al. 2009). This suggests that subgenomic sequences originating from recombination of smaller sections of the mitochondrial genome do exist (Arrieta-Montiel et al. 2009 ).

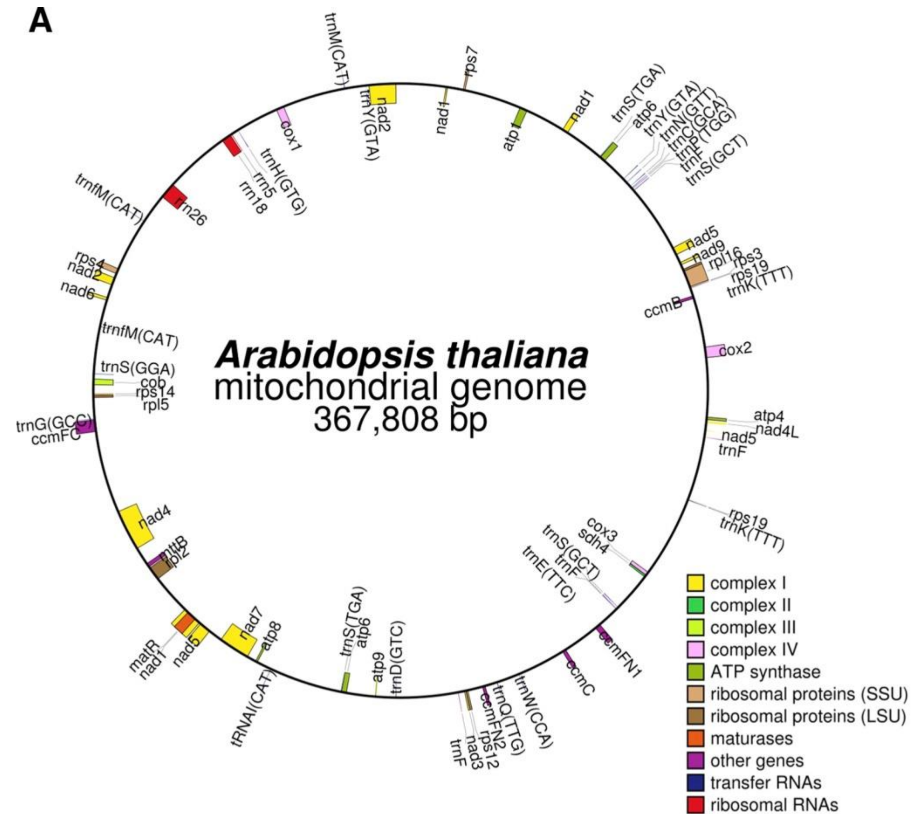


**Figure 1.** Mitochondrial genome of *Arabidopsis thaliana*, as originally published as Figure 1A in Johnston (2019) Circular mapping of mtDNA (NCBI NC_037304), encoding some (not all) subunits of Complexes I–IV, ATP synthase, the mitochondrial ribosome, tRNAs, and rRNAs. The genome is much larger than the familiar 16 kb mammalian mtDNA, with large non-coding regions.

The plant mitochondrial genome includes a large number of non-tandem repeated sequences throughout that are frequently involved in recombination events that alter the overall arrangement of the genome. The large repeat regions in the mitochondrial genomes allow for multiple genomic arrangements to persist, with there being an even higher frequency of repeats at sizes under 1 kb in length. These have been generally grouped into large repeat regions that are several kb, intermediate size repeat regions that are between 50 base pairs (bp) and half a kb, and the common microhomology regions at less than 50 bp (Unseld et al 1977; Arrieta-Montiel et al 2009; Christensen 2018). To distinguish it, the middle of these two classifications is sometimes referred to as repeats of unusual size (ROUS) (Wynn and Christensen 2018). Impairment in genome repair mechanisms have been confirmed to increase the ectopic recombination rate, whereby crossing over occurs between non-tandem repeats, regions of identical sequence located in nonhomologous locations. Thus, being able to properly describe the functions of the repair mechanisms requires an understanding of existing repeat regions generally found across plant mitochondrial genomes (Klein et al. 1994; Wynn and Christensen 2018). While recombination involving the large repeats is frequent, such recombination events are less common for the smaller ROUS and microhomology regions. Furthermore, the rearrangements involving the large repeats can be reversed from subsequent recombination events involving them, but this has not been observed to occur from rearrangements involving smaller repeat regions (Maréchal and Brisson 2010).

This diversity in recombination can also lead to extensive copy number variation for small mitochondrial genomic molecules, particularly those found in chimeric forms, due to generational and cellular duplications and losses of specific gene regions. This results in a large number of structural variations, as noted, that can interconvert within cells (Wu et al. 2020a). Even a small number of generations can see massive changes in the predominant structural form of the mitochondrial genome across individual species populations, with such rapid alterations being termed substoichiometric shifting (Small et al. 1989).

The high number of small repeat sequences appear to be critical to the major rearrangements observed between the mitochondrial lineages of different plant species and in regards to observed genome differences across populations due to their involvement in the formation of large repeats and the overall evolution of genome structure. Additionally, these repeats can in turn potentially lead to not only the creation of further small repeats, but also the formation of large repeats responsible for the greater mitochondrial genome sizes observed in the plant kingdom in general versus other kingdoms of life (Andre et al. 1992). Despite the massive discrepancies in size and overall expansions of the plant mitochondrial genome, there are relatively few nucleotide substitutions in coding regions, though these rates have been shown to be highly variable between species, especially with the observed higher rates of substitutions, indels, and rearrangements in non-coding regions (Wolfe et al. 1987; Christensen 2014; Chen et al. 2017). This lower substitution rate leaves the molecular evolution of plant mitochondria

to be a result of accumulated rearrangements over time within the plant mitochondrial genome rather than due to mutations (Wu et al. 2020b).

A side effect of this, as noted previously, is that the genome is not evenly distributed across the mitochondria in a plant cell. Any given mitochondrion is likely not to have a full copy of the genome and can often have none of the genomic sequences at all. Thus, the multiple isoforms from alternative stoichiometry can be recognized as subgenomic molecules that are dispersed throughout the mitochondria dependent on recombination events and prior fusion and fission of the mitochondria as a whole. Despite this sub-genomic distribution to each individual mitochondrion, the entire genome is nonetheless faithfully inherited by subsequent generations, though the exact mechanisms of how this inheritance occurs remains poorly understood (Preuten et al. 2010; Sloan 2013).

### 1.1.1 Identity of plant mitochondrial genes

While the core genes found within the plant mitochondrial genome have been largely unchanged across the plant kingdom (Figure 2), there remain specialized characteristics of promoter sequences along with intergenic regions that frequently include species-specific sequences (Kubo and Newton 2008). Many of the coding genes utilize multiple promoter sequences that are poorly conserved and imply a non-stringent initiation mechanism that allows for easy transcription of mitochondrial genes to occur. The genetic identity of these gene expression and regulation sequences is still lacking, with only a few mitochondria targeted genes, such as pentatricopeptide repeat (PPR) proteins, having

been identified to have activities involved in mitochondrial RNA processing as in other organisms (Binder and Kühn 2015).

The identity of the genes involved in regulating gene expression, fusion and fission of individual mitochondrion, and DNA repair and maintenance remain under investigation. The genome is packaged in DNA-protein complexes known as nucleoids (Arimura et al. 2004). This packaging is controlled by a double-stranded DNA (dsDNA) binding protein called mitochondrial transcription factor A (TFAM) in humans, but whose identity in plants is an area of ongoing research (Kukat et al. 2015). Other than these larger complexes, the only candidate gene functions that have been directly identified thus far are for the pathways involved in DNA repair and homologous recombination (Lupold et al. 1999; Arimura et al. 2004).

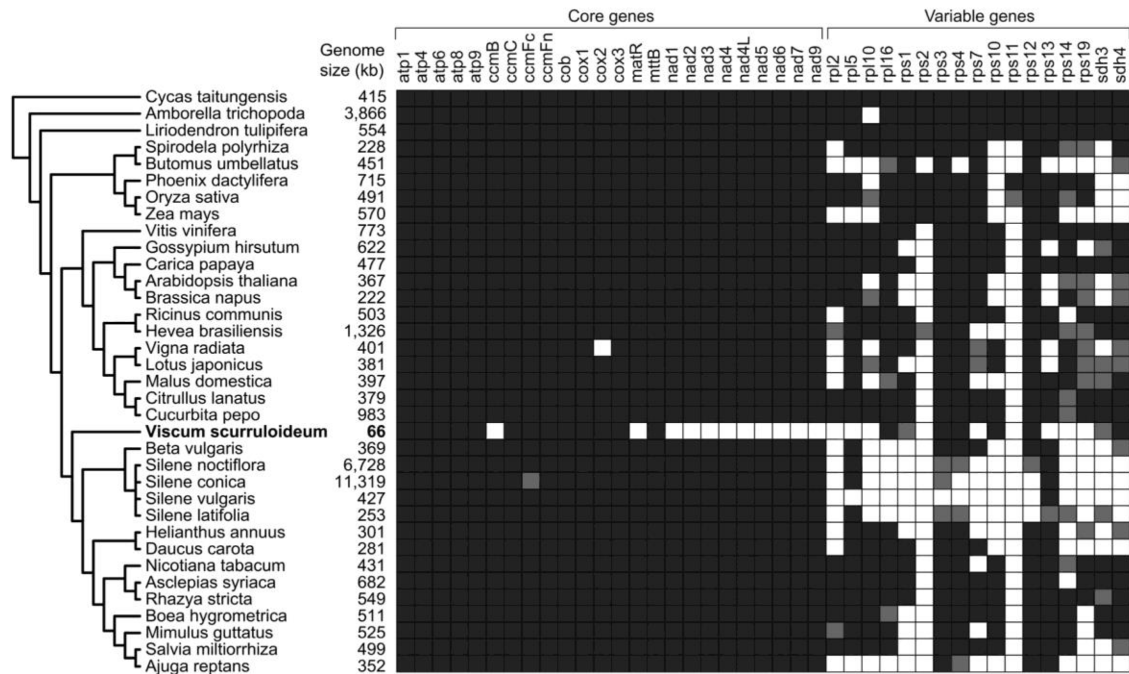


**Figure 2.** Genome size and protein-gene content of 34 sequenced angiosperm mitochondrial genomes and outgroup *Cycas taitungensis*, as originally published as Figure 2 in Skippington et al (2015). Intact genes are dark gray, pseudogenes are light gray, and absent genes are white.

Previous functional studies by an array of authors have succeeded in revealing parts of the structural and maintenance functions of some genes, including those targeted to the mitochondria from the nucleus. These include two DNA polymerases (Parent et al. 2011), a DNA helicase named TWINKLE after the human mitochondrial homologue (Diray-Arce et al. 2014), two genes that are homologous to single-stranded binding (ssb) proteins (Elo et al. 2003; Edmondson et al. 2005), and a DNA gyrase (Wall et al. 2004). Several additional ssb proteins connected to particular gene families have been observed (Zaegal et al. 2006; Janicka et al. 2012), along with proteins homologous to the bacterial RecA (Khazi et al. 2003). The last major gene of note is the homologue to a DNA mismatch protein in bacteria named MutS, whose involvement in the DNA repair systems of plant mitochondria may be greater than is currently confirmed. This gene was originally referred to as chloroplast mutator (*CHM*), but was renamed to MutS homologue 1 (*MSH1*) once its homology to *mutS* was identified (Abdelnoor et al. 2003; Abdelnoor et al. 2006).

Additional DNA-binding proteins with unique functionality to plants were identified through affinity chromatography, showing that Organellar DNA-Binding protein 1 (ODB1) was able to bind with sequences found throughout the mitochondrial genome with a preference for single-stranded DNA (ssDNA) (Janicka et al. 2012). These characteristics may be due to its similarity with the recombination gene Radiation Sensitive 52 (RAD52) found in the nucleus of non-plant species (Janicka et al. 2012). The ssDNA binding protein related to the Whirly family, WHY2, which is involved in

gene repair, may also have a broader involvement in repair through active denaturation of dsDNA (Cappadocia et al. 2010). The usage of both these proteins in RNA metabolism due to their binding affinities remains unclear, though WHY2 has been shown to be associated with complexes involved in splicing of RNA transcripts (Cappadocia et al. 2010).

### 1.1.2 Repeats in the *Arabidopsis* mitochondrial genome

The internal repeats within the plant mitochondrial genome, both large and small, create rearrangements that affect genome structure and organization throughout the genome itself (Klein et al. 1994). The two large repeats in the *A. thaliana* mitochondrial genome consist of sequences that make up 6.6 and 4.2 kb in length, respectively (Table 1). Portions of other genes are included in these repeats and in the unique case of atp6, different extensions of the gene can be transcribed and cleaved to produce the same working protein (Unseld et al. 1997).

An additional set of repeats exist on a smaller scale, ranging from 53 to 556 bp in length (Table 1). In total, these repeats make up approximately 14 kb of the total mitochondrial genome (Unseld et al. 1997; Arrieta-Montiel et al. 2009). There are no repeats between the sizes of 556 bp and 4.2 kb (Wynn and Christensen 2018). Suppression of crossing over events among the small repeats is hypothesized to be due to the activity of recombination control genes including *MSH1* and *RECA3* (Shedge et al. 2007).

The naming scheme of all repeats in the Arabidopsis mitochondrial genome follows an alphabetical format, A to Z, that leads into double letters, AA, for extended numbers. The recombination of these repeats has been suggested to correlate directly to nucleotide length with larger repeats having a correspondingly greater rate of recombination, though there has yet to be measurements to confirm this hypothesis. Clustering of repeats has also been observed generally in relation to highly transcribed genomic regions that may result in higher rates of recombination and formation of repeat sequences (Arrieta-Montiel et al. 2009).

| Start | End | Repeat Name | Length |
|---|---|---|---|
| 49820 | 56409 | Large1-1 | 6590 |
| 258638 | 265227 | Large1-2 | 6590 |
| 1 | 4193 | Large2-1 | 4193 |
| 194198 | 198390 | Large2-2 | 4193 |
| 19682 | 20237 | A-1 | 556 |
| 346208 | 346763 | A-2 | 556 |
| 41464 | 41996 | B-1 | 533 |
| 321435 | 321967 | B-2 | 533 |
| 143953 | 144409 | C-1 | 457 |
| 36362 | 36818 | C-2 | 457 |
| 6118 | 6569 | D-1 | 452 |
| 84089 | 84540 | D-2 | 452 |
| 269864 | 270304 | E-1 | 441 |
| 332187 | 332616 | E-2 | 430 |
| 206095 | 206444 | F-1 | 350 |
| 246766 | 247115 | F-2 | 350 |
| 30938 | 31272 | G-1 | 335 |
| 271061 | 271395 | G-2 | 335 |
| 31725 | 32066 | H-1 | 342 |
| 269864 | 270199 | H-2 | 336 |
| 332181 | 332522 | H-3 | 342 |
| 30442 | 30722 | I-1 | 281 |
| 254842 | 255122 | I-2 | 281 |
| 134636 | 134920 | J-1 | 285 |
| 257621 | 257897 | J-2 | 277 |
| 23253 | 23503 | K-1 | 251 |
| 331581 | 331831 | K-2 | 251 |
| 270775 | 271023 | L-1 | 249 |
| 331877 | 332125 | L-2 | 249 |
| 234172 | 234429 | M-1 | 258 |
| 270573 | 270830 | M-2 | 258 |
| 110303 | 110596 | N-1 | 294 |
| 48899 | 49185 | N-2 | 287 |
| 332660 | 332955 | O-1 | 296 |
| 358982 | 359292 | O-2 | 311 |
| 138910 | 139374 | P-1 | 465 |
| 258129 | 258558 | P-2 | 430 |
| 66858 | 67062 | Q-1 | 205 |
| 269570 | 269775 | Q-2 | 206 |
| 182729 | 182934 | R-1 | 206 |
| 207329 | 207534 | R-2 | 206 |
| 134964 | 135193 | S-1 | 230 |
| 257923 | 258143 | S-2 | 221 |
| 68660 | 68844 | T-1 | 185 |
| 360869 | 361049 | T-2 | 181 |
| 165948 | 166134 | U-1 | 187 |

| | | | |
|---|---|---|---|
| 307932 | 308118 | U-2 | 187 |
| 49243 | 49418 | V-1 | 176 |
| 247494 | 247673 | V-2 | 180 |
| 265698 | 265867 | V-3 | 170 |
| 80177 | 80360 | W-1 | 184 |
| 278458 | 278640 | W-2 | 183 |
| 288315 | 288518 | X-1 | 204 |
| 306969 | 307164 | X-2 | 196 |
| 50976 | 51150 | Y-1 | 175 |
| 263897 | 264071 | Y-2 | 175 |
| 265507 | 265681 | Y-3 | 175 |
| 11394 | 11535 | Z-1 | 142 |
| 87937 | 88078 | Z-2 | 142 |
| 247484 | 247706 | AA-1 | 223 |
| 265688 | 265896 | AA-2 | 209 |
| 1956 | 2100 | BB-1 | 145 |
| 36473 | 36619 | BB-2 | 147 |
| 144152 | 144298 | BB-3 | 147 |
| 196153 | 196297 | BB-4 | 145 |
| 79223 | 79370 | CC-1 | 148 |
| 233646 | 233789 | CC-2 | 144 |
| 113664 | 113791 | DD-1 | 128 |
| 276492 | 276619 | DD-2 | 128 |
| 73611 | 73737 | EE-1 | 127 |
| 65547 | 65673 | EE-2 | 127 |
| 165832 | 165932 | GG-1 | 101 |
| 207225 | 207332 | GG-2 | 108 |
| 23851 | 23952 | HH-1 | 102 |
| 222608 | 222705 | HH-2 | 98 |
| 8921 | 9013 | II-1 | 93 |
| 172944 | 173036 | II-2 | 93 |
| 171686 | 171800 | JJ-1 | 115 |
| 274990 | 275104 | JJ-2 | 115 |
| 128290 | 128402 | KK-1 | 113 |
| 286705 | 286808 | KK-2 | 104 |
| 358722 | 358822 | KK-3 | 101 |
| 36656 | 36763 | LL-1 | 108 |
| 144008 | 144115 | LL-2 | 108 |
| 333070 | 333174 | LL-3 | 105 |
| 134500 | 134580 | MM-1 | 81 |
| 257511 | 257580 | MM-2 | 70 |
| 96728 | 96797 | NN-1 | 70 |
| 243328 | 243397 | NN-2 | 70 |
| 88445 | 88535 | OO-1 | 91 |
| 284284 | 284369 | OO-2 | 86 |
| 358524 | 358613 | OO-3 | 90 |
| 234168 | 234228 | PP-1 | 61 |

| | | | |
|---|---|---|---|
| 270774 | 270830 | PP-2 | 57 |
| 331877 | 331932 | PP-3 | 56 |
| 200801 | 201039 | QQ-1 | 239 |
| 347347 | 347575 | QQ-2 | 229 |
| 234413 | 234466 | RR-1 | 54 |
| 287032 | 287085 | RR-2 | 54 |
| 332814 | 332867 | RR-3 | 54 |
| 359076 | 359129 | RR-4 | 54 |
| 60912 | 60988 | SS-1 | 77 |
| 234045 | 234124 | SS-2 | 80 |
| 238391 | 238445 | TT-1 | 55 |
| 288619 | 288668 | TT-2 | 50 |
| 223469 | 223531 | UU-1 | 63 |
| 285128 | 285190 | UU-2 | 63 |
| 286705 | 286887 | VV-1 | 183 |
| 358726 | 358887 | VV-2 | 162 |

**Table 1.** Non-tandem repeats within the *Arabidopsis* mitochondrial genome identified by size and location, arranged in BLAST score order and lettered name, as obtained from the BK010421 Arabidopsis genome accession.

## 1.2 DNA repair pathways in plant mitochondria

Due to the high copy number of genomes within plant and animal organelles, it was originally expected that DNA repair was non-existent and unnecessary within them, since damage to any single copy would have minimal impact due to the undamaged copies being available for transcription (Boesch et al. 2011). This assumption was overturned, however, with evidence that DNA repair does happen in organelles, but in a more limited fashion than repair pathways in the nucleus (Boesch et al. 2011). For plant mitochondria, base excision repair (BER) and homologous recombination (HR) have been definitively shown to occur, but the other methods of repair remain lacking in evidence (Spampinato 2016; Chevigny et al. 2020).

Animal mitochondrial genomes are known for their high synonymous substitution rates and a high level of conservation of gene order. That would normally be expected thanks to the oxidative damage within the mitochondrial matrix that is constantly harming the mitochondrial DNA (Brown et al. 1979). A disparate characteristic of plant mitochondria is that they feature few mutations and thus few changes to coding sequences. Therefore, they have a slow evolutionary rate, possibly due to their robust HR repair systems, but have such variety in structure regardless due to recombination and non-homologous processes (Palmer and Herbon 1988). It was hypothesized that part of the explanation within angiosperm mitochondrial genomes is that their synonymous substitutions are not completely neutral and this non-neutrality keeps the synonymous substitution rate low (Wynn and Christensen 2015). However, it was found that the synonymous substitutions

are close to neutral, leaving the double strand break repair (DSBR) pathway as the cause for both a low mutation rate and a relatively high rearrangement rate (Sloan and Taylor 2010; Christensen 2013).

### 1.2.1 DNA repair excision pathways

There are multiple forms of harm that can occur to DNA, including direct damage, mismatches of nucleotides, and chemical alterations to the helical backbone such as alkylation. When direct damage is caused to DNA, such as with ultraviolet radiation, this can lead to the formation of lesions known as cyclobutane pyrimidine dimers. This UV damage has been shown in some species to be corrected with the application of blue light through a photolyase enzyme. Some plant species such as rice have been identified as having a nuclear photolyase targeted at the mitochondria (Takahashi et al. 2011). However the activity of the protein has not been fully confirmed in regards to repair of UV-induced deformation (Takahashi et al. 2011).

For mismatches between nucleotides, a mismatch repair (MMR) complex is expected to correct the conflict both in bacteria and eukaryotic nuclei. In bacteria, this would involve repair proteins centered around a MutS complex binding to the mismatched DNA (Lahue et al. 1989; Schofield and Hsieh 2003). There is only a single homologue for this system known in plant mitochondria, MSH1, and there is no evidence of other DNA mismatch repair factors such as MutL or MutH.

A protein structural analysis of MSH1 has found that it has an additional C-terminal GIY-YIG homing endonuclease domain which MutS lacks (Abdelnoor et al. 2006). This would theoretically give it the same capabilities of incising DNA for nucleotide replacement like MutH, but this has not been confirmed experimentally. Furthermore, when tested in vitro, the MSH1 endonuclease domain was found to not have endonuclease activity on its own and only had the capability to bind to branched DNA, leaving that part of its mismatch repair an ongoing question (Fukui et al. 2018). It has been suggested that the full MSH1 protein with the mismatch-binding domain included is required for DNA incision to occur (Fukui et al. 2018). It has been hypothesized that MSH1 could recruit and promote the correction of mismatches by using its incision domain to create a DSB at the location of the mismatched nucleotide, thus allowing the normal recombination repair mechanisms of DSBs to remove the mismatch (Christensen 2014).

Lesion damage to DNA in the nucleus can also be corrected via nucleotide excision repair (NER) that removes not only the lesions, but also the structural malformations that impair the transcriptional machinery. This pathway usually is conducted via hetero-tetramers that scan for lesions and then cleave them (Wirth et al. 2016) or with a translocase called transcription-repair-coupling factor that instead scans for machinery that has been stalled due to the lesion (Selby and Sancar 1990). This is the system within prokaryotes, but this pathway is completely lacking in plant mitochondria. There are no homologs for any of the factors required in the mitochondrial genome and no evidence of nuclear targeted

proteins either. Therefore, it is proposed that this repair pathway is instead covered by other pathways in plant mitochondria (Christensen 2014).

When oxidative damage or other base modifications occur, BER can be used to remove the harmed nucleotide base and replace it. There are multiple BER pathways based around the different glycosylases used for excision of the base and each one prioritizes a certain form of damage. A commonly studied factor is uracil-DNA glycosylase (UNG or UDG) for recognizing the incorrect formation of uracil bases in DNA caused by cytosine deamination. The presence of UNG in plant mitochondria has already been experimentally confirmed through GFP fusions, *in vitro* and *in organello* uracil glycosylase assays, along with direct gene knockouts (Boesch et al. 2009; Wynn et al. 2020).

Other pathways include 8-oxoguanine glycosylase (OGG1) for excision of oxidized guanine bases and the multiple members making up formamidopyrimidine glycosylase/endonuclease VIII (FPG/NEI) for excision of oxidized pyrimidines. The two protein groups also function together, with FPG/NEI proteins identifying the same oxidative products as OGG1 in *A. thaliana* (Córdoba-Cañero et al. 2014). While both have been confirmed to exist in plants, they appear to be localized to the nucleus and no evidence of mitochondrial targeting has been shown. However, both protein groups have been characterized in the mitochondria of potatoes and knockout mutants have shown

increased oxidative damage in both the nuclear and mitochondrial genomes, suggesting their activity in the latter (Ferrando et al. 2018).

### 1.2.2 DNA repair recombination pathways

Recombination repair is an essential part of both repairing DNA damage and of the DNA replication process itself. Therefore, it is found as a pathway in all forms of life, even in viruses. When DSBs occur, recombination is needed to repair the damage with an accurate template, along with rescuing replication forks that have been stalled. HR relies upon high similarity between a template strand sequence and the sequence to be repaired. In plant mitochondria, their large genome copy number allows for such templates to be readily available (Maréchal and Brisson 2010). Other forms of DSBR are non-homologous in nature and include non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ). Eukaryotes use these for DSBs despite their propensity for causing rearrangements and indels (Christensen 2014).

The HR pathway is a critical component in plant mitochondria, possibly serving as the main mechanism, whereas the non-homologous forms are rarely seen and experiments have yet to confirm the occurrence of NHEJ in plant mitochondria. The large amount of repeated sequences in the plant mitochondrial genome can result in rearrangements over generations, especially due to multiple isoforms of the genome being available for recombination. While the mechanisms and protein complexes involved in HR are well

known in bacteria, very little is yet understood about the process in plant mitochondria (Gualberto and Newton 2017).

What is known about HR in plant mitochondria is that, after the initial step of end resectioning where the 5' ends are nucleolytically degraded to expose the 3' ends, the single-stranded ends should be protected from further damage and nuclease activity by the ssDNA binding proteins and this may include activity by the organellar single-stranded binding (OSB) proteins and the Whirly family of proteins (Edmondson et al. 2005; Zaegel et al. 2006; Cappadocia et al. 2010). Both cause an increase in ectopic recombination, which is recombination of homologous sequences occurring at non-homologous chromosomal loci, if knocked out, implying their required presence for HR . The Whirly proteins are incapable of associating with the POL1B polymerase, which has led to a hypothesis that the ss-DNA binding proteins are the ones to recruit POL1B during repair and the alternative binding proteins, such as the Whirly family, inhibit the capability of the ssDNA binding proteins and the polymerase to bind to the DNA undergoing HR (García-Medel et al. 2019). Both ODB1 and WHY2 have the capability of then loading the RECA3, or RECA2, recombinase enzyme onto the ssDNA after displacing the ss-DNA binding proteins and promoting the annealing of the RECA enzyme to the ssDNA. The multiplicity of combinations of ss-DNA binding enzymes available for this process in each step also implies the potential existence of alternative pathways for conducting HR using different ss-DNA binding proteins (Janicka et al. 2012; Miller-Messmer et al. 2012; Gualberto and Kuhn 2014).

Following regulation of recombination steps by the RECAs and RECX, branch migration allows the formed D-loops to be extended to ensure accurate homologous recombination (Odahara and Sekine 2018). The protein activity in this step remains unknown, as no RuvAB pathway is known in plants and the RECG1 protein lacks the RuvAB-like translocase activity of its bacterial homologue (Wallet et al. 2015). It is possible that RADA or RECA3 is used for branch migration due to the involvement of other RECA proteins in early parts of the branch migration step (Chevigny et al. 2019).

The final resolution step once again requires the RuvAB pathway and resolvases like RuvC in bacteria, but a separate process is required for plants due to the lack of this pathway. Alternative resolvases, such as a homologue for YqgF, may take the place for this step (Chevigny et al. 2020). It has been suggested that HR may resolve the same manner as in the nucleus, via dissolution from the branch migration factors and topoisomerase I, as has been indirectly shown with RECG in bacteria. Thus far, no direct evidence for the resolution process in plant mitochondria has been identified (Kobayashi et al. 2017).

## 1.3 DNA degradation during pollen development

Unlike in the nucleus where multiple robust signaling and repair pathways exist, plant mitochondria are left with only a small number of known repair options for DSBs and DNA lesions. Therefore these pathways must be maintained at high levels of activity and

are critical for mitochondrial DNA upkeep (Gualberto and Newton 2017; Fu et al. 2020). But there are periods of plant development where this maintenance is not upheld, during the process of pollen formation and flowering. It is during this period that the copy number of the mitochondrial genome begins to decline across all plant tissues except for ongoing stem cell production in meristematic tissues (Bendich et al. 2013) and it is general organelle DNA decline within the male pollen that may be the reason for maternal inheritance of the mitochondrial genome (Izumi 2018).

Due to this copy number decrease, determining if the decrease is a passive reduction over time or if there are enzymes that could be responsible for enacting the genome degradation has been paramount. When testing quantitative levels of plastid DNA in pollen vegetative cells, the genomic DNA is unable to be observed cytologically (Matsushima et al. 2011). Meaning that even the pollen cells not involved in fertilization lack meaningfully detectable levels of mitochondrial DNA, despite having high numbers of plastid organelles in general. This implies there is activity related to degradation of the plastid DNA in these cells on a molecular level (Matsushima et al. 2008).

The aging of plant leaves, known as leaf senescence, is a key component of plant development and entering into the fertilization stage and includes many signaling hormones and altered nutrient distribution. Degradation of cellular organelles and macromolecules is an additional method to use less energy within the leaf tissues. This abandonment of DNA repair in order to reduce energy costs has been proposed by

Bendich (2013) to be a main reason why uniparental inheritance of mitochondria and chloroplasts first evolved. An additional study has revealed that DPD1 exhibits increased expression in senescing leaves as well, implying activity in breaking down mitochondrial and chloroplast genomes alongside similar activity in the nucleus for recycling of the DNA components. Other factors, including M20, have yet to be confirmed as upregulated in senescing leaves (Bendich 2013; Sakamoto and Takami 2014).

## 1.4 Cytoplasmic male sterility

While sterility of one sex would be evolutionarily catastrophic for many organisms, it plays a key role in many of the flowering plants and is of great importance to human agriculture. The trait itself is conferred entirely through alterations to the mitochondrial genome, though the exact mechanism remains clouded and several different factors, such as chimeric genes, appear to play a role (Touzet and Meyer 2014). From a population point of view there must be a mix of females (male steriles) and hermaphrodites. This is referred to as gynodioecy (Darwin 1877; Richards 1997). The trait can persist if it provides a fitness advantage to the female-only plants, such as producing more seed than their hermaphroditic counterparts. This fitness advantage requires that pollen not be limiting in the environment, so the trait can never go to fixation (McCauley and Taylor 1997).

Cytoplasmic male sterility (CMS) is a form of plant sterility that causes pollen development to be aborted and prevents the combination of gametes to create subsequent

generations. This allows for precise control of breeding among a field population by only allowing plant crossing to occur when controlled and regulated by growers and preventing self-crossing. Because of this, CMS has great agricultural importance in promoting retainment of high quality traits, such as yield and stress resistance, particularly in the production of F1 hybrid crops with hybrid vigor traits that are lost if uncontrolled crossing occurs. The explicit genetic identity of the trait is a topic of speculation, as there are a variety of factors that appear to be able to cause CMS. Similarly, knocking out those factors when they occur can cause rescue of male fertility in the plant (Kazama et al. 2019). In addition to the formation of chimeric genes, increased rearrangements in the mitochondrial genome can also induce the CMS trait, such as by knocking out MSH1. This form of CMS is also heritable and reproducible through transgenic induction of rearrangements (Sandhu et al. 2007).

Identifying candidate genes for creating CMS have been a major focus of study in most agriculturally important crop species, including cotton, beans, and rapeseed. Research is ongoing to determine the basic requirements for producing the CMS trait and a greater understanding of the plant mitochondrial genome's structure remains highly relevant toward that end (Sarria et al. 1998; Kang et al. 2017; Li et al. 2018).

### 1.5 DSBR, Stress, and Repeat Recombination

Recombination events between the smaller repeats at several hundred bp are rare to occur under non-stressful conditions. This rarity suggests that only certain conditions, such as

DNA damage or repair gene interruption, are responsible for these events to occur. This can both be directly tested and observed through experiments using chemical inhibitors that disturb DNA replication, including fluoroquinolones like ciprofloxacin that inhibit the activity of DNA gyrase (Wallet et al. 2015; Wynn and Christensen 2019). Additionally, gene knockouts of components involved in DNA repair, such as RECA3 or MSH1 (Shedge et al. 2007), result in increased recombination between short repeats and alteration in the stoichiometric makeup of the plant mitochondrial genome (Arrieta-Montiel et al. 2009; Odahara et al. 2015). Recombination of short repeats occurs primarily when double-strand breakage is increased, such as by ciprofloxacin, or when the DSBR system is defective, such as in the mutants. Thus recombination at a repeat is diagnostic of the level of damage or the inadequacy of repair.

One of the earliest repeats identified, which would come to be known as Repeat L (Arrieta-Montiel et al. 2009), was first identified from *Bam*HI fragmentation of the plant mitochondrial genome and the use of a genomic probe that identified the *atp9* gene (Sakamoto et al. 1996). It was already known at the time that the CMS phenotype was associated with novel chimeric sequences having formed from recombination events that included fragments of genes, particularly those such as *atp9* (Hanson 1991). Repeat L, 249 nucleotides in length, can act as a representative for recombination events across the range of small repeats when stress and damage is applied during plant growth. In turn, this can act as a diagnostic tool to investigate DSBR pathway functionality, especially because recombination at repeat L has been used as a marker for DSB repair defects and

increased levels of breakage (Shedge et al. 2007; Miller-Messmer et al. 2012; Wallet et al. 2015).

## 1.6 Purpose and Objectives

The first aim of this investigation of the mitochondrial genome of Arabidopsis is to confirm the transformation of a restriction enzyme into the plant nucleus and targeting of this enzyme to the mitochondria in order to cause controlled DSB cuts. The second aim is to investigate the effects of PvuII cutting on mitochondrial genome maintenance, by detecting ectopic recombination at a pair of repeats in the genome. Our lab's working hypothesis is that most natural DNA damage in the plant mitochondrial genome is converted to DSBs and that the DSBR pathways are responsible for the observed low mutation rate and high rearrangement rate in the plant mitochondrial genome. Therefore, the induction of DSBs allows for an investigation of the capability of the mitochondrial genome to repair such damage, particularly at repeat sequences that are known to be involved in recombination and expansion events in the mitochondrial genome. Additionally, stressful growing conditions for the plants during the induction of double strand breaks will be applied to investigate how stress and forced DSBs affect the DSBR system. This will be accomplished in vivo by using light stress and cold stress along with a transgenically inserted restriction enzyme to induce double strand breaks. The repeat sequences in their original locations, referred to as parentals, will be measured against the quantitative formation of recombinant sequences between the copies of the repeat. This recombination rate will be ascertained using qPCR measurements of the aforementioned

L-1 and L-2 repeats, to determine if the amount of recombinants increases under stress and DNA damage as compared to control groups.

# CHAPTER 2: DSBR, LIGHT AND COLD STRESS, AND REPEAT RECOMBINATION

## 2.1 Introduction

Plant mitochondria have major roles inside the plant cell that include ATP production through the electron transport chain, components of which are produced using proteins encoded by the atp synthase genes, cox genes for electron transport, and nad genes for respiratory complexes (Figure 2). A full scientific grasp of the plant mitochondrial genome, its structure, its non-coding regions, and its methods for DNA repair, replication, and recombination are of importance for all of plant biology research. But the latter methods of DNA processing, how damage such as DSBs are repaired, and to what extent damage is repairable under stressful conditions are not fully understood.

An investigation of these systems requires the ability to induce DNA damage and DSBs on command and at precise, known locations. Because of this, the use of genotoxic chemicals or high energy radiation such as UV is unsuitable due to the general damage caused randomly across the genome, including the nuclear and plastid genomes. Another method that would allow for precise cutting is to use a restriction enzyme expressed from a nuclear transgene that would target the mitochondrial genome. This would allow for an exact number of cuts to be known and the genomic location of said cuts due to restriction enzymes acting only on certain nucleotide sequences. Causing DSBs would also let the

formation of recombinant sequences be measured directly, particularly at regions of repeat sequences that are prone to recombination during the DNA repair process.

The restriction enzyme *Pvu*II creates blunt end cuts in the middle of the nucleotide sequence 5'-CAGCTG-3' and causes 107 cuts in total across the mitochondrial genome of the model plant *Arabidopsis thaliana*. This restriction enzyme was chosen due to the number of cuts being hypothesized to be significant enough to show a phenotype on the plant due to devoting energy and resources to repairing the DSBs caused, but possibly not enough to overwhelm the DNA repair system of the mitochondria and result in death of the host cell and the plant as a whole. The results of this hypothesis and usage of a transgenic Arabidopsis with an inserted *Pvu*II gene was initiated by prior work in the Christensen lab (M. Bonnett and E. Kahlandt, personal communication, 2016-7).

To create a transgenic *Pvu*II insertion plant, a gene cassette and plasmid was constructed by prior lab researchers (Figure 3). This construct includes the *Pvu*II endonuclease gene obtained from Robert Blumenthal, University of Toledo, (Blumenthal et al, 1985) as well as the *Pvu*II methylase gene (Blumenthal et al. 1985) driven by the *tac* promoter from pUC19. The methylase was required to enable cloning in *E. coli* and *A. tumefaciens* because it methylates *Pvu*II sites in those bacterial genomes and prevents readthrough expression of the endonuclease from killing the cell. The *Pvu*II endonuclease gene is fused at the N-terminal end to a mitochondrial targeting peptide from the AOX1A gene (Saisho et al. 1997). This 62 amino acid peptide was included so that the *Pvu*II restriction

enzyme would be shuttled to the mitochondria after translation of the protein in the plant cytoplasm. These components were inserted into the plant transformation vector pArt27 (Gleave 1992). This vector contains a promoter sequence and transcription factor named ALCR/alcA that acts as a gene switch that is inducible through exposure to ethanol (Roslan et al. 2001). Lastly, two antibiotic resistance genes were included in the vector, spectinomycin and kanamycin. The first allows for selection of transformants in bacteria (Gleave 1992) and the second for selection in transgenic plants (Weigel and Glazebrook 2006).

The plasmid was first transformed into *A. thaliana* by Michaela Bonnett (M. Bonnett and A. Christensen, personal communication). The plant transformation was conducted using the floral dip method (Clough and Bent 1998). The plants were then selfed and tested on kanamycin in order to identify plants with the plasmid insertion. From the progeny of these plants, three homozygous lines were chosen, but they were replicate transformants. Plants were grown on soil and the *Pvu*II transgene was induced by watering with a 1% ethanol solution in order to determine if there were any observed phenotypic or genotypic changes after inducing the *Pvu*II restriction enzyme. There was no obvious phenotype and Illumina sequencing found no meaningful whole genome changes in the induced plants (M. Bonnett, E. Kahlandt, and A. Christensen, unpublished data, 2016-7)

A serendipitous discovery by M. Bonnett (Bonnett and Christensen, unpublished data) was that ethanol induction of *Pvu*II when the seedlings were very crowded produced a

phenotype, if the plants were also subjected to drought following the induction. Using transgenic lines 8, 10, and 11 (which were later determined to have identical insertion locations and were not true independent lines), a browning and purpling of leaf tissue and a failure to grow rachises (floral shoots) was observed that did not occur in the wild type Col-0 controls (Figure 4; M. Bonnett, E. Kahlandt and F. Cassidy, unpublished data, 2017). This suggested the hypothesis that combining induction of *Pvu*II with stress could disrupt the capability of Arabidopsis plants to fully recover from the DSB damage and could give insight into how robust these repair systems are and how their attempted repair would cause recombination to occur. However, additional stresses would also introduce extra variables that had to be considered, especially as ethanol itself is already a stress on plant metabolisms (Kuo et al. 2000).

More experiments were tried using constant ethanol exposure as a 1% solution in water given every three days over multiple generations, but no phenotype was observed in those attempts. A genetic analysis using DNA samples taken from the constant ethanol plants was done using two non-tandem repeats, repeats B and D, in order to determine if the constant exposure altered recombination at those sides, with repeat B containing a *Pvu*II cut site and the other repeat not containing one. The results were inconsistent and showed a lower recombination rate in the transgenic plants as compared to the wild type, but this difference was observed at both repeat locations, making it unclear if this difference had any connection to the *Pvu*II restriction enzyme and was instead damage resulting from the ethanol exposure itself, though this damage was not observed in the wild type. Thus,

the damage caused by the DSBs specifically from the *Pvu*II enzyme was hypothesized to be wholly repairable by the plant mitochondria on their own despite constant induction of cutting and that only by adding in other forms of DNA damage or non-optimum growing and energy production conditions would a change in recombination be measurable (F. Cassidy and A. Christensen, unpublished data, 2017-8).

Other stresses were considered for their effect on plant growth and development, including reduced light to inhibit photosynthetic capabilities, hot and cold stress to activate temperature stress responses, and osmotic and ionic stress with high salt concentrations. It was hypothesized that reducing photosynthesis would lower available glucose to fuel the electron transport chain and inhibit the production of ATP and this would have a greater likelihood of reducing the mitochondrial DNA repair mechanisms' capabilities to repair DSB damage. The plants were grown from initial planting to final leaf tissue harvesting with a shade cloth over the dome covering to reduce the light received by approximately 40-45%. An additional experiment used a 72 hour cold (4°C) treatment to determine if cold stress would have an impact similar to reduced light, as cold temperature exposure has been shown to reduce ATP synthesis and the overall efficiency of respiratory pathways (Kerbler et al. 2018). This would reduce energy availability for repairing DSB damage caused by ethanol induction of *Pvu*II.

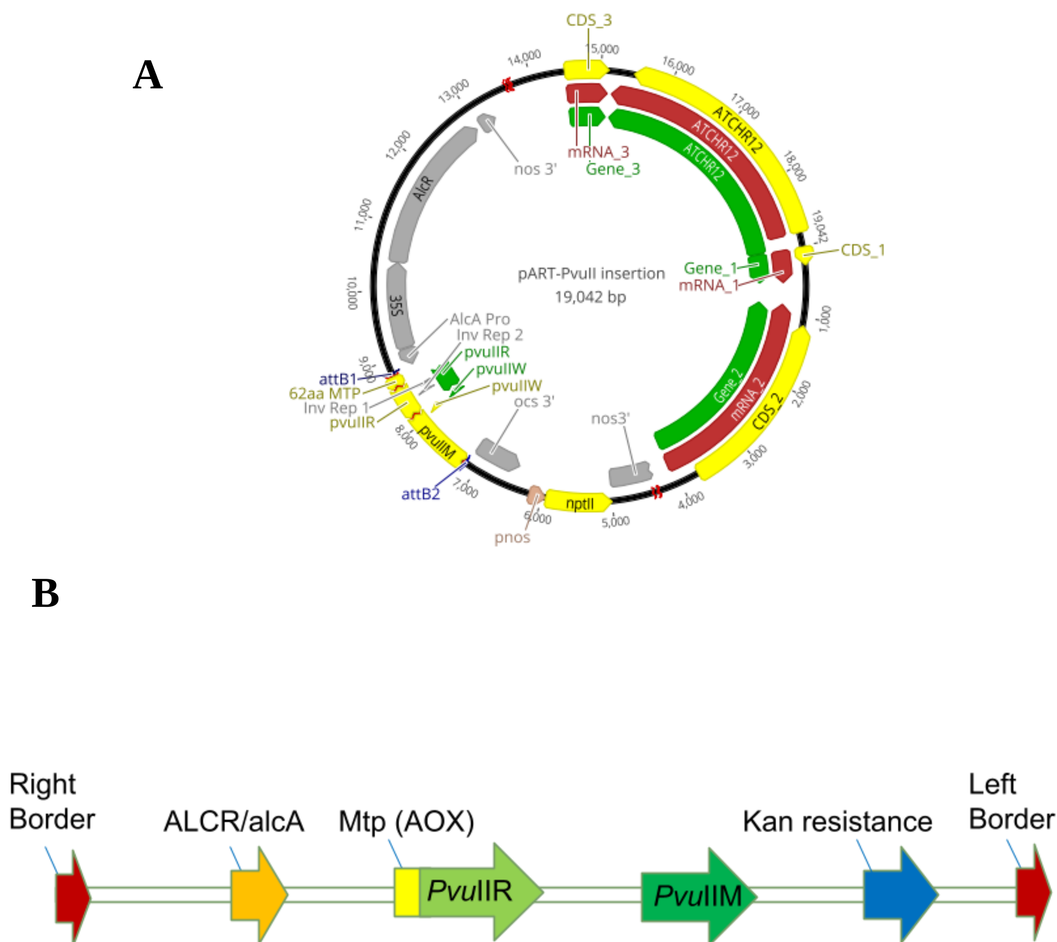


**Figure 3.** pART insertion sequence map and *Pvu*II cassette construction. (A) Sequence map for the pART27 plasmid with inserted gene cassette as transformed into Agrobacterium as a bacterial intermediary. (B) The gene cassette containing the *Pvu*II gene fused to the 62 amino acid mitochondrially targeted peptide sequence from the AOX1A gene, a *Pvu*II methylase gene to prevent expression in Agrobacterium, the ALCR/alcA promoter inducible with ethanol, a Kanamycin resistance gene for selection of transformed plants, and T-DNA insertion border repeats.

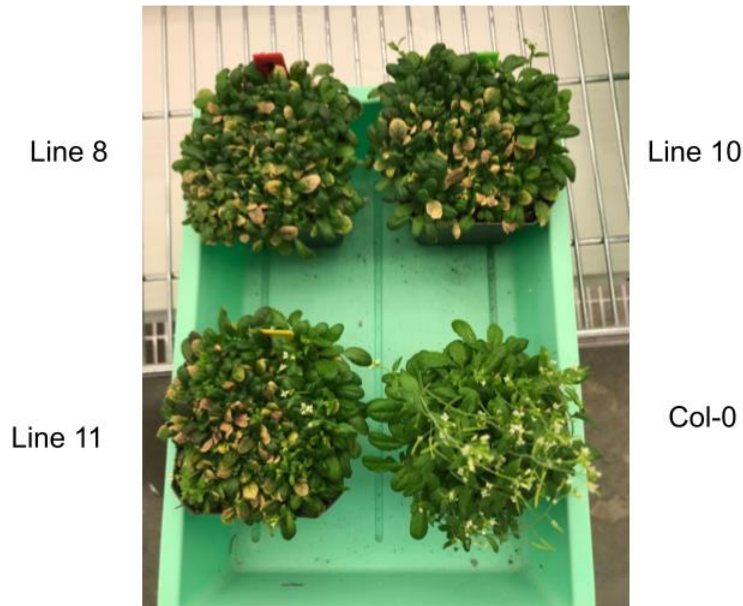


**Figure 4**. Drought phenotype response from *Pvu*II induction (M. Bonnett, unpublished data, 2017). Wild type Col-0 plants were grown in a pot along with separate pots for transgenic *Pvu*II insertion lines 8, 10, and 11. The seeds were sown to crowd the soil in the pot and the plants were ethanol induced for 72 hours after three weeks of growth and then put into drought conditions. The Col-0 proceeded to produce stems and flowers, but the transgenic lines resulted in browning and purpling of leaf tissue with minimal stem and flower growth.

## 2.2 Materials and Methods

### 2.2.1 *Arabidopsis thaliana* seeds and growth conditions

The Arabidopsis plants were grown from a package of Lehle Seeds (Accession: Col-0/Redel-L214-500). These were propagated through selfing again to build a supply of seeds. Seeds were sown onto potting soil and cold treated at 4° C for a minimum of two days before being moved to a Percival AR66L growth chamber. The growth chamber was set at 22° C on a 16 hour light cycle with 14% humidity and plants were covered in a clear plastic dome for the entire length of growth, with a light intensity under the dome of 167 µmol/m²/s at soil height. Plants grown under a single shade cloth received a light intensity of 96 µmol/m²/s at soil height and plants under a double layer of shade cloth received a light intensity of 49 µmol/m²/s at soil height. Classification of light intensity as high, medium, or low were based on light levels determined by Schumann et al. (2017). High light intensity is also referred to as normal, due to this being the normal light intensity within a Percival AR66L growth chamber, and in the University of Nebraska Plant Growth Facility. Plants grown under cold temperature conditions were placed into a cold room at 4° C and non-treated plants were kept in the growth chamber at 22° C.

### 2.2.2 AOX-*Pvu*II ethanol inducible transgenic mutant

A transgenic insertion line was developed using a gene cassette and plasmid containing the endonuclease *PvuII* sequence, a *Pvu*II methylase, an ethanol inducible ALCR/alcA genetic switch and the 62 amino acid sequence of the mitochondrial targeting peptide from the AOX1A gene (Figure 3). The spectinomycin resistance gene in the vector is

used for bacterial selection of transformants, and the kanamycin resistance gene is used for selection of transgenic plants.

### 2.2.3 Ethanol induction

The plants were grown for 21-24 days after three day cold room exposure, and then induction was done by giving a 1% ethanol solution every 24 hours for a 72 hour period. The solution was allowed to remain in the potted trays for approximately 2 hours before being removed to ensure uptake of the solution and saturation of the soil.

### 2.2.4 Leaf tissue processing and DNA extraction

Leaf tissue was collected from the plants after the 72 hour period of ethanol induction. Tissue was removed as whole leaves from each biological replicate and placed into 2.0 mL conical screw cap tubes along with a single bead of zirconia silica with a diameter of 23 mm. Initial homogenization of the leaf tissue was conducted using a grinding buffer (Strehle et al. 2018) and a Mini-BeadBeater 16 from Biospec Products, with two grinding sequences done for 45 seconds. The subsequent DNA extraction procedure followed the methodology described in Rowan et al. (2015).

#### 2.2.4.1 Extraction buffer

The extraction buffer is used to extract and remove organelles from the rest of the leaf tissue, inactivate denaturing proteins and other compounds, and is made up of two solutions, one with sodium dodecyl sulfate (SDS) and one without in order to control when cellular lysis occurs (Goldenberger et al. 1995).

#### 2.2.4.2 Potassium acetate (KOAc) solution

The lysed solution is renatured with an acidic acetate solution and the potassium acts to precipitate out SDS via formation of potassium dodecyl sulfate that is poorly water soluble (Sokolov et al. 1989)

#### 2.2.4.3 SPRI beads for DNA binding

The solid phase reversible immobilization (SPRI) beads are size selectable paramagnetic beads that are able to bind to DNA selectively by strandedness type and by fragment size in order to extract and purify DNA samples for PCR, qPCR, and other sequencing procedures. The solution of beads was made and used according to the protocol in Rowan et al. (2015).

### 2.2.5 Primers and DNA analysis methods

#### 2.2.5.1 Quantitative PCR

Quantitative PCR was conducted with equivalent 1.5 uL of DNA and 13.5 uL of a SsoAdvanced Universal SYBR Green Supermix kit from Bio-Rad. The data analysis used a C1000 Touch thermal cycler running a CFX96 Real-Time System from Bio-Rad. The samples were run in triplicate for all three biological replicates in each experimental group. The primer sequences used for the real time analysis of the *Pvu*90P and L repeat regions, along with the rrn18 housekeeping gene as a control, were rrn18-qF2 (5'-CCTTGAGCTAGGAGCCTCTTT-3'), rrn18-qR2 (5'-CATGCAAGTCGAACGTTGTT-3'), *Pvu*90P-qF1 (5'-GGCAGCTCATCAACTACGAG-3'), *Pvu*90P-qR1 (5'-CAAGTAGCCCGGTCTGGTAA-3'), L1-qF1 (5'-GTTATGCCATTTTGGGCTTTGCTC-3'), L1-qR1 (5'-CGCTCGACCGAAGAAATGAGTAAC-3'), L2-qF1 (5'-

CCGGTTGAAAGCTAAGCCGGAG-3'), and L2-qR1 (5'-CCCTCACTGAACCGACTTGAATCTG-3').

### 2.2.5.2 Standard curve efficiencies

The standard curves for each primer pair were conducted using DNA dilutions starting at a concentration of 500 picograms per microliter and diluted ten-fold three times down to 0.5 picograms per microliter. A log base 10 analysis was used. The rrn18 primers had a regression slope of -3.298 and an efficiency of 101.008%. The L1 primers had a regression slope of -3.497 and an efficiency of 93.172%. The L2 primers had a regression slope of -3.579 and an efficiency of 90.275%. The Pvu90P primers had a regression slope of -3.295 and an efficiency of 101.118%.

### 2.2.5.3 Two-way ANOVA

A two-way ANOVA analysis was done with Microsoft Excel using a fixed effect model with interaction between variables considered. A significance level (α) of 0.05 was used with a medium effect size of 0.25. Variable A was assigned to plant type, either wild type Col-0 or transgenic PvuII, and Variable B was assigned to the different stress groups, including ethanol induction.

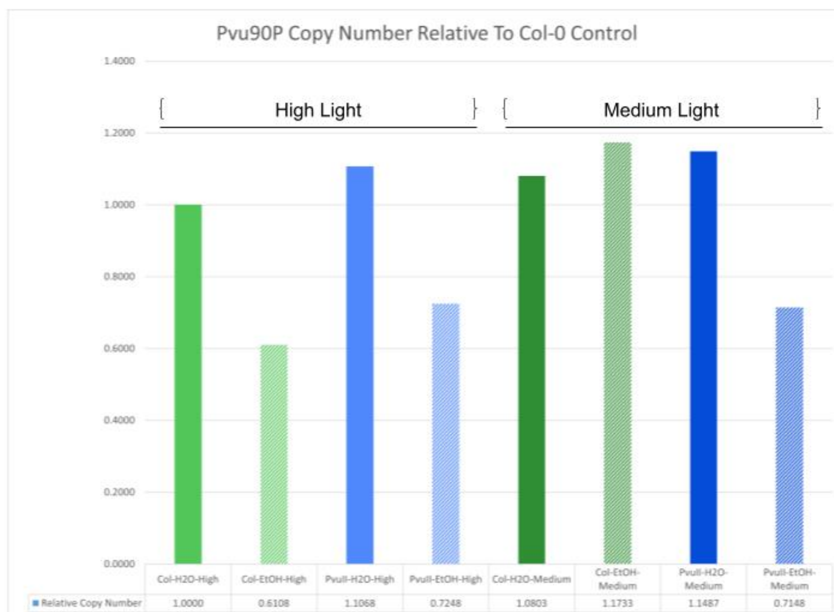


**Figure 5.** Relative copy numbers, measured by ΔΔCq calculations, of the 90th *Pvu*II site in the Arabidopsis mitochondrial genome in Col-0 wild type and transgenic *Pvu*II under differing stress conditions of ethanol induction and high and medium light. The Col-0 groups are presented in green and the *Pvu*II groups in blue, with a darker shade indicating reduced light. Solid bars are uninduced groups and hatched bars are ethanol induced groups.

## 2.3 Results

### 2.3.1 The Pvu90P cut site confirms ethanol-induced reduction of relative copy number in transgenic Arabidopsis *Pvu*II mutants

In the Arabidopsis plants with a transgenically inserted *Pvu*II restriction enzyme in the nucleus with a mitochondrial-targeting peptide, successful cutting at *Pvu*II sites in the mitochondrial genome was shown using primers for the $90^{th}$ *Pvu*II location in the Arabidopsis mitochondrial genome, referred to as *Pvu*90P. This site is in a part of the genome with no coded genes or repeat sequences, removing any variable impacts those features may have on DNA repair after a DSB. The copy number of the sequence was calculated using the ΔΔCq calculation method for determining relative sequence amounts in both Col-0 control plants and *Pvu*II transgenic mutants (Livak and Schmittgen 2001). Each biological replicates' Cq mean had the replicates' Cq mean obtained from the housekeeping gene rrn18 subtracted from it, normalizing the resulting number to rrn18 and creating the ΔΔCq. Relative copy numbers were then expressed as 2^(-ΔΔCq). The control Col-0 group under normal conditions of high light was used as a baseline for comparing the copy number, equal to1.0000.

The copy numbers of the sequence region across the $90^{th}$ *Pvu*II cut site (Figure 5) showed little alteration between the Col-0 wild type in reduced light (1.0803), and transgenic *Pvu*II plants in high and reduced light without ethanol induction (1.1068 and 1.1487, respectively). There were observed expected decreases in the transgenic plants for the

group that only underwent ethanol induction (0.7248) and the group with ethanol induction in reduced light (0.7148). This indicated that the ethanol induction resulted in cutting at the *Pvu*II site that were not being fully repaired.

Unexpectedly, the ethanol induction group in Col-0 wild type plants also saw a significant decrease in relative copy number of the *Pvu*II cut site, decreasing to 0.6108, lower than even the transgenic plants. This decrease, however, was not consistently shown, as the ethanol induction in reduced light plants had a copy number that returned to approximately control levels (1.1733). Such a difference implies that ethanol exposure in wild type plants increases non-specific DNA damage that leads to increased ectopic recombination at Repeat L, but that this DNA damage only occurs at the higher light level, and not in reduced light.

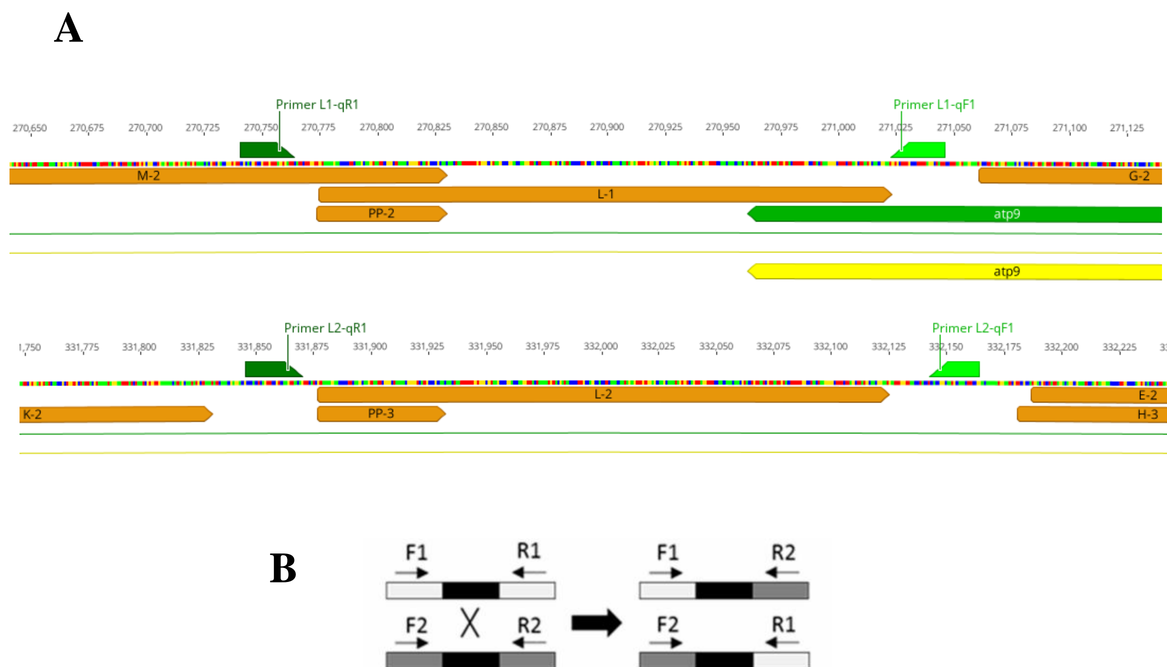


**Figure 6.** Locations of the L1 and L2 repeats and related qPCR primers in the BK010421 Arabidopsis genome accession. (A) The BK010421 Arabidopsis genome accession representation of the L1 repeat and its qPCR primer sequences, located from 270,741 bp to 271,046 bp in the accession, and the L2 repeat and its qPCR primer sequences, located from 331,846 bp to 332,164 bp in the accession. Other features include additional repeats in the region and the *atp9* gene partially included in the L1 repeat sequence.

(B) Representation of the primer scheme for showing parental sequences, F1-R1 and F2-R2, and the recombinant sequences, F1-R2 and F2-R1, at the L repeat sites

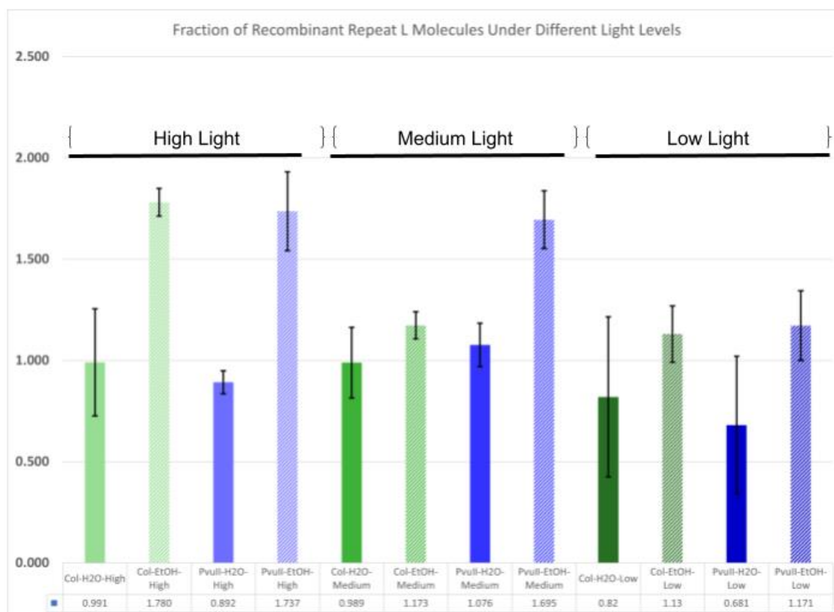


**Figure 7.** Fraction of recombinant Repeat L molecules under high light (167 µmol/m²/s ), medium light (96 µmol/m²/s ), and low light (49 µmol/m²/s). The fraction of recombinant molecules at the L Repeat site in the Arabidopsis mitochondrial genome in Col-0 wild type and transgenic *Pvu*II plants under ethanol induction and reduced light conditions, as compared to the amount of recombinants in the control set to an average of 1.000. The groups are divided into plant type, Col-0 or *Pvu*II, whether ethanol induction was conducted or not, shown as H2O or EtOH, and what level of light was applied, either high light, medium light, or low light. The Col-0 groups are presented in green and the *Pvu*II groups in blue, with a darker shade indicating reduced light. Solid bars are uninduced groups and hatched bars are ethanol induced groups.  Standard deviations from the three biological replicates included in each experimental group are represented by the included error bars.

### 2.3.2 The L Repeat region saw increased recombination after ethanol induction in reduced light

Prior work has shown that repeat L is a sensitive indicator of repair defects or increased DSBs (Shedge et al. 2007; Miller-Messmer et al. 2012; Wallet et al. 2015). For reasons that are still not understood, repeat L appears to be more sensitive to these effects than are other repeats. This may be due to its smaller size at 249 bp (Table 1), though there are many other repeats at this size and smaller. Other features of repeat L include several other repeats in and around the L1 and L2 repeat locations, along with a portion of the atp9 gene (Figure 6).

The fraction of recombinant configurations at repeat L (compared to the total copy number of parentals and repeats) was calculated by measuring the relative copy number of the two recombinant configurations and the two parental configurations, each normalized to the standard of the rrn18 gene. The sum of the relative copy numbers of the recombinants was divided by the total copy number of all configurations around repeat L, and multiplied by 100 to get a percent recombinants measurement. The percentage of these ectopic recombinants was taken as an indicator of double-strand breakage and repair. Both plant types, Col-0 and the transgenic *Pvu*II lines were used. Ethanol induction was compared to controls not treated with ethanol. Reduced light and cold stress were used in addition. Under reduced levels of light, ethanol treatment caused an increase in ectopic recombination in both Col-0 and the *Pvu*II transgenic line (Figure 7). This indicates that ethanol treatment alone, regardless of its effect in inducing *Pvu*II

synthesis, causes some amount of either DNA damage or impairment of DSBR. This was an unexpected result, and complicates all further controls.

Surprisingly, in the reduced light level from the shade cloth, ethanol treatment of Col-0 plants did not have this effect (Figure 7). The un-induced *Pvu*II lines had a similar fraction of recombinants at repeat L to the un-induced Col-0 group, and ethanol treatment caused an increase in the *Pvu*II lines (Figure 7). These lower light levels reflect the expected result that induction of *Pvu*II will induce higher levels of ectopic recombination, and this effect is not due to the reduced light, nor to the ethanol treatment itself. This result shows that under some environmental conditions ethanol induction of the *Pvu*II enzyme does produce double-strand breaks in the mitochondrial genome, and ectopic recombination at the 249bp repeat L is one outcome of that increased level of damage and repair.

An additional set of plants were tested under low light conditions at a light intensity of 49 $\mu mol/m^2/s$ . There was an observed increase in recombinant variation between biological replicates of the low light only group, as evidenced by the error bars in Figure 7. This may be due to the low light having a greater impact on photosynthesis capabilities of the plants and increasing individual variation due to other variables. Ethanol induction showed an increase in recombinants for both Col-0 wild type and transgenic *Pvu*II plants, as previously noted in the medium light experiment (Figure 7). But this accumulation in the ethanol and low light groups were greatly reduced for both Col-0 and *Pvu*II plants, only slightly above the controls. A complicating factor of the low light data was the large

standard deviations observed on the biological replicates and technical replicates due to low amounts of DNA amplification with qPCR that prevented statistically significant differences from being observed.

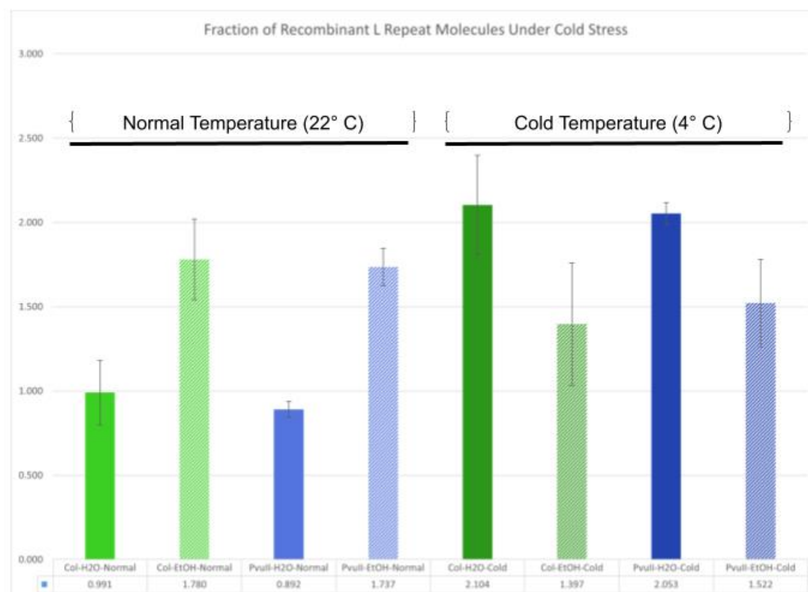


**Figure 8**. Fraction of recombinant Repeat L molecules under cold stress. The fraction of recombinant molecules at the L Repeat site in the Arabidopsis mitochondrial genome in Col-0 wild type and transgenic *Pvu*II plants under ethanol induction and cold stress conditions, as compared to the amount of recombinants in the wild type control. The groups are divided into plant type, Col-0 or *Pvu*II, whether ethanol induction was conducted or not, shown as H2O or EtOH, and whether cold stress was applied, labeled as cold or normal temperatures. The Col-0 groups are presented in green and the PvuII groups in blue, with a darker shade indicating cold temperature application. Solid bars are uninduced groups and hatched bars are ethanol induced groups. Standard deviations from the three biological replicates included in each experimental group are represented by the included error bars.

### 2.3.3 Recombination at the L repeat region increased after cold stress as compared to ethanol exposure

Further testing of the L repeat region and its sensitivity to DSBs was conducted by using a cold room set to 4 degrees Celsius. Under conditions of cold stress, ectopic recombination was observed to increase in both the Col-0 wild type and *Pvu*II transgenic lines (Figure 8). This occurred in cold stress only conditions and in ethanol induction with cold stress, with both plant types seeing increases similar to each other. Cold stress had a recombinant percent average of 2.104 in the Col-0 plants and 2.053 in the *Pvu*II plants, This fraction decreased in both plant types when ethanol was applied, the mock induction in Col-0 having an average of 1.397 and the ethanol induction in *Pvu*II having an average of 1.522. These results were less than those for ethanol only, with those averages being 1.780 and 1.737 for the Col-0 and PvuII plants, respectively.

The effect of cold stress only creating a greater accumulation of recombinants at repeat L as compared to the data for ethanol induction with and without reduced light (Figure 8) is an unexpected result. However, the increase being seen in both the wild type and transgenic plants to an almost identical level shows that cold stress appears to cause recombinant formation, which is reduced when ethanol is applied. The temperature condition is seen to have an impact on DNA repair in general and that recombination at the repeat L sites is hypothesized to be a result of the effect that cold has on mitochondrial repair mechanisms and their functionality for accurate repair.

| ANOVA - High Light | | | | | | |
|---|---|---|---|---|---|---|
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 5.44E-06 | 1 | 5.44E-06 | 1.213434 | 0.302688 | 5.317655 |
| Treatment | 0.000141 | 1 | 0.000141 | 31.40475 | 0.000508 | 5.317655 |
| Interaction | 3.33E-06 | 1 | 3.33E-06 | 0.743343 | 0.413688 | 5.317655 |
| Within | 3.59E-05 | 8 | 4.48E-06 | | | |
| Total | 0.000185 | 11 | | | | |

**Table 2**. ANOVA analysis of wild type and transgenic plants in high light (167 µmol/m²/s) conditions

| ANOVA - Medium Light | | | | | | |
|---|---|---|---|---|---|---|
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 2.69E-05 | 1 | 2.69E-05 | 9.597621 | 0.01471 | 5.317655 |
| Treatment | 4.58E-05 | 1 | 4.58E-05 | 16.31574 | 0.00374 | 5.317655 |
| Interaction | 1.56E-05 | 1 | 1.56E-05 | 5.551656 | 0.046232 | 5.317655 |
| Within | 2.25E-05 | 8 | 2.81E-06 | | | |
| Total | 0.000111 | 11 | | | | |

**Table 3**. ANOVA analysis of wild type and transgenic plants in medium light (96 μmol/m²/s) conditions

| ANOVA - Low Light | | | | | | |
|---|---|---|---|---|---|---|
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 0.00176 | 1 | 0.00176 | 0.059984 | 0.812686 | 5.317655 |
| Treatment | 0.117458 | 1 | 0.117458 | 4.003429 | 0.080409 | 5.317655 |
| Interaction | 0.005907 | 1 | 0.005907 | 0.201333 | 0.665552 | 5.317655 |
| Within | 0.234714 | 8 | 0.029339 | | | |
| Total | 0.359838 | 11 | | | | |

**Table 4**. ANOVA analysis of wild type and transgenic plants in low light (49 µmol/m²/s) conditions

| ANOVA - Cold Temps | | | | | | |
|---|---|---|---|---|---|---|
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 6.35E-06 | 1 | 6.35E-06 | 1.295621 | 0.287952 | 5.317655 |
| Treatment | 0.000356 | 1 | 0.000356 | 72.6104 | 2.76E-05 | 5.317655 |
| Interaction | 2.68E-06 | 1 | 2.68E-06 | 0.546936 | 0.480708 | 5.317655 |
| Within | 3.92E-05 | 8 | 4.9E-06 | | | |
| Total | 0.000404 | 11 | | | | |

**Table 5**. ANOVA analysis of wild type and transgenic plants in cold temperature (4° C) conditions without ethanol induction

| ANOVA - Ethanol In Cold | | | | | | |
|---|---|---|---|---|---|---|
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 6.88E-07 | 1 | 6.88E-07 | 0.065616 | 0.804294 | 5.317655 |
| Treatment | 1.38E-05 | 1 | 1.38E-05 | 1.315438 | 0.284555 | 5.317655 |
| Interaction | 1.79E-06 | 1 | 1.79E-06 | 0.170276 | 0.690704 | 5.317655 |
| Within | 8.39E-05 | 8 | 1.05E-05 | | | |
| Total | 0.0001 | 11 | | | | |

**Table 6**. ANOVA analysis of wild type and transgenic plants in cold temperature (4° C) conditions with ethanol induction

### 2.3.4 Two-way ANOVA analysis on the effects of light level and cold stress on recombination with and without ethanol induction

A two-way ANOVA analysis was used to compare the levels of repeat L recombination in the conditions of high, medium, and low light levels, along with cold temperatures. The results are shown in Tables 2 through 6. The variables assessed were differences between the genotypes of Col-0 wild type plants and the transgenic *Pvu*II plants, the differences between the induced and uninduced plants, the light level or temperature and interaction between induction and those stresses that influenced the recombination outcomes. The significance of the comparison was determined by reaching a p-value below 0.05.

Each level of light was compared between Col-0 and *Pvu*II plants to determine if ethanol induction had a significant difference in percent recombination. At high light (Table 2), there was no observed difference due to genotype (P-value: 0.303); both genotypes were affected by ethanol treatment to the same degree and no interaction was observed between genotype and treatment (P-value: 0.414). The ethanol treatment itself, however, had a significant difference in percent recombination (P-value: $5.08 \times 10^{-4}$) compared to the untreated plants, indicating that ethanol stress alone caused an increase in recombination events and not to a lesser degree than *Pvu*II cutting in the transgenic lines. Therefore, the impact of cuts in the transgenic lines cannot be distinguished as having an effect in the high light group beyond the stress of ethanol itself.

At medium light (Table 3), significance was observed in all factors. The impact of plant genotype and the change in percent recombination was significant (P-value: 0.015), showing that transgenic *Pvu*II plants have an increase in percent recombination that was not observed in the wild type. Similarly, ethanol induction produced a significant increase (P-value: $3.74 \times 10^{-3}$) in the *Pvu*II plants as compared to water, which did not occur in the Col-0 plants. This difference in interaction between plant genotype and ethanol induced state was significant (P-value: 0.046), indicating that the type of plant and the use of ethanol were connected variables under medium light conditions. Unlike in high light, *Pvu*II cutting did result in an increase in recombination and the effect of ethanol stress as an extra variable was not shown in the wild type under medium light conditions.

At low light (Table 4), the amount of individual variance among biological replicates and low DNA amplification resulting in high Cq means made analysis of significance difficult. There was no observed significant difference between genotype, treatment, or an interaction between the two variables (P-values: 0.813, 0.08, and 0.666, respectively) that was observed. The reduction of impact of ethanol treatment under low light conditions being similar to control groups cannot be confirmed due to this variance, so while it is possible that low light does reduce recombinant accumulation due to lowering ATP synthesis rates, the current data do not support this hypothesis.

For cold conditions, the experimental groups were analyzed with two-way ANOVA for application of cold stress only and for application of ethanol induction during cold stress as separate measurements, in order to better visualize the impact that cold has on percent recombination at the L repeat. In warm or cold temperatures only with no ethanol induction (Table 5), there were no significant differences observed between genotypes (P-value: 0.288), as the cold was shown to have similar increases in recombination for both the wild type and transgenic plants. The use of cold treatment, however, was found to be extremely significant (P-value: $2.76 \times 10^{-5}$), indicating that cold alone heavily impacts the amount of recombination at repeat L as compared to regular growth chamber temperatures. No interaction was observed between plant type and the effects of cold temperatures (P-value: 0.481).

In warm or cold temperatures with ethanol induction (Table 6), there were no significant differences observed regardless of genotype, ethanol induction, or any interaction between those variables (P-values: 0.804, 0.285, and 0.691, respectively). This indicates that when under cold conditions, the use of ethanol has no significant difference for wild type or transgenic plants with *Pvu*II induced cutting and only the application of cold treatment is significant to how the DSBR pathways are affected. Recombination under cold conditions is increased regardless of additional DSBs or general DNA damage that is applied, at least under the methods used for this experiment.

## 2.4 Discussion

**Ectopic recombination rate was hypothesized to be altered after PvuII expression and mitochondrial repair of DSBs:** The use of a transgenic *Pvu*II restriction enzyme that acted on the Arabidopsis mitochondrial genome was expected to cause DSBs that resulted in a higher ectopic recombination rate between non-tandem repeat sequences, even when there was not a *Pvu*II site in a repeat sequence. How accurate and robust the DSB repair mechanisms are and how frequently recombinants would be formed was unknown. The hypothesis included the possibility of a highly robust repair system correcting all DSB damage without forming recombinants even under additional stress responses. In such an event, quantitative analysis would reveal no change in the amount of recombinant sequences observed as compared to wild type controls. Preliminary data in previous experiments had revealed no phenotype (Figure 4; M. Bonnett and A. Christensen, unpublished data, 2016-2017) and only after drought and crowding were applied as additional stresses was a phenotype observed (M. Bonnett and F. Cassidy, unpublished data, 2017). Induction of *Pvu*II did not appear to lead to detectable rearrangements, as assayed by Illumina sequencing (E. Kahlandt and A. Christensen, unpublished data, 2017). This could be because cutting is inefficient in the transgenic plants or repair is very efficient or both. This leads to the first aims of confirming the cutting activity of *Pvu*II in transformed plants and to the following aims of examining the effects of those cuts.

**Confirmation of PvuII activity at expected cut sites:** A possible explanation for the prior lack of a phenotype and detectable rearrangements after induction of the *Pvu*II restriction enzyme is that its cutting activity was impaired and not occurring. Despite previous testing confirming the insertion of the gene cassette into the plant nuclear genome using the antibiotic resistance selectable marker, there was the potential for the mitochondrially targeted peptide to not direct the restriction enzyme to the mitochondrial genome or for there to be some other obstruction preventing cutting at the *Pvu*II targeted nucleotide sequences. In addition, the previous phenotype observed when crowding and drought were added to the induction suggested that the enzyme was cutting, but that repair was surprisingly robust unless the plants were also stressed. However, testing for copy number at the $90^{th}$ *Pvu*II cut site presented a decrease in observed copy number under ethanol induction conditions and not in the non-ethanol groups, suggesting that the restriction enzyme was actively causing DSBs.

**The fraction of recombinant sequences increased at the L repeat under ethanol induction:** When measuring the amount of recombinant sequences at the L repeat against parental sequences, the transgenic plants under ethanol induction of the *Pvu*II enzyme revealed a higher fraction of ectopic recombinants than in the non-induced controls, showing that the recombinant sequences were accumulating in these experimental groups. This was true for the group with ethanol induction only and the group with ethanol induction and reduced light, though the plants undergoing induction and reduced light had a larger standard deviation in this fraction of recombinants among the biological

replicates than in the plants only under ethanol induction. This may imply that reduced photosynthetic activity has a broader range of impact on the plants that can create a fraction of recombinants with different amounts of recombinant accumulation among individual biological replicates than ethanol induction causes alone, depending on individual plant responses and other factors influencing DNA repair mechanisms.

**The wild type under ethanol induction had an increase in recombinants, but not under reduced light:** An outlier that remained consistent within the wild type group under ethanol induction in the Pvu90P experiment and the L repeat experiments was the copy number and amount of recombinants, respectively, being close to the results for the transgenic *Pvu*II plants under ethanol induction than the non-induced controls. Furthermore, the wild type Col-0 had this decrease in copy number at the Pvu90P cut site and an increase in accumulated recombinants at the L repeat when exposed to ethanol, but this difference disappeared under medium and low light. A potential explanation for this is that ethanol treatment is in itself a stress that causes reactive oxygen species (ROS) formation which then creates random DNA damage throughout the mitochondrial genome. The decrease in copy number at the $90^{th}$ *Pvu*II site supports this hypothesis because random damage would also occur at this site and the increase in the ethanol induction and medium light could be due to the lowered photosynthetic activity producing fewer ROS and causing less DNA damage. The fraction of recombinants would also be affected, as less DNA damage at the L repeat sites would cause less recombination to occur during repair, causing less recombinant sequences to be formed.

**Cold stress increased recombinants at repeat L in the wild type and transgenic plants without ethanol induction**: When cold stress was applied to the plants, there was an increase in recombination at the repeat L sites for both wild type Col-0 and transgenic *Pvu*II plants, having a recombination percentage exceeding what was seen with ethanol induction only and with ethanol induction applied alongside cold stress. Because cold temperatures reduce ATP synthesis and the availability of ATP for DNA repair processes, we hypothesize that DSBR functionality is impacted and inaccurate repair of normal DNA damage results in increased recombination events under cold temperatures at 4° C as compared to normal conditions at 22° C. However, the addition of ethanol induction, both as a stress for the wild type and as an activator of *Pvu*II cutting in the transgenic lines, appears to not have a significant effect on recombination that is measurable beyond the effect that cold temperatures were observed to have.

# CHAPTER 3: OVERALL CONCLUSIONS AND POSSIBLE FUTURE DIRECTIONS

The effect of stress on recombination during DSBR, both in wild type Col-0 plants and transgenic *Pvu*II plants with inducible DSB cutting, is an important facet to investigating how DNA damage is repaired in plant mitochondrial genomes and how altering the normal growth conditions can change how that repair occurs and the accumulation of recombinant sequences. The use of light stress, high and low, has helped refine our hypotheses on how photosynthetic mechanisms and ATP production will impact the capability of plant mitochondria to continue their repair pathways even while under non-optimal conditions, providing a not entirely expected result of how high light stress increases recombination after DSBs caused by *Pvu*II cutting and that as light is reduced, the amount of recombinants formed goes down as well. This has in its own turn also provided insight into how the use of ethanol as an inducible mechanism can have unintended consequences in wild type plants due to acting as its own source of potential stress and DSB formation. The application of cold stress, meanwhile, has indicated that cold by itself, without the application of ethanol, creates an increase in recombinant accumulation. This possibly occurs through inhibition of cellular processes and lowering the activity rate of enzymes, including those involved in DSBR, resulting in a higher rate of inaccurate repair that forms recombinants instead.

The creation of DSBs in the Arabidopsis mitochondrial genome has been shown through past experimentation to not create a phenotype or measurable rearrangements in the genome through this damage alone. Only after the application of additional stress factors alongside causing DSBs do phenotypic changes occur, as previously shown through the use of drought and crowding stresses (M. Bonnett, E. Kahlandt, and F. Cassidy, unpublished data, 2017-2018). This raised the questions of how robust the repair system is in the face of PvuII digestion, and the effect of stress on that robustness. Altered environmental conditions studied included reduction of photosynthetic activity and the subsequent reduction in energy and carbon availability.

Other forms of stress are available that would impact the plant growth and development in varying ways. While cold temperatures have been investigated here, the effect of heat stress and activation of heat shock proteins have not. It is known that heat shock and thermotolerance proteins have connections to activating DNA repair responses (Han et al. 2020) and so testing with high heat may result in a stronger DSBR response than under other conditions. Using deficient mutants for heat shock genes may allow for a greater understanding of DNA repair responses under higher temperatures. Outside of temperature, changes to the chemical uptake of the plants can also play a role in altering DNA repair pathways. Recent research has shown that excess salinity can cause oxidative stress that in turn forms DSBs, along with changes to the cell cycle and nuclear endoreduplication (Mahapatra and Roy 2021). Genotoxic chemical application, such as hydrogen peroxide, salicylic acid, and camptothecin, have been observed for their DNA

damaging effects in the nuclear genome and the repair following removal of the harmful chemical application (O'brien et al. 1998), but their effects on the repair mechanisms in the mitochondrial genome have only begun to be studied. The use of heavy metals in the soil have also been shown to cause nuclear damage, such as with the application of cadmium (Fojtova 2002), and DNA repair and recovery observed after removal. Such metals have been shown to induce high production of ROS in plant mitochondria and increase DNA damage to the point of apoptosis (Bi et al. 2009).

An important conclusion from this work is that the transgenic *Pvu*II plants have a major flaw due to the use of ethanol as an induction medium. Ethanol induction is a form of stress itself and is likely to produce ROS that cause DNA damage, including in any wild type controls. This inherent source of damage makes comparing damage from *Pvu*II cutting difficult. Because of this, research in the Christensen lab has focused on developing an alternative series of transgenic *Pvu*II Arabidopsis plants without a ethanol inducible promoter. A different plasmid referred to as pMDC7 has been combined with the *Pvu*II gene cassette and features an estradiol inducible promoter. The human hormone estradiol can be applied directly to the plant's external tissues rather than being included in the watering solution that is taken up by the plant as a whole. The hormone only causes induction in treated cells and it is not transferred to other parts of the plant, meaning that it can be selectively applied to only particular leaves and only be expressed in those leaf cells. This would allow for comparisons to be made even within individual biological replicates, as leaf tissue that has been treated with estradiol can have its formation of

recombinants be contrasted against leaves on the same plant that have had not been treated, allowing for changes to be observed on the effects of DSBs and stress responses.

Beyond stress responses, other research has also focused on the components of the DSBR pathways in plant mitochondria and the extent of DNA repair that is possible before cell death occurs. Knockout mutants for individual genes both hypothesized and confirmed to be involved in DSBR have been tested for their impact on mutation and recombination rates, including MSH1 (Wu et al. 2020) and uracil-N-glycosylase (UNG) (Wynn et al. 2020). Crosses of these knockout mutants with the transgenic *Pvu*II insertion plants could yield further information on the DNA repair mechanisms and the limits and robustness of the system itself. Along with knockouts, chemical inhibition of genetic components of the repair pathways has the opportunity to present similar results, while also potentially identifying more genes and proteins involved in DSBR. Previous research (Wallet et al. 2015) has looked into the impact of chemicals such as ciprofloxacin, which inhibits DNA gyrase, and experiments using CRISPR-Cas9 in the Christensen lab have been successful in developing mutants as discussed that are lacking component genes for the mitochondrial repair system. Future experiments are planned to apply ciprofloxacin to those mutants in order to induce DSBs and measure repair phenotypes and responses (Campbell et al. 2020).

The components of the DSBR pathways can be looked at more broadly as well, focusing on particular pathways other than HR, such as NHEJ. The existence of this DNA repair pathway in plant mitochondria has yet to be definitively confirmed through experimental data, but there is some research that implies its existence. NHEJ is mediated in other organisms, including humans, by Ku proteins and their interactions with DNA (Fattah et al. 2010). Research into plant cells have found the existence of Ku-like proteins that appear to have similar activity as in metazoan cells, but this has only been investigated on a whole cell level and their activity within the plant mitochondria has not been confirmed (Tuteja and Tuteja 2000; Tamura et al. 2002). While direct analysis of activity within the plant mitochondria may be difficult, making confirming Ku-like protein targeting to the mitochondrial genome a multi-step endeavor, there are also new molecular compounds aimed at targeting the Ku-DNA interaction and preventing the binding from occurring for NHEJ mediation (Gavande et al. 2020). If applied to plant cells, it may be possible to see the effects of inhibiting NHEJ in plant mitochondria and indirectly confirm it as an active pathway of DSBR.

There are multiple avenues of research still available to be studied on DSBR in plant mitochondria and new opportunities are made an option every year as biotechnology and bioengineering expand as fields of interest. At the same time, there remain many mysteries about how DSBR pathways function and what genetic components are involved their mechanisms. Future experiments using the availability of stress testing, Agrobacterium insertions and CRISPR constructs, and nuclear and chloroplastic

molecules shuttled to the mitochondria will help to reveal the answers to these questions, including those that have yet to be thought of.

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## APPENDIX A: ABBREVIATIONS/DEFINITIONS

| | |
|---|---|
| Atp6 | Mitochondrial ATP synthase membrane subunit 6 gene |
| Atp9 | ATP synthase subunit 9, mitochondrial |
| BER | Base Excision Repair |
| Bp | Base pairs, 1 nucleotide base |
| CHM | Chloroplast mutator gene |
| CMS | Cytoplasmic male sterility |
| DPD1 | Defective In Pollen Organelle DNA Degradation gene |
| DSB | Double strand break |
| DSBR | Double strand break repair |
| dsDNA | Double-stranded DNA |
| FPG/NEI | Formamidopyramidine glycosylase/endonuclease VIII |
| FRIENDLY: | Arabidopsis mutant (AT3G52140.4) with irregular mitochondrial fusion |
| GFP | Green Fluorescent Protein |
| HR | Homologous Recombination |
| Kb | Kilobases, 1,000 nucleotide bases |
| M20 | 20-kiloDalton Mitochondrial endonuclease in maize, *AtM20* as *Arabidopsis* homologue |
| Mb | Megabases, 1,000,000 nucleotide bases |
| $Mg^{2+}$ | Magnesium 2+ ion |

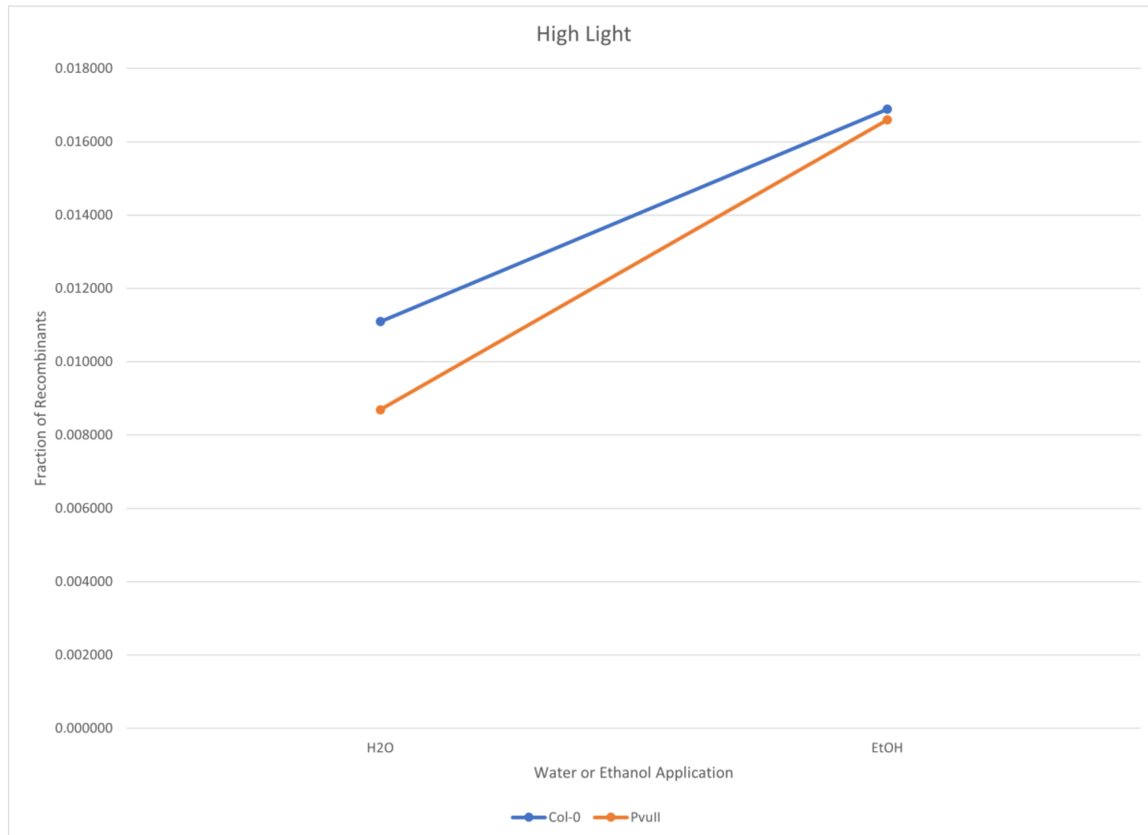
| | |
|---|---|
| MMEJ | Microhomology-mediated end joining |
| MMR | Mismatch Repair |
| MSH1 | Mutator S Homologue 1, plant mitochondrial gene |
| mtDNA | Mitochondrial DNA |
| MutH | Mutator H, DNA mismatch repair endonuclease gene |
| MutL | Mutator L, ATP-dependent DNA mismatch repair gene |
| MutS | Mutator S, DNA mismatch repair gene |
| NER | Nucleotide excision repair |
| NHEJ | Non-homologous end joining |
| ODB1 | Organellar DNA-Binding protein 1 |
| OGG1 | 8-oxoguanine glycosylase 1, excises oxidated guanine bases |
| OSB | Organellar single-stranded binding proteins |
| POL1B | DNA-directed DNA polymerase |
| PPR | Pentatricopeptide repeat proteins |
| qPCR | Quantitative Polymerase Chain Reaction |
| RADA | Radiation Sensitive A, DNA-dependent ATPase involved in DNA break repair |
| RAD52 | Radiation Sensitive 52, homologous recombination-related protein |
| RecA | DNA Recombinase A, homologous recombination repair gene |
| RECA3 | Recombinase A Homologue 3 protein |
| RECG/1 | DNA Recombinase G, ATP-dependent DNA helicase |
| RECX | DNA Recombinase X, homologous recombination transcription factor |

| | |
|---|---|
| Rf | Restorer-of-fertility genes |
| ROUS | Repeats of Unusual Size |
| rRNA | Ribosomal RNA |
| RuvA/B/C | Holliday junction ATP-dependent DNA helicase complex |
| ssb | Single-stranded binding |
| ssDNA | Single-stranded DNA |
| TFAM | Mitochondrial transcription factor A |
| tRNA | Transfer RNA |
| TWINKLE | Mitochondrial DNA helicase |
| UNG/UDG | Uracil-DNA-glycosylase, corrects for deaminations and uracil-forming processes |
| UV | Ultraviolet light |
| WHY2 | Whirly 2, single-stranded DNA binding protein |
| YqgF | rRNA nuclease, structurally similar to RuvC |

# APPENDIX B: SUPPLEMENTARY ANOVA DATA AND FIGURES

| Anova: Two-Factor With Replication | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| SUMMARY | H2O | EtOH | Total | | | |
| *Col-0* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 0.033279 | 0.050669 | 0.083948 | | | |
| Average | 0.011093 | 0.01689 | 0.013991 | | | |
| Variance | 5.54E-06 | 8.59E-06 | 1.57E-05 | | | |
| | | | | | | |
| *PvuII* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 0.026077 | 0.049791 | 0.075868 | | | |
| Average | 0.008692 | 0.016597 | 0.012645 | | | |
| Variance | 3.38E-07 | 3.46E-06 | 2.03E-05 | | | |
| | | | | | | |
| *Total* | | | | | | |
| Count | 6 | 6 | | | | |
| Sum | 0.059356 | 0.100461 | | | | |
| Average | 0.009893 | 0.016743 | | | | |
| Variance | 4.08E-06 | 4.85E-06 | | | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 5.44E-06 | 1 | 5.44E-06 | 1.213434 | 0.302688 | 5.317655 |
| Treatment | 0.000141 | 1 | 0.000141 | 31.40475 | 0.000508 | 5.317655 |
| Interaction | 3.33E-06 | 1 | 3.33E-06 | 0.743343 | 0.413688 | 5.317655 |
| Within | 3.59E-05 | 8 | 4.48E-06 | | | |
| Total | 0.000185 | 11 | | | | |
| | | | | | | |
| Fraction | H2O | EtOH | | | | |
| Col-0 | 0.011093 | 0.01689 | | | | |
| PvuII | 0.008692 | 0.016597 | | | | |

**Supplementary Table 1**. Two-way ANOVA analysis of Col-0 and *Pvu*II plant percent recombination data in high light conditions comparing with and without ethanol induction




**Supplementary Figure 1**. A graph depicting the fraction of recombinants from ANOVA analysis of high light data for Col-0 and *Pvu*II plants with and without ethanol induction.

Anova: Two-Factor With Replication

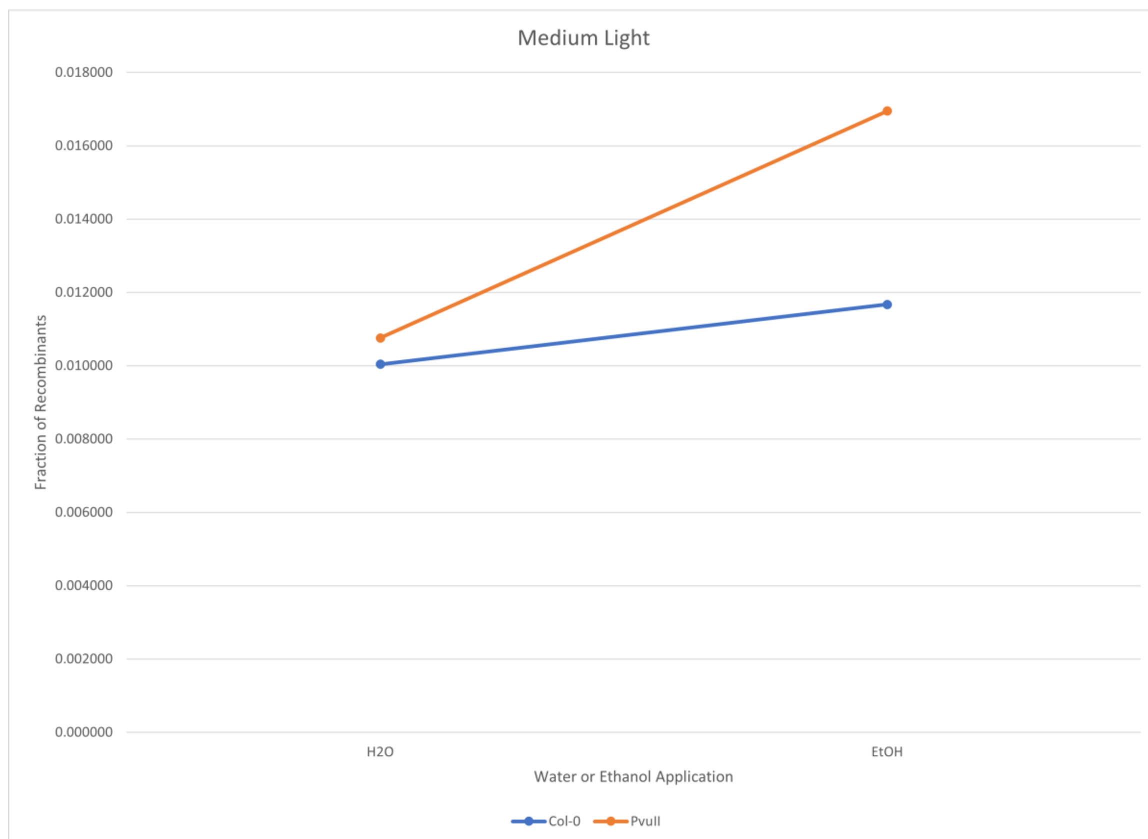
| SUMMARY | H2O | EtOH | Total |
|---|---|---|---|
| *Col-0* | | | |
| Count | 3 | 3 | 6 |
| Sum | 0.030128 | 0.035012 | 0.065141 |
| Average | 0.010043 | 0.011671 | 0.010857 |
| Variance | 6E-06 | 4.53E-07 | 3.38E-06 |
| *PvuII* | | | |
| Count | 3 | 3 | 6 |
| Sum | 0.032281 | 0.05084 | 0.083121 |
| Average | 0.01076 | 0.016947 | 0.013853 |
| Variance | 1.74E-06 | 3.04E-06 | 1.34E-05 |
| *Total* | | | |
| Count | 6 | 6 | |
| Sum | 0.062409 | 0.085852 | |
| Average | 0.010402 | 0.014309 | |
| Variance | 3.25E-06 | 9.75E-06 | |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Genotype | 2.69E-05 | 1 | 2.69E-05 | 9.597621 | 0.01471 | 5.317655 |
| Treatment | 4.58E-05 | 1 | 4.58E-05 | 16.31574 | 0.00374 | 5.317655 |
| Interaction | 1.56E-05 | 1 | 1.56E-05 | 5.551656 | 0.046232 | 5.317655 |
| Within | 2.25E-05 | 8 | 2.81E-06 | | | |
| Total | 0.000111 | 11 | | | | |

| Fraction | H2O | EtOH |
|---|---|---|
| Col-0 | 0.010043 | 0.011671 |
| PvuII | 0.01076 | 0.016947 |

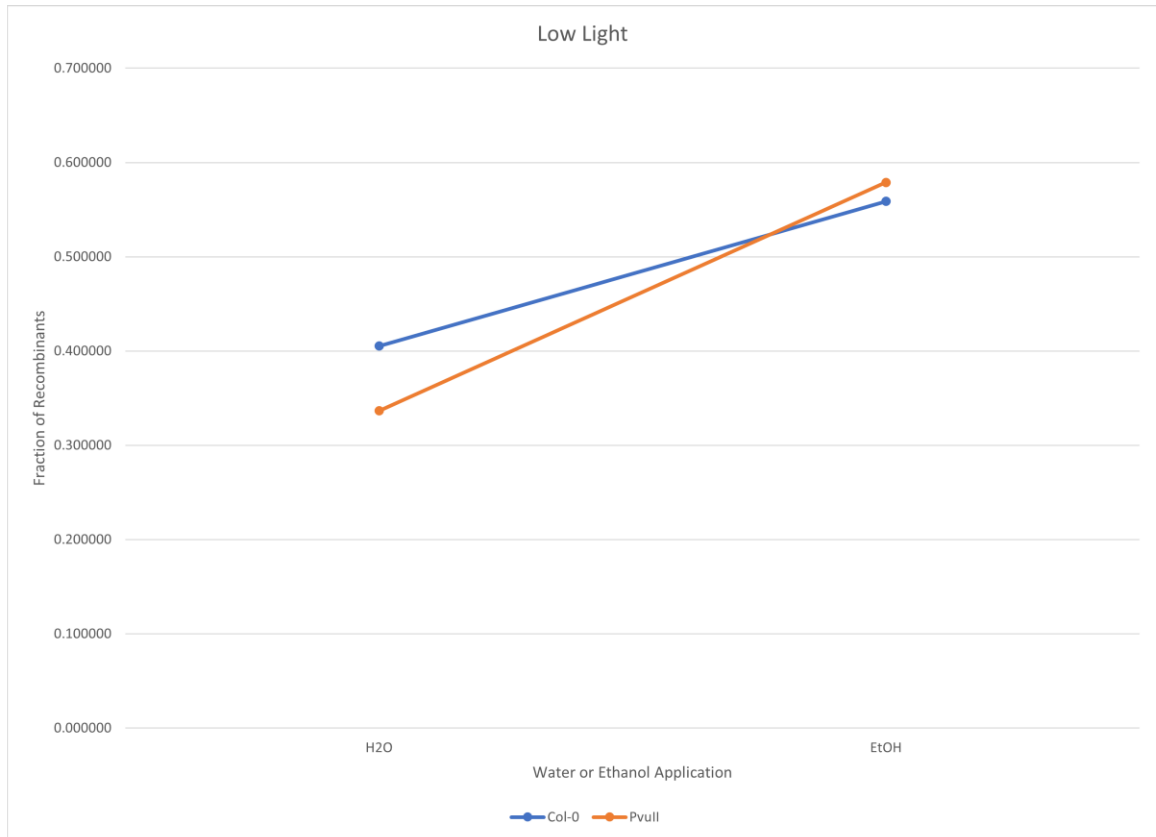
**Supplementary Table 2**. Two-way ANOVA analysis of Col-0 and *Pvu*II plant percent recombination data in medium light conditions comparing with and without ethanol induction




**Supplementary Figure 2**. A graph depicting the fraction of recombinants from ANOVA analysis of medium light data for Col-0 and *Pvu*II plants with and without ethanol induction.

| Anova: Two-Factor With Replication | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| SUMMARY | H2O | EtOH | Total | | | |
| *Col-0* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 1.215611 | 1.676101 | 2.891712 | | | |
| Average | 0.405204 | 0.5587 | 0.481952 | | | |
| Variance | 0.057024 | 0.007075 | 0.032708 | | | |
| | | | | | | |
| *PvuII* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 1.00983 | 1.73656 | 2.746389 | | | |
| Average | 0.33661 | 0.578853 | 0.457732 | | | |
| Variance | 0.042389 | 0.010869 | 0.038908 | | | |
| | | | | | | |
| *Total* | | | | | | |
| Count | 6 | 6 | | | | |
| Sum | 2.225441 | 3.412661 | | | | |
| Average | 0.370907 | 0.568777 | | | | |
| Variance | 0.041177 | 0.0073 | | | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 0.00176 | 1 | 0.00176 | 0.059984 | 0.812686 | 5.317655 |
| Treatment | 0.117458 | 1 | 0.117458 | 4.003429 | 0.080409 | 5.317655 |
| Interaction | 0.005907 | 1 | 0.005907 | 0.201333 | 0.665552 | 5.317655 |
| Within | 0.234714 | 8 | 0.029339 | | | |
| Total | 0.359838 | 11 | | | | |
| | | | | | | |
| Fraction | H2O | EtOH | | | | |
| Col-0 | 0.405204 | 0.5587 | | | | |
| PvuII | 0.33661 | 0.578853 | | | | |

**Supplementary Table 3**. Two-way ANOVA analysis of Col-0 and *Pvu*II plant percent recombination data in low light conditions comparing with and without ethanol induction




**Supplementary Figure 3**. A graph depicting the fraction of recombinants from ANOVA analysis of low light data for Col-0 and *Pvu*II plants with and without ethanol induction.

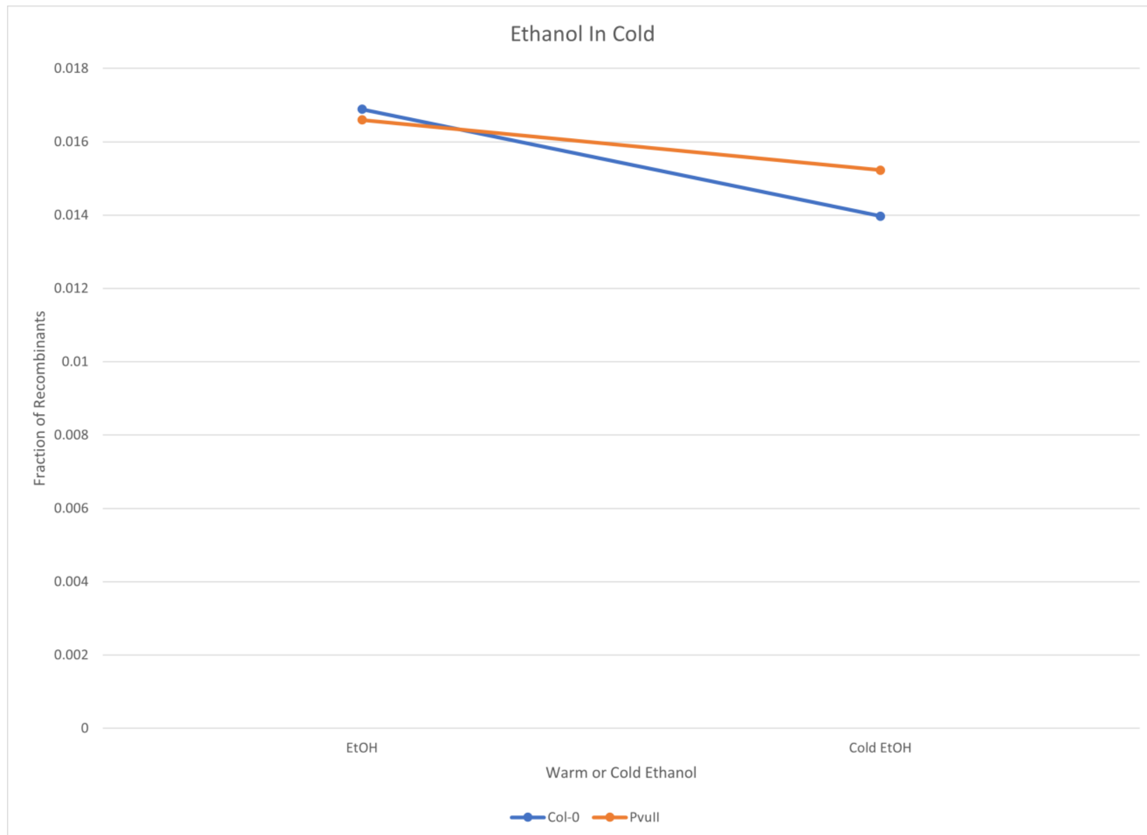
| Anova: Two-Factor With Replication | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| SUMMARY | H2O | Cold H2O | Total | | | |
| *Col-0* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 0.033279 | 0.063124 | 0.096403 | | | |
| Average | 0.011093 | 0.021041 | 0.016067 | | | |
| Variance | 5.54E-06 | 1.31E-05 | 3.71E-05 | | | |
| | | | | | | |
| *PvuII* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 0.026077 | 0.061595 | 0.087672 | | | |
| Average | 0.008692 | 0.020532 | 0.014612 | | | |
| Variance | 3.38E-07 | 6.34E-07 | 4.24E-05 | | | |
| | | | | | | |
| *Total* | | | | | | |
| Count | 6 | 6 | | | | |
| Sum | 0.059356 | 0.124718 | | | | |
| Average | 0.009893 | 0.020786 | | | | |
| Variance | 4.08E-06 | 5.57E-06 | | | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 6.35E-06 | 1 | 6.35E-06 | 1.295621 | 0.287952 | 5.317655 |
| Treatment | 0.000356 | 1 | 0.000356 | 72.6104 | 2.76E-05 | 5.317655 |
| Interaction | 2.68E-06 | 1 | 2.68E-06 | 0.546936 | 0.480708 | 5.317655 |
| Within | 3.92E-05 | 8 | 4.9E-06 | | | |
| Total | 0.000404 | 11 | | | | |
| | | | | | | |
| Fraction | H2O | Cold H2O | | | | |
| Col-0 | 0.011093 | 0.021041 | | | | |
| PvuII | 0.008692 | 0.020532 | | | | |

**Supplementary Table 4**. Two-way ANOVA analysis of Col-0 and *Pvu*II plant percent recombination data in warm and cold temperatures without ethanol induction

**Supplementary Figure 4**. A graph depicting the fraction of recombinants from ANOVA analysis of warm and cold temperature data for Col-0 and *Pvu*II plants without ethanol induction

| Anova: Two-Factor With Replication | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| SUMMARY | EtOH | Cold EtOH | Total | | | |
| *Col-0* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 0.050669 | 0.04192 | 0.092589 | | | |
| Average | 0.01689 | 0.013973 | 0.015432 | | | |
| Variance | 8.59E-06 | 1.98E-05 | 1.39E-05 | | | |
| | | | | | | |
| *PvuII* | | | | | | |
| Count | 3 | 3 | 6 | | | |
| Sum | 0.049791 | 0.045672 | 0.095463 | | | |
| Average | 0.016597 | 0.015224 | 0.015911 | | | |
| Variance | 3.46E-06 | 1.01E-05 | 6E-06 | | | |
| | | | | | | |
| *Total* | | | | | | |
| Count | 6 | 6 | | | | |
| Sum | 0.100461 | 0.087592 | | | | |
| Average | 0.016743 | 0.014599 | | | | |
| Variance | 4.85E-06 | 1.24E-05 | | | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Genotype | 6.88E-07 | 1 | 6.88E-07 | 0.065616 | 0.804294 | 5.317655 |
| Treatment | 1.38E-05 | 1 | 1.38E-05 | 1.315438 | 0.284555 | 5.317655 |
| Interaction | 1.79E-06 | 1 | 1.79E-06 | 0.170276 | 0.690704 | 5.317655 |
| Within | 8.39E-05 | 8 | 1.05E-05 | | | |
| Total | 0.0001 | 11 | | | | |
| | | | | | | |
| Fraction | EtOH | Cold EtOH | | | | |
| Col-0 | 0.01689 | 0.013973 | | | | |
| PvuII | 0.016597 | 0.015224 | | | | |

**Supplementary Table 5**. Two-way ANOVA analysis of Col-0 and *Pvu*II plant percent recombination data in warm and cold temperatures with ethanol induction




**Supplementary Figure 5**. A graph depicting the fraction of recombinants from ANOVA analysis of warm and cold temperature data for Col-0 and *Pvu*II plants with ethanol induction

# APPENDIX C: RAW LIGHT LEVEL AND COLD TEMPERATURE qPCR DATA

| Target | Primers | Sample | Plant | Replicate # | EtOH or H2O | Stress or No | Cq Mean | Cq Std. Dev |
|---|---|---|---|---|---|---|---|---|
| L1F - L2R | 1. Recomb 12 | Control Col 1 | Col-0 | 1 | H2O | Normal | 27.73 | 0.06 |
| L2F - L1R | 2. Recomb 21 | Control Col 1 | Col-0 | 1 | H2O | Normal | 31.48 | 0.65 |
| L1 F-R | 3. Parent 11 | Control Col 1 | Col-0 | 1 | H2O | Normal | 21.67 | 0.09 |
| L2 F-R | 4. Parent 22 | Control Col 1 | Col-0 | 1 | H2O | Normal | 21.83 | 0.11 |
| rrn18 F-R | 5. rrn18 | Control Col 1 | Col-0 | 1 | H2O | Normal | 22.64 | 0.10 |
| L1F - L2R | 1. Recomb 12 | Control Col 2 | Col-0 | 2 | H2O | Normal | 26.86 | 0.08 |
| L2F - L1R | 2. Recomb 21 | Control Col 2 | Col-0 | 2 | H2O | Normal | 30.69 | 0.07 |
| L1 F-R | 3. Parent 11 | Control Col 2 | Col-0 | 2 | H2O | Normal | 21.41 | 0.27 |
| L2 F-R | 4. Parent 22 | Control Col 2 | Col-0 | 2 | H2O | Normal | 21.38 | 0.05 |
| rrn18 F-R | 5. rrn18 | Control Col 2 | Col-0 | 2 | H2O | Normal | 22.34 | 0.08 |
| L1F - L2R | 1. Recomb 12 | Control Col 3 | Col-0 | 3 | H2O | Normal | 26.71 | 0.04 |
| L2F - L1R | 2. Recomb 21 | Control Col 3 | Col-0 | 3 | H2O | Normal | 30.92 | 0.33 |
| L1 F-R | 3. Parent 11 | Control Col 3 | Col-0 | 3 | H2O | Normal | 21.21 | 0.13 |
| L2 F-R | 4. Parent 22 | Control Col 3 | Col-0 | 3 | H2O | Normal | 21.56 | 0.11 |
| rrn18 F-R | 5. rrn18 | Control Col 3 | Col-0 | 3 | H2O | Normal | 22.54 | 0.15 |
| L1F - L2R | 1. Recomb 12 | Medium Only Col 1 | Col-0 | 1 | H2O | Medium Light | 26.96 | 0.32 |
| L2F - L1R | 2. Recomb 21 | Medium Only Col 1 | Col-0 | 1 | H2O | Medium Light | 29.82 | 0.33 |
| L1 F-R | 3. Parent 11 | Medium Only Col 1 | Col-0 | 1 | H2O | Medium Light | 21.41 | 0.42 |
| L2 F-R | 4. Parent 22 | Medium Only Col 1 | Col-0 | 1 | H2O | Medium Light | 21.61 | 0.13 |
| rrn18 F-R | 5. rrn18 | Medium Only Col 1 | Col-0 | 1 | H2O | Medium Light | 22.15 | 0.07 |
| L1F - L2R | 1. Recomb 12 | Medium Only Col 2 | Col-0 | 2 | H2O | Medium Light | 28.22 | 0.23 |
| L2F - L1R | 2. Recomb 21 | Medium Only Col 2 | Col-0 | 2 | H2O | Medium Light | 30.40 | 0.14 |
| L1 F-R | 3. Parent 11 | Medium Only Col 2 | Col-0 | 2 | H2O | Medium Light | 21.85 | 0.13 |
| L2 F-R | 4. Parent 22 | Medium Only Col 2 | Col-0 | 2 | H2O | Medium Light | 22.19 | 0.19 |
| rrn18 F-R | 5. rrn18 | Medium Only Col 2 | Col-0 | 2 | H2O | Medium Light | 22.65 | 0.18 |
| L1F - L2R | 1. Recomb 12 | Medium Only Col 3 | Col-0 | 3 | H2O | Medium Light | 28.01 | 0.13 |
| L2F - L1R | 2. Recomb 21 | Medium Only Col 3 | Col-0 | 3 | H2O | Medium Light | 30.60 | 0.33 |
| L1 F-R | 3. Parent 11 | Medium Only Col 3 | Col-0 | 3 | H2O | Medium Light | 21.94 | 0.24 |
| L2 F-R | 4. Parent 22 | Medium Only Col 3 | Col-0 | 3 | H2O | Medium Light | 22.12 | 0.15 |
| rrn18 F-R | 5. rrn18 | Medium Only Col 3 | Col-0 | 3 | H2O | Medium Light | 22.43 | 0.07 |
| L1F - L2R | 1. Recomb 12 | Low Only Col 1 | Col-0 | 1 | H2O | Low Light | 41.38 | 2.29 |
| L2F - L1R | 2. Recomb 21 | Low Only Col 1 | Col-0 | 1 | H2O | Low Light | 37.52 | 0.66 |
| L1 F-R | 3. Parent 11 | Low Only Col 1 | Col-0 | 1 | H2O | Low Light | 39.42 | 7.74 |
| L2 F-R | 4. Parent 22 | Low Only Col 1 | Col-0 | 1 | H2O | Low Light | 39.33 | 1.14 |
| rrn18 | 5. rrn18 | Low Only Col 1 | Col-0 | 1 | H2O | Low Light | 37.25 | 2.49 |
| L1F - L2R | 1. Recomb 12 | Low Only Col 2 | Col-0 | 2 | H2O | Low Light | 40.53 | 2.29 |
| L2F - L1R | 2. Recomb 21 | Low Only Col 2 | Col-0 | 2 | H2O | Low Light | 38.22 | 0.65 |
| L1 F-R | 3. Parent 11 | Low Only Col 2 | Col-0 | 2 | H2O | Low Light | 36.82 | 0.35 |
| L2 F-R | 4. Parent 22 | Low Only Col 2 | Col-0 | 2 | H2O | Low Light | 36.81 | 0.58 |
| rrn18 | 5. rrn18 | Low Only Col 2 | Col-0 | 2 | H2O | Low Light | 38.00 | 4.39 |
| L1F - L2R | 1. Recomb 12 | Low Only Col 3 | Col-0 | 3 | H2O | Low Light | 40.86 | 0.44 |
| L2F - L1R | 2. Recomb 21 | Low Only Col 3 | Col-0 | 3 | H2O | Low Light | 35.43 | 1.15 |
| L1 F-R | 3. Parent 11 | Low Only Col 3 | Col-0 | 3 | H2O | Low Light | 37.40 | 0.81 |
| L2 F-R | 4. Parent 22 | Low Only Col 3 | Col-0 | 3 | H2O | Low Light | 34.87 | 0.53 |
| rrn18 | 5. rrn18 | Low Only Col 3 | Col-0 | 3 | H2O | Low Light | 41.18 | 4.58 |
| L1F - L2R | 1. Recomb 12 | Eth Only Col 1 | Col-0 | 1 | EtOH | Normal | 25.92 | 0.285 |
| L2F - L1R | 2. Recomb 21 | Eth Only Col 1 | Col-0 | 1 | EtOH | Normal | 29.57 | 0.439 |
| L1 F-R | 3. Parent 11 | Eth Only Col 1 | Col-0 | 1 | EtOH | Normal | 20.84 | 0.237 |
| L2 F-R | 4. Parent 22 | Eth Only Col 1 | Col-0 | 1 | EtOH | Normal | 20.62 | 0.174 |
| rrn18 | 5. rrn18 | Eth Only Col 1 | Col-0 | 1 | EtOH | Normal | 22.08 | 0.049 |
| L1F - L2R | 1. Recomb 12 | Eth Only Col 2 | Col-0 | 2 | EtOH | Normal | 26.79 | 0.074 |
| L2F - L1R | 2. Recomb 21 | Eth Only Col 2 | Col-0 | 2 | EtOH | Normal | 29.58 | 0.324 |
| L1 F-R | 3. Parent 11 | Eth Only Col 2 | Col-0 | 2 | EtOH | Normal | 21.68 | 0.084 |
| L2 F-R | 4. Parent 22 | Eth Only Col 2 | Col-0 | 2 | EtOH | Normal | 21.62 | 0.006 |
| rrn18 | 5. rrn18 | Eth Only Col 2 | Col-0 | 2 | EtOH | Normal | 22.84 | 0.049 |
| L1F - L2R | 1. Recomb 12 | Eth Only Col 3 | Col-0 | 3 | EtOH | Normal | 27.57 | 0.148 |
| L2F - L1R | 2. Recomb 21 | Eth Only Col 3 | Col-0 | 3 | EtOH | Normal | 30.87 | 0.756 |
| L1 F-R | 3. Parent 11 | Eth Only Col 3 | Col-0 | 3 | EtOH | Normal | 22.76 | 0.225 |
| L2 F-R | 4. Parent 22 | Eth Only Col 3 | Col-0 | 3 | EtOH | Normal | 22.9 | 0.161 |
| rrn18 | 5. rrn18 | Eth Only Col 3 | Col-0 | 3 | EtOH | Normal | 23.18 | 0.092 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| L1F - L2R | 1. Recomb 12 | Medium Eth Col 1 | Col-0 | 1 EtOH | Medium Light | 27.56 | 0.36 |
| L2F - L1R | 2. Recomb 21 | Medium Eth Col 1 | Col-0 | 1 EtOH | Medium Light | 30.56 | 0.71 |
| L1 F-R | 3. Parent 11 | Medium Eth Col 1 | Col-0 | 1 EtOH | Medium Light | 21.62 | 0.04 |
| L2 F-R | 4. Parent 22 | Medium Eth Col 1 | Col-0 | 1 EtOH | Medium Light | 22.27 | 0.35 |
| rrn18 F-R | 5. rrn18 | Medium Eth Col 1 | Col-0 | 1 EtOH | Medium Light | 22.49 | 0.16 |
| L1F - L2R | 1. Recomb 12 | Medium Eth Col 2 | Col-0 | 2 EtOH | Medium Light | 27.83 | 0.217 |
| L2F - L1R | 2. Recomb 21 | Medium Eth Col 2 | Col-0 | 2 EtOH | Medium Light | 29.61 | 0.494 |
| L1 F-R | 3. Parent 11 | Medium Eth Col 2 | Col-0 | 2 EtOH | Medium Light | 21.63 | 0.225 |
| L2 F-R | 4. Parent 22 | Medium Eth Col 2 | Col-0 | 2 EtOH | Medium Light | 22.62 | 0.023 |
| rrn18 F-R | 5. rrn18 | Medium Eth Col 2 | Col-0 | 2 EtOH | Medium Light | 22.70 | 0.17 |
| L1F - L2R | 1. Recomb 12 | Medium Eth Col 3 | Col-0 | 3 EtOH | Medium Light | 27.56 | 0.08 |
| L2F - L1R | 2. Recomb 21 | Medium Eth Col 3 | Col-0 | 3 EtOH | Medium Light | 29.46 | 0.31 |
| L1 F-R | 3. Parent 11 | Medium Eth Col 3 | Col-0 | 3 EtOH | Medium Light | 21.49 | 0.11 |
| L2 F-R | 4. Parent 22 | Medium Eth Col 3 | Col-0 | 3 EtOH | Medium Light | 22.48 | 0.21 |
| rrn18 F-R | 5. rrn18 | Medium Eth Col 3 | Col-0 | 3 EtOH | Medium Light | 22.60 | 0.16 |
| L1F - L2R | 1. Recomb 12 | Low Eth Col 1 | Col-0 | 1 EtOH | Low Light | 43.51 | 5.47 |
| L2F - L1R | 2. Recomb 21 | Low Eth Col 1 | Col-0 | 1 EtOH | Low Light | 36.16 | 0.67 |
| L1 F-R | 3. Parent 11 | Low Eth Col 1 | Col-0 | 1 EtOH | Low Light | 40.38 | 5.45 |
| L2 F-R | 4. Parent 22 | Low Eth Col 1 | Col-0 | 1 EtOH | Low Light | 36.86 | 0.26 |
| rrn18 | 5. rrn18 | Low Eth Col 1 | Col-0 | 1 EtOH | Low Light | 36.36 | 1.10 |
| L1F - L2R | 1. Recomb 12 | Low Eth Col 2 | Col-0 | 2 EtOH | Low Light | 42.19 | 3.73 |
| L2F - L1R | 2. Recomb 21 | Low Eth Col 2 | Col-0 | 2 EtOH | Low Light | 35.28 | 0.65 |
| L1 F-R | 3. Parent 11 | Low Eth Col 2 | Col-0 | 2 EtOH | Low Light | 38.10 | 4.40 |
| L2 F-R | 4. Parent 22 | Low Eth Col 2 | Col-0 | 2 EtOH | Low Light | 36.30 | 0.47 |
| rrn18 | 5. rrn18 | Low Eth Col 2 | Col-0 | 2 EtOH | Low Light | 42.51 | 4.98 |
| L1F - L2R | 1. Recomb 12 | Low Eth Col 3 | Col-0 | 3 EtOH | Low Light | 43.13 | 5.10 |
| L2F - L1R | 2. Recomb 21 | Low Eth Col 3 | Col-0 | 3 EtOH | Low Light | 35.29 | 1.11 |
| L1 F-R | 3. Parent 11 | Low Eth Col 3 | Col-0 | 3 EtOH | Low Light | 37.68 | 1.72 |
| L2 F-R | 4. Parent 22 | Low Eth Col 3 | Col-0 | 3 EtOH | Low Light | 35.32 | 0.35 |
| rrn18 | 5. rrn18 | Low Eth Col 3 | Col-0 | 3 EtOH | Low Light | 32.44 | 0.49 |
| L1F - L2R | 1. Recomb 12 | Con Pvu 1 | PvuII | 1 H2O | Normal | 27.38 | 0.43 |
| L2F - L1R | 2. Recomb 21 | Con Pvu 1 | PvuII | 1 H2O | Normal | 30.49 | 0.26 |
| L1 F-R | 3. Parent 11 | Con Pvu 1 | PvuII | 1 H2O | Normal | 21.68 | 0.27 |
| L2 F-R | 4. Parent 22 | Con Pvu 1 | PvuII | 1 H2O | Normal | 21.27 | 0.21 |
| rrn18 F-R | 5. rrn18 | Con Pvu 1 | PvuII | 1 H2O | Normal | 22.44 | 0.22 |
| L1F - L2R | 1. Recomb 12 | Con Pvu 2 | PvuII | 2 H2O | Normal | 26.81 | 0.14 |
| L2F - L1R | 2. Recomb 21 | Con Pvu 2 | PvuII | 2 H2O | Normal | 29.83 | 0.14 |
| L1 F-R | 3. Parent 11 | Con Pvu 2 | PvuII | 2 H2O | Normal | 20.86 | 0.18 |
| L2 F-R | 4. Parent 22 | Con Pvu 2 | PvuII | 2 H2O | Normal | 20.83 | 0.15 |
| rrn18 F-R | 5. rrn18 | Con Pvu 2 | PvuII | 2 H2O | Normal | 22.13 | 0.23 |
| L1F - L2R | 1. Recomb 12 | Con Pvu 3 | PvuII | 3 H2O | Normal | 27.05 | 0.17 |
| L2F - L1R | 2. Recomb 21 | Con Pvu 3 | PvuII | 3 H2O | Normal | 29.98 | 0.38 |
| L1 F-R | 3. Parent 11 | Con Pvu 3 | PvuII | 3 H2O | Normal | 20.98 | 0.11 |
| L2 F-R | 4. Parent 22 | Con Pvu 3 | PvuII | 3 H2O | Normal | 20.87 | 0.06 |
| rrn18 F-R | 5. rrn18 | Con Pvu 3 | PvuII | 3 H2O | Normal | 22.12 | 0.14 |
| L1F - L2R | 1. Recomb 12 | Medium Only Pvu 1 | PvuII | 1 H2O | Medium Light | 27.72 | 0.10 |
| L2F - L1R | 2. Recomb 21 | Medium Only Pvu 1 | PvuII | 1 H2O | Medium Light | 30.71 | 0.62 |
| L1 F-R | 3. Parent 11 | Medium Only Pvu 1 | PvuII | 1 H2O | Medium Light | 21.88 | 0.12 |
| L2 F-R | 4. Parent 22 | Medium Only Pvu 1 | PvuII | 1 H2O | Medium Light | 22.17 | 0.10 |
| rrn18 F-R | 5. rrn18 | Medium Only Pvu 1 | PvuII | 1 H2O | Medium Light | 22.36 | 0.12 |
| L1F - L2R | 1. Recomb 12 | Medium Only Pvu 2 | PvuII | 2 H2O | Medium Light | 27.74 | 0.29 |
| L2F - L1R | 2. Recomb 21 | Medium Only Pvu 2 | PvuII | 2 H2O | Medium Light | 30.81 | 0.19 |
| L1 F-R | 3. Parent 11 | Medium Only Pvu 2 | PvuII | 2 H2O | Medium Light | 21.75 | 0.19 |
| L2 F-R | 4. Parent 22 | Medium Only Pvu 2 | PvuII | 2 H2O | Medium Light | 22.00 | 0.04 |
| rrn18 F-R | 5. rrn18 | Medium Only Pvu 2 | PvuII | 2 H2O | Medium Light | 22.21 | 0.15 |
| L1F - L2R | 1. Recomb 12 | Medium Only Pvu 3 | PvuII | 3 H2O | Medium Light | 27.92 | 0.16 |
| L2F - L1R | 2. Recomb 21 | Medium Only Pvu 3 | PvuII | 3 H2O | Medium Light | 31.19 | 0.25 |
| L1 F-R | 3. Parent 11 | Medium Only Pvu 3 | PvuII | 3 H2O | Medium Light | 22.29 | 0.20 |
| L2 F-R | 4. Parent 22 | Medium Only Pvu 3 | PvuII | 3 H2O | Medium Light | 22.58 | 0.13 |
| rrn18 F-R | 5. rrn18 | Medium Only Pvu 3 | PvuII | 3 H2O | Medium Light | 22.60 | 0.14 |
| L1F - L2R | 1. Recomb 12 | Low Only Pvu 1 | PvuII | 1 H2O | Low Light | 44.93 | 3.24 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| L2F - L1R | 2. Recomb 21 | Low Only Pvu 1 | PvuII | 1 H2O | Low Light | 34.88 | 0.53 |
| L1 F-R | 3. Parent 11 | Low Only Pvu 1 | PvuII | 1 H2O | Low Light | 34.50 | 0.57 |
| L2 F-R | 4. Parent 22 | Low Only Pvu 1 | PvuII | 1 H2O | Low Light | 34.13 | 0.57 |
| rrn18 | 5. rrn18 | Low Only Pvu 1 | PvuII | 1 H2O | Low Light | 32.67 | 0.13 |
| L1F - L2R | 1. Recomb 12 | Low Only Pvu 2 | PvuII | 2 H2O | Low Light | 44.34 | 4.11 |
| L2F - L1R | 2. Recomb 21 | Low Only Pvu 2 | PvuII | 2 H2O | Low Light | 36.46 | 0.41 |
| L1 F-R | 3. Parent 11 | Low Only Pvu 2 | PvuII | 2 H2O | Low Light | 35.20 | 1.11 |
| L2 F-R | 4. Parent 22 | Low Only Pvu 2 | PvuII | 2 H2O | Low Light | 35.48 | 0.32 |
| rrn18 | 5. rrn18 | Low Only Pvu 2 | PvuII | 2 H2O | Low Light | 35.32 | 0.93 |
| L1F - L2R | 1. Recomb 12 | Low Only Pvu 3 | PvuII | 3 H2O | Low Light | 40.59 | 2.97 |
| L2F - L1R | 2. Recomb 21 | Low Only Pvu 3 | PvuII | 3 H2O | Low Light | 34.82 | 0.55 |
| L1 F-R | 3. Parent 11 | Low Only Pvu 3 | PvuII | 3 H2O | Low Light | 38.37 | 0.86 |
| L2 F-R | 4. Parent 22 | Low Only Pvu 3 | PvuII | 3 H2O | Low Light | 35.38 | 0.26 |
| rrn18 | 5. rrn18 | Low Only Pvu 3 | PvuII | 3 H2O | Low Light | 28.68 | 0.20 |
| L1F - L2R | 1. Recomb 12 | Eth Only Pvu 1 | PvuII | 1 EtOH | Normal | 25.84 | 0.36 |
| L2F - L1R | 2. Recomb 21 | Eth Only Pvu 1 | PvuII | 1 EtOH | Normal | 29.67 | 0.10 |
| L1 F-R | 3. Parent 11 | Eth Only Pvu 1 | PvuII | 1 EtOH | Normal | 20.67 | 0.03 |
| L2 F-R | 4. Parent 22 | Eth Only Pvu 1 | PvuII | 1 EtOH | Normal | 20.67 | 0.05 |
| rrn18 F-R | 5. rrn18 | Eth Only Pvu 1 | PvuII | 1 EtOH | Normal | 22.02 | 0.11 |
| L1F - L2R | 1. Recomb 12 | Eth Only Pvu 2 | PvuII | 2 EtOH | Normal | 25.39 | 0.21 |
| L2F - L1R | 2. Recomb 21 | Eth Only Pvu 2 | PvuII | 2 EtOH | Normal | 30.79 | 3.66 |
| L1 F-R | 3. Parent 11 | Eth Only Pvu 2 | PvuII | 2 EtOH | Normal | 20.45 | 0.13 |
| L2 F-R | 4. Parent 22 | Eth Only Pvu 2 | PvuII | 2 EtOH | Normal | 20.52 | 0.06 |
| rrn18 F-R | 5. rrn18 | Eth Only Pvu 2 | PvuII | 2 EtOH | Normal | 21.58 | 0.09 |
| L1F - L2R | 1. Recomb 12 | Eth Only Pvu 3 | PvuII | 3 EtOH | Normal | 25.17 | 0.14 |
| L2F - L1R | 2. Recomb 21 | Eth Only Pvu 3 | PvuII | 3 EtOH | Normal | 28.81 | 0.35 |
| L1 F-R | 3. Parent 11 | Eth Only Pvu 3 | PvuII | 3 EtOH | Normal | 20.14 | 0.14 |
| L2 F-R | 4. Parent 22 | Eth Only Pvu 3 | PvuII | 3 EtOH | Normal | 20.52 | 0.03 |
| rrn18 F-R | 5. rrn18 | Eth Only Pvu 3 | PvuII | 3 EtOH | Normal | 21.60 | 0.03 |
| L1F - L2R | 1. Recomb 12 | Medium Eth Pvu 1 | PvuII | 1 EtOH | Medium Light | 28.21 | 0.27 |
| L2F - L1R | 2. Recomb 21 | Medium Eth Pvu 1 | PvuII | 1 EtOH | Medium Light | 31.08 | 0.45 |
| L1 F-R | 3. Parent 11 | Medium Eth Pvu 1 | PvuII | 1 EtOH | Medium Light | 22.91 | 0.16 |
| L2 F-R | 4. Parent 22 | Medium Eth Pvu 1 | PvuII | 1 EtOH | Medium Light | 23.06 | 0.17 |
| rrn18 F-R | 5. rrn18 | Medium Eth Pvu 1 | PvuII | 1 EtOH | Medium Light | 22.43 | 0.21 |
| L1F - L2R | 1. Recomb 12 | Medium Eth Pvu 2 | PvuII | 2 EtOH | Medium Light | 28.06 | 0.05 |
| L2F - L1R | 2. Recomb 21 | Medium Eth Pvu 2 | PvuII | 2 EtOH | Medium Light | 31.25 | 0.08 |
| L1 F-R | 3. Parent 11 | Medium Eth Pvu 2 | PvuII | 2 EtOH | Medium Light | 22.98 | 0.24 |
| L2 F-R | 4. Parent 22 | Medium Eth Pvu 2 | PvuII | 2 EtOH | Medium Light | 23.35 | 0.34 |
| rrn18 F-R | 5. rrn18 | Medium Eth Pvu 2 | PvuII | 2 EtOH | Medium Light | 22.17 | 0.05 |
| L1F - L2R | 1. Recomb 12 | Medium Eth Pvu 3 | PvuII | 3 EtOH | Medium Light | 27.18 | 0.16 |
| L2F - L1R | 2. Recomb 21 | Medium Eth Pvu 3 | PvuII | 3 EtOH | Medium Light | 30.60 | 0.56 |
| L1 F-R | 3. Parent 11 | Medium Eth Pvu 3 | PvuII | 3 EtOH | Medium Light | 22.22 | 0.21 |
| L2 F-R | 4. Parent 22 | Medium Eth Pvu 3 | PvuII | 3 EtOH | Medium Light | 22.30 | 0.30 |
| rrn18 F-R | 5. rrn18 | Medium Eth Pvu 3 | PvuII | 3 EtOH | Medium Light | 21.71 | 0.18 |
| L1F - L2R | 1. Recomb 12 | Low Eth Pvu 1 | PvuII | 1 EtOH | Low Light | 46.56 | 0.36 |
| L2F - L1R | 2. Recomb 21 | Low Eth Pvu 1 | PvuII | 1 EtOH | Low Light | 36.72 | 1.00 |
| L1 F-R | 3. Parent 11 | Low Eth Pvu 1 | PvuII | 1 EtOH | Low Light | 43.16 | 4.18 |
| L2 F-R | 4. Parent 22 | Low Eth Pvu 1 | PvuII | 1 EtOH | Low Light | 36.89 | 0.51 |
| rrn18 | 5. rrn18 | Low Eth Pvu 1 | PvuII | 1 EtOH | Low Light | 41.63 | 4.26 |
| L1F - L2R | 1. Recomb 12 | Low Eth Pvu 2 | PvuII | 2 EtOH | Low Light | 46.27 | 2.23 |
| L2F - L1R | 2. Recomb 21 | Low Eth Pvu 2 | PvuII | 2 EtOH | Low Light | 35.50 | 0.34 |
| L1 F-R | 3. Parent 11 | Low Eth Pvu 2 | PvuII | 2 EtOH | Low Light | 42.06 | 4.06 |
| L2 F-R | 4. Parent 22 | Low Eth Pvu 2 | PvuII | 2 EtOH | Low Light | 36.75 | 0.30 |
| rrn18 | 5. rrn18 | Low Eth Pvu 2 | PvuII | 2 EtOH | Low Light | 40.97 | 4.86 |
| L1F - L2R | 1. Recomb 12 | Low Eth Pvu 3 | PvuII | 3 EtOH | Low Light | 44.16 | 4.48 |
| L2F - L1R | 2. Recomb 21 | Low Eth Pvu 3 | PvuII | 3 EtOH | Low Light | 35.47 | 0.61 |
| L1 F-R | 3. Parent 11 | Low Eth Pvu 3 | PvuII | 3 EtOH | Low Light | 39.95 | 2.84 |
| L2 F-R | 4. Parent 22 | Low Eth Pvu 3 | PvuII | 3 EtOH | Low Light | 35.60 | 1.18 |
| rrn18 | 5. rrn18 | Low Eth Pvu 3 | PvuII | 3 EtOH | Low Light | 37.76 | 2.98 |

| Target | Primers | Sample | Plant | Replicate # | EtOH or H2O | Stress or No | Cq Mean | Cq Std. Dev |
|---|---|---|---|---|---|---|---|---|
| L1F - L2R | 1. Recomb 12 | Cold Only Col 1 | Col-0 | 1 | H2O | Cold | 26.75 | 0.146 |
| L1F - L2R | 1. Recomb 12 | Cold Only Col 2 | Col-0 | 2 | H2O | Cold | 24.72 | 0.075 |
| L1F - L2R | 1. Recomb 12 | Cold Only Col 3 | Col-0 | 3 | H2O | Cold | 24.29 | 0.249 |
| L2F - L1R | 2. Recomb 21 | Cold Only Col 1 | Col-0 | 1 | H2O | Cold | 30.86 | 0.618 |
| L2F - L1R | 2. Recomb 21 | Cold Only Col 2 | Col-0 | 2 | H2O | Cold | 28.47 | 0.33 |
| L2F - L1R | 2. Recomb 21 | Cold Only Col 3 | Col-0 | 3 | H2O | Cold | 28.29 | 0.459 |
| L1 F-R | 3. Parent 11 | Cold Only Col 1 | Col-0 | 1 | H2O | Cold | 22.46 | 0.218 |
| L1 F-R | 3. Parent 11 | Cold Only Col 2 | Col-0 | 2 | H2O | Cold | 19.88 | 0.227 |
| L1 F-R | 3. Parent 11 | Cold Only Col 3 | Col-0 | 3 | H2O | Cold | 19.86 | 0.067 |
| L2 F-R | 4. Parent 22 | Cold Only Col 1 | Col-0 | 1 | H2O | Cold | 21.43 | 0.192 |
| L2 F-R | 4. Parent 22 | Cold Only Col 2 | Col-0 | 2 | H2O | Cold | 20.29 | 0.233 |
| L2 F-R | 4. Parent 22 | Cold Only Col 3 | Col-0 | 3 | H2O | Cold | 19.94 | 0.137 |
| rrn18 F-R | 5. rrn18 | Cold Only Col 1 | Col-0 | 1 | H2O | Cold | 23.18 | 0.184 |
| rrn18 F-R | 5. rrn18 | Cold Only Col 2 | Col-0 | 2 | H2O | Cold | 21.93 | 0.03 |
| rrn18 F-R | 5. rrn18 | Cold Only Col 3 | Col-0 | 3 | H2O | Cold | 22.02 | 0.058 |
| L1F - L2R | 1. Recomb 12 | Cold Eth Col 1 | Col-0 | 1 | EtOH | Cold | 26.48 | 0.167 |
| L1F - L2R | 1. Recomb 12 | Cold Eth Col 2 | Col-0 | 2 | EtOH | Cold | 24.9 | 0.55 |
| L1F - L2R | 1. Recomb 12 | Cold Eth Col 3 | Col-0 | 3 | EtOH | Cold | 24.35 | 0.222 |
| L2F - L1R | 2. Recomb 21 | Cold Eth Col 1 | Col-0 | 1 | EtOH | Cold | 28.71 | 0.158 |
| L2F - L1R | 2. Recomb 21 | Cold Eth Col 2 | Col-0 | 2 | EtOH | Cold | 28.57 | 0.253 |
| L2F - L1R | 2. Recomb 21 | Cold Eth Col 3 | Col-0 | 3 | EtOH | Cold | 28.26 | 0.285 |
| L1 F-R | 3. Parent 11 | Cold Eth Col 1 | Col-0 | 1 | EtOH | Cold | 20.4 | 0.068 |
| L1 F-R | 3. Parent 11 | Cold Eth Col 2 | Col-0 | 2 | EtOH | Cold | 19.62 | 0.013 |
| L1 F-R | 3. Parent 11 | Cold Eth Col 3 | Col-0 | 3 | EtOH | Cold | 19.47 | 0.127 |
| L2 F-R | 4. Parent 22 | Cold Eth Col 1 | Col-0 | 1 | EtOH | Cold | 20.52 | 0.035 |
| L2 F-R | 4. Parent 22 | Cold Eth Col 2 | Col-0 | 2 | EtOH | Cold | 19.81 | 0.113 |
| L2 F-R | 4. Parent 22 | Cold Eth Col 3 | Col-0 | 3 | EtOH | Cold | 19.52 | 0.093 |
| rrn18 F-R | 5. rrn18 | Cold Eth Col 1 | Col-0 | 1 | EtOH | Cold | 22.06 | 0.069 |
| rrn18 F-R | 5. rrn18 | Cold Eth Col 2 | Col-0 | 2 | EtOH | Cold | 21.69 | 0.038 |
| rrn18 F-R | 5. rrn18 | Cold Eth Col 3 | Col-0 | 3 | EtOH | Cold | 21.67 | 0.041 |
| L1F - L2R | 1. Recomb 12 | Cold Only Pvu 1 | PvuII | 1 | H2O | Cold | 25.12 | 0.314 |
| L1F - L2R | 1. Recomb 12 | Cold Only Pvu 2 | PvuII | 2 | H2O | Cold | 24.06 | 0.218 |
| L1F - L2R | 1. Recomb 12 | Cold Only Pvu 3 | PvuII | 3 | H2O | Cold | 24.25 | 0.101 |
| L2F - L1R | 2. Recomb 21 | Cold Only Pvu 1 | PvuII | 1 | H2O | Cold | 28.54 | 0.117 |
| L2F - L1R | 2. Recomb 21 | Cold Only Pvu 2 | PvuII | 2 | H2O | Cold | 27.74 | 0.363 |
| L2F - L1R | 2. Recomb 21 | Cold Only Pvu 3 | PvuII | 3 | H2O | Cold | 27.57 | 0.363 |
| L1 F-R | 3. Parent 11 | Cold Only Pvu 1 | PvuII | 1 | H2O | Cold | 20.28 | 0.15 |
| L1 F-R | 3. Parent 11 | Cold Only Pvu 2 | PvuII | 2 | H2O | Cold | 19.52 | 0.037 |
| L1 F-R | 3. Parent 11 | Cold Only Pvu 3 | PvuII | 3 | H2O | Cold | 19.55 | 0.05 |
| L2 F-R | 4. Parent 22 | Cold Only Pvu 1 | PvuII | 1 | H2O | Cold | 20.46 | 0.182 |
| L2 F-R | 4. Parent 22 | Cold Only Pvu 2 | PvuII | 2 | H2O | Cold | 19.36 | 0.155 |
| L2 F-R | 4. Parent 22 | Cold Only Pvu 3 | PvuII | 3 | H2O | Cold | 19.49 | 0.239 |
| rrn18 F-R | 5. rrn18 | Cold Only Pvu 1 | PvuII | 1 | H2O | Cold | 22.07 | 0.124 |
| rrn18 F-R | 5. rrn18 | Cold Only Pvu 2 | PvuII | 2 | H2O | Cold | 21.59 | 0.02 |
| rrn18 F-R | 5. rrn18 | Cold Only Pvu 3 | PvuII | 3 | H2O | Cold | 21.62 | 0.144 |
| L1F - L2R | 1. Recomb 12 | Cold Eth Pvu 1 | PvuII | 1 | EtOH | Cold | 26.07 | 0.009 |
| L1F - L2R | 1. Recomb 12 | Cold Eth Pvu 2 | PvuII | 2 | EtOH | Cold | 24.53 | 0.018 |
| L1F - L2R | 1. Recomb 12 | Cold Eth Pvu 3 | PvuII | 3 | EtOH | Cold | 24.91 | 0.263 |
| L2F - L1R | 2. Recomb 21 | Cold Eth Pvu 1 | PvuII | 1 | EtOH | Cold | 29.23 | 0.416 |
| L2F - L1R | 2. Recomb 21 | Cold Eth Pvu 2 | PvuII | 2 | EtOH | Cold | 27.96 | 0.131 |
| L2F - L1R | 2. Recomb 21 | Cold Eth Pvu 3 | PvuII | 3 | EtOH | Cold | 27.86 | 0.031 |
| L1 F-R | 3. Parent 11 | Cold Eth Pvu 1 | PvuII | 1 | EtOH | Cold | 20.5 | 0.013 |
| L1 F-R | 3. Parent 11 | Cold Eth Pvu 2 | PvuII | 2 | EtOH | Cold | 19.61 | 0.04 |
| L1 F-R | 3. Parent 11 | Cold Eth Pvu 3 | PvuII | 3 | EtOH | Cold | 19.47 | 0.017 |
| L2 F-R | 4. Parent 22 | Cold Eth Pvu 1 | PvuII | 1 | EtOH | Cold | 20.8 | 0.2 |
| L2 F-R | 4. Parent 22 | Cold Eth Pvu 2 | PvuII | 2 | EtOH | Cold | 19.79 | 0.071 |

| L2 F-R | 4. Parent 22 | Cold Eth Pvu 3 | PvuII | 3 | EtOH | Cold | 19.76 | 0.207 |
|---|---|---|---|---|---|---|---|---|
| rrn18 F-R | 5. rrn18 | Cold Eth Pvu 1 | PvuII | 1 | EtOH | Cold | 22.51 | 0.064 |
| rrn18 F-R | 5. rrn18 | Cold Eth Pvu 2 | PvuII | 2 | EtOH | Cold | 21.76 | 0.118 |
| rrn18 F-R | 5. rrn18 | Cold Eth Pvu 3 | PvuII | 3 | EtOH | Cold | 21.37 | 0.198 |