TRANSGENERATIONAL INHERITANCE OF EPIGENETIC RESPONSE TO ABIOTIC STRESS IN *ARABIDOPSIS THALIANA*

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Abstract

Abiotic stresses are one of the major limiting factors of plant growth and thus crop productivity. Exposure to these stresses, including temperature and UV, cause physiological and epigenetic changes in plants. Such changes may be inherited in the progeny of stressed plants, and may change their ability to respond to stress. To understand the ability of plants to inherit an epigenetic stress memory as well as the physiological manifestations of such a memory, we propagated both stressed and control plants and compared the progeny under both normal and stressed conditions. In addition to wild-type plants we used Dicer-like mutants dcl2, dcl3 and dcl4, as Dicers have been linked to RNA-directed DNA methylation, a form of epigenetic memory. These studies revealed that leaf number decreases in the progeny of stressed plants, and bolting occurs earlier in the progeny of temperature stressed plants but later in the progeny of UV-C stressed plants. Transposons were also re-activated in the progeny of stressed plants. While heat shock transcription factor 2A increased expression in the progeny of heat stressed plants, many genes involved in DNA repair and histone modifications decreased. DCL2 and DCL3 appeared to be more important in transgenerational stress memory than DCL4. However, all dcl plants were generally not significantly different than wild-type plants, indicating that a single DCL deficiency may be compensated for by another DCL.

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List of Abbreviations

(-)- grown at normal conditions

(+)- grown at stressed conditions

C_1- progeny of plants grown at normal conditions in F0

C_1S_1- progeny of plants grown at normal conditions in F0 and exposed to stress in F1

C_2- progeny of plants grown at normal conditions for two generations

CBFs- C-REPEAT BINDING FACTORS

CCN- central cell nuclei

ChIP-chromatin immunoprecipitation assay

CMT3- CHROMOMETHYLASE3

COR- COLD-RESPONSIVE genes

CSPs- Cold shock proteins

DBD- DNA binding domain

DCL- Dicer-like gene

DCL- Dicer-like protein

DME- DEMETER DNA gylcosylase

dpg- days post germination

DRM2- DOMAINS REARRANGED METHYLTRANSFERASE 2

EC- egg cell

GN- generative nucleus

gDNA- genomic DNA

H3K27me- histone 3 lysine 27 methylation

H3K9me2-histone H3 lysine 9 dimethylation

HDAC- histone deacetylation

Hop- HSP70 and HSP90 organizing protein

HS- Heat stress

HSEs- heat shock elements

HSFA2- Heat shock transcription factor A-2

HSFs- heat stress transcription factors

HSPs- heat shock proteins

ICE1- INDUCER OF CBF EXPRESSION 1

KO- gene knock-out

LHP1- LIKE HETEROCHROMATIN PROTEIN1

m5C- 5-methylcytosine

mee1-maternal expressed in embryo 1

MET1- DNA METHYLTRANSFERASE 1

miRNAs- microRNAs

MSI1- MULTICOPY SUPPRESSOR of IRA1

ONSEN- a copia-type retrotransposon

piRNAs- PIWI RNAs

qPCR- Real-Time quantitative PCR

RBR1- RETINOBLASTOMA RELATED 1

RdDM- RNA-directed DNA methylation

ROS1- REPRESSOR OF SILENCING1

S_1- progeny of plants exposed to stress in F0

S_1C_1- progeny of plants exposed to stress in F0 and grown at normal conditions in F1

S_2- progeny of plants exposed to stress for two generations

SAR- systemic acquired resistance

SC- sperm cells

sHSPs- small heat shock proteins

siRNAs- small interfering RNAs

sRNAs- small RNAs

SUVH2- histone methyltransferase

SUVH5- histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH5

SUVH6- histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH6

SUVH8- histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH8

TEs- transposable elements

TMV- tobacco mosaic virus

TSI- transcriptionally silent information

UV- ultraviolet radiation

VN- vegetative nuclei

1. Literature Review

1.1. Epigenetic Modifications During Angiosperm Gametogenesis

Flowering plants do not contain a distinct germline and instead maintain undifferentiated cells in the sporophyte, which continually produces vegetative tissues and organs. These stem cells serve as the gametophyte initials, which undergo meiosis and give rise to microspores and megaspores. The spores develop into male and female gametophytes that are then responsible for the production of gametes (Yadegari and Drews, 2004; Twell, 2011).

The male gametophyte in *Arabidopsis thaliana* undergoes only two divisions to generate gametes, the first of which produces a vegetative cell as well as a germ cell. The germ cell then divides a second time. The result is a male gamete that contains two sperm cells (SC) and a vegetative nuclei (VN) (Twell, 2011). The female gametophyte consists of four different cell types for a total of seven cells. Critically, it contains the egg cell (EC) that gives rise to the embryo, as well as the two central cell nuclei (CCN) from which the endosperm develops. In addition the female gametophyte contains two accessory cells, synergid cells, which are crucial for pollen tube attraction and three antipodal cells of unknown function (Sundaresan and Alandete-Saez, 2010).

The sperm cells present in pollen are responsible for a double fertilization event, in which one fuses with the two nuclei of central cell, resulting in a triploid (3n) endosperm nucleus which is a terminally differentiated tissue analogous to the placenta in mammals. The second sperm cell fuses with the egg cell, producing an embryo that

develops into a mature plant (Law and Jacobsen, 2010). We will discuss the epigenetic processes operating in plant gametes including DNA methylation, small RNAs and histones modifications.

1.1.1. DNA Methylation

De novo methylation of DNA is catalyzed predominately by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), and maintained via three different pathways. These pathways are DNA METHYLTRANSFERASE 1 (MET1) - , CHROMOMETHYLASE (CMT3) - , and DRM2-dependent (Chan et al., 2005; Law and Jacobsen, 2010). For details on how maintenance methylation occurs in plants see Table 1.

In *Arabidopsis*, the protein DEMETER (DME) is responsible for active demethylation in reproductive organs. DME is a bifunctional DNA glycosylase/lyase that works with the base excision repair pathway to demethylate DNA by removing 5-methylcytosine and replacing it with cytosine (Choi et al., 2002; Gehring, et al., 2006). In addition to active demethylation, as described in Table 2, passive demethylation may also occur.

MULTICOPY SUPPRESSOR of IRA1 (MSI1) transcriptionally represses MET1 during female gametogenesis, a process that also requires RETINOBLASTOMA RELATED 1 (RBR1). MSI1/RBR1-dependent passive demethylation compliments DME-dependent active demethylation (DME) in the activation of imprinted genes (Jullien et al., 2008).

Gene imprinting occurs when the parent of origin impacts the expression level of a particular allele, resulting in differential expression of maternal and paternal (Gehring et al., 2004). The exact number of imprinted genes in plants is unknown, but recent

analysis has discovered that more than 200 genes that appear to show imprinting (Gehring et al., 2011; Hsieh et al., 2011; Wolff et al., 2011).

While it is accepted that the majority of loci are only imprinted in the endosperm, there are conflicting viewpoints on the number of genes with parentally biased expression in the embryo. Jahnke and Scholten (2009) found the first reported incident of imprinting in a plant embryo when they described *maternal expressed in embryo 1* (*mee1*) in maize. *mee1* is only expressed following fertilization and is unique to the embryo and endosperm. *in situ* hybridization found *mee1* expression throughout the embryo (Jahnke and Scholten, 2009). Recent work by Hsieh et al. (2011) saw no examples of imprinting in the *Arabidopsis* embryo and while a study by Gehring et al. (2011) found 18 imprinted genes in the embryo, it was concluded that all 18 might have been false positives.

In mammals, imprints are established following reprogramming of the germline during which the methylation status of the genome is reset (Migicovsky and Kovalchuk, 2011). Plants behave very differently, and one possible explanation for this is that control of imprinting in angiosperms such as *Arabidopsis* is maintained via the endosperm, a tissue that does not contribute to the germline, meaning that there is no need to erase and then apply imprints each generation (Xiao et al., 2003; Scott and Spielman, 2004).

1.1.1.1. DNA Methylation: Male Gametogenesis

Male gametogenesis in *Arabidopsis* results in the production of pollen grains containing a vegetative nucleus as well as two sperm cells (Twell, 2011). Transposons, which are methylated and therefore silent in most tissues, are demethylated and expressed in the vegetative nucleus of the pollen but not in SCs (Table 3) (Slotkin et al., 2009). Decreased methylation in pollen is supported by the down regulation of RdDM

components as well as the absence of DDM1 in the vegetative nucleus. Reduction of DNA methylation in the VN is likely an active process, as it is separated from sperm cells by only two cell divisions (Slotkin et al., 2009).

Slotkin et al. (2009) suspected the existence of a DNA demethylase active only in the VN, where it is responsible for removing DNA methylation from some transposable elements (TEs). It is crucial to protect the genome of SCs from transposons but not the genome of the VN, as it does not contribute genetic material to the progeny (Law and Jacobsen, 2010). In addition, an increase of 21nt siRNAs that do not correspond to transposons expressed in sperm cells has been found in VN. It is thought that siRNAs produced in the vegetative nucleus may have the ability to travel to sperm cells, reinforcing silencing (Slotkin et al., 2009). The impact of methylation on transposable elements is discussed further in section 3.

In addition, the DNA methyltransferase MET1 plays an important role during male gametogenesis in maintaining silencing. This has been demonstrated on genes such as FWA and FIS2, which are only expressed in the maternal genome of the endosperm. The use of maintenance methylation as opposed to *de novo* makes the establishment of imprints distinct in plants versus animals (Jullien et al., 2006).

1.1.1.2. DNA Methylation: Female Gametogenesis

In *Arabidopsis*, paternal imprinting results from demethylation in the CCN prior to fertilization, which combines with a sperm cell to form the endosperm, resulting in maternal-only expression in the endosperm (Hsieh et al., 2009; Law and Jacobsen, 2010). Cases of paternal-only expression may also occur, although there are fewer examples of this (Hsieh et al., 2011). DME is essential in the CCN and seeds with a mutant maternal

dme have CpG methylation levels similar to other tissues and do not complete development (Choi et al., 2002; Hsieh et al., 2009; Hsieh et al., 2011). However, the enzyme disappears following fertilization, resulting in an almost entirely demethylated maternal genome in the endosperm (Hsieh et al., 2009; Hsieh et al., 2011) (Table 3). The end result is that the endosperm inherits two genomes with differing epigenetic states, a necessary requirement for imprinting (Bauer and Fischer, 2011).

So far only one imprinted gene has been found in the embryo, emphasizing that the majority of plant imprinting occurs in the endosperm. In addition, the majority of imprinting results in maternal-only expression, as was seen with 52 of the 65 imprinted genes identified in a recent study by Wolff et al. (2011). Another study by Gehring et al. (2011) found maternal only expression for 165 of the 208 genes uncovered. Finally, work by Hsieh et al. (2011) found that among 43 imprinted genes, 34 of them were maternally expressed. Even among those that are paternally expressed, maternal demethylation may occur; for example demethylation at the tandem repeats downstream of *PHE1* gene allows for PcG-mediated silencing of the maternal allele (Villar et al., 2009; Hsieh et al., 2011).

This maternal demethylation is likely the cause of the hypomethylation seen in endosperm tissue in comparison to the embryo. These findings correspond to previous observations that chromatin in the endosperm is less tightly bound, a characteristic often caused by hypomethylated DNA (Baroux et al., 2007; Gehring et al., 2009). In fact, studying differentially methylated regions (DMRs) in the endosperm and embryo has shown that approximately 90% are more methylated in the embryo. Many of these imprinted regions are those enriched for TEs, which are also the best candidates for

imprinting as they show increased expression in the endosperm and low levels of transcription in other parts of the plants (Gehring et al., 2009; Kohler and Weinhofer-Molisch, 2010).

Recent work by Luo et al. (2011) in rice found that only one of 56 imprinted loci occurred in the embryo, and it also showed maternal-only expression in the endosperm. The locus identified was Os10g0750, which is a homolog of *Ole e 1*, the major allergen in olive and thought to control pollen tube emergence and production (de Dios Alche et al., 2004; Luo et al., 2011). Zemach et al. (2010) showed many genes that are hypomethylated and preferentially expressed in the endosperm are involved in rice endosperm biogenesis, such as starch synthesizing enzymes, indicating the crucial role of DNA methylation on gene expression. In addition, short TEs lost the most CHH methylation in the rice endosperm, while showing hypermethylation in the embryo, further suggesting the enhancement of transposon silencing via the endosperm, similar to what occurs in *Arabidopsis* (Zemach et al., 2010).

Despite a decrease of methylation in the endosperm, findings by Hsieh et al. (2009) actually indicated increased levels of CpNpN methylation in both endosperm and embryo tissues in comparison to adult plant tissue. They suggested that this might be the result of RdDM activity. In fact, endosperm hypermethylation has been shown to be a highly specific process performed by targeted siRNA (Hsieh et al., 2009). It was suggested that the high levels of maternally-derived siRNAs that accumulate in the endosperm as a result of hypomethylation may be generated in the CCN, and possibly responsible for local hypermethylation and silencing in the EC as well as the developing embryo, a process similar to the one described in pollen (Hsieh et al., 2009).

1.1.2. Regulation of Transposable Elements via Small RNAs

TEs are usually heterochromatic and transcriptionally silent, however they play a key role in the structure of the eukaryotic genome. If a transposon is activated the result may be extremely negative for the host, as it could be inserted into important regions of the genome, or support rearrangements causing genomic instability. In response to this possibility, many eukaryotes have developed the ability to target transposons and ensure they are inactivated (Biemont, 2009; Calarco and Martienssen, 2011).

However, TEs do have several important roles in the genome including chromatin formation and centromere function (Chueh et al., 2009; van der Heijen and Bortvin, 2009). This means that although TEs are maintained because of their positive contributions, the fact that they continue to be present in the genome makes further invasion possible, even though genomes may attempt to silence them using epigenetic processes such as small RNAs (Biemont, 2009).

Demethylation during gametogenesis, as described above, may help reveal TEs within the genome that could be expressed and ensure they are silenced. The benefit of this system is that even newly integrated transposons would be expressed, resulting in siRNA production and therefore silencing (Law and Jacobsen, 2010). Work by Teixeira et al. (2009), demonstrated that unlike other regions of the *Arabidopsis* genome, loci responsible for the production of siRNAs can be re-methylated even when methylation is lost in previous generations (Teixeira et al., 2009). It is possible that reactivation of TEs in surrounding cells which are not a part of the germline allows for improved siRNA silencing in the plant germline, a role suggestive of PIWI proteins in animals. In the animal germ line, piRNAs are responsible for silencing TEs through a mechanism in

which active TEs result in significant production of siRNAs that are then responsible for TE inactivation. It is possible siRNAs function similarly in plants (Aravin et al., 2008; Armenta-Medina et al., 2011). The role of small RNAs in silencing TEs clearly indicates their importance for maintaining genome integrity in angiosperm gametes (Mosher and Melnyk, 2010; Trionnaire et al., 2011).

1.1.2.1. Regulation of Transposable Elements via Small RNAs: Male Gametogenesis

Two distinct nuclei exist in pollen, and DNA hypomethylation is seen in the pollen VN but not the SC. Sperm cells maintain high levels of methylation, which prevent TEs from being activated, a mechanism that is reinforced by siRNAs from the VN, which is a terminal tissue (Slotkin, et al., 2009). As DDM1 (Table 1) is downregulated in the VN of *Arabidopsis*, reactivation of TEs occurs. The VN degenerates in the pollen tube prior to the SCs entering the ovule and so the cells responsible for fertilization lack active TEs, resulting in paternal imprinting (Slotkin et al., 2009).

Small RNA pathway components that show a strong increase in sperm cells include AGO9, DDM1, DRB4, MET1 AND SUVH5, and their presence in pollen is likely a result of enrichment in SCs (Borges et al., 2008). Many of the enriched transcripts are involved in RdDM including DDM1, as previously described, and SUVH5, a histone methyltransferase that helps maintain non-CG methylation (Ebbs and Bender, 2006; Borges et al., 2008) (Table 3). Although DCL3 was not found to be expressed in sperm cells, DCL1 was, as well as the AGO1-homolog AGO5. This suggests that although RdDM and maintenance of DNA methylation are important in sperm cells, they likely occur as a result of a novel small RNA pathway due to the absence of certain important

transcripts such as DCL3 (Borges et al., 2008).

1.1.2.2. Regulation of Transposable Elements via Small RNAs: Female Gametogenesis

Demethylation of the maternal genome in the endosperm supports transposon reactivation while TEs remain silenced in the embryo (Hsieh et al., 2009). However, studying the transcriptome of female gametophytes is much more difficult than it is for males, due to its location and the relative rarity of female gametophytic cells (Trionnaire et al., 2011). Still, there is early evidence that similar to males, components of small RNA pathways may be present in female gametophytes. It has been speculated that down-regulation of MET1 early during female gametogenesis results in the production of Pol IV-dependent siRNAs, also known as p4 siRNAs (Bourc'his and Voinnet, 2010). However, it is also currently thought that DME-directed genome demethylation is what allows the transcription and production of p4-siRNAs (Mosher and Melnyk, 2010). A recent study by Mosher et al. (2011) indicated that locus 08002 in *Arabidopsis* does not require differential DNA methylation for maternal-specific expression of p4 siRNAs. More work is still needed to investigate this potential link.

Work in *Arabidopsis* revealed several different AGO transcripts (AGO1, AGO2, and AGO5), which bind to siRNAs and direct chromatin remodeling, as well as *DCL1* transcripts indicating that small RNA pathways are definitely present in the female gametophyte (Wuest et al., 2010; Le Trionnaire et al., 2011). AGO9 plays a crucial role in silencing of transposons in the ovule; *ago9* exhibits reactivation of transposons in the ovule (Feng et al., 2010; Olmedo-Monfil et al., 2010). While *ago9* mutants can initiate gametophyte development in somatic tissue, their ortholog in maize, *ago104* results in the

megaspore mother cell producing unreduced gametophytes (Grossniklaus, 2011; Singh et al., 2011). For this reason, AGO104 is required for the production of male and female spores in maize. In fact, *ago104* showed reduced methylation at non-CG sites as well as increased transcription of repeats (Singh et al., 2011). Finally, in rice, MEL1, another AGO protein, has a crucial role in sporogenesis that is likely achieved via small RNA-mediated gene silencing (Nomura et al., 2007).

Two functionally uncharacterized paralogs of PAZ/Piwi-domain encoding genes, AT5G21150 and AT5G21030 have also been uncovered, providing further evidence that small RNAs play an important role in female gametogenesis, potentially protecting against TEs in a manner similar to that which occurs in males (Wuest et al., 2010; Trionnaire et al., 2011).

1.1.3. Histone Modifications and Histone Replacement

Histones help control essential processes in the genome such as transcription, replication, chromosome condensation and segregation, as well as DNA repair (Hauser et al., 2011). Among the modifications of histones, in particular H3 and H4, which help to control gene expression, are acetylation and methylation of histone lysine residues (Hauser et al., 2011). In addition to changes in DNA methylation, imprinted genes in *Arabidopsis* may be regulated via histone 3 lysine 27 methylation (H3K27me) by Polycomb Group Activity (Jullien and Berger, 2009). Histones can also be replaced by histone variants that may cause changes in expression.

1.1.3.1. Histone Modifications and Histone Replacement: Male Gametogenesis

Histones are an extremely important form of epigenetic control, and specific histone H3 variants characterize the angiosperm male germline. Despite a single cell division separating the VN from the GN, they show significant differences in chromatin structure, a differentiation that depends on histones (Ueda and Tanaka, 1995).

For example, *Lilium longiflorum* has been shown to possess three novel histone genes; gH2A, gH2B and gH3; that only recognize the nuclei of the male gametic (both generative and sperm) cells and appear to be correlated to chromatin structure due to their association with the nucleosome (Ueda and Tanaka, 1995; Ueda et al., 2000). In addition, a gradual decrease in histone H1 levels has been shown to exist in only the VN of *Lilium* resulting in mature pollen where the VN contains very little histone H1. In comparison, histone H2B is maintained at a similar level in both the VN and GN (Tanaka et al., 1998). It is thought that histone H1 is involved not only in chromatin structure, but also the

condensation of chromosomes during the mitotic phase (Woodcock et al., 2006). Therefore it is possible that the decrease of H1 in the VN helps to specify the developmental fate of a cell that, unlike the GN, does not divide again (Tanaka, 1997; Tanaka et al., 1998).

In *Arabidopsis*, the H3 variant HTR10 is expressed in the germline and mature sperm, but is completely replaced by other H3.3 variants in the zygote upon fertilization (Ingouff et al., 2007) (Table 3). The impact of this replacement is not yet fully understood, however it is hypothesized that it is a part of global reprogramming events, similar to the H3.3 replacement that occurs in the mammalian germline. It is unique from DNA methylation reprogramming because it occurs in the zygote, as opposed to being limited to accessory cells in plants (Hajkova et al., 2008; Feng et al., 2010; Twell, 2011). This is likely an active process as paternal HTR10 signals are completely removed from the sperm cell that fertilizes the egg only hours before S phase of the first zygote division, suggesting it occurs in a replication-independent manner (Ingouff et al., 2007; Feng et al., 2010).

1.1.3.2. Histone Modifications and Histone Replacement: Female Gametogenesis

Histone modifications in female gametes remain significantly less well understood than in males. However, an epigenetic dimorphism between the egg cell and central cell is known to exist, including the level of specific H3.3 isoforms found in each cell.

Only one H3.3 isoform (HTR5) present in sperm cells has also been found in egg cells, while two- HTR8 and HTR14- have been found in central cells. This dimorphism appears to be eliminated following fertilization, potentially indicating that it is established in order to distinguish the EC from the CCN during gametogenesis. The resetting of H3

variants in zygote chromatin suggests that these epigenetic marks are not transmitted to progeny. However, it is still possible that some loci may escape remodeling following fertilization, resulting in a maternal imprint, and further work is needed to determine whether or not this occurs (Ingouff et al., 2010; Baroux et al., 2011).

Recent work has provided further evidence of histone modifications resulting in epigenetic dimorphism between EC and CCN late during female gametogenesis in *Arabidopsis*. EC accumulates high levels of histone H3 lysine 9 dimethylation (H3K9me2) and LIKE HETEROCHROMATIN PROTEIN1 (LHP1) localization in comparison to the CCN. In addition, there is selective depletion of histone H2B in the egg cell and synergids of mature gametophytes (Pillot et al., 2010). These changes result in differences in transcriptional activity, due to the association of LHP1 with loci enriched in H3K27me3 and of a transcriptionally repressed state (Exner et al., 2009; Pillot et al., 2010).

In maize, variation in histone marks including H3K9ac and H3K4me3, both of which are associated with active transcription states, showed a much higher level of repressed chromatin in the EC versus the CCN, consistent with the pattern of the repressive H3K9me (Garcia-Aguilar et al., 2010). In fact, the ability of the RdDM pathway to silence gene expression is reinforced by methylation of H3K9me in *Arabidopsis*, indicating a link between changes in histones and the DNA methylation pathways previously discussed (Jackson et al., 2002).

1.1.4. Concluding Remarks

DNA methylation, regulation of transposons via small RNAs, and histone modifications all represent effective ways of controlling the epigenetic status of plant gametes, efficiently altering the way plants develop. Although knowledge of the role that these mechanisms play in developing male and female plant gametes remains limited, it is already recognized how essential they are for successful plant reproduction. In particular, there is a strong need for more research with regards to the role that epigenetics plays during female gametogenesis, an area which remains difficult to study due to the deep embedding of the female gametophyte within maternal tissues. Ultimately, comprehending the ability of epigenetic mechanisms to modify inheritance and gene expression during gametogenesis is an important step in understanding the way plant reproduction works.

1.1.5. References

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Table 1. Maintenance Methylation in Plants

Methylation Type	**Method of Maintenance**
CpG methylation	Requires MET1 DNA methyltransferase (orthologous to Dnmt1) and DDM1 chromatin remodeling factor.[1, 2] Approximately 1/3 of coding regions in *Arabidopsis* contain CpG methylation which is maintained by MET1.[3]
Non-CpG methylation	Appears to require an active signal to continuously target regions.[4] CpNpG methylation is thought to use a histone and DNA methylation-reinforcing loop which requires methyltransferase CMT3 as well as histone H3K9 dimethylation.[3, 5] Asymmetric DNA methylation is maintained by constant *de novo* methylation by DRM2 and RNA-directed DNA Methylation (RdDM), and at some loci by CMT3 and DRM2.[6, 7]

[1]Finnegan and Dennis, 1993 [2]Jeddeloh et al., 1999 [3]Law and Jacobsen, 2010 [4]Chan et al., 2005 [5]Johnson et al., 2007 [6]Bartee et al., 2001 [7]Feng and Jacobsen, 2011

Table 2. Active DNA Demethylation in Plants

DNA Glycosylases	**Role**
DME	Expressed in CCN during gametogenesis. Demethylation of maternal alleles persist in the endosperm, while paternal alleles remain methylated, making DME responsible for parental imprinting.[1]
ROS1, DML2, DML3	Expressed in vegetative tissues.[2, 3] All three appear to function redundantly although some locus specificity has been observed.[4] Act at normally silenced loci as well as euchromatin/heterochromatin boundaries where they may protect genes targeted for methylation via RdDM by removing DNA methylation.[5]

[1]Huh et al., 2008 [2]Gong et al., 2002 [3]Ortega-Galisteo et al., 2008 [4]Penterman et al., 2007 [5]Law and Jacobsen, 2010

Table 1. Summary of epigenetic changes during angiosperm gametogenesis

Epigenetic Change: **Location**	**Methylation**	**Small RNAs**	**Histones**
Sperm Cells	Maintain high levels of methylation preventing activation of TEs.[1]	Increased amount of components involved in siRNA biogenesis such as AGO9, DDM1, DRB4, MET1 AND SUVH5.[2] Increased amount of siRNAs that do not correspond to TEs expressed.[1]	HTR10 expressed in germline but replaced by other H3.3 variants following fertilization. Specific H2A, H2B and H3 proteins determine chromatin structure.[3]
Vegetative Nucleus	TEs are demethylated as a result of the absence of DDM1 and the downregulation of RdDM components, which is likely an active process. It is hypomethylated.[1, 4]	Reactivated TEs result in corresponding siRNA biogenesis; these siRNAs then travel to the SCs to reinforce silencing there.[1]	Decrease in H1 levels as well as difference in H3 methylation levels compared to SC.[5, 6]
Central Cell Nuclei	Almost entirely demethylated by DME, resulting in an overall reduction of DNA methylation in endosperm, as well as maternally-expressed imprinting [4, 7, 8]	High level of maternal-derived siRNAs found in endosperm as a result of prior demethylation in CCN.[9]	Two H3.3 isoforms found in SC also seen (HTR8 and HTR14). Possibly responsible for differentiation from EC. Reset following fertilization.[10]
Egg Cell	Increased level of methylation in comparison to CCN, reinforced at TEs by siRNAs.[11]	siRNAs generated in CCN may result in local hypermethylation and silencing especially at TEs.[7]	One H3.3 isoform found in SC also seen (HTR5). Possibly responsible for differentiation from CCN. Reset following fertilization.[10]

[1]Slotkin *et al.*, 2009 [2]Borges *et al.*, 2008 [3]Ingouff et al., 2007 [4]Law and Jacobsen, 2010 [5]Tanaka *et al.*, 1998 [6]Okada *et al.*, 2006 [7]Hsieh et al., 2009 [8]Hsieh et al., 2011 [9]Mosher *et al.,* 2009 [10]Ingouff et al., 2010 [11]Gehring *et al.*, 2009

1.2. Plant Physiological Response to Temperature Extremes

Plants frequently encounter external environmental conditions, known as stresses, that negatively impact their growth, survival, and ultimately crop yield (Bray et al., 2000; Mittler and Blumwald, 2010). Biotic stresses consist of living stresses such as bacteria, fungi, viruses and parasitic plants, while abiotic stress occurs due to an excess or insufficiency in the physical or chemical environment, including temperature, water and nutrient extremes. Stress characteristics such as severity, duration, number of exposures and combination of stresses all play a role in stress response, as do the organ or tissue in question, the stage of development as well as the genome and epigenome of the plant (Bray et al., 2000; Sung et al., 2003). In response to a stress there are two possible outcomes for a plant: resistance or susceptibility. When a plant is susceptible to the stress either substantial impairment in development, or death, will occur. The ability to resist stress relies on either avoidance, which requires minimizing the amount of stress encountered, or tolerance, which means maximizing its ability to withstand stress (Bray et al., 2000; Vinocur and Altman, 2005; Puijalon et al., 2011). Acclimation occurs when a plant is able to change in response to a stress resulting in either avoidance or tolerance. Acclimation is the result of resistance mechanisms at an individual level, while adaption occurs at a population level, as an evolutionary response to environmental factors (Bray et al., 2000).

Environmental stress is the leading cause of crop losses, reducing yields for most major crops by over 50% (Bray et al., 2000). The specific impact of stress on a plant

varies and depends not only on plant species, but also on the origin of the plant, as it may possess local adaptations (Dudley, 1996; Ackerly et al., 2000).

Previous research on hot and cold stress has identified changes in both plant physiology and the genome as being modified under stress, with many genes being cold or heat-inducible allowing for immediate plant response (Thomashow, 1999). Establishing acquired tolerance requires many changes in gene expression, as well as metabolite composition, but it ultimately results in plants being able to rapidly modify their homeostasis, acclimating in response to a changing environment (Vinocur and Altman, 2005; Boyko et al., 2010).

Plant stress-avoidance mechanisms include restricting vegetative growth to favourable seasons, thereby avoiding unnecessary environmental stress (Jung and Muller, 2009; Chew and Halliday, 2011). This may be accomplished through vernalization, a protective measure that uses temperature as an indicator in order to prevent plants that germinate in the fall from flowering until a long period of cold has passed, thereby insuring they wait until spring (for a complete review on this topic see Amasino, 2010). Another example is transpiration cooling or leaf shading of sensitive tissues, which can help plants maintain their internal temperature (Xu et al., 2011). Avoidance is usually the result of whole-plant changes, both morphological and physiological, while cellular and molecular biochemical changes cause tolerance. Therefore, manipulation at a genetic or epigenetic level is more feasible with regards to stress tolerance, not avoidance, and presents the strongest possibility for improved stress resistance (Vinocur and Altman, 2005). For this reason, the processes detailed in this paper will focus on the methods plants have for tolerating stress when it is unavoidable.

Plants have the ability to recognize stress at a cellular level, initiating a stress response which helps cope with the hostile environment. Various pathways are induced, depending on the stress, and these results in changes in gene expression, which may modify growth, development, and even influence reproduction (Bray et al., 2000). Temperature extremes may also directly impact the plant by altering cellular components such as membrane fluidity, conformation of proteins and nucleic acid, metabolic activity including biochemical reaction rates, and cellular structure (Bray et al., 2000; Margesin et al., 2007). This paper discusses the responses that plants have available for temperature stress in order to prevent and repair such damage.

1.2.1. Heat Stress

High temperature stress impacts plant productivity around the world, often in combination with drought or other stresses, and results in extensive agricultural losses (Mittler, 2006; Kotak et al., 2007). Temperatures above the optimum are sensed as heat stress (HS) and disturb cellular homeostasis (Kotak et al., 2007). Temperatures that rise 5-10 °C above the optimal growing temperature, may cause irreversible damage to plant function and development, or alteration of metabolism resulting in a reduction in growth as well as plant yield (Bray et al., 2000; Porter, 2005; Xu et al., 2011). This occurs because by increasing the rate of plant development, heat stress may shorten the life cycle of a plant; reducing the length of the growing period, and thereby decreasing seed and plant yield (Porter, 2005).

Plants in the field are regularly exposed to high temperatures, usually on a daily basis during the hours surrounding midday (Howarth, 2005). Fortunately, it is possible for plants to acquire thermotolerance if they undergo high, but non lethal (or permissive), temperatures for several hours allowing them to acclimate before being exposed to what would otherwise be a lethally high temperature (Bray et al., 2000; Sung et al., 2005). This process is mediated at least in part by induced heat-shock proteins (Bray et al., 2000).

1.2.1.1. Heat-Shock Proteins (HSPs)

Heat stress triggers the production of heat-shock proteins (HSPs), a process accomplished by the recognition and binding of heat stress transcription factors (HSFs) to heat shock elements (HSEs) located in the promoters of HSPs (Kotak et al., 2007; von Koskull-Doring et al., 2007; Mahmood et al., 2010). HSPs have important physiological functions as molecular chaperones and are regulated at the transcription level. They fold

proteins following translation and transform proteins into a structure suited to membrane transport. HSPs also prevent the aggregation of denatured proteins and promote the renaturation of aggregated protein molecules. As a result, they are strongly linked to resistance to both hot and cold temperature stress, as well as other abiotic stresses such as salinity and drought (Boston et al., 1996; Sun et al., 2001; Iba, 2002). The classes of heat shock proteins in plants are outlined in Table 4.

Interestingly, high molecular weight HSPs differ in terms of specific functions, even among homologs which belong to the same family but function in different cellular compartments, despite showing high levels of sequence similarity (Vierling, 1997; Hua, 2009; Mahmood et al., 2010).

1.2.1.1.1. Small Heat Shock Proteins (sHSP)

Small heat shock proteins (sHSPs) are the most complex group of HSPs in plants. sHSPs belong to a super-family of chaperones that have a conserved carboxyl-terminal domain of approximately 90 amino acids (Nakamoto and Vigh, 2007). Despite their small size, most sHSPs form large oligomers complexes of at least 8 subunits, or approximately 200-800 kDa in size, depending on the specific protein (Bray et al., 2000; Kirschner et al., 2000; Scharf et al., 2001). sHSPs are thought to be involved in the targeted protection of nearly all cellular compartments, as indicated in Table 4 (Sun et al., 2002; Hu et al., 2010).

In plants, sHSPs are the most prevalent form of heat shock protein, making them unique from other eukaryotes. For example, *Arabidopsis* contains 19 sHSPs and plant sHSPs are divided into 6 classes, three of which CI, CII and CIII are located in the

cytosol or nucleus, while the other three (CIV, CV and CVI) are found in plastids, endoplasmic reticulum, and mitochondria. Other eukaryotes contain only a single class of sHSPs (Bray et al., 2000; Sun et al., 2002; Wang et al., 2003).

Heat stress, as well as other form of abiotic stress such as salt and cold, trigger expression of sHSPs in plants. Unlike many other HSPs, they are not expressed constitutively (Boston et al., 1996; Wang et al., 2003; Wang et al., 2004; Grigorova et al., 2011). sHSPs, in combination with HSP70, function to prevent aggregation of cellular proteins, and they are therefore important factors for stress tolerance, especially thermotolerance (Grigorova et al., 2011).

1.2.1.1.2. HSP60

HSP60s are thought to function as molecular chaperones and thus are often referred to as chaperonins (Boston et al., 1996; Wang et al., 2004). Abundant even at normal temperatures, the major role of HSP60 proteins is to facilitate protein folding (Efeoglu, 2009; Xu et al., 2011). However, under heat stress the amount of HSP60 in the mitochondria is increased, thereby improving thermal tolerance through the formation of complexes with proteins, stabilizing them and protecting them from thermal inactivation during heat stress (Martin et al., 1992; Xu et al., 2011). One HSP60 protein, chaperonin-60 (cpn60b), has been shown to associate with Rubisco activase in the chloroplast during heat stress, with the association increasing with duration and intensity of stress, and decreasing following recovery. This indicates that perhaps cpn60b shifts from protein assembly to protection of thermally unstable proteins during heat stress, preventing Rubisco from denaturation and helping to acclimate photosynthesis to heat stress (Salvucci, 2008).

1.2.1.1.3. HSP70

HSP70 proteins have essential roles in protein metabolism and are therefore required for cell function, even under normal conditions. However, while some *HSP70s* are expressed at a constant rate, others may be induced by hot or cold temperature stress (Hartl, 1996; Bray et al., 2000). HSP70s play a central role as molecular chaperones and folding catalysts in which they help prevent aggregation. They also assist under normal and stressed conditions with refolding denatured proteins (Hartl, 1996; Frydman, 2001; Huang and Xu, 2008). Finally, HSP70s are involved in membrane translocation as well as ensuring the degradation of unstable proteins (Hartl, 1996).

1.2.1.1.4. HSP90

The HSP90 chaperone complex consists of five proteins, including HSP90 and HSP70, as well as their co-chaperones, HSP40, Hop (HSP70 and HSP90 organizing protein) and a 23-kDa acidic protein (p23), and is required for the activation and stabilization of several signalling proteins (Pratt et al., 2001; Zhang et al., 2003; Sharkey and Schrader, 2006; Zhang et al., 2010). HSP90 proteins are essential for cell viability and growth. Some are constitutively expressed, while others are either induced or up-regulated by stress (Reddy et al., 1998; Krishna and Gloor, 2001; Stephanou and Latchman, 2011). HSP90 proteins functions include managing protein folding, as well signal-transduction, cell-cycle control, protein degradation and protein trafficking (Wang et al., 2004).

1.2.1.1.5. HSP100

HSP100s are involved in removal of non-functional but potentially detrimental polypeptides arising from misfolding, denaturation, or aggregation; a role crucial for

maintaining cellular homeostasis (Wang et al., 2004). One such protein, HSP101, interacts with the sHSP chaperone system in order to resolubilize proteins after heat stress, in a process involving complex interactions between HSP101 and the protein domains (Lee et al., 2005a). In fact, while HSP101 is dispensable for normal growth, it is crucial for plant tolerance to high temperatures (Hong and Vierling, 2001).

1.2.1.2. Heat Stress Transcription Factors (HSFs)

Heat stress transcription factors (HSFs) recognize the binding motifs of heat stress elements (HSEs), which are conserved in the promoters of HS-inducible genes of all eukaryotes (Bray et al., 2000; von Koskull-Doring et al., 2007). HSFs are constantly expressed, but are only able to bind to DNA as trimers and heat stress is required for trimerization, enabling HSFs to recognize HSEs (Bray et al., 2000; Zhu et al., 2006). In plants, HSFs consist of three evolutionary classes: class A, class B, and class C; which include more than 20 HSFs, remarkable diversification in comparison to other eukaryotes. For example Drosophila contains only one HSF, while vertebrates have four (Baniwal et al., 2004**;** von Skull-Doring, et al., 2007; Akerfelt et al., 2010). Despite significant diversity in size and sequence, all HSFs maintain a similar basic structure, including a highly conserved N-terminal DNA binding domain (DBD) required for the recognition of HSEs (Baniwal et al., 2004; Akerfelt et al., 2010; Lin et al., 2011).

In tomato, *HSFA1a*, *HSFA2*, and *HSFB1* appear to form a regulatory network responsible for the expression of heat-stress inducible genes (Baniwal et al., 2004). *HSFA1a* is the master regulator of heat stress response in tomato, due to its constitutive expression and regulation of HS-induced expression of *HSFA2* and *HSFB1* (Mishra et al., 2002; Baniwal et al., 2004). In addition, HSFA1a works as a nuclear retention factor and

co-activator of HSFA2 by forming a HSFA1a-HSFA2 hetero-oligomeric complex, as class B and C HSFs have no activator function on their own (Kotak et al., 2004; Kotak et al., 2007; Chan-Schaminet et al., 2009). As a result, even though HSFA1 is functionally equivalent to HSFA2, HSFA2 expression is dependent on HSFA1. Therefore, HSFA2 cannot be substituted as the master regulator (Mishra et al., 2002). Studies using HSF mutant lines have also indicated that *HSFA1a* and *HSFA1b* are important for the initial phase of HS-responsive gene expression. In contrast, *HSFA2* controls expression under prolonged heat stress and recovery conditions, as indicated by the rapid decline of thermotolerance following repeated heat stress treatments in *HSFA2* knockout mutants (Schramm et al., 2006; Charng et al., 2007).

HSFA2 is also a dominant HSF in *Arabidopsis*, as well as tomato, due to its high activator potential for transcription of HSP genes and its continued accumulation during repeated cycles of heat stress and recovery. This is true regardless of tissue (Scharf et al., 1998; Baniwal et al., 2004; Schramm et al., 2006; Kotak et al., 2007). It was thought that *HSFA1a* and *HSFA1b* did not regulate HS-induced expression of *HSFA2* in *Arabidopsis*, because the *HSFA1a/1b* double KO mutant does not show substantial defects in thermotolerance (Busch et al., 2005; Kotak et al., 2007). Recent work using a quadruple mutant *HSFA1a/b/d/e* showed that *HSFA1*s do function as master regulators of HSR. A triple mutant *HSF1a/b/d* was sufficient for impaired thermotolerance, but all four proteins were needed for normal growth and development, as indicated by the substantial changes in growth and development seen in quadruple mutants (Liu et al., 2011; Yoshida et al., 2011).

1.2.1.3. Other Components in Thermotolerance

In addition to their molecular profile, plants may modify their physiology to help tolerate heat stress better. For example, increasing water extraction in response to high temperature. This response is possible because high temperatures increase transpiration, which helps convert heat energy into latent energy, thereby keeping the plant tissue cooler than air temperature. Unfortunately, the same mechanism can also cause water deficit, and a shortage of water in the soil can limit the potential of this heat dissipating process, thus exacerbating high temperatures. The ability of heat stress to induce drought stress via increased transpiration is one of the reasons the two stresses often occur in combination, especially during the daytime (Tsukaguchi et al., 2003; Howarth, 2005; Wahid et al., 2007). However, if the soil is well-watered, drought stress is not a serious concern and this may be an effective mechanism for helping to tolerate heat stress (Hightshoe, 1987).

The region surrounding a leaf is the boundary layer, and it is where the leaf surface area influences the movement of the surrounding vapour molecules, and which controls heat transfer by convection. As a result, the boundary layer is partially controlled by the size and shape of a leaf. Small leaves can transfer heat more effectively than large leaves, making them better adapted to situations of heat stress, especially when water and air movement are limited. When water is also a limiting factor, heat loss through transpiration is prohibited, making small leaves ideal under hot and dry conditions (Smith, 1978; Yates et al., 2010). Small leaves are also better able to move in the wind, resulting in a smaller boundary layer, and as such a plant may wish to mimic this morphology under heat stress conditions (Chapin et al., 2000a). For example, Musaceae plants are

able to undergo leaf tearing in response to the environment, allowing leaves to tear more under greater sunlight. As a result, leaves less than 10 cm wide have been shown not to be subjected to critical heat stress (Taylor and Sexton, 1972). In addition, lobbing may increase on a large leaf under heat stress, resulting in a morphology similar to having many smaller leaves (Chapin et al., 2000a).

Finally, the production of the volatile organic carbon isoprene, particularly in trees, may help photosynthesis manage with high temperatures below 45 °C by reducing the level of fluorescence at high temperatures. Therefore, the presence of isoprene can increase the thermotolerance of leaves, explaining the large amount produced by trees such as oak (*Quercus* sp.) and aspen (*Populus* sp.) (Chapin et al., 2000b; Sharkey et al., 2001).

1.2.2. Cold Stress

Another extremely important environmental factor that influences the global distribution of organisms, including agricultural crops, is cold stress. Low temperatures damage plants by both chilling, which is classified as temperatures less than 20 °C, and freezing, which is less than 0 °C. Chilling leads to physiological and developmental abnormalities, while freezing causes cellular damage directly or via cellular dehydration (Rehman et al., 2005; Chinnusamy et al., 2010).

Cold stress limits plants by inhibiting metabolic reactions, as well as potentially inducing osmotic, oxidative, and other stresses (Chinnusamy et al., 2007). As a result, periods of cold may lead to decreased plant yield, as well as decreased survival rates (Chew and Halliday, 2011). Fortunately, plants have the ability to sense low temperatures and respond by activating mechanisms that increase freezing tolerance. Hundreds of genes for various transcription factors are associated with cold acclimation (Lee et al., 2005b). Deacclimation to cold is primarily controlled by transcriptional repression of many cold-inducible genes (Oono et al., 2006). The induction of these genes influences plant metabolic and regulatory pathways, corresponding to the new temperature range (Chinnusamy et al., 2007; Chew and Halliday, 2011). Ultimately, plants may not only be able to withstand cold, but actually acquire freezing tolerance, a trait caused by exposure to chilling temperatures and known as cold acclimation (Chinnusamy et al., 2007).

1.2.2.1. C-REPEAT BINDING FACTORS (CBFs) Cold Response Pathway

The C-REPEAT BINDING FACTORS (CBFs) cold response pathway in *Arabidopsis thaliana* is the principal source of transcription factors that help combat the impact of low temperatures (Gilmour et al., 2000; Chew and Halliday, 2011). There are three CBF genes, *CBF1*, *CBF2* and *CBF3*, which are located in tandem on chromosome 4 and are induced under cold temperatures (Gilmour et al., 1998; Shinwari et al., 1998).

Genetic systems, such as CBF cold response pathway in *Arabidopsis*, allow for the transcription of genes with functions including the synthesis of cryoprotectants that protect the tissue from freezing damage. The amino acid proline and the sugars glucose, fructose, inositol, galactinol, raffinose and sucrose, many of which have cryoprotective properties, have all been shown to increase in *Arabidopsis* plants upon exposure to low temperature (Cook et al., 2004). The soluble sugar content of plant tissue is closely correlated with freezing tolerance, allowing plants with larger reserves of cryoprotective sugars to maintain cold hardiness (Ogren et al., 1997).

COLD-RESPONSIVE (*COR*) genes are induced by the expression of CBFs, which follow exposure to low temperature and are able to bind to the cis-elements in the promoters of *COR* genes (Thomashow, 1999; Chinnusamy et al., 2007). The transcription factor *INDUCER OF CBF EXPRESSION 1* (*ICE1*) enhances CBF expression in a temperature-dependent manner (Chinnusamy et al., 2003; Chinnusamy et al., 2007). In fact, within 15 minutes of exposure to low temperatures plants show an increase in CBF transcript levels, followed by accumulation of transcripts belonging to *COR* genes within two hours (Gilmour et al., 1998).

Expression of *CBF1* and *CBF3* in transgenic *Arabidopsis* plants induced expression of *COR* genes and resulted in enhanced freezing tolerance in nonacclimated plants. Subsequent work demonstrated that overexpression of *CBF3* increased freezing tolerance, even if plants were already cold-acclimated (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000). These plants also mimicked multiple biochemical changes associated with cold acclimation, such as increased levels of proline and sugars including sucrose and raffinose. Overexpression of *CBF3* also caused elevated levels of *P5CS* transcripts, indicating that increased expression of the essential proline biosynthetic enzyme 1-pyrroline-5-carboxylate synthase was at least partially responsible for the higher level of proline. Together, these results indicate the important role that CBF regulatory genes play in cold acclimation (Gilmour et al., 2000).

The CBF pathway is highly conserved in flowering plants including temperate cereals, such as wheat, and species completely unable to tolerate freezing such as rice (Jaglo et al., 2001; Nakashima and Yamaguchi-Shinozaki, 2006). In the future, manipulating this pathway may allow for improved survival rates and yield, as well as increasing the ability of plants to withstand sudden cold spells. This is particularly relevant when it is considered that the CBF pathway also boosts protection from drought and salinity, both major abiotic stresses (Chew and Halliday, 2011). In fact, expression of the CBF pathway even improves resistance to extreme heat, through activation of the heat shock pathway (Schramm et al., 2008).

1.2.2.2. Other Reponses to Cold

Plants contain a large amount of cold-regulated genes, including approximately 4% of the genome in *Arabidopsis*, and the result is significant transcriptome changes

under stress, leading to substantial metabolome modifications as well (Cook et al., 2004; Lee et al., 2005b). In one study, 75% of the 434 metabolites monitored in *Arabidopsis* increased in response to cold stress (Cook et al., 2004). Another study found similar values, in which approximately 70% of the cold-regulated genes they studied were up-regulated (Lee et al., 2005b).

One of the most damaging aspects of cold stress is its ability to induce membrane rigidity. In fact, temperature-induced changes in membrane fluidity are what allow plant cells to sense cold stress (Chinnusamy et al., 2007). Thus, among the modifications crucial in developing freezing tolerance are changes in membrane lipid composition, such as enhanced fatty acid desaturation in membrane phospholipids (Bray et al., 2000; Chinnusamy et al., 2010). The desaturation of fatty acids in the membrane is ideal under cold stress conditions, because lipids containing more saturated fatty acids solidify faster and at higher temperatures than those with a higher proportion of unsaturated to saturated fatty acids (Uemura and Steponkus, 1994; Yadav, 2010). In addition the amount of cerebrosides has been shown to decrease under cold stress (Uemura and Steponkus, 1994; Bray et al., 2000).

Plants also cope with cold stress through thermal hysteresis proteins that depress the freezing point but not the melting point of water, averting the formation of ice crystals (Urrutia et al., 1992). Phenotypic changes evident in plants exposed to cold stress included a decrease in leaf expansion, wilting and chlorosis (yellowing) of leaves, and potentially necrosis of the tissue (Yadav, 2010).

1.2.3. Cross-Protection Theory

The cross-protection theory states that exposure to one moderate stress may induce tolerance to another stress, even if the plant has not been exposed to it (Sabehat et al., 1998). Several transcription factors are key players in many different stress response pathways, for both abiotic and biotic stresses (Fujita et al., 2009). For example, dehydration and osmotic stress often accompany temperature stress, and as such temperature extremes may elicit a molecular response to protect from them. This includes the production of dehydrins, cold-induced genes that offer protection from dehydration and osmotic stress (Chew and Halliday, 2011).

Comparing heat- and cold-shock response patterns has shown that the majority of heat-shock responses are shared with those initiated under cold stress. Approximately two-thirds of the heat shock metabolite response was shared with that of cold stress, indicating that the majority of heat-responsive metabolites are not heat-specific (Kaplan et al., 2004). Another study determined *Arabidopsis HOT2,* which encodes a chitinase-like protein AtCTL1, is needed not only for heat acclimation but also in response to salt and drought stresses as well as for proper development (Kwon et al., 2007). These are only a few of the many examples indicating the linkage between response to one stress and the ability it has to help protect a plant from other stresses.

1.2.4. Concluding Remarks

At first glance it would seem that plants face great difficulty in surviving due to their inability to escape from environmental conditions, but as a result they have developed remarkable systems for withstanding such stress. Through both specific and general stress responses plants are able to acclimate to extreme temperature conditions in unique and complex ways, allowing them to tolerate what should be lethal heat or cold. The temperature-induced triggering of HSFs and CBFs are especially important.

As we continue to further our understanding of plant response to temperature extremes, there is the potential to develop plants able to better tolerate some of the most significant causes of decreased crop productivity.

1.2.5. References

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Table 4. Heat shock proteins in plants, named according to their molecular weight

Protein Class	Size (kDa)	Location
sHSP	15-42[1, 2]	Cytoplasm/nucleus, plastids, mitochondria, endoplasmic reticulum[2]
HSP60	~60	Cytoplasm, chloroplast, mitochondria[3, 4, 5]
HSP70	~70	Cytoplasm, chloroplast, mitochondria, endoplasmic reticulum[4, 6]
HSP90	~90	Cytosol, chloroplast, mitochondria, endoplasmic reticulum[7]
HSP100	84 -104[8]	Cytoplasm/nucleus, chloroplast, mitochondria, plasma membrane [4, 9, 10]

[1]Mahmood et al., 2010 [2]Sun et al., 2002 [3]Kruft et al., 2001 [4]Bray et al., 2000 [5]Hill and Hemmingsen, 2001 [6]Marshall et al., 1990 [7]Krishna and Gloor, 2001 [8]Grigorova et al. 2011 [9]Constan et al., 2004 [10]Singh and Grover, 2010

1.3. Epigenetic Changes and Inheritance in Response to Stress in Plants

Epigenetic systems are both important and unique, due to their dynamic ability to be rapidly induced in response to stimuli both developmental and environmental in nature. The gene expression changes that result from such epigenetic modifications may be short or long-term in nature (Grant-Downton and Dickinson, 2006; Zhu, 2008; Chinnusamy and Zhu, 2009a). Epigenetic mechanisms are a crucial part of stress response and can be achieved in a single generation. In contrast, natural selection and breeding programs take considerable time and rely on existent characteristics in order to form crosses between plants (Persley and Siedow, 1999).

The limitations of traditional plant breeding are evident when considering that the freezing tolerance of wheat varieties today are only slightly better than those available a century ago (Fowler and Gusta, 1979). Producing more stress-tolerant plants through epigenetic modifications would not only greatly reduce the time required for the creation of such plants, but also increase the potential obtainable traits. Epigenetic modifications do not require genetic manipulation of the organism and because there is significant global opposition to agricultural applications of genetic engineering throughout the world provides an excellent and efficient alternative for the creation of stress-tolerant crops (Bonny, 2003). Epigenetics does not rely on insertion of genes from other organisms, nor does it have the potential environmental consequences involved with altering the genome, as epigenetic changes are not permanent and reverse after several generations in the absence of stress (Boyko et al., 2010). Traditional crop development will be insufficient to adapt crops in response to changing global temperatures and increasing agricultural demand, and considering global opposition to genetic engineering, the understanding and

integration of epigenetic mechanisms for coping with temperature stress will only increase in importance (Ainsworth and Ort, 2010).

Epigenetic alterations in response to stress discussed in this review include changes in DNA methylation, histone modifications and changes in small RNA species, all of which may be inherited by the progeny of plants as a form of epigenetic stress memory and improve the stress tolerance of progeny.

1.3.1. DNA Methylation

DNA methylation, or the presence of a 5-methylcytosine (m5C) nucleotide instead of a normal cytosine in DNA, is a stable gene silencing mechanism essential for epigenetic regulation of gene expression in plants (Sharma et al., 2011; Uthup et al., 2011). In plants, DNA cytosine methylation may occur at CG, CHG or CHH (Mirouze and Paszkowski, 2011). Demethylation and hypermethylation of genomic DNA may occur in response to abiotic stress (Qiao and Fan, 2011). For example, plants may increase DNA methylation on a genome-wide level in response to stress in order to increase genome stability, by decreasing transposon activity as well as suppressing homologous recombination events (Lukens and Zhan, 2007). In other cases, environmental stresses have been shown to result in genomic hypomethylation (Sano, 2010). These changes may be either short-term in response to a stress, or stably inherited (Uthup et al., 2011). In fact, some patterns of DNA methylation can be passed down for many generations, even withstanding backcrossing to wild-type plants (Johannes et al., 2009; Daxinger and Whitelaw, 2010).

A recent study by Verhoeven et al. (2010) in dandelions (*Taraxacum officinale*) provided evidence of epigenetic variation being transmitted to progeny as a result of abiotic and biotic stress. As dandelions reproduce asexually, their offspring are assumed to be genetically identically. The offspring of stressed and not stressed plants were compared for genome-wide DNA methylation changes and the results indicated that not only are stress-induced changes common, but they are mostly inherited by the next generation (Verhoeven et al., 2010).

In tobacco, abiotic stresses including low temperature and salt, induced demethylation in the coding region, and therefore transcriptional activation, of a glycerophosphodiesterase-like protein that is silent in the plant under both normal and pathogenic conditions (Choi and Sano, 2007). Work with the tropical tree species *Hevea brasiliensis* also showed direct impact of abiotic stress on the epigenome via changes in DNA methylation (Uthup et al., 2011). While pea root tips exposed to drought stress were found to have a hypermethylated genome in response (Labra et al., 2002).

Cold-stress of maize seedlings lead to genome-wide demethylation in root tissues, including a fragment designated *ZmMI1*, which was transcribed only under cold stress (Steward et al., 2002). Low temperature has also been correlated with demethylation in *Antirrhinum majus*, while higher temperatures resulted in hypermethylation (Hashida et al., 2003). Work in wheat also showed vernalization-induced demethylation in colder temperatures, including demethylation related to flower induction as well as more general changes unrelated to flowering (Sherman and Talbert, 2002). For a more complete review on the role of epigenetics in controlling flowering time in plants exposed to stress, see Yaish et al., 2011.

Work on biotic stresses, such as pathogen-infected plants, has also found transgenerational changes in genome stability and methylation as induced by tobacco mosaic virus (TMV)-infected tobacco plants. Progeny of stressed plants had global genome hypermethylation as a part of a general protective mechanism against stress, while locus-specific hypomethylation allowed for higher frequency of rearrangements at loci, such as those involved in disease resistance, that would help allow for an adaptive response (Boyko et al., 2007; Boyko and Kovalchuk, 2011a). Similarly, work on tomato

plants showed that viral infection caused DNA methylation changes at several marker loci associated with defense and stress responses (Mason et al., 2008).

CG DNA methylation is coupled to DNA replication, making it a stable epigenetic mark (Lauria and Rossi, 2011). In fact, CG methylation is a crucial coordinator for stable epigenetic memory and transgenerational inheritance in *Arabidopsis* (Mathieu et al., 2007). Work by Boyko et al. (2010), provides evidence of the importance of DNA methylation passed on stress memory to untreated progeny. Transgenerational memory of stress in *Arabidopsis* was seen in response to a variety of abiotic stresses including salt, UV-C, cold, heat, and flood (Boyko et al., 2010). Ultimately, DNA methylation appears to play a crucial role in stress adaptation, as well as transgenerational inheritance of stress memory (Boyko and Kovalchuk, 2011b; Mirouze and Paszkowski, 2011).

1.3.2. Histone Modifications

Histone modifications play a key role in chromatin structure and are a strong contributor in determining the transcriptional state and expression level of genes. Different histones modifications may either increase or decrease transcription (Chinnusamy and Zhu, 2009a; Qiao and Fan, 2011). For example, post-translation modification of histones such as acetylation, and sometimes phosphorylation and ubiquitination, enhance transcription. Other changes to histones including biotinylation and sumoylation are responsible for repressing gene expression (Chinnusamy and Zhu, 2009a (and references therein); Lauria and Rossi, 2011; Qiao and Fan, 2011).

Histone changes are an important form of epigenetic regulation (Chinnusamy and Zhu, 2009a). Like DNA methylation, histone modifications play a crucial role in stress-induced gene regulation and may be regulated by environmental conditions (Boyko and Kovalchuk, 2008). Work by van Dijk et al. (2010) in *Arabidopsis* showed that dehydration causes dynamic changes to histones H3K4me1, H3K4me2, and especially H3Kme3. In tomato plants, drought induced the linker histone variant H1-S, which appears to be involved in reducing stomatal conductance, thereby minimizing water loss at a crucial time (Scippa et al., 2004).

A recent study by Lang-Mladek et al. (2010) in *Arabidopsis* also showed transgenerational inheritance of epigenetic changes as a result of temperature and UV-B stress. In this case, the stress-mediated release of gene silencing in the studied transgene was a result of modifications in chromatin conformation and histone H3 acetylation but not DNA methylation, emphasizing how crucial the role of histones is in passing on epigenetic memory. However, while the impact of stress on gene silencing was heritable,

it was only passed to a limited number of cells and restricted to two generations of non-stressed progeny (Lang-Mladek et al., 2010).

Work by Pecinka et al. (2010), found heat stress caused transcriptional activation of several classes of repetitive elements in *Arabidopsis*, despite DNA methylation remaining the same. In addition to minor histone modifications, an overall reduction in nucleosome occupancy resulted in the partial lost of H3 association in all sequences as well as heterochromatin decondensation. Transcriptional and nucleosome changes were transient and returned to normal following heat stress but decondensation did not. The presence of epigenetic changes in response to stress, even those that are transient, provides potential for more permanent changes, which may be transmitted to progeny (Pecinka et al., 2010).

Dynamic changes in histone H3 Ser-10 phosphorylation, H3 phosphorylation and histone H4 acetylation occurred in both *Arabidopsis* and tobacco in response to cold and salt stress, and were associated with the induction of stress-specific genes (Sokol et al., 2007). Drought stress conditions in *Arabidopsis* lead to H3K4 trimethylation and H3K9 acetylation, which correlated with gene activation of stress-inducible genes (Kim et al., 2008).

In rice, genes belonging to the histone deacetylation (HDAC) family have distinct expression patterns to abiotic stresses including cold and salt (Fu et al., 2007). Dynamic changes to histone H3-Lys4 methylation and H3 acetylation at stress-responsive loci also occur in rice in response to submergence, resulting in increased expression although levels returned to normal following exposure (Tsuji et al., 2006).

In addition, recent work on pathogens in *Arabidopsis* showed that the systemic acquired resistance (SAR) to *Pseudomonas syringae* pv tomato DC3000 (PstDC3000), could be passed on to one generation of non-stressed progeny. Luna et al. (2012) found that SAR was associated with changes in DNA methylation, as well as chromatin remodeling. Chromatin immunoprecipitation (ChIP) analyses showed that the SA-inducible promoter regions of genes such as PATHOGENESIS-RELATED GENE1, WRKY6, and WRKY53, were enriched in the permissive state acetylated histone H3 at lysine 9, in the progeny of stressed plants (Luna et al., 2012). By controlling expression of important, stress-induced genes, histone modifications pay a pivotal role in the epigenetic memory that may be passed on by stressed plants.

1.3.3. Small RNAs (sRNAs)

Small non-coding RNAs are 20-24 nucleotides (nt) in length and critical regulators of gene expression. sRNAs function in a sequence-specific manner, often by interfering with mRNA translation (Padmanabhan et al., 2009; Khraiwesh et al., 2011). As such, they are a core component of epigenetic modifications in plants as a part of the dynamic relationship between sRNAs, DNA methylation and histone changes (Simon and Meyers, 2011). In plants, sRNAs are divided based on their biogenesis and function (Khraiwesh et al., 2011). microRNAs (miRNAs) are 21 nt in length and involved in post-transcriptional silencing (Simon and Meyers, 2011). In contrast, small interfering RNAs (siRNAs) are usually 24 nt and play a role in the formation of heterochromatin and gene silencing via DNA and histone methylation using the RNA-directed DNA methylation (RdDM) pathway (Gao et al., 2010; Simon and Meyers, 2011). Recent work by Gao et al. (2010), suggests that the protein RDM1 functions with RNA polymerase II, AGO4 and DRM2, resulting an RdDM effector complex that plays a role in linking siRNA production with *de novo* DNA methylation (Gao et al., 2010).

Studying the *Arabidopsis* transcriptome has revealed a strong correlation between sRNAs and DNA methylation, siRNA-directed DNA methylation covering approximately 30% of the genome. Both methylation and demethylation help control sRNAs levels, as hypomethylated areas of the genome also had reduced sRNAs (Lister et al., 2008; Simon and Meyers, 2011). Many of the sRNAs produced originate from transposable elements (TEs) and play a crucial role in silencing them (For more details on this phenomenon refer to Migicovsky and Kovalchuk, 2012). In fact, what makes sRNAs so unique is their ability, unlike DNA methylation and histone modifications, to be

mobile and carry a short-range signal from one cell to another, allowing them to guide processes like DNA methylation (Molnar et al., 2010).

The enzyme Dicer is a member of the ribonuclease III family that cleaves at least partially double-stranded RNA precursors into small RNAs in worms, fungi, mammals and plants (Bernstein et al., 2001; Khraiwesh et al., 2011). The Dicer-like (DCL) proteins found in *Arabidopsis*, as well as their functions, are described in Table 5.

Stress-induced sRNAs have already been implicated in events corresponding to changes in physiology and development. Several biotic and abiotic stress-induced miRNAs have already been discovered in plants (Zhou et al., 2009; Khraiwesh et al., 2011). For example, work by Liu et al. (2008) found 10 high-salinity, 4 drought- and 10 cold-regulated miRNAs induced in response to their respective stresses in *Arabidopsis*. The result was a total of 14 stress-induced miRNAs out of the possible 117 found on the probes used (Liu et al., 2008). In addition to *Arabidopsis*, expression changes in miRNAs have also been found in response to cold stress *Populus* and *Brachypodium*, including the upregulation of *miR397* and *miR169* in all three species (Liu et al., 2008; Lu et al., 2008; Zhang et al., 2009; Khraiwesh et al., 2011). Heat stress in wheat has also been shown to result in changes in miRNAs, including a recent study by Xin et al. (2010), in which nine of the 32 miRNA families were heat responsive. Other abiotic stresses also impact miRNAs include UV-B radiation, which was shown experimentally in *Populus* and computationally in *Arabidopsis* (Zhou et al., 2007; Jia et al., 2009).

Like miRNAs, siRNAs are involved in plant stress response. In one critical example, Borsani et al. (2005), discovered that salt stress of *Arabidopsis* results in

expression of P5CDH, a stress-related gene, and SR05, a gene of unknown function, and that when both transcripts are present, a 24-nt siRNA is formed in a DCL2-dependent pathway. Cleavage of the P5CDH transcript is guided by this 24-nt siRNA, leading to a subsequent generation of 21-nt siRNAs produced by DCL1, as well as further cleavage of P5CDH. By downregulating P5CDH, proline degradation is reduced. Accumulation of proline results in improved salt-stress tolerance. As SR05 is induced by salt, this siRNA pathway is reliant on salt stress for initiation (Borsani et al., 2005). In addition, four different siRNAs were responsive to abiotic stress treatments including heat, cold, salt and dehydration in wheat seedlings (Yao et al., 2010).

Evidence of the critical role of small RNAs in cold adaptation is available from WCSP1, a wheat cold shock domain protein induced by cold stress that has RNA-binding as well as nucleic acid melting properties (Karlson et al., 2002; Nakaminami et al., 2005; Nakaminami et al., 2006; Nakaminami et al., 2011). Cold shock proteins (CSPs) function as RNA chaperones that regulate transcription and are considered the most ancient form of RNA binding protein in bacteria. They are also found in eukaryote proteins as a cold shock domain, an RNA-binding domain (Graumann and Marahiel, 1998). In *Arabidopsis*, knock out *AtCSP3* mutants resulted in freezing-sensitivity, and overexpression of the protein allowed for enhanced freezing tolerance, demonstrating the key role of RNA chaperones in cold stress response of higher plants (Kim et al., 2009).

Small RNAs have also been shown to be involved in the establishment of transgenerational stress memory in response to biotic stresses. For example, a recent study by Rasmann et al. (2012) showed that the herbivory in a parental generation, primes progeny for enhanced insect resistance in both *Arabidopsis* and tomato. However,

this trait was not passed on in *Arabidopsis* mutants such as *dcl2*, *dcl3*, and *dcl4*, which are involved in the biogenesis of siRNAs, indicating the important role of small RNAs play in epigenetic stress memory and heritable resistance to insect herbivory (Rasmann et al., 2012).

Consequently, because RdDM is a key mechanism for repressing transposons it is particularly important in cases where abiotic stresses may activate TEs through DNA demethylation, thereby impacting genome stability (Chinnusamy and Zhu, 2009b). Abiotic stresses that induce hypo- or hyper-methylation, as described earlier, can change gene expression. A reduction in methylation, or increase in demethylation, may result in hypomethylation, while RdDM and heterochromatic histone modifications may cause DNA hypermethylation (Chinnusamy and Zhu, 2009b). For example, cold stress causes hypomethylation resulting in TE activation in both maize root tips and *Antirrhinum majus* (Steward et al., 2000; Hashida et al., 2006**).** The sequence-specific nature of such changes in methylation may be partially directed by small RNAs, such as ROS3, which is required for DNA demethylation in *Arabidopsis* (Zheng et al., 2008). Therefore, although sRNAs are not passed on to progeny directly due to their transient nature, they may still play an important role in making epigenetic changes that lead to transgenerational stress memory.

1.3.4. Concluding Remarks

Modifying the epigenome is a dynamic way for plants to respond to environmental cues, and an important mechanism for passing on their stress response to offspring (Yaish et al., 2011). Epigenetic states that can be inherited hold much evolutionary significance as transgenerational inheritance of stress-induced epigenetic marks may play an important role in plant adaption and survival (Paszkowski and Grossniklaus, 2011; Richards, 2011).

Unlike an organism's genome, an epigenome can be altered in response to developmental and environmental cues. As a result, many epigenomes need to be sequenced for a single organism, making the endeavor even more challenging than genomic sequencing (Zhu, 2008). Although the major focus thus far has been on changes in DNA methylation, it is clear the histone modifications as well as small RNA populations are extremely important with regards to epigenetic inheritance (Richards, 2011).

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Table 5. Dicers found in *Arabidopsis* as well as the function, class, and size of sRNAs they produce.

Dicer	sRNA class	sRNA size (nt)	Function
DCL1	miRNA[1]	21[2]	miRNAs for endogenous gene silencing [3, 4]
DCL2	siRNA[1]	22[5, 6]	siRNAs from transgenes and double-stranded plant viruses. [5, 6] Can substitute for DCL4.[7]
DCL3	siRNA[1]	24[8]	Mainly transposon and other repeated sequence siRNAs.[1] Can substitute for DCL4.[7]
DCL4	siRNA[9]	21[9]	*trans*-acting siRNAs for endogenous gene silencing.[9, 10]Also siRNAs from transgenes and viruses. [6, 11]

[1]Xie et al., 2004 [2]Simon and Meyers, 2011 [3]Schauer et al., 2002 [4]Chitwood and Timmermans, 2010 [5]Mlotshwa et al., 2008 [6]Molnar et al., 2011 [7]Bouché et al., 2006 [8]Qi et al., 2005 [9]Dunoyer et al., 2005 [10]Gasciolli et al., 2005 [11]Deleris et al., 2006

2. Methods and Materials

2.1. Plant Growth Conditions

Arabidopsis thaliana lines *15d8*, *dcl2*, *dcl3*, and *dcl4* mutants (Columbia ecotype) were planted in 7:1 all-purpose potting soil to vermiculite. This soil was prepared using water containing a generic fertilizer to field capacity and then stratified for 96 hours at 4°C. Plants were then grown in growth chambers (BioChambers) at long day conditions of 16 hours light (22°C) and 8 hours dark (18°C), at high light conditions (32.8 $\mu Em^{-2}s^{-1}$), under a constant humidity of 65%. Approximately 5 days post germination (dpg) plants were transplanted into pots containing the same soil ratio and fertilizer, for a total of twelve plants per pot, and two pots per sample group, resulting in approximately 24 plants per treatment group. This included ~24 plants of the same mutant type and parental treatment, grown continuously under normal conditions, as a measure of the impact that stress had on each sample group. At approximately one-week dpg plants underwent the abiotic stress being studied. Seven days post-stress, physiological measurements were taken and tissue was collected. Tissue was taken from rosette leaves on both stressed and non-stressed (control) plants, flash-frozen using liquid nitrogen and stored at -80°C. Four samples of approximately 100mg of tissue were collected from each treatment group. Plants from which tissue was collected were killed in order to prevent bias in progeny due to mechanical stress.

2.1.1. Stress Treatment

Plants undergoing heat stress were incubated at 50°C for 3 hours, on five consecutive days.

Plants undergoing cold stress were incubated at 4°C for 12 hours, on seven consecutive days.

Plants undergoing UV stress were exposed to 4 minutes of UV-C irradiation (G30T8) of 30.5 watts and 99 volts, and a UV output of 13.9 W.

2.2. Measurement of Transgenerational Effects

To analyze whether progeny of stressed plants acquired higher tolerance to the same stress, and whether the stress response varied among *dcl* mutants, measurements assessing physical fitness were taken a week following the stress.

2.2.1. Physiological Measurements

The number of leaves on each plant was counted (excluding cotyledons) and measurements of the length and width were taken of the third youngest leaf on each plant at approximately three weeks of age. At approximately four-weeks of age bolting was assessed on each plant in order to determine the percentage of plants in each pot that had bolted.

Seeds were collected from plants and photographed under the microscope. Seed length was measured using Image J for approximately 100-200 seeds per treatment.

2.2.2. Molecular Techniques

2.2.2.1. RNA Isolation

Approximately 100 mg of plant tissue was ground to a fine powder in liquid nitrogen. 1 mL of Trizol reagent (Invitrogen) was added per sample, and centrifuged at 12,000xg for 10 minutes at 4°C. Supernatant was transferred to a new tube, and incubated for 5 minutes at room temperature. 0.2 mL of chloroform was added to each sample, and then shaken by hand for 15 seconds. Samples were incubated at room temperature for an additional 2-3 minutes. Samples were centrifuged at 12,000xg for 15 minutes at 4°C, and the colourless aqueous phase was transferred to a new tube. 0.5 mL of isopropyl alcohol was added, and samples were incubated at -80°C overnight. Afterwards, samples were centrifuged at 12,000xg for 10 minutes at 4°C, until a gel-like pellet formed on side and bottom of tube. The supernatant was discarded; 1 mL of 75% ethanol (in DepC DW) was added. The tube was flicked to re-suspend the RNA pellet, and sample was centrifuged at 7,500xg for 5 minutes at 4°C. The ethanol was then discarded, and the RNA pellet was allowed to dry at room temperature for 10 minutes, before being dissolved in 100 uL RNAse free water. Sample concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific). In some cases, mRNA was purified and concentrated using Oligotex mRNA Mini Kit (Qiagen) according to manufacturer's protocol.

2.2.2.1.1. cDNA Preparation

cDNA was prepared from mRNA using the iScript Select cDNA synthesis kit (Bio-Rad) according to manufacturer's protocol.

2.2.2.2. DNA Isolation

Total genomic DNA was prepared by homogenizing approximately 100 mg of frozen tissue in liquid nitrogen. DNA extraction buffer consisted of 31.8 g Sorbitol, 6 g Trizma base (tris), 0.84 g EDTA, and DDW to nearly 500 mL, from which pH was adjusted to 7.5 with HCl, bringing final volume to 500 mL. Nucleic lysis buffer was prepared using 200 mL Tris 1M (pH= 7.5), 200 mL EDTA 0.25 M, 400 mL NaCl 5M, 20g CTAB, and approximately 200 mL of DDW was added. pH was adjusted to 7.5, resulting in a final volume of 1L. Total extraction solution consisted of 1 volume of DNA extraction buffer (with Na-biSulfite (38mg/10mL) added before use), 1 volume of nucleic lysis buffer and 0.4 volume of 5% sarkosyl. 800 uL of total extraction buffer was per sample. Samples were incubated at 65°C for one hour and inverted periodically. 800 uL of chloroform was added, and sample was shaken by hand for 5 minutes. Samples were centrifuged at 16,000xg for 10 minutes at 4°C. The supernatant phase was transferred to a new tube, and chloroform steps repeated. 2 to 3 times sample volume of isopropanol was added. DNA was precipitated overnight at -80°C. Samples were centrifuged at 12,000xg for 15 minutes, rinsed twice with 70% ethanol, and air-dried at room temperature for about 10 minutes. DNA pellets were dissolved in water and concentration was determined using NanoDrop 2000c spectrophotometer (Thermo Scientific).

2.2.2.3. qPCR

Quantitative real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad), with promoter- or gene-specific primers. Amplification occurred under the following conditions: (1) 98°C for 2 min for one cycle; 98°C for 5 s, Tm+1°C for 5 s, for 40 cycles; (2) melt-curve analysis – 65°C to 95°C for 5 s, with a 0.5°C increment. Tm+1°C was altered according to the primers. Each reaction was repeated three times.

2.2.2.3.1. Transposons

The transcriptional activity of ONSEN and TSI was determined by amplifying the genes from cDNA, as well as the standard ubiquitous primer tubulin. Primer sequences are listed in Table 6.

2.2.2.3.2. Epigenetic Genes

The transcriptional activity of several histone modifier genes (SUVH2, SUVH5, SUVH6, and SUVH8) as well as a demethylase ROS1, mismatch repair gene MSH6, and heat shock transcription factor HSFA2, were measured using quantitative Real Time PCR. The genes were amplified from cDNA, and the ubiquitous tubulin primers used to generate a standard, compensating for any variation in the amount of cDNA present. Primers were designed using Beacon Designer7 software, and are listed in Table 6.

2.2.2.4. ChIP

ChIP analysis of histone modifications in the progeny of 15D8, *dcl2* and *dcl3* *Arabidopsis* plants grown under normal and heat-stressed conditions, was done according to a previously described protocol (Saleh et al., 2008) with minor modifications previously described by Bilichak et al. (2012). Precipitated gDNA fragments were amplified according using described qPCR protocol. Quantitative measurements of DNA precipitated through MeDIP, followed by qPCR using coding sequence primers for SUVH2, SUVH5 and HSFA2 and SUVH2 promoter region as listed in Table 6.

2.2.2.5. Cytosine-Extension Assay

Global genome methylation changes were detected using the cytosine-extension assay, following the protocol described by Boyko and Kovalchuk (2010).

2.3. Statistical analysis

Statistical analysis of physiological data including leaf number, length, width, and seed length, were performed using R software. A 95% confidence interval was calculated for each measurement, under both stressed and normal conditions, and confidence intervals were compared with bootstrap x10,000 to determine if two intervals were significantly different from each other.

The response of plants under stress was determined by dividing the measurement of stressed plants by the measurement under normal conditions, in order to determine if a significant change occurred. If the resulting value overlapped with 1 at 95% confidence ($p=0.05$) no significant change occured. All ratios were calculated using bootstrap x10,000. Comparisons were done between wild-type and *dcl* mutants in order to determine if the difference between the two responses was significantly different at a confidence interval of 95%. These results were graphed using Prism (Graphpad) software. Bolting time was determined as a percentage, calculated by dividing the number of bolted plants by the total number of plants measured, and graphed using Microsoft Excel.

Transcription results for transposons, epigenetic genes and ChIP results were graphed and analyzed using Prism (Graphpad) software. Standard deviations and standard errors of the mean were given from Q-PCR (BioRad) results and a t-test was performed to determine differences, where significance was given to a result with a p-value ≤ 0.05.

Global genome methylation results were graphed, along with standard deviations, in Microsoft Excel. As only two repeats were performed, significant differences could not be calculated.

2.4. References

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Table 6. Genes tested and corresponding primer sequences.

Gene Information	Gene Region	Forward Primer	Reverse Primer
at2g26150-HSFA2	Coding Sequence	AGGTTGAGAGGTTGAAGAGG	GTCATCATCTGCTGCTGTC
at4g02070-MSH6	Coding Sequence	GGTAATGTGGAAGAAGATA	ATTCTCATCAACCAACTC
ONSEN	Coding Sequence	CCACAAGAGGAACCAACGAA	TTCGATCATGGAAGACCGG
at2g36490-ROS1	Coding Sequence	ACCTGCTTCTCTAATGTC	AACTTCAACTCGTCCTAA
at2g33290-SUVH2	Coding Sequence	TTATTCGTATCTCAGAGC	CAGAATCCAATCCGTATA
at2g33290-SUVH2	Promoter	ACCAAATAATTAGTACAGAAGAA	GTATGAACTTAAGATCGGAAT
at2g35160-SUVH5	Coding Sequence	ACGACATTACAATCATCAG	CTTGAAGACGAGTTTACC
at2g23740-SUVH6	Coding Sequence	TTAACAGTGTAGGTGAAC	AGGTGAAGAAATCTAAGTC
at2g24740-SUVH8	Coding Sequence	TCAAGGATAACCGAAGCAGAG	GCATAACCGCCGTCTAACC
TSI	Coding Sequence	ACCATCAAAGCCTTGAGAAGCA	CCGTATGAGTCTTTGTCTTTGTATCTT
Tubulin	Coding Sequence	ACAGAAGCGGAGAGCAACAT	TCCTCATCCTCGTAGTCACCTT

3. Results

3.1. Physiological Results

3.1.1. Changes to Leaf Phenotype

In our study, we measured the physiological response of plants to stress using parameters such as leaf size and number. Plants stressed included the progeny of plants, which had undergone the same stress, as well as the progeny of genetically identical non-stressed plants. Abiotic stresses tested were heat, cold and UV-C. Plants deficient in specific dicer proteins *dcl2*, *dcl3*, and *dcl4*, were compared to wild-type 15D8, of the same background (Columbia cultivar) in order to determine the potential role of dicers in transgenerational stress response.

Our experiment began with seeds (F1) from stressed (S_1) and control (C_1) lines, and was repeated for two generations, as described in Figure 1. These seeds, consisting for wild-type (15D8) and three *dcl* mutants, were grown under both stressed (+) and normal (-) conditions and measurements were taken for each of the resulting four F1 groups. Seeds (F2) were collected from stressed progeny of stressed plants (S_2), control progeny of stressed plants (S_1C_1), stressed progeny of control plants (C_1S_1), and control progeny of control plants (C_2). The F2 plants were grown under both stressed and normal conditions as well, and corresponding measurements were taken for all resulting eight groups, as described in Figure 1.

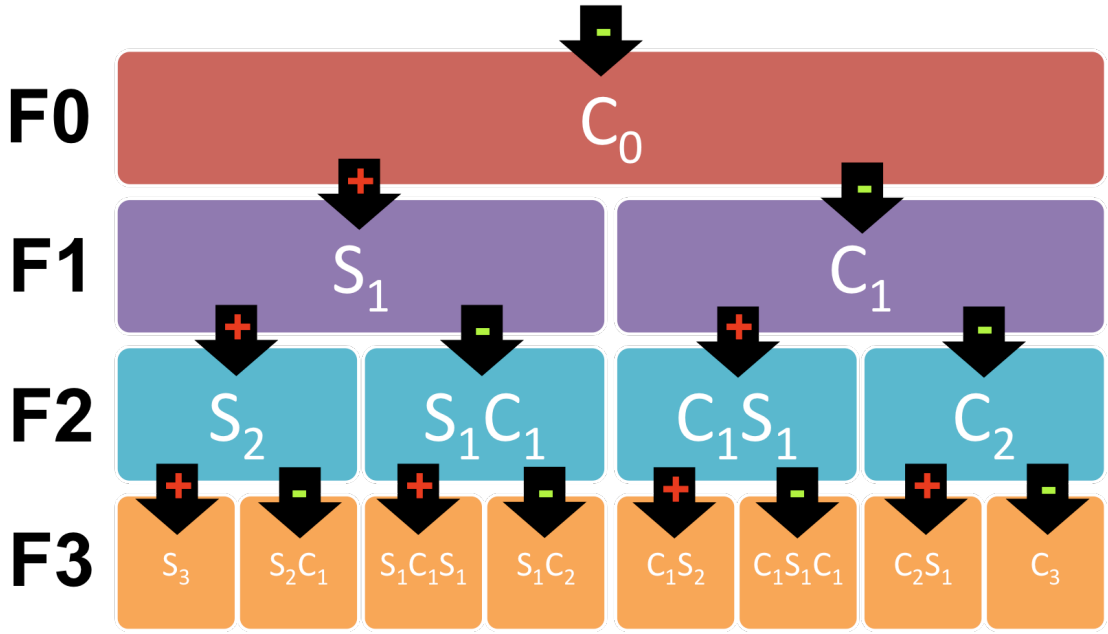

Figure 1. Project began with F1 seeds, which were the progeny of exposed (S0) and control non-exposed (C0) plants. F1 plants were grown under either stressed or normal conditions. F2 seeds were collected and grown for another generation under either stressed or normal conditions, from which F3 seeds were collected. Each step was completed for 15D8, *dcl2*, *dcl3* and *dcl4*. The complete project was repeated for heat, cold, and UV stress.

In order to determine the impact of stress on plant phenotype, as opposed to natural variation, response to stress was calculated as a ratio indicating the difference between a physiological factor under stress compared to under normal growth conditions. This ratio was calculated using a 10,000x bootstrap and a confidence interval of 95%.

3.1.1.1. F1 Generation

3.1.1.1.1. Heat Stress

The number of leaves on heat stressed plants that were progeny of control plants (C_1+) was overall higher than heat stressed progeny of stressed plants (S_1+), in particular for *dcl* plants (Figure 2). In addition, the number of leaves present on the progeny of stressed grown under normal conditions (S_1-), was consistently lower than the progeny of control plants grown under normal conditions (C_1-). The number of leaves in progeny of

stressed plants in comparison to progeny of control plants, decreased for all *dcl* plants, regardless of F1 treatment.

In general, leaves that were stressed (+) were longer than leaves grown under normal conditions (-) (Figure 3). However, while heat stress increased leaf length for 15D8, parental heat stress also had an impact, as the progeny of stressed plants (S_1) had longer leaves than the progeny of control (C_1) 15D8 plants under both stressed and normal conditions. S_1 *dcl4* plants leaves were significantly shorter than those belonging to 15D8, regardless treatment, while C_1 *dcl4* were quite similar.

Leaf width tended to increase with F1 heat stress (+) (Figure 4). However, the difference between the progeny of stressed and the progeny of control plants was not substantial, except for a decrease in leaf width for S_1+ *dcl3* and *dcl4* progeny, in comparison to C_1+. Also, while the leaf width of C_1- *dcl2* was significantly higher than 15D8, it was still significantly lower in the progeny of stressed plants grown under normal conditions (S_1-). *dcl3* decreased leaf width under normal conditions in the progeny of stressed plants, and both 15D8 and *dcl4* increased.

Heat stress resulted in significantly fewer leaves in the progeny of stressed (S_1) *dcl2* and *dcl3* plants, in contrast to 15D8 and *dcl4* (Figure 5). Leaf length was not significantly influenced by parental treatment. However, leaf width showed a significant difference between all other plant types and *dcl3*, which was significantly wider under stress. In nearly all cases, leaf number and size increased in response to heat stress resulting in a ratio greater than 1.

3.1.1.1.2. Cold Stress

Cold stress decreased leaf number, and the number of leaves present on the progeny of cold stressed plants under cold stress (S_1+), was significantly lower than the progeny of control plants (C_1+) (Figure 6). In addition, the offspring of cold stressed plants, grown under normal conditions (S_1-), had significantly fewer leaves than the progeny of control plants (C_1-), in the case of 15D8 and *dcl4*. While the number of leaves on C_1+ *dcl2* and *dcl4* was significantly higher than wild-type, S_1+ *dcl4* was significantly lower than 15D8. Non-stressed *dcl3* (both (C_1- and S_1-), had significantly fewer leaves than 15D8 but there was no significant difference when the same plants were stressed (+). S_1 *dcl4* had significantly fewer leaves than 15D8, regardless of F1 treatment.

The progeny of control plants increased leaf length in response to cold stress (C_1+) (Figure 7). In contrast, the progeny of stressed plants maintained a similar length (S_1+). Both S_1- and C_1+ *dcl4* had significantly shorter leaves than wild-type.

Both the progeny of control and the progeny of stressed plants increased leaf width in response to cold stress; however, the progeny of control (C_1+) leaves were longer than those belonging to the progeny of stressed plants (S_1+) (Figure 8). Non-stressed plants (C_1- and S_1-) had leaves that were similar in width, with the exception of 15D8, in which case S_1- leaves were slightly wider.

Response to cold showed that while leaf number decreased with cold stress, leaf width and length increased stress (Figure 9). The change in leaf number in response to cold stress in the progeny of control (C_1) *dcl* plants was significantly greater than wild-type. However, the progeny of stressed plants (S_1) line did not result in a significantly different response. Changes to leaf length and width are very similar in response to cold

stress in the progeny of non-stressed plants, while the progeny of stressed plants show some interesting differences. In particular, the response of leaf length and width of *dcl2* and *dcl4* in the progeny of stressed plants appear to be much greater than that of 15D8 and *dcl3*.

3.1.1.1.3. UV Stress

UV-stressed progeny of stressed plants (S_1+) have significantly less leaves than UV-stressed progeny of control plants (C_1+) (Figure 10). However, while C_1+ *dcl3* had significantly fewer leaves than 15D8, S_1+ *dcl3* had a similar number, and S_1+ *dcl4* had significantly fewer than 15D8. Non-stressed progeny of stressed plants (S_1-) had fewer leaves than the non-stressed progeny of control plants (C_1-) for both 15D8 and *dcl2*, a similar amount for *dcl4*, and higher value for *dcl3*. Both *dcl2* had significantly more leaves than 15D8 under normal conditions, regardless of parental treatment.

The progeny of UV stressed 15D8 plants had longer leaves under normal conditions (S_1-) than the progeny of control (C_1-). *dcl2* and *dcl4* both increased leaf length slightly as well, but *dcl3* had shorter leaves (Figure 11). While UV stress (+) resulted in leaves of a similar length for all *dcl* plants, regardless of parental treatment, S_1+ 15D8 leaves were longer than C_1+. While all three S_1- *dcl* groups had significantly shorter leaves than 15D8, C_1- conditions resulted in *dcl2* and *dcl4*, but not *dcl3*, leaves that were significantly shorter than 15D8.

The progeny of UV stressed plants, grown under normal conditions (S_1-) resulted in *dcl* plants with significantly narrower leaves than 15D8 (Figure 12). However, the progeny of control plants, when grown under normal conditions, resulted in *dcl2* and *dcl4*,

but not *dcl3*, leaves that were significantly narrower than 15D8. In addition, parental stress significantly increased the width of 15D8 leaves when grown under normal conditions (S_1- in comparison to C_1-), as well as *dcl2* and *dcl4*. The leaf width of all UV stressed plants was similar, but those that had been parentally stressed (S_1+) were slightly wider than those that had not been, especially in the case of 15D8.

UV stressing progeny of control plants increased leaf number, with the exception of *dcl2*, which was significantly different from wild-type response (Figure 13). Progeny of all stressed (S_1) dicer mutant plants had fewer leaves in response to UV-C stress, and *dcl4* was significantly different from 15D8. Interestingly, while leaf number in response to stress did not change much with differing parental treatments of wild-type and *dcl2*, parental stress did have a strong impact on the leaf number for *dcl3* and *dcl4* mutants. Changes to leaf morphology in C_1 plants were similar in wild-type and *dcl3* plants, while *dcl2* (both length and width), and *dcl4* (length) showed significant differences from wild-type in response to stress. Changes to leaf shape in response to UV-C stress were consistent among parentally stressed wild-type, *dcl2* and *dcl4*. However, the length and width response of *dcl3* increased with parental stressing, in comparison to *dcl3* C_1 where both factors decreased in response to stress. As a result, all three S_1+ dicer mutants had longer, and significantly wider leaves than 15D8.

3.1.1.2. F2 Generation

3.1.1.2.1. Heat Stress

F1 heat stress alone did not result in significantly more leaves in plants grown under normal conditions (C_2- versus C_1S_1-) (Figure 14). However, F0 heat stress did impact leaf number, as S_2- and S_1C_1-, had significantly more leaves than C_2- and C_1S_1- for all mutant types. With all parental treatments, *dcl3* plants had significantly fewer leaves than 15D8, as did *dcl4* for C_2 and C_1S_1 under normal conditions. Though there was some minor fluctuation in leaf number between S_2- and S_1C_1-, in particular 15D8 and *dcl2* both having more leaves in S_2-, the differences were not significant.

In F2 heat stressed plants (+), there was also a division between those that had been stressed in F0, and those that had been grown under normal conditions in F0 (Figure 14). Plants that had been stressed in F0 (S_2 and S_1C_1) had significantly more leaves than those that had not been. In addition, plants that had been stressed for two previous generations (S_2) had significantly fewer leaves under heat stress than those that had been grown under normal conditions for F1 (S_1C_1) for 15D8, *dcl2* and *dcl3*, while *dcl4* actually increased in leaf number. Also, similar to plants grown under normal conditions, *dcl3* plants frequently had significantly fewer leaves under heat stress than 15D8, the only exception being C_2 plants. Finally, while 15D8 did increase leaf number in stressed C_1S_1 in comparison to C_2, both *dcl2* and *dcl3* maintained a similar number of leaves, and *dcl4* actually decreased leaf number. The result was that all *dcl* plants had significantly fewer leaves than 15D8.

The response of leaf number to heat stress in the F2 generation was determined by dividing the number of leaves under stress (+) by the number of leaves in plants with the

same parental treatment, but grown under normal conditions (-). For example: S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2-.

The response of leaf number of *dcl2* to heat stress was consistent regardless of parental treatment (Figure 15). The *dcl3* and *dcl4* progeny of control plants, grown under control conditions (C_2) had a significantly greater response to heat stress than 15D8. *dcl3* was also significantly different for C_1S_1. The number of leaves in response to heat stress never changed significantly for *dcl4* and only changed significantly for 15D8 with S_2 parental treatment. C_2 and C_1S_1 parental treatments for *dcl2* and *dcl3* resulted in a significant change with heat stress, but not S_2 and S_1C_1.

Under normal growth conditions (-), *dcl* plants tended to have longer leaves than 15D8, when they had been grown under normal conditions for at least one previous generation (Figure 16). Following two generations of heat stress (S_2) *dcl* plants had shorter leaves than 15D8, with the difference being significant for *dcl2* and *dcl3*. When plants were heat stressed, the variation in length was less significant among mutant types. Though the heat stressed F2 progeny of F0 controls (both C_2 and C_1S_1) tended to result in *dcl* plants with leaves that were shorter than 15D8, the difference was only significant for C_1S_1 *dcl3*. Heat stress during F1 resulted in F2 15D8 leaves that were longer under heat stress (C_1S_1 in comparison to C_2, and S_2 in comparison to S_1C_1).

The change in leaf length in response to heat stress varied with F0 treatment (Figure 17). Among plants that had undergone F0 growth in normal conditions (both C_1S_1 and C_2) only 15D8 showed a significant change in response to heat stress, in which leaves became longer under stress. These 15D8 plants increased length significantly more than

dcl plants, with the exception of C_2 *dcl4*. In contrast, S_1C_1 and S_2 15D8 plants did not show a significant difference under stress, although all *dcl* S_1C_1 plants, and S_2 *dcl3*, did. Both S_1C_1 *dcl4* and S_2 *dcl3* had a significantly different response than 15D8.

Amid the fluctuation in leaf width for F2 heat stressed plants, a few trends appeared (Figure 18). When grown under normal conditions, *dcl* plants tended to have wider leaves than 15D8, the exception being plants that had been heat stressed for two previous generations (S_2) in which case *dcl* leafs were less wide than 15D8, a difference that was significant for *dcl2* and *dcl3*. The leaf width of *dcl4* plants grown under normal conditions remained consistent regardless of parent treatment. When heat stressed, 15D8 plants that had been heat stressed in F1 (S_2 and C_1S_1) had wider leaves than those that had not been. F0 heat stress (S_2 and S_1C_1) resulted in F2 *dcl3* leaves that were wider following heat stress than those in which F0 treatment had been growth under normal conditions (C_2 and C_1S_1). While the differences were not always significant, the widest *dcl3* leaves were the parental treatment S_2 under heat stress (+). The same was also true of *dcl2*, *dcl4*, and 15D8.

Heat stress also impacted leaf width. All parental treatments except S_2 resulted in 15D8 increasing leaf width in response to heat stress (Figure 19). In addition, while F0 treatments (C_1S_1 and C_2, versus S_1C_1 and S_2) varied in the response of *dcl2* and *dcl3*, the F1 treatment of stress (C_1S_1 and S_2) or control (C_2 and S_1C_1) did not seem to impact F2 leaf width response to heat stress.

3.1.1.2.2. Cold Stress

Parental treatment influenced the number of leaves in F2 plants, both under normal conditions and cold stress (Figure 20). C_2 plants grown under normal conditions (-) had the most leaves, and there was a steady decrease with increasing generations of parental cold stress. Cold stress (+) of further decreased leaf number, especially if parental plants had been stressed as well, with the lowest leaf number being found on either S_1C_1 + or S_2+ plants, depending on the mutant type. In general, F0 treatment was more important than F1 treatment in influencing number of leaves. Under normal and stressed conditions, those with the same F0 treatment (C_2 and C_1S_1, and S_1C_1 and S_2) tended to be more similar to each other than treatment groups with the opposite F0 treatment, though in many cases F1 cold stress did further decrease leaf number.

Cold stress resulted in a significant decrease in leaf number, regardless of mutant type or parental treatment (Figure 21). 15D8 plants with F0 stress had a larger decrease in leaf number under stress than those with F0 control conditions, but F1 treatment did not appear to impact response. The response of *dcl2* did not vary with parental treatment, but was significantly less than 15D8 for S_1C_1 and S_2. In addition, *dcl3* shows little variation in response regardless of parental treatment, with the only exception being S_1C_1. *dcl3* also had significantly less of a response than 15D8 with S_2 parental treatment. *dcl4* C_1S_1 and C_2 plants had less of a change under stress than S_1C_1 and S_2, F1 stress lessened the response among *dcl4* plants with the same F0 treatment. In other words, C_1S_1 showed less of a change under stress than C_2, and S_2 had a smaller change under stress than S_1C_1. For C_1S_1, *dcl4* had significantly less of a response than 15D8. Overall, the most similarity in

responses is seen between *dcl2* and *dcl3* in which the response to cold stress is nearly identical with all parental treatments.

In general, leaf length was lower in plants that had been exposed to cold stress (+), than those under normal conditions (-) (Figure 22). While *dcl4* plants decreased length under normal conditions with increasing parental stressing (S_2 was lower than C_1S_1 and S_1C_1, which were lower than C_2), when stressed their length actually increased with additional parental stressing, and all treatment groups under stress are longer than S_2 -. Under normal conditions, the longest leaves in all groups were *dcl3*.

Changes in leaf length for 15D8 in response to cold stress were not influenced by parental treatment, and were not altered significantly under stress (Figure 23). In contrast *dcl2* and *dcl3* significantly decreased by stress in every case except for S_1C_1. The response of *dcl4* was only significant for S_2, where cold stress increased leaf length. Thus, 15D8 and *dcl4* had similar responses, as did *dcl2* and *dcl3*, with the same parental treatments. The only exception was S_2, in which two generations of parental cold stress result in an increase in leaf length in response to stress for *dcl3*, while *dcl2* decreased.

Leaf width tended to be lower in plants that were cold stressed in comparison to those grown under normal conditions, with the exception of *dcl4* (Figure 24). Under normal growth conditions, *dcl3* had the widest leaves, which were significantly larger than 15D8 in the case of treatment group C_1S_1. 15D8 leaves were significantly wider than *dcl2* under cold stress in C_2 and S_2 treatment groups.

Leaf widths of *dcl*, but not 15D8, plants were impacted by cold stress (Figure 25). *dcl3* was significantly impacted by cold stress with parental treatments besides S_2 and

was significantly lower than 15D8 for C_1S_1. Like *dcl3*, *dcl2* decreased with 3 of the parental conditions, although in this case the exception was C_1S_1. The response of *dcl2* was significantly lower than 15D8 for S_2. *dcl4* response significant for S_2. Overall, the most similarity occurred between *dcl2* and *dcl3* leaf width responses to cold.

3.1.1.2.3. UV Stress

There was not a significant difference in leaf number among mutant types grown at normal conditions, following 2 generations of growth at normal conditions (C_2) (Figure 26). However, among plants that had been UV-stressed in F1 (C_1S_1), *dcl4* had significantly more leaves than 15D8. *dcl4* plants that had been stressed for F0 and F1 (S_2-) also had significantly more leaves than 15D8. Under both control and UV stress, the S_1C_1 *dcl4* had significantly fewer leaves than 15D8. C_1S_1+ *dcl* plants had significantly more leaves than 15D8, but after two generations of stress (S_2 +), there were no significant differences in leaf number. In general, treatment groups in which plants had been UV stressed in F0 (S_1C_1), or stressed in F0 and F1 (S_2) had fewer leaves than those in which no parental stressing had occurred (C_2) or it had only occurred in F1 (C_1S_1).

All significant changes to leaf number in response to UV stress were positive (Figure 27). *dcl4* did not have a significant response, while 15D8 only had a significant change to leaf number for S_2. For *dcl2*, the exception was C_2, although it did have a significantly lower change in leaf number than 15D8 for the S_2 group. Finally, *dcl3* only had a significant change for C_1S_1, a response that was also significantly higher than 15D8. The most consistency was within S_1C_1 plants, which showed little variation in leaf number regardless of mutant type.

Under normal conditions there was not significant variation in leaf length, although plants that were the progeny of two generations of UV-stress (S_2) had slightly shorter leaves than C_2 plants (Figure 28). UV stress resulted in some fluctuation in leaf length. For example, while C_2 and C_1S_1 *dcl3* had significantly longer leaves than 15D8 under UV stress, S_1C_1 *dcl3* had significantly shorter leaves. Parental treatment did not impact leaf length under UV stress for 15D8 and *dcl4*.

Changes to leaf length in response to UV stress were not significant (Figure 29). However, C_2 *dcl3* had significantly longer leaves than 15D8, as did S_2 *dcl2*. F0, but not F1, *dcl3* parental treatment impacted leaf length, lowering it under stressed conditions.

Leaf width was consistent among 15D8 plants (Figure 30). However, *dcl3* C_2 and C_1S_1 had significantly wider leaves than S_2 and S_1C_1. C_2 and C_1S_1 *dcl3* were significantly wider than 15D8 under UV stress. *dcl4* plants had the least wide leaves in all groups except S_1C_1+, where *dcl3* leaves were smaller. Like leaf length, the response of leaf width to UV stress was not significant in most cases (Figure 31). The exceptions were C_2 *dcl3* and *dcl2*, which were significantly wider in response to UV than 15D8. F0 treatment impacted *dcl3* response, as C_1S_1 and C_2 had larger leaf width responses than S_2 and S_1C_1.

Natural phenotypic variation exists among different mutant types, and may be more pronounced with additional generations. For example, *dcl3* plants had significantly fewer and longer leaves than wild-type in C_1, but the variation diminished by the C_2 generation (Figure 32). While 15D8 maintained similar values in C_1 and C_2, *dcl* had fewer, longer, and wider leaves in C_2, providing strong evidence of the natural phenotypic variation present among mutant *dcl* plants.

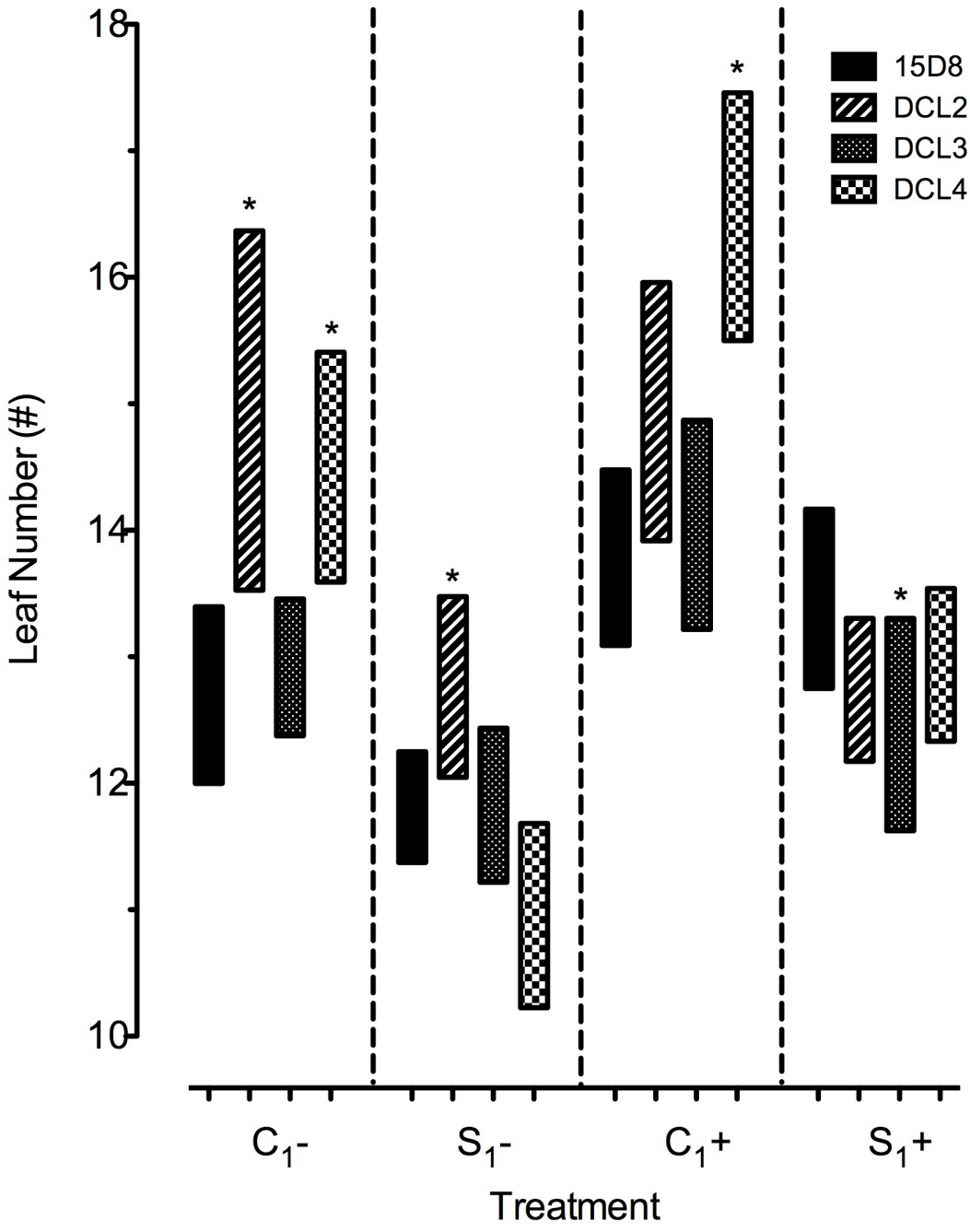

Figure 2. Leaf number in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 5 to observe which responses to stress were statistically significant.

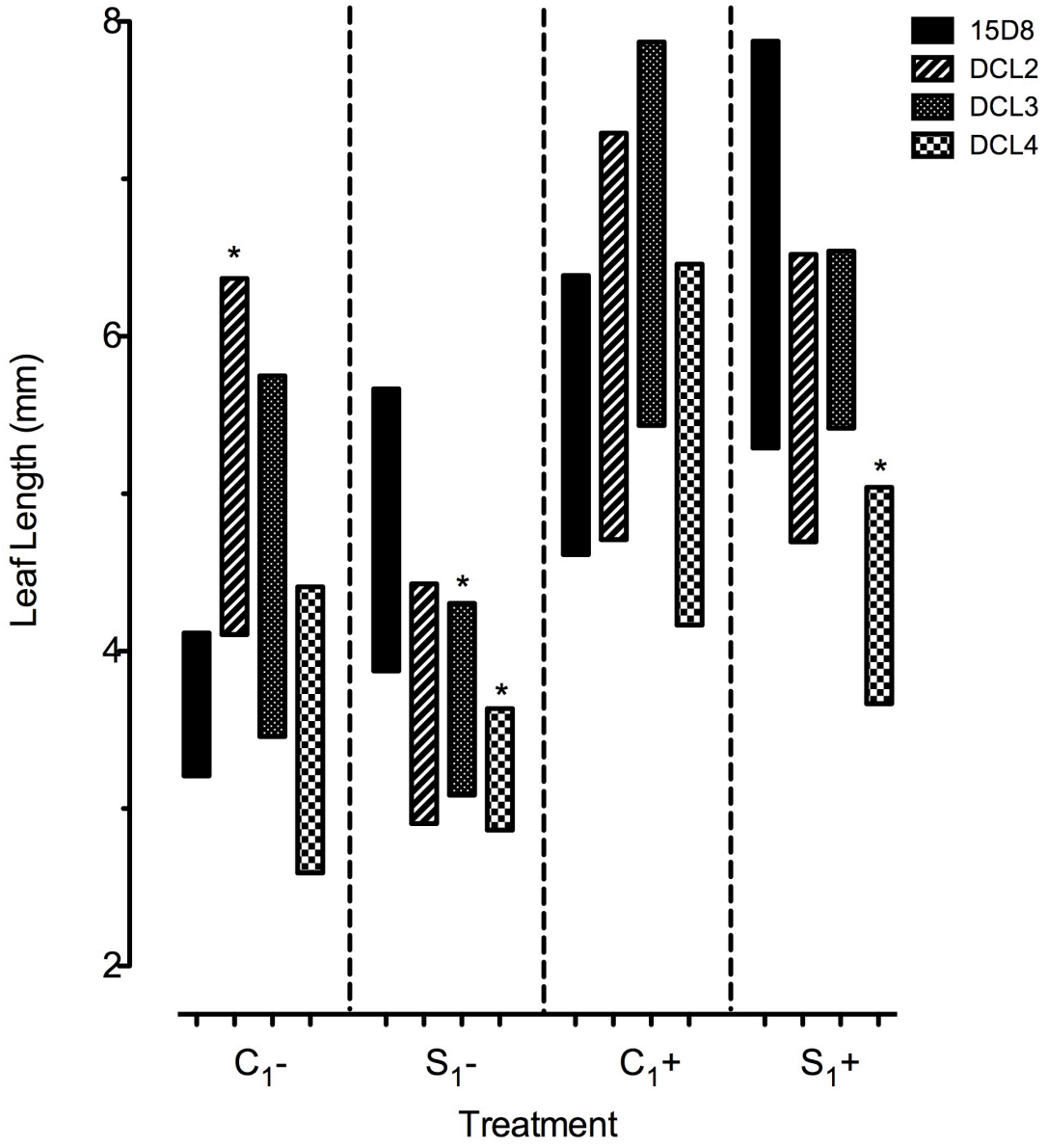

Figure 3. Leaf length in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 5 to observe which responses to stress were statistically significant.

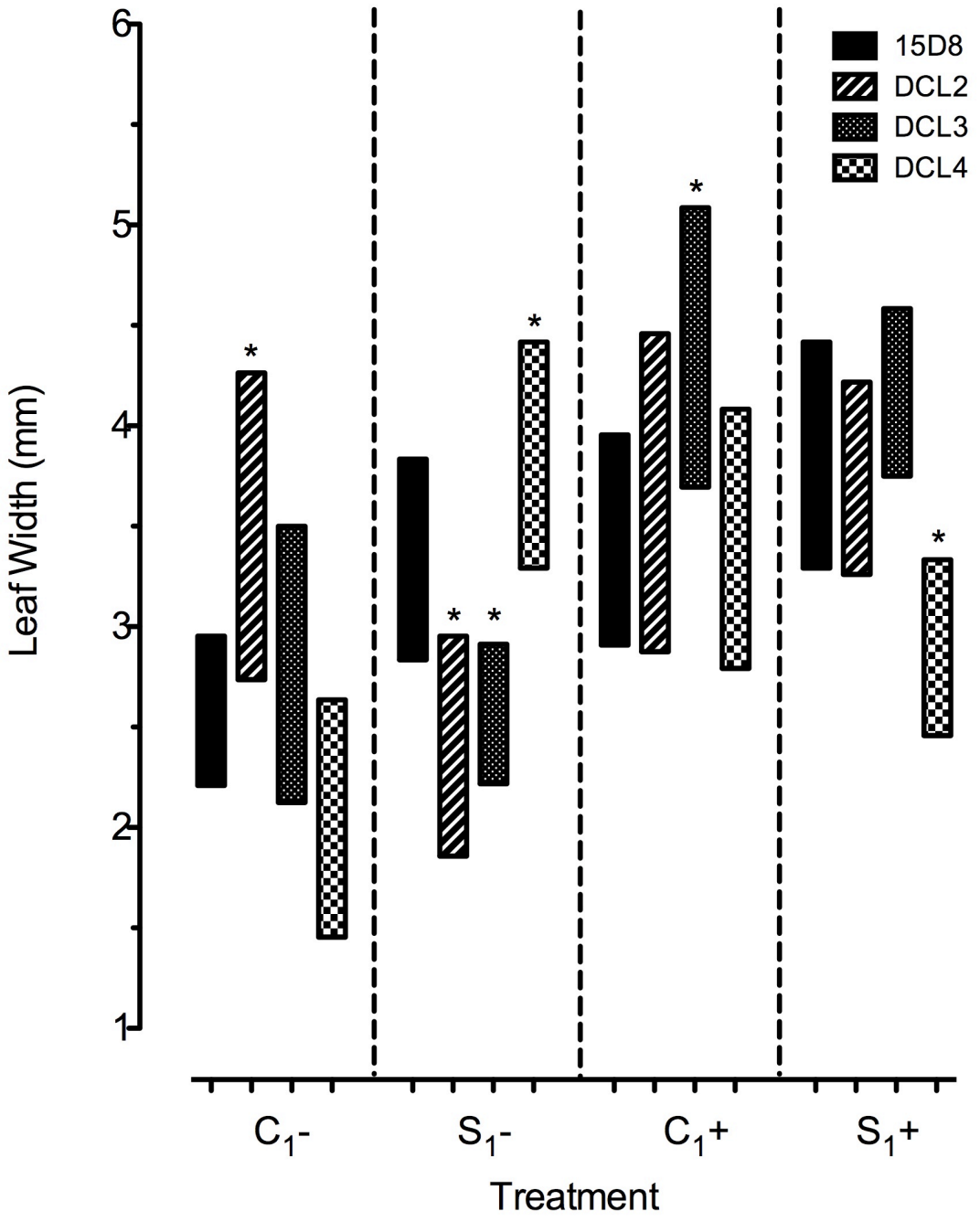

Figure 4. Leaf width in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 5 to observe which responses to stress were statistically significant.

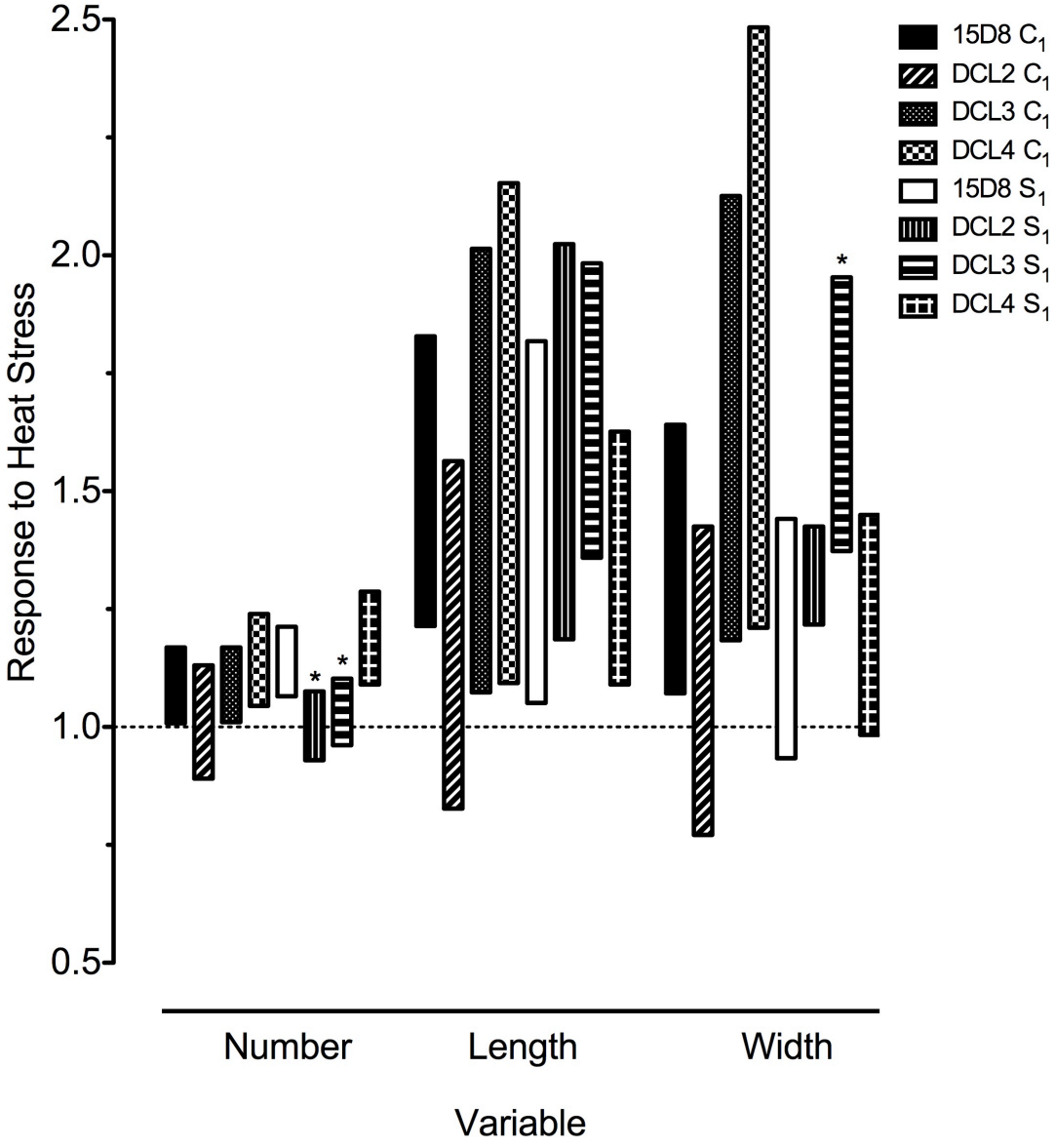

Figure 5. Bars represent ratio of change in F1 plants in response to heat stress (S+/S- or C+/C-) at 95% confidence, p-value of 0.05. Ratios are the result of 10,000 bootstrap analysis. Four different plant types were used. Plants were stressed at 50°C for 3 hours, on five consecutive days. Ratios which overlap with the value of 1 indicate no significant change under heat stress. Measurements were taken for leaf number, length, and width. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same treatment. Legend indicates parental treatment.

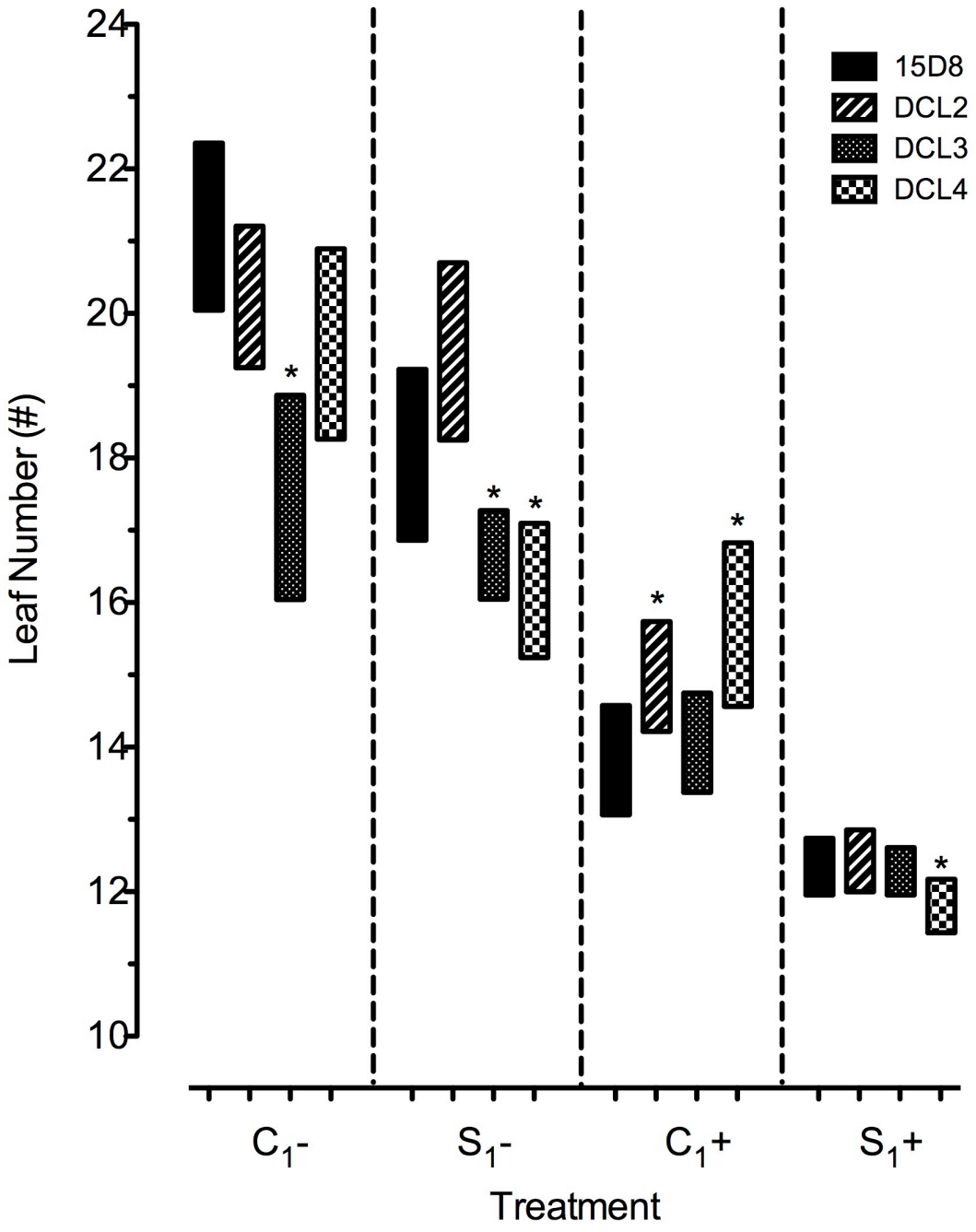

Figure 6. Leaf number in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 9 to observe which responses to stress were statistically significant.

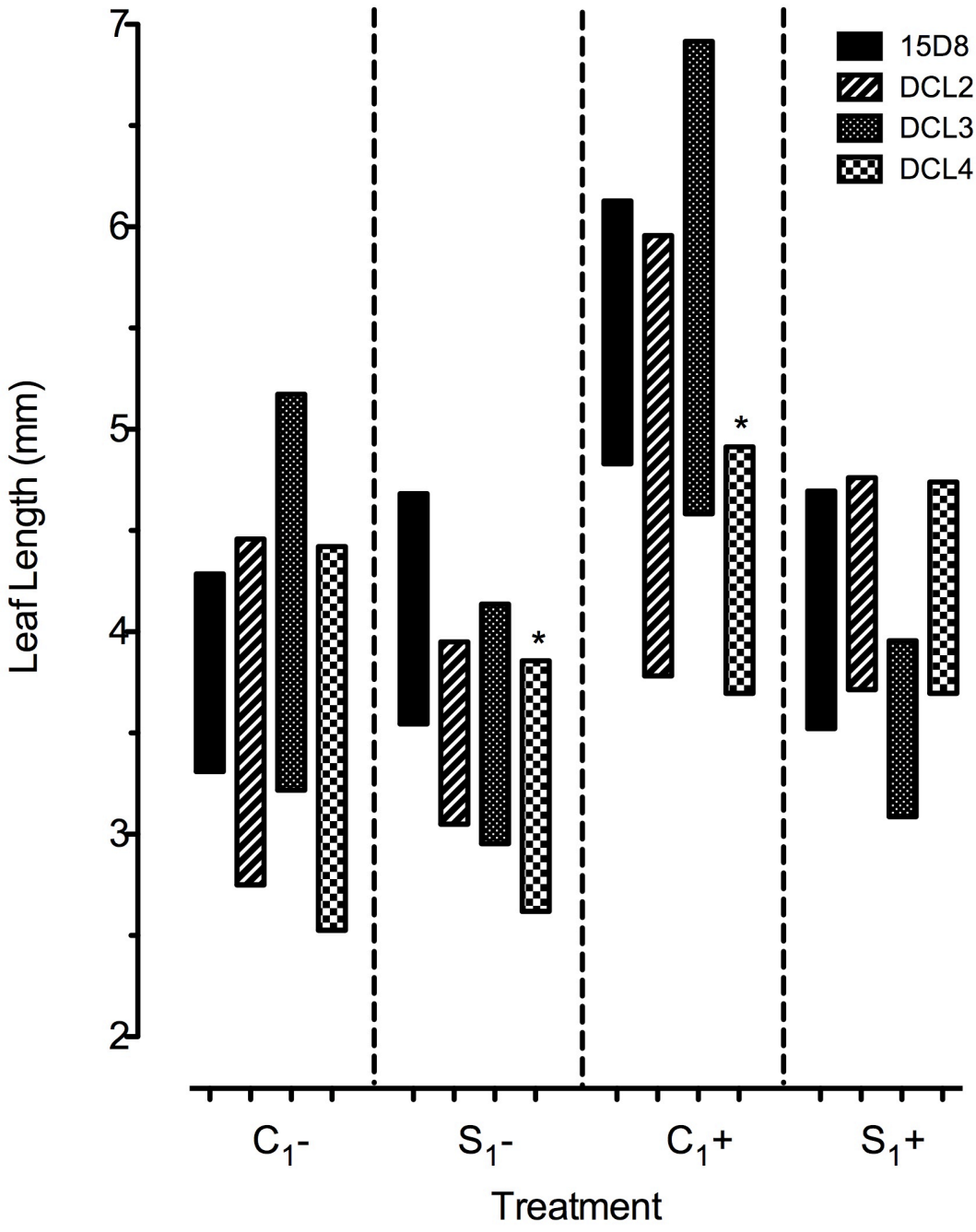

Figure 7. Leaf length in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 9 to observe which responses to stress were statistically significant.

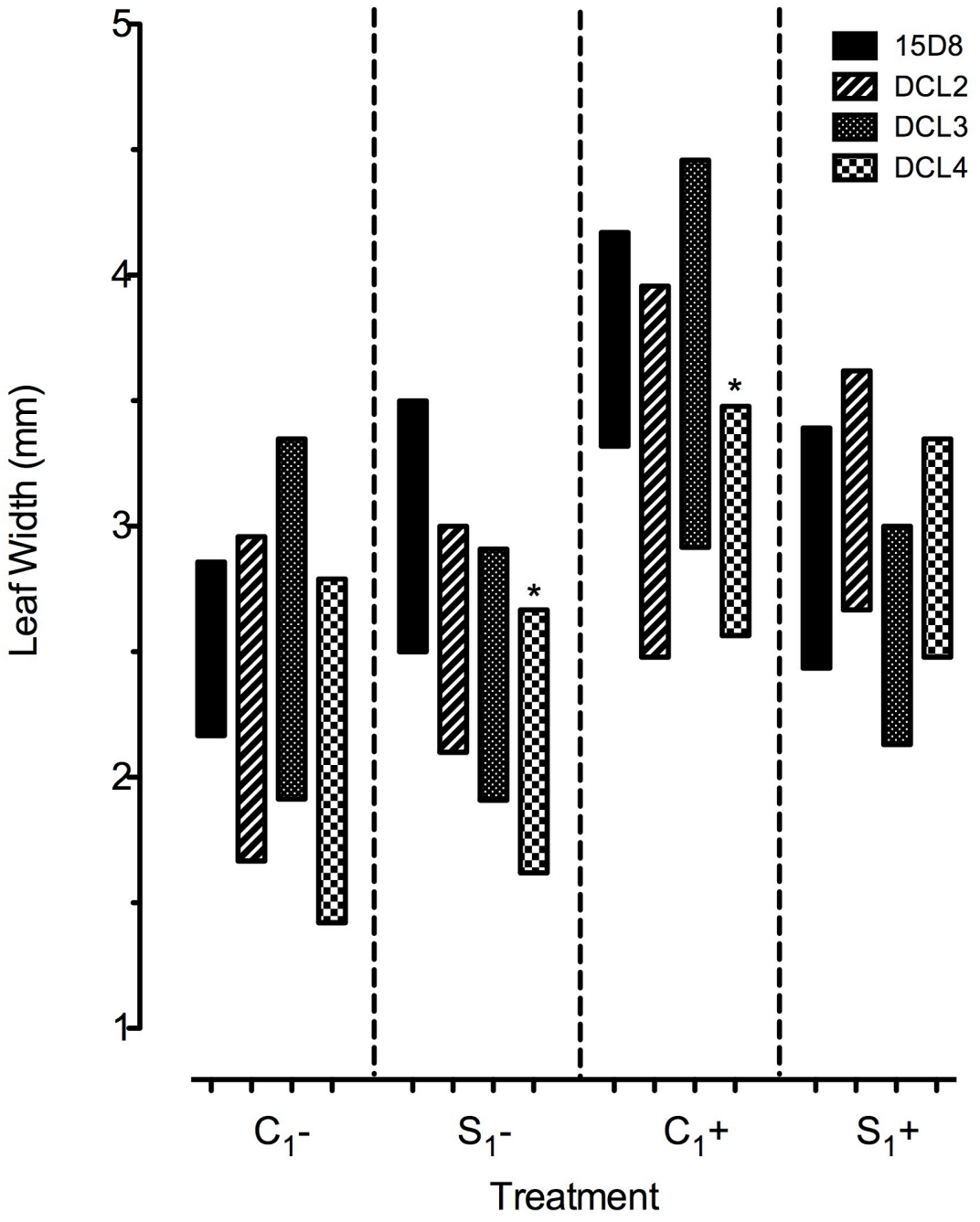

Figure 8. Leaf width in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 9 to observe which responses to stress were statistically significant.

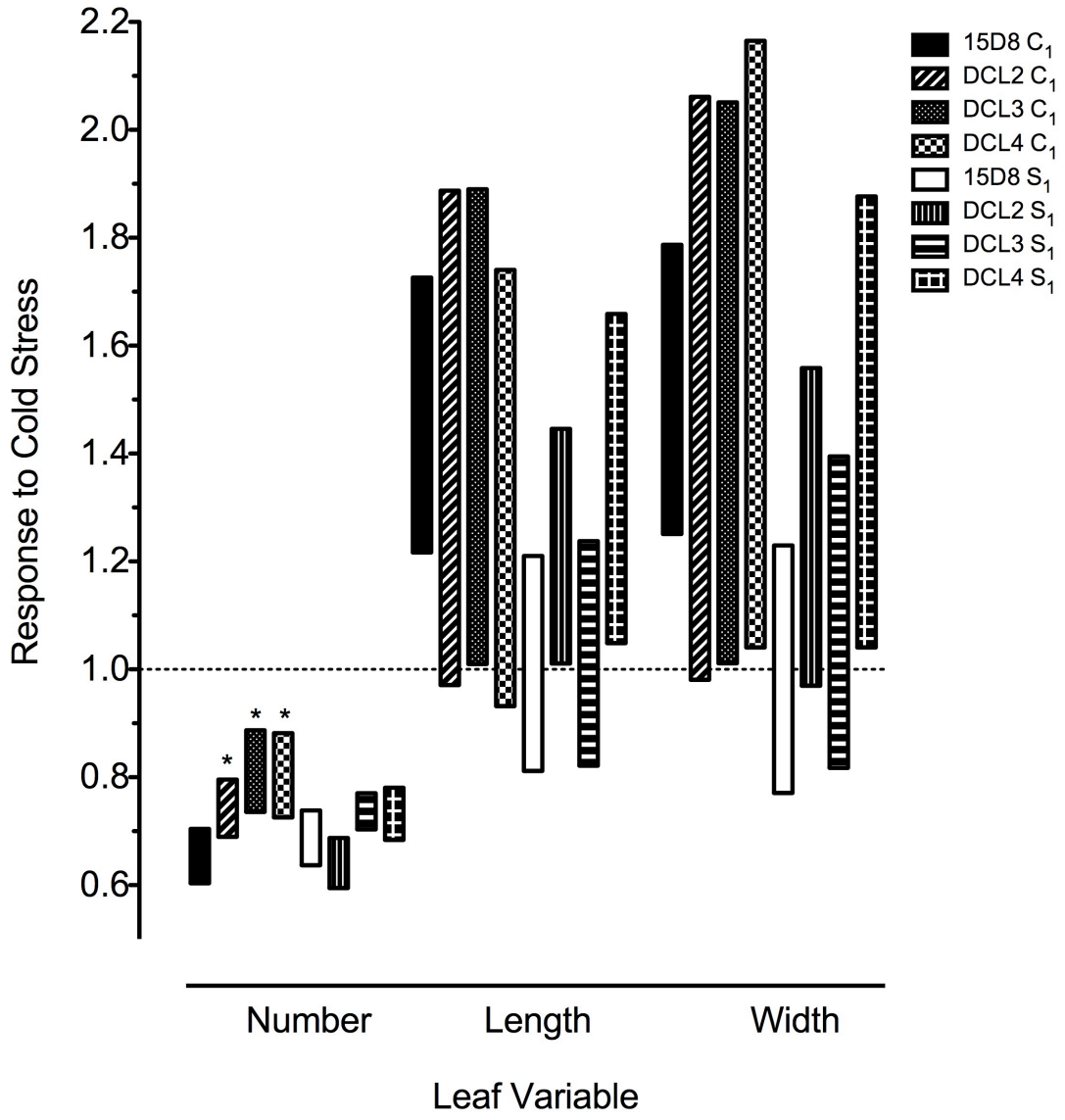

Figure 9. Bars represent ratio of change in F1 plants in response to cold stress (S+/S- or C+/C-) at 95% confidence, p-value of 0.05. Ratios are the result of 10,000 bootstrap analysis. Four different plant types were used. Plants were stressed at 4°C for 12 hours, on seven consecutive days. Ratios which overlap with the value of 1 indicate no significant change under cold stress. Measurements were taken for leaf number, length, and width. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same treatment. Legend indicates parental treatment.

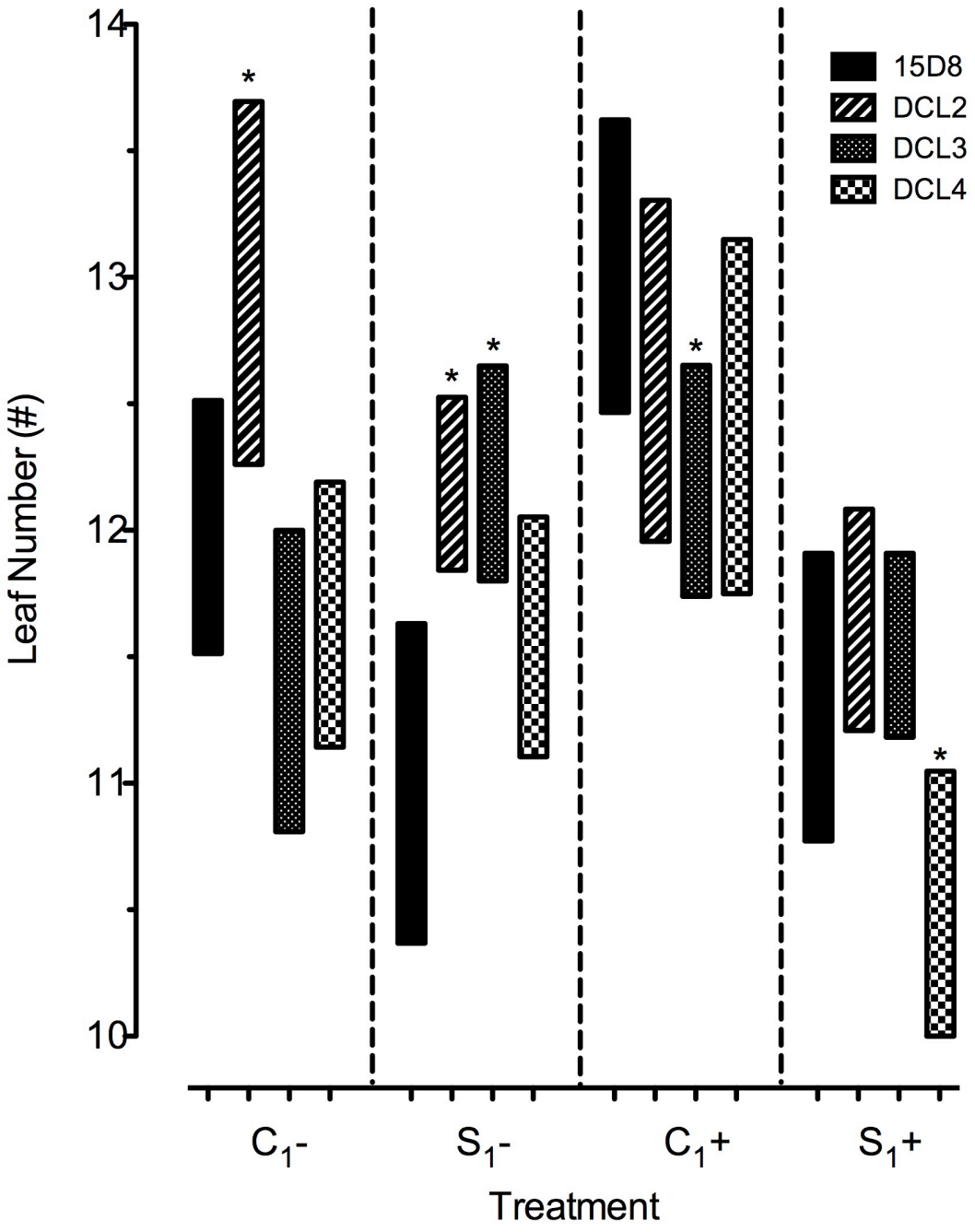

Figure 10. Leaf number in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to UV in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 13 to observe which responses to stress were statistically significant.

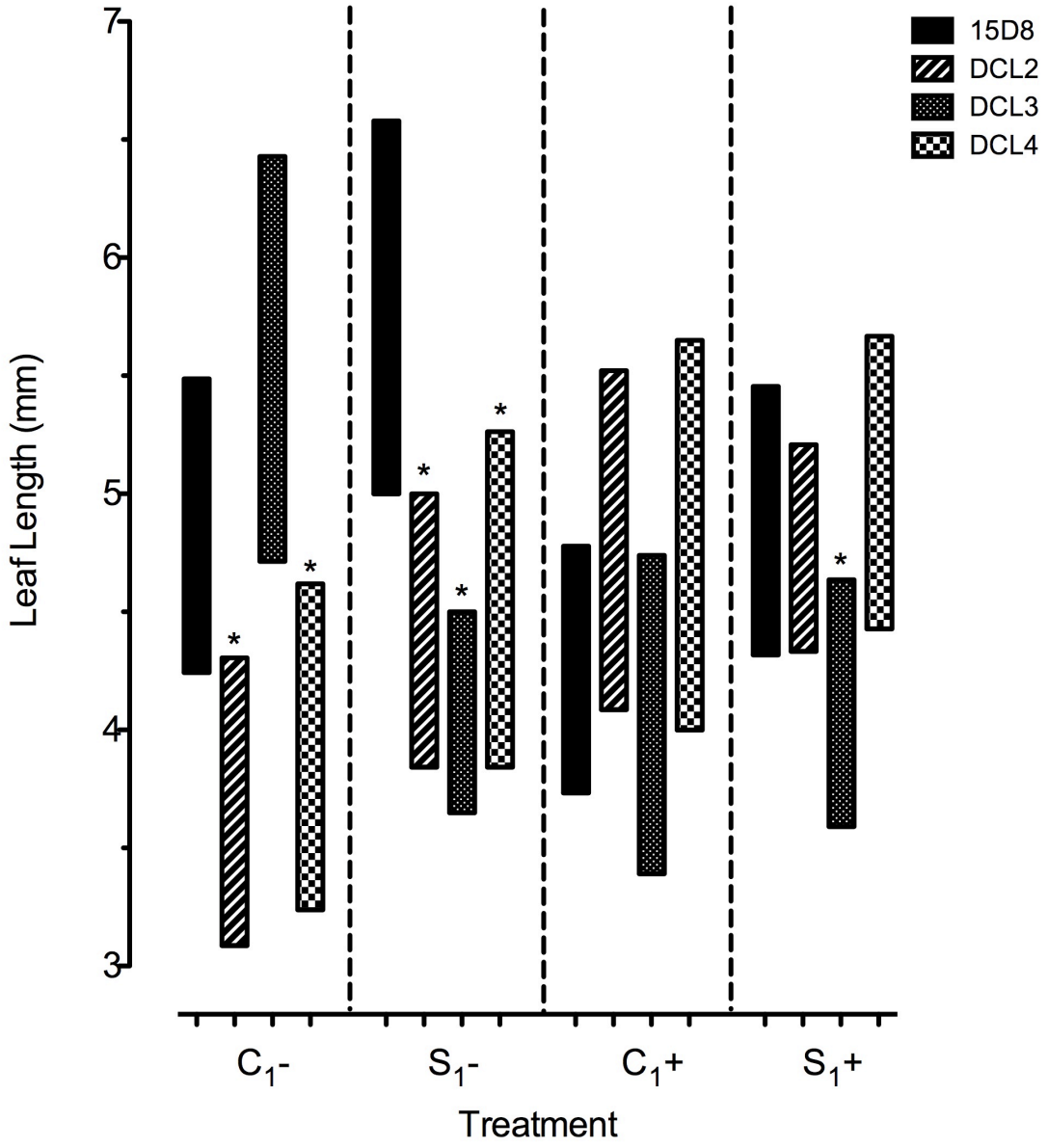

Figure 11. Leaf length in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to UV in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 13 to observe which responses to stress were statistically significant.

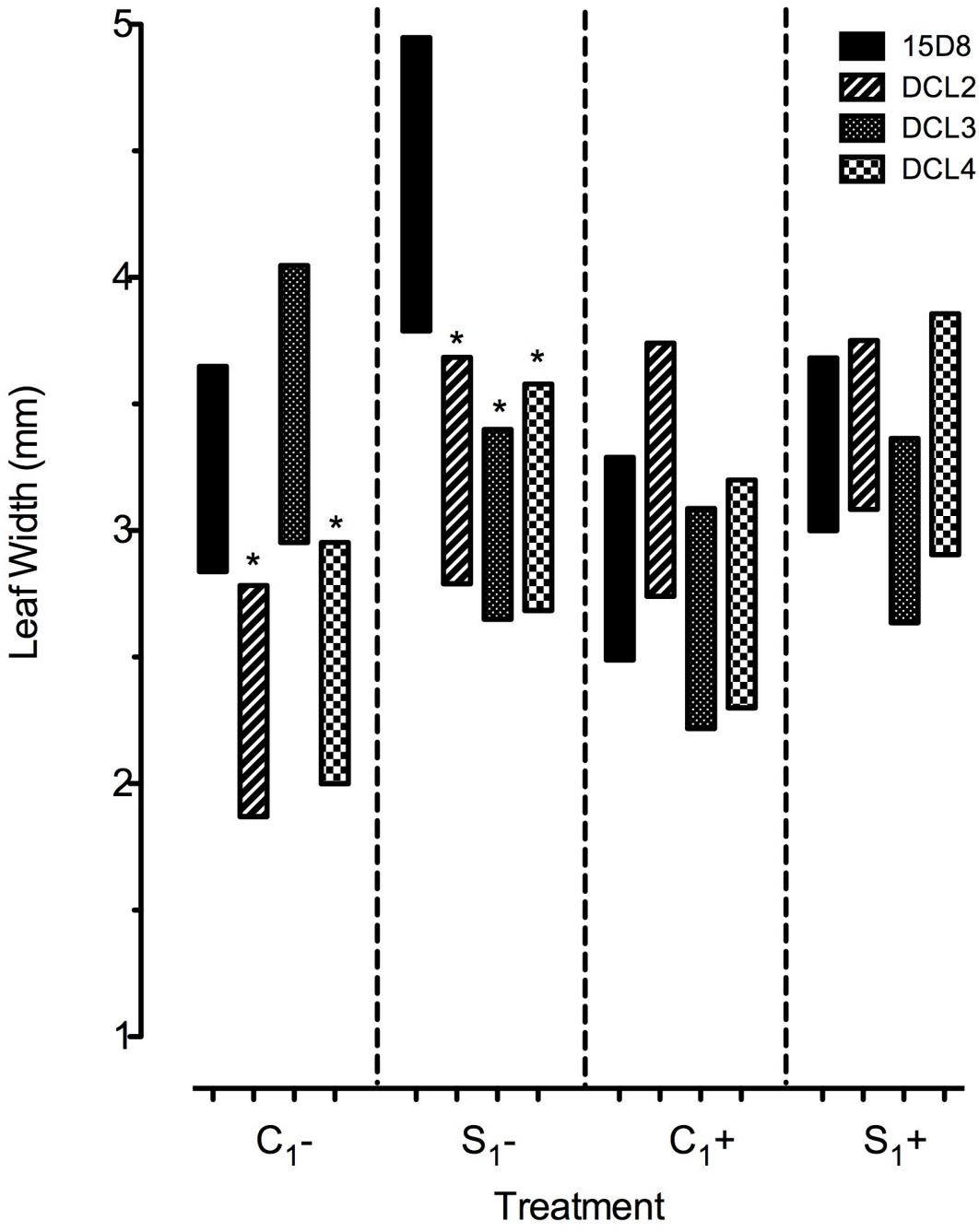

Figure 12. Leaf width in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to UV in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 13 to observe which responses to stress were statistically significant.

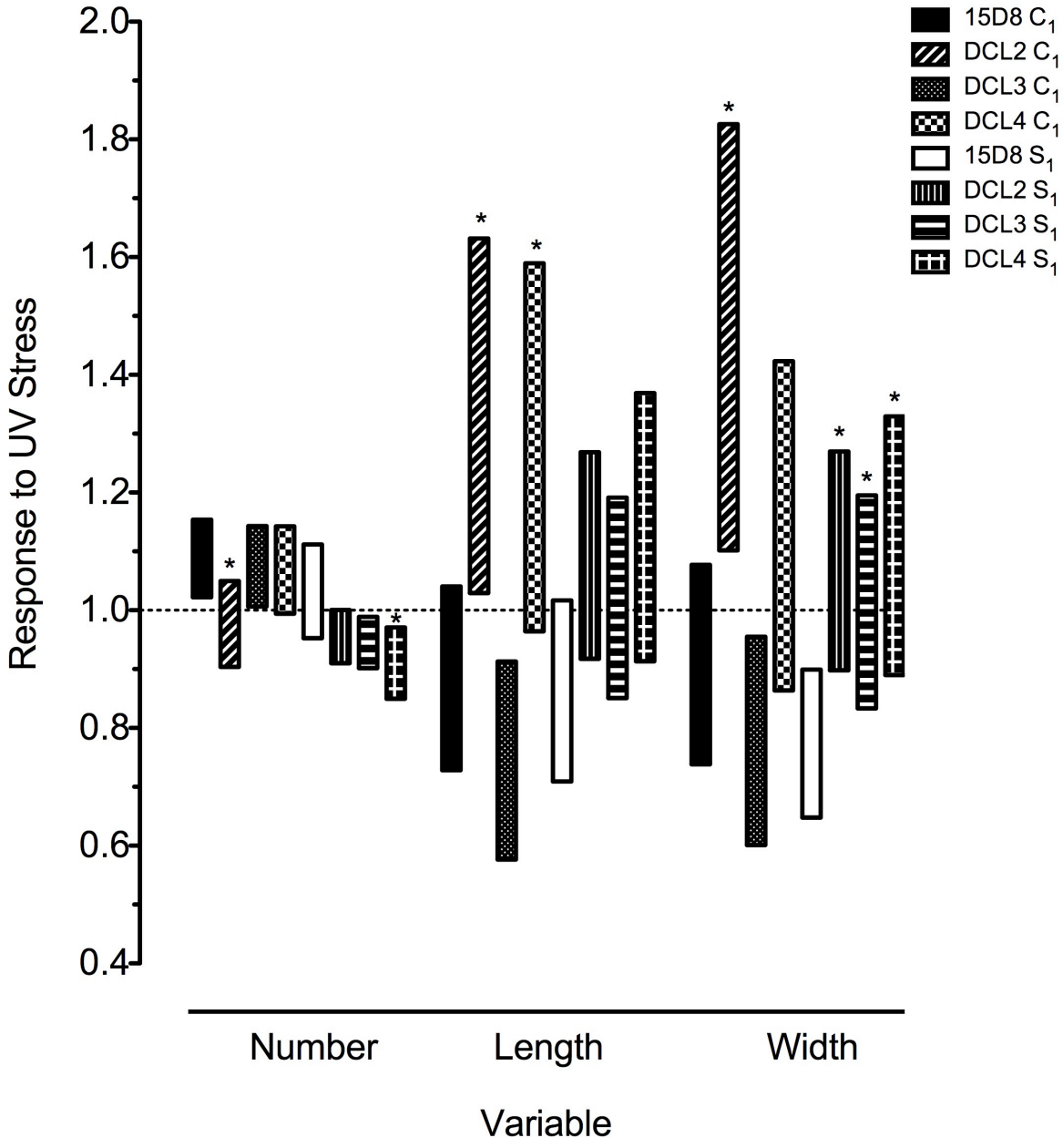

Figure 13. Bars represent ratio of change in F1 plants in response to UV stress (S+/S- or C+/C-), at 95% confidence, p-value of 0.05. Ratios are the result of 10,000 bootstrap analysis. Four different plant types were used. Plants were stressed 4 minutes of UV-C irradiation with an output of 13.9 W. Ratios which overlap with the value of 1 indicate no significant change under UV stress. Measurements were taken for leaf number, length, and width. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same treatment. Legend indicates parental treatment.

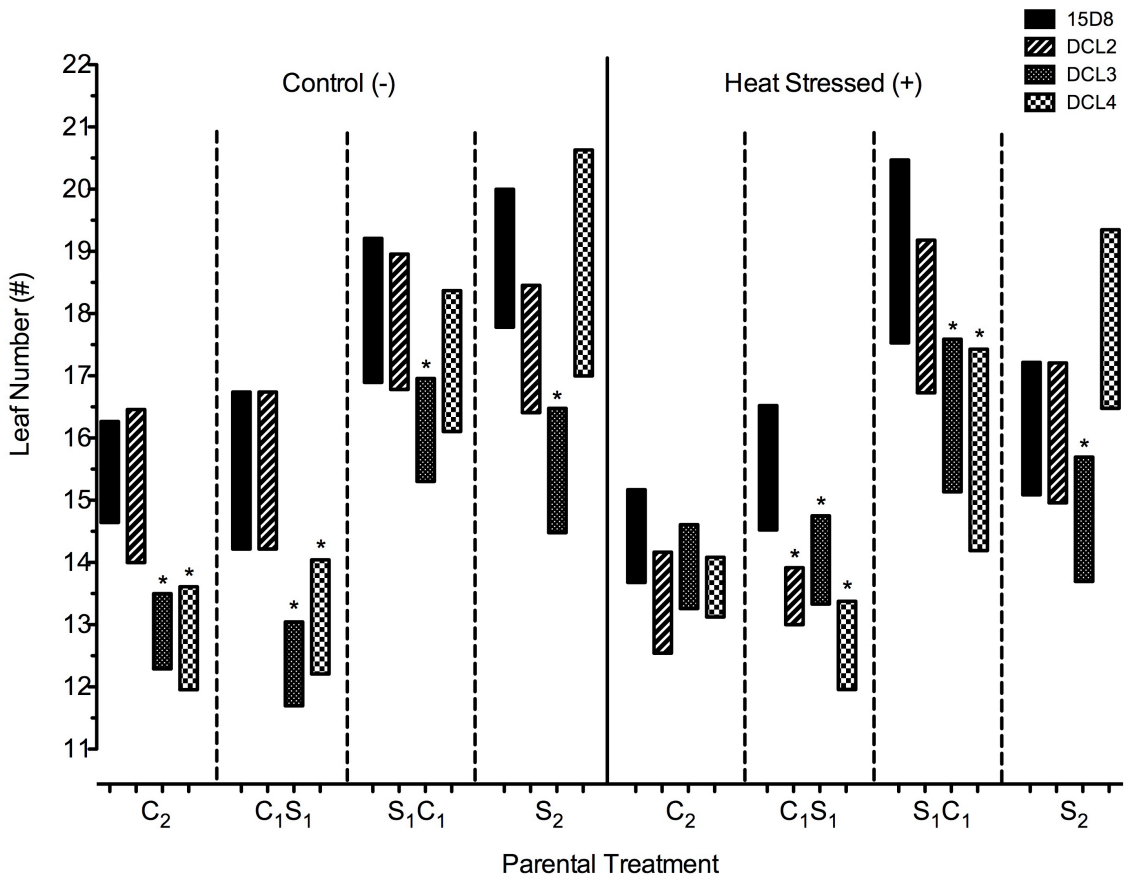

Figure 14. Leaf number in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to heat in F1. "S_1C_1" – the progeny of plants exposed to heat in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to heat in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 15 to observe which responses to stress were statistically significant.

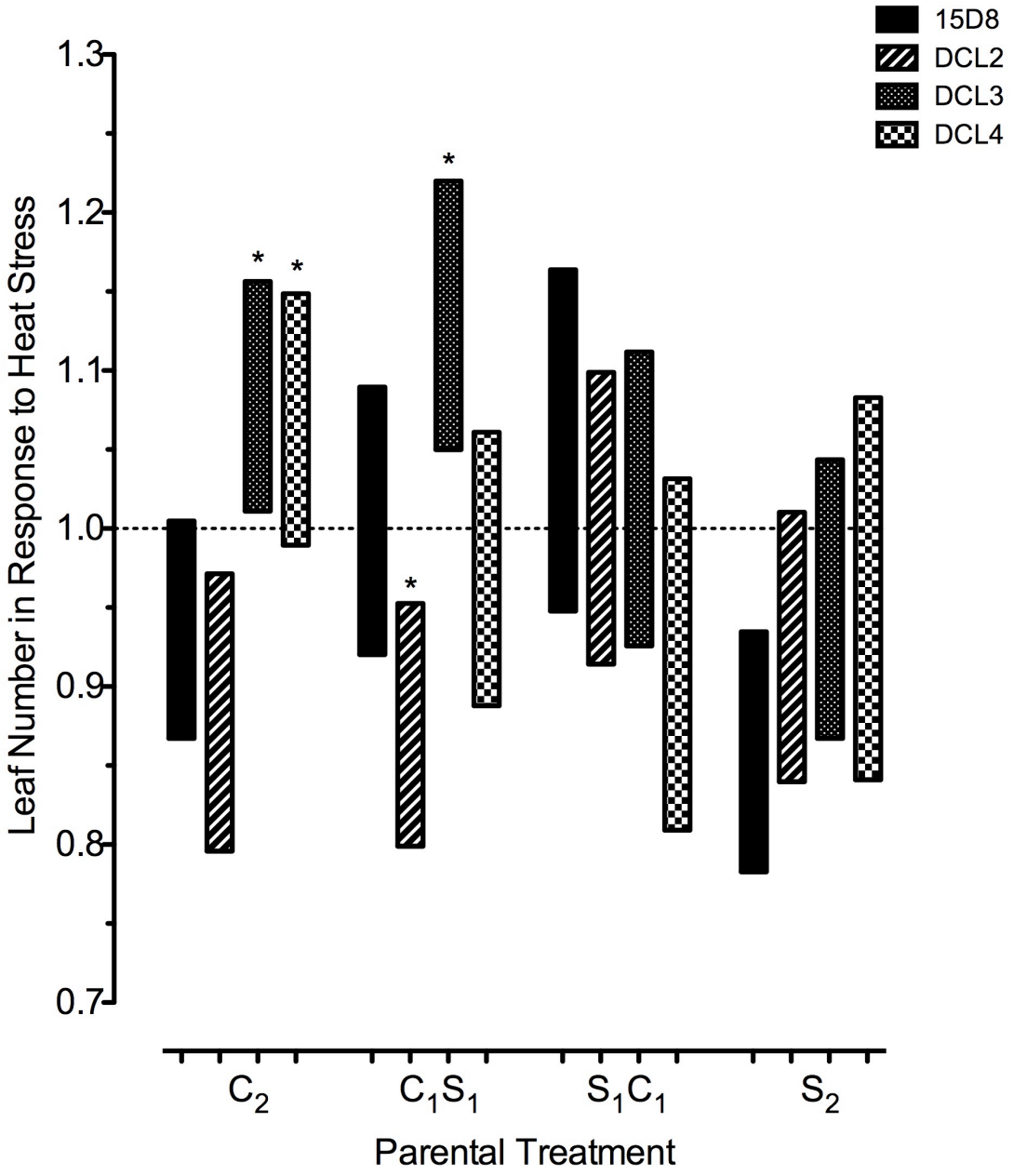

Figure 15. Bars represent ratio of change in leaf number in response to heat stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2-. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were stressed at 50°C for 3 hours, on five consecutive days. Ratios which overlap with the value of 1 indicate no significant change under heat stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

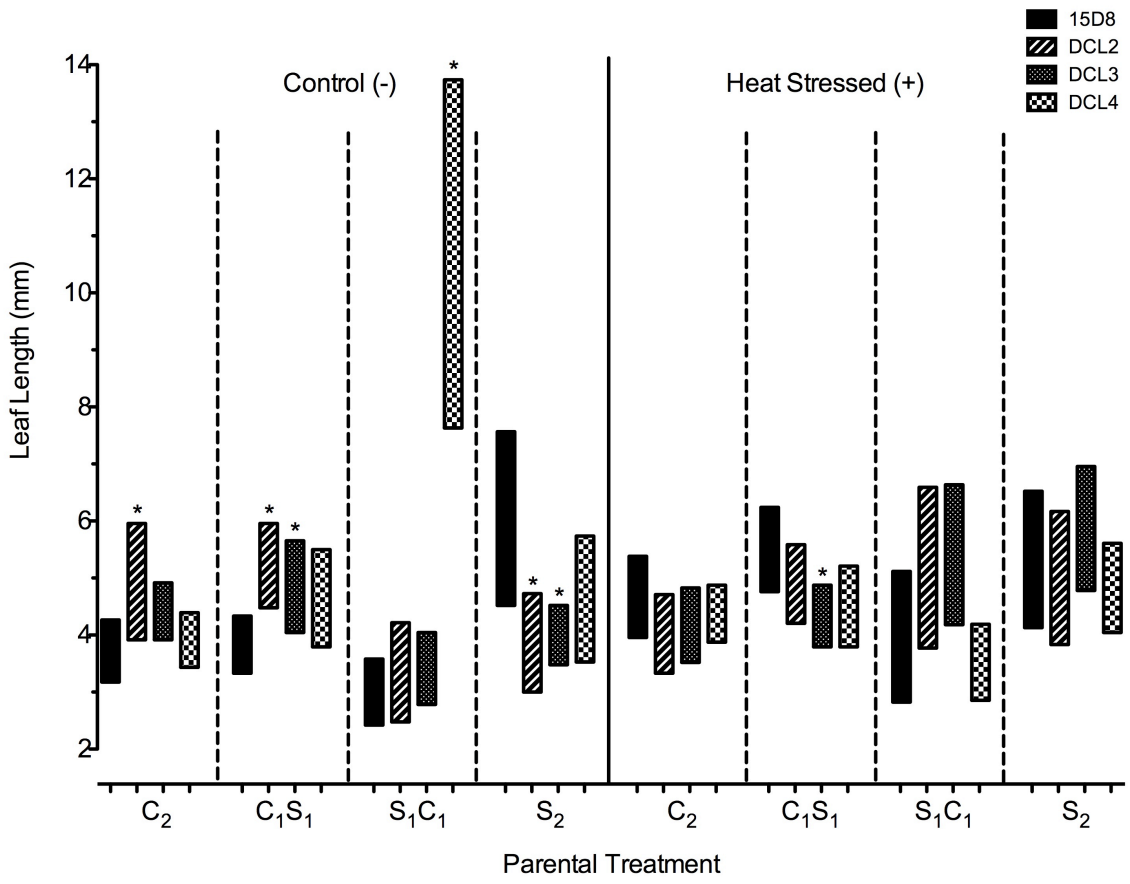

Figure 16. Leaf length in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to heat in F1. "S_1C_1" – the progeny of plants exposed to heat in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to heat in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 17 to observe which responses to stress were statistically significant.

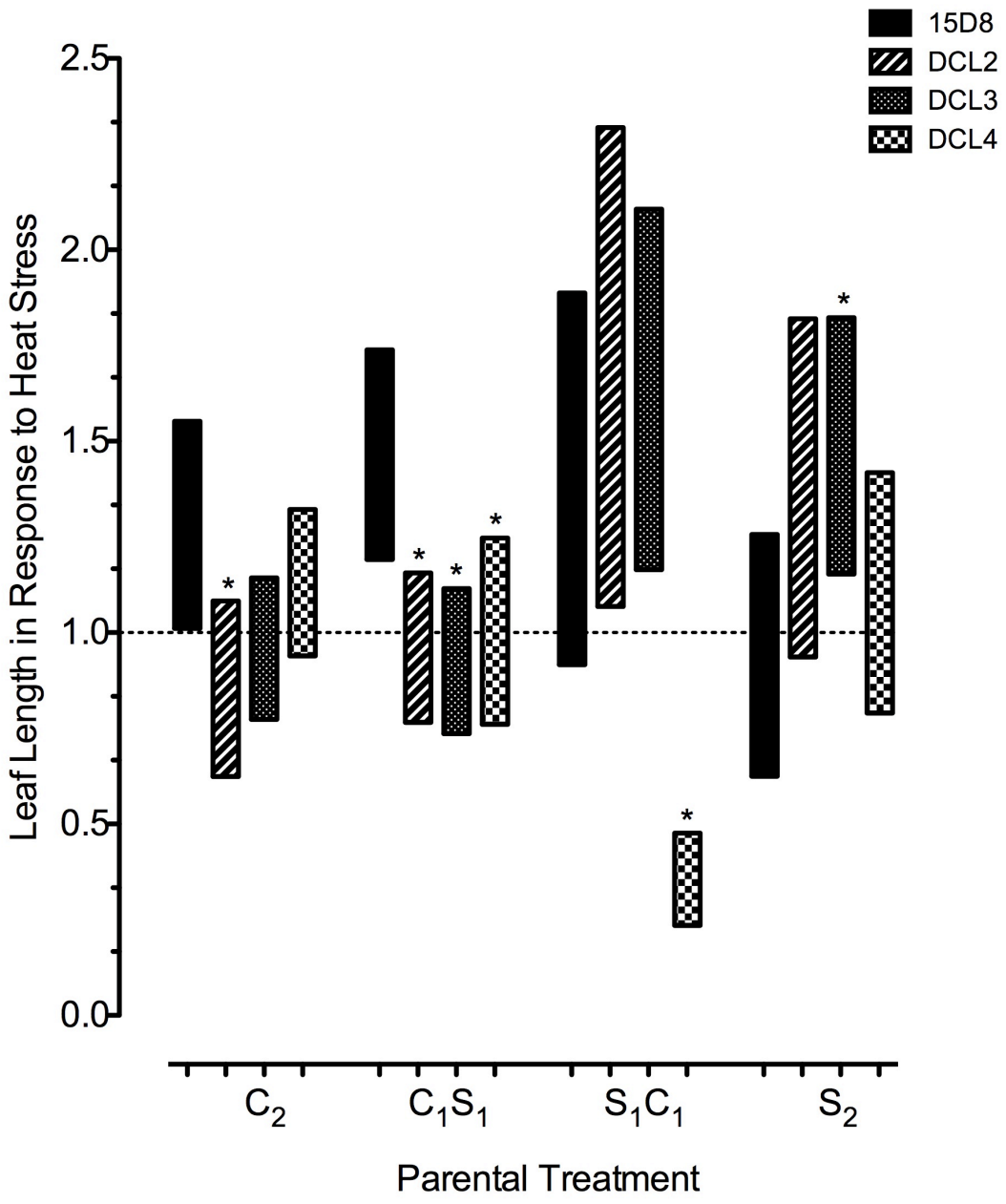

Figure 17. Bars represent ratio of change in leaf length in response to heat stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were stressed at 50°C for 3 hours, on five consecutive days. Ratios which overlap with the value of 1 indicate no significant change under heat stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

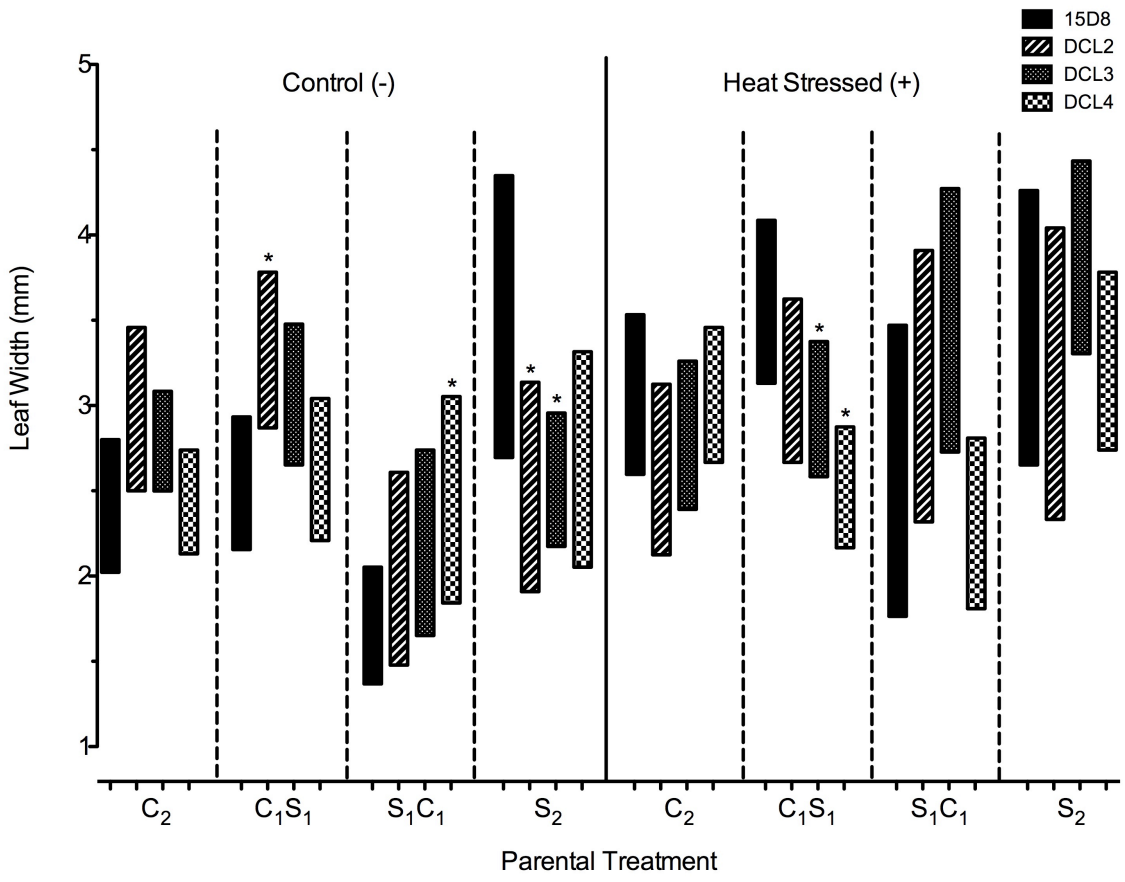

Figure 18. Leaf width in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to heat in F1. "S_1C_1" – the progeny of plants exposed to heat in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to heat in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 19 to observe which responses to stress were statistically significant.

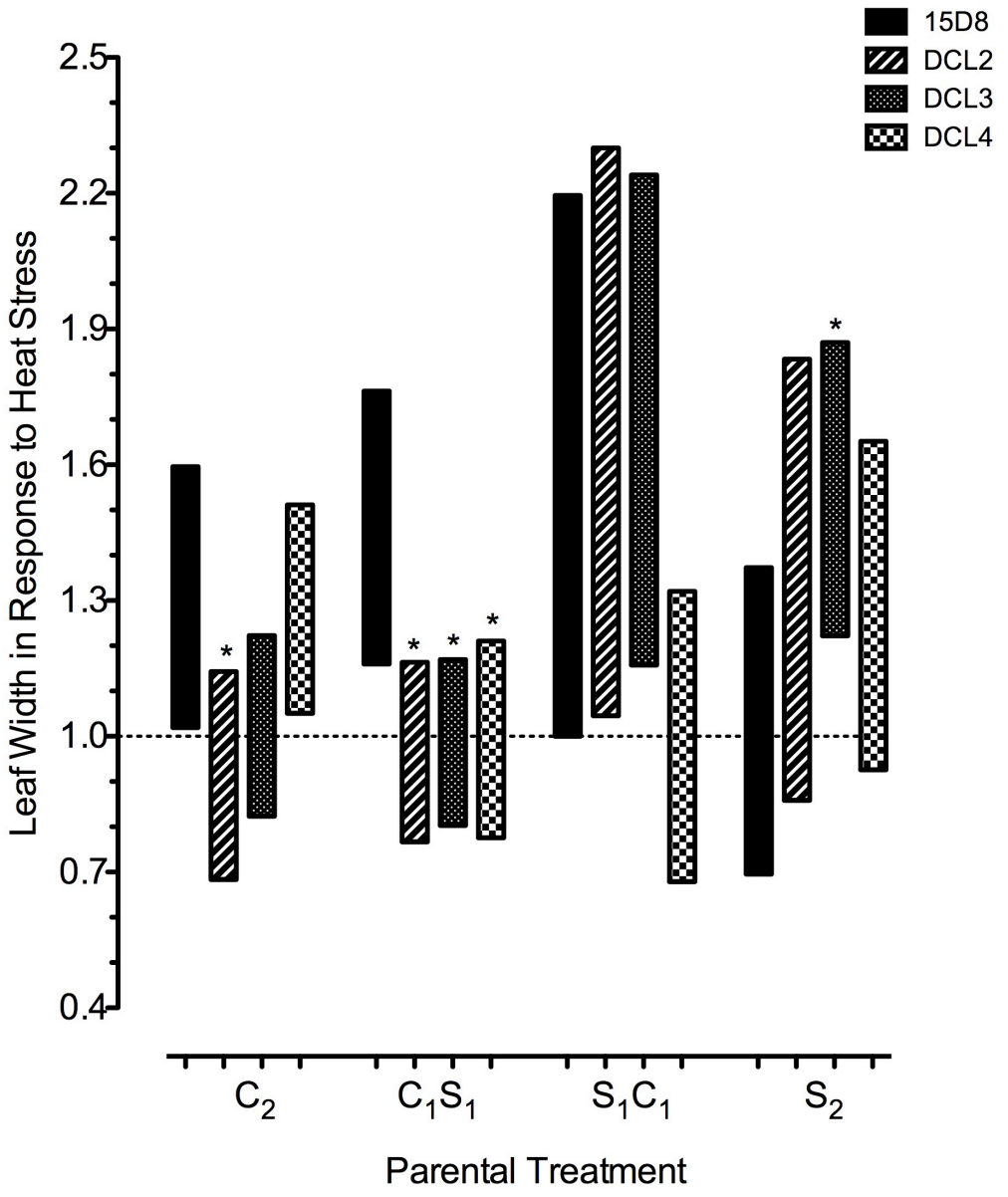

Figure 19. Bars represent ratio of change in leaf width in response to heat stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were stressed at 50°C for 3 hours, on five consecutive days. Ratios which overlap with the value of 1 indicate no significant change under heat stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

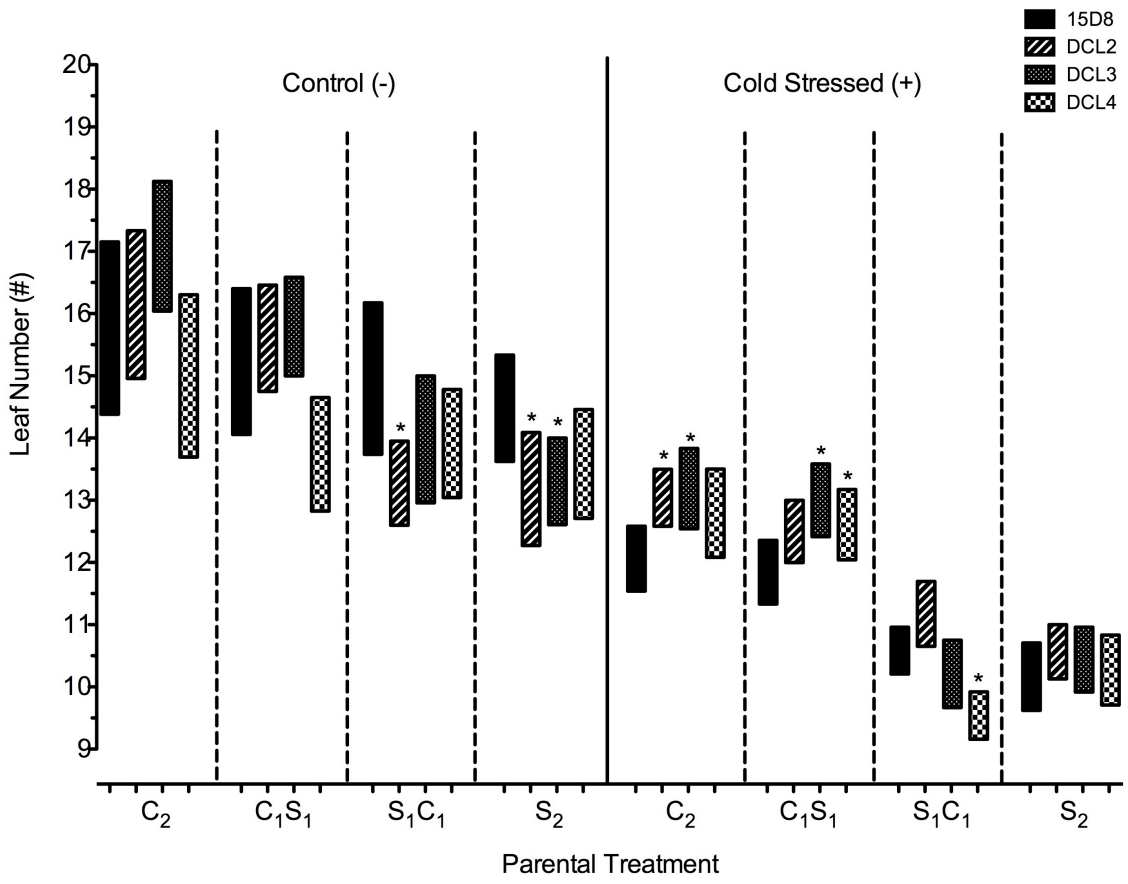

Figure 20. Leaf number in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to cold in F1. "S_1C_1" – the progeny of plants exposed to cold in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to cold in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 21 to observe which responses to stress were statistically significant.

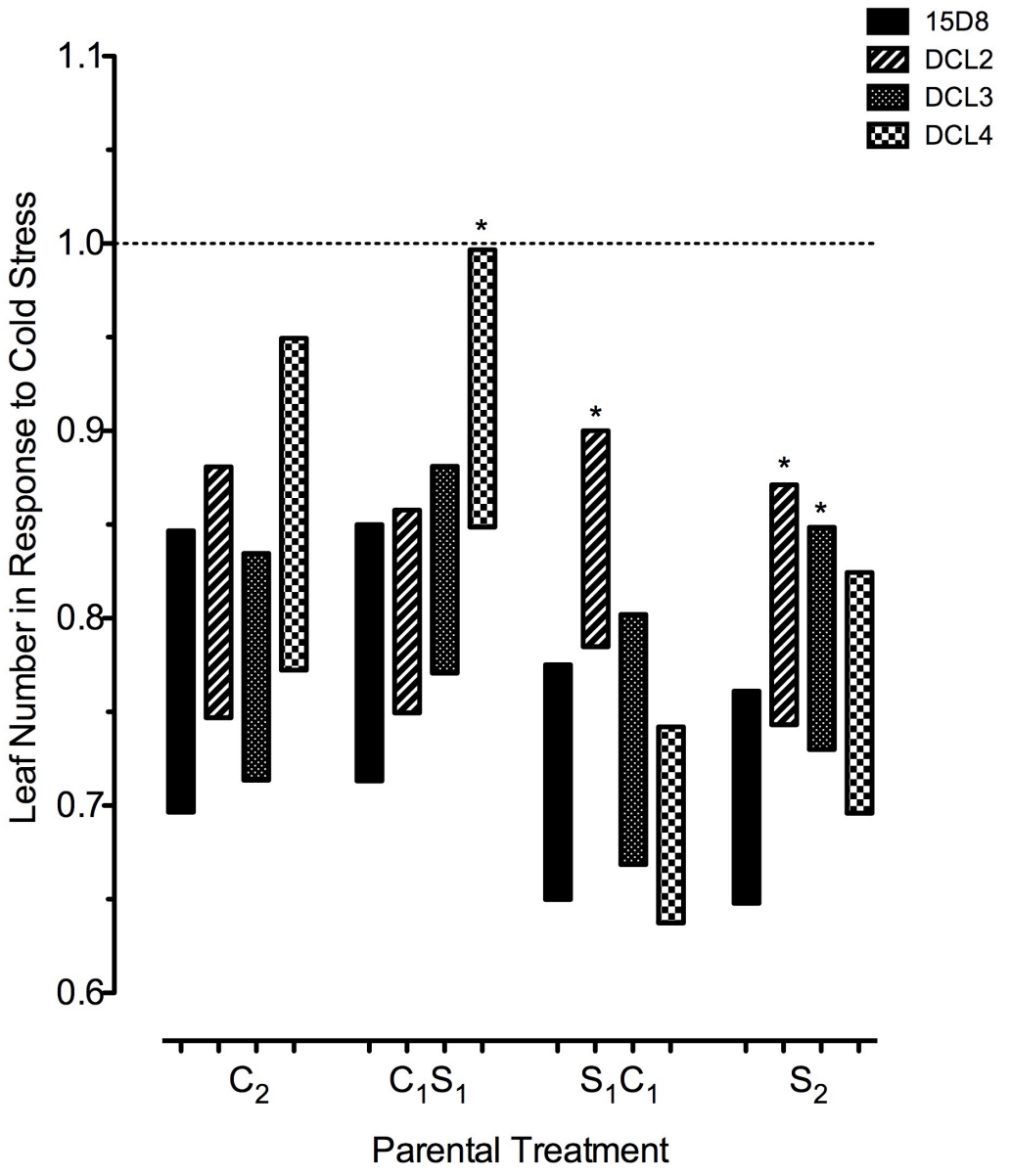

Figure 21. Bars represent ratio of change in leaf number in response to cold stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were stressed at 4°C for 12 hours, on seven consecutive days. Ratios which overlap with the value of 1 indicate no significant change under cold stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

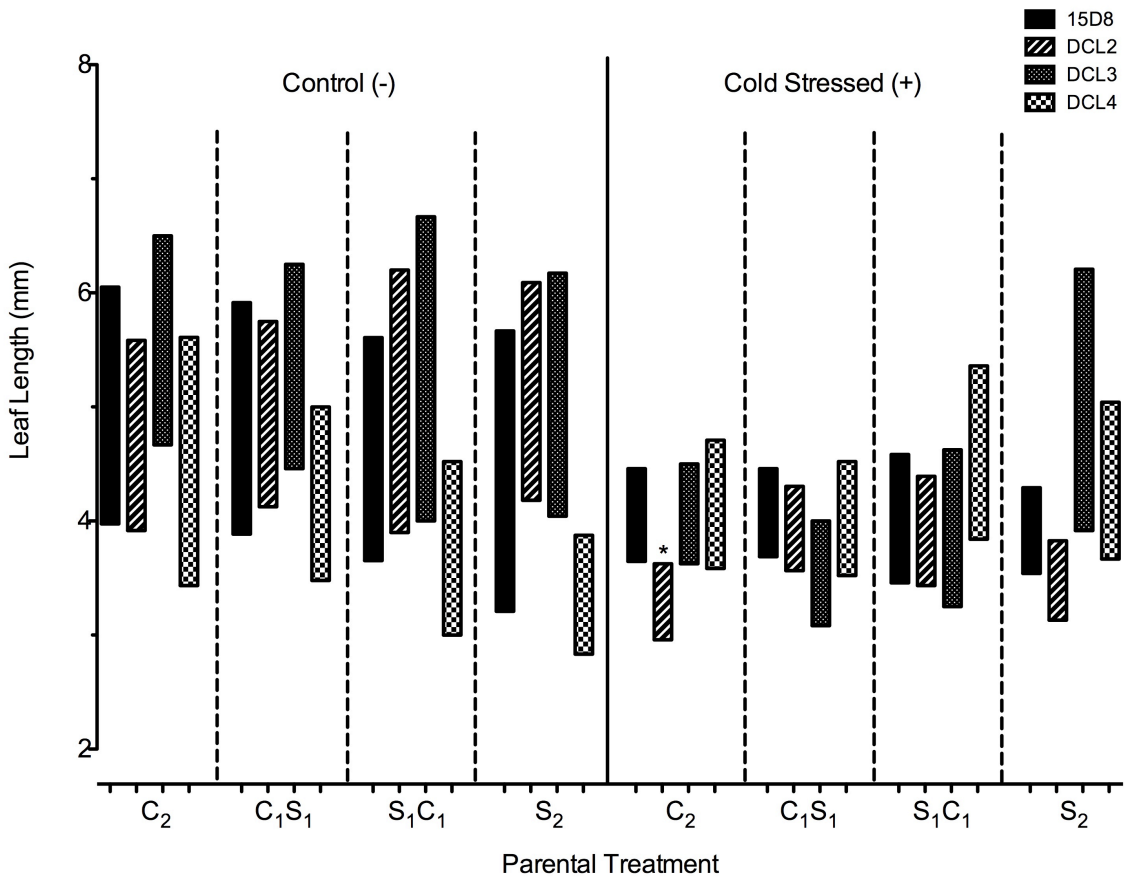

Figure 22. Leaf length in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to cold in F1. "S_1C_1" – the progeny of plants exposed to cold in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to cold in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 23 to observe which responses to stress were statistically significant.

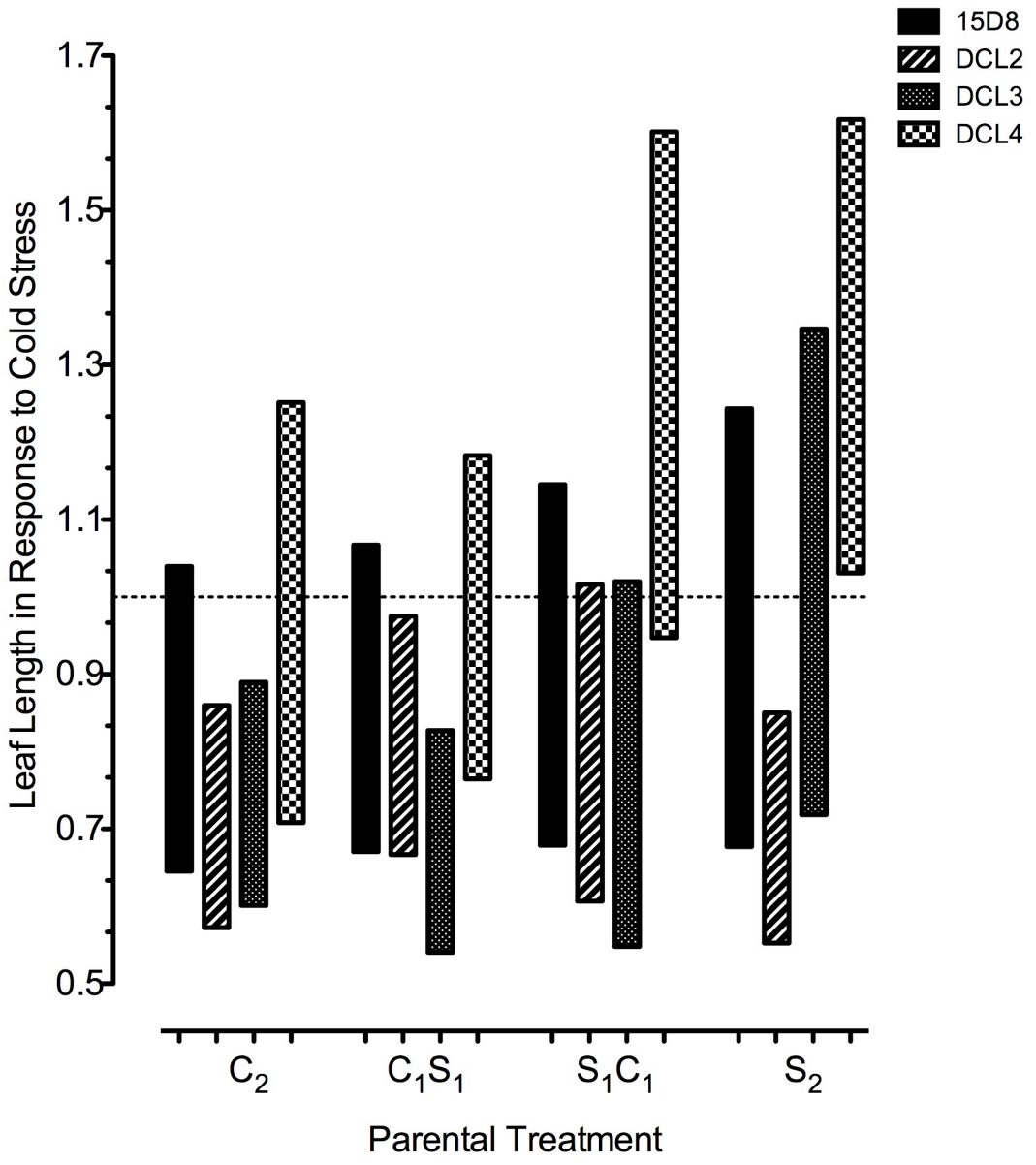

Figure 23. Bars represent ratio of change in leaf length in response to cold stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were stressed at 4°C for 12 hours, on seven consecutive days. Ratios which overlap with the value of 1 indicate no significant change under cold stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

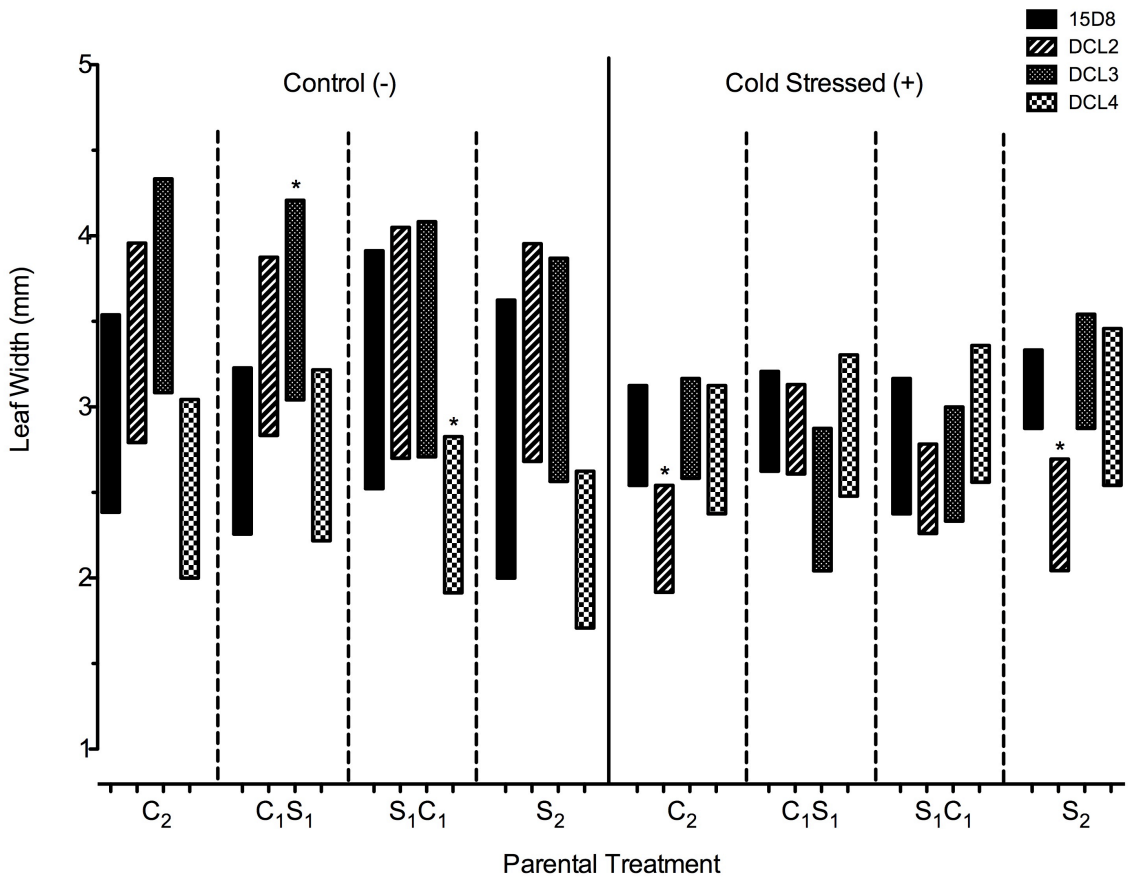

Figure 24. Leaf width in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to cold in F1. "S_1C_1" – the progeny of plants exposed to cold in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to cold in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 25 to observe which responses to stress were statistically significant.

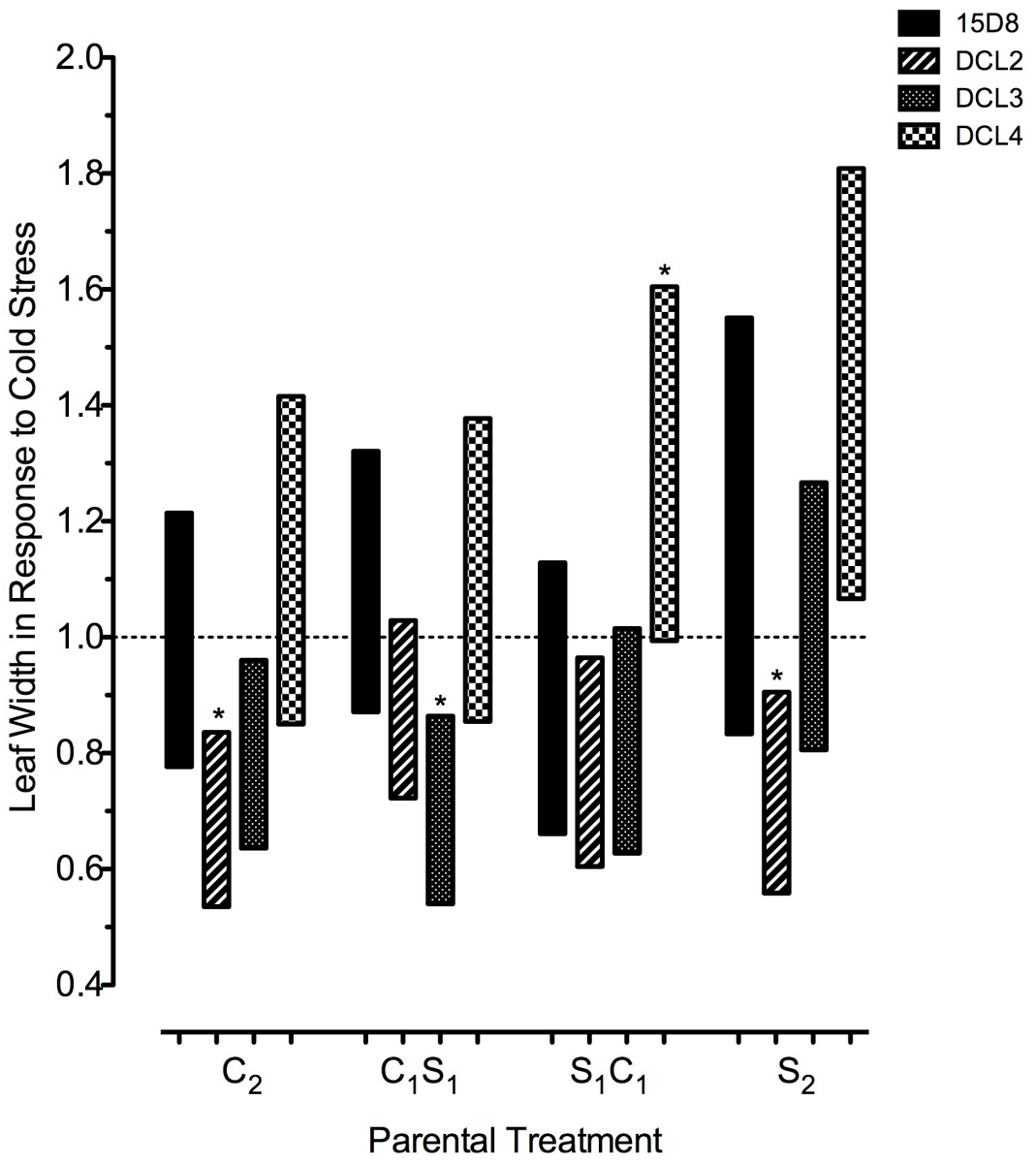

Figure 25. Bars represent ratio of change in leaf width in response to cold stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were stressed at 4°C for 12 hours, on seven consecutive days. Ratios which overlap with the value of 1 indicate no significant change under cold stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

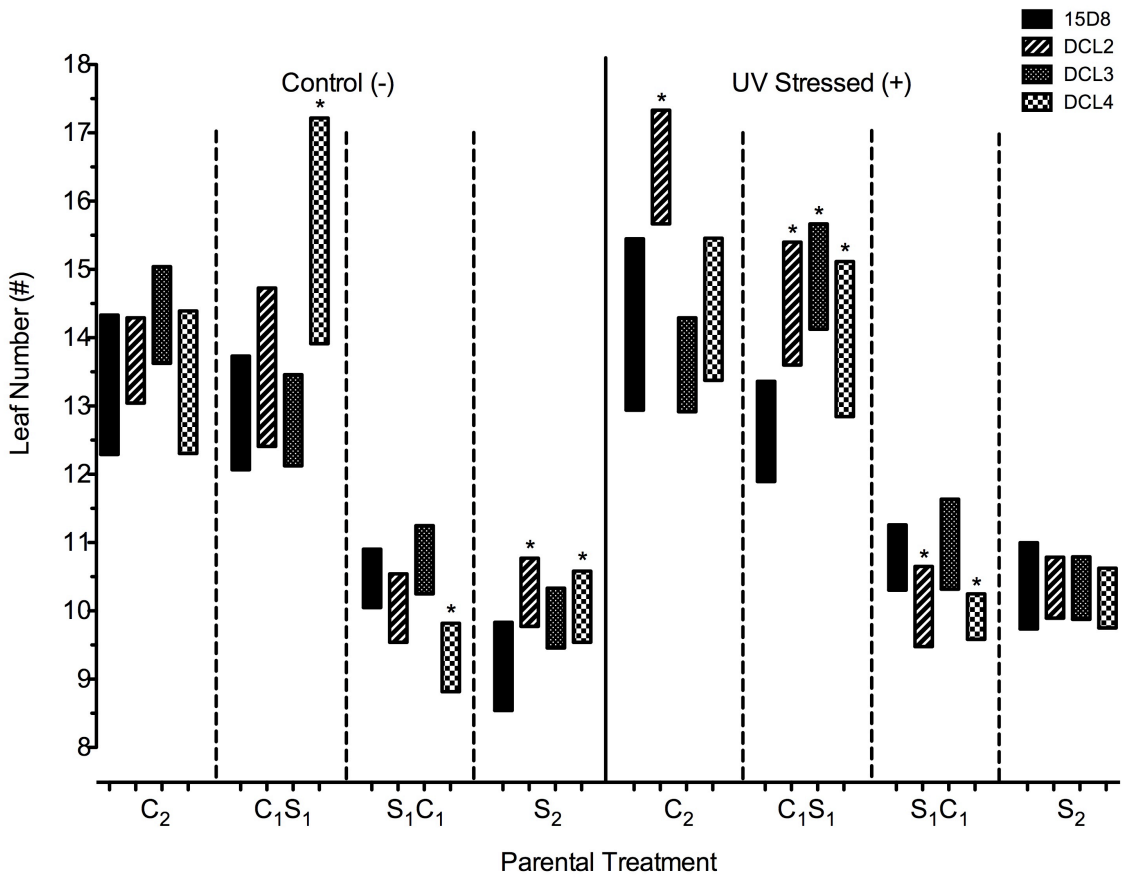

Figure 26. Leaf number in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to UV in F1. "S_1C_1" – the progeny of plants exposed to UV in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to UV in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 27 to observe which responses to stress were statistically significant.

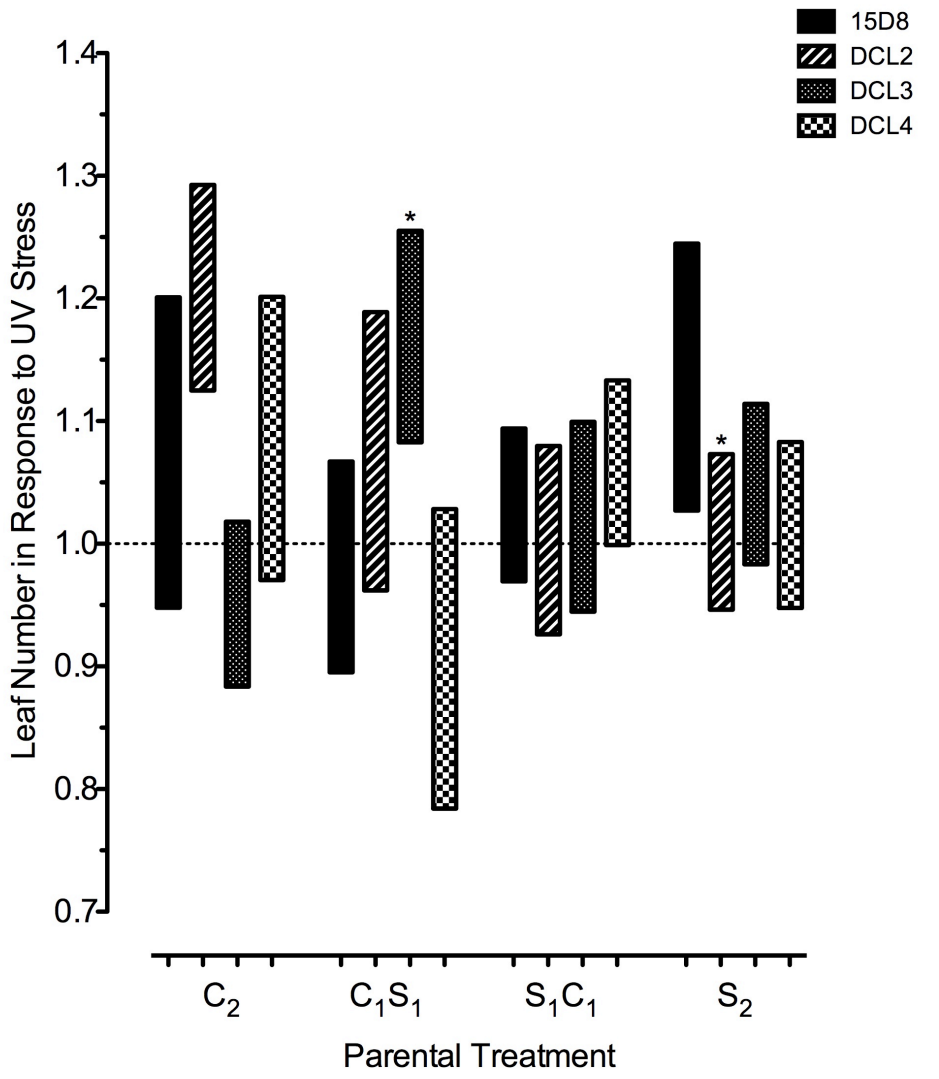

Figure 27. Bars represent ratio of change in leaf number in response to UV-C stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2-. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were exposed to 4 minutes of UV-C irradiation with an output of 13.9 W. Ratios which overlap with the value of 1 indicate no significant change under UV stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

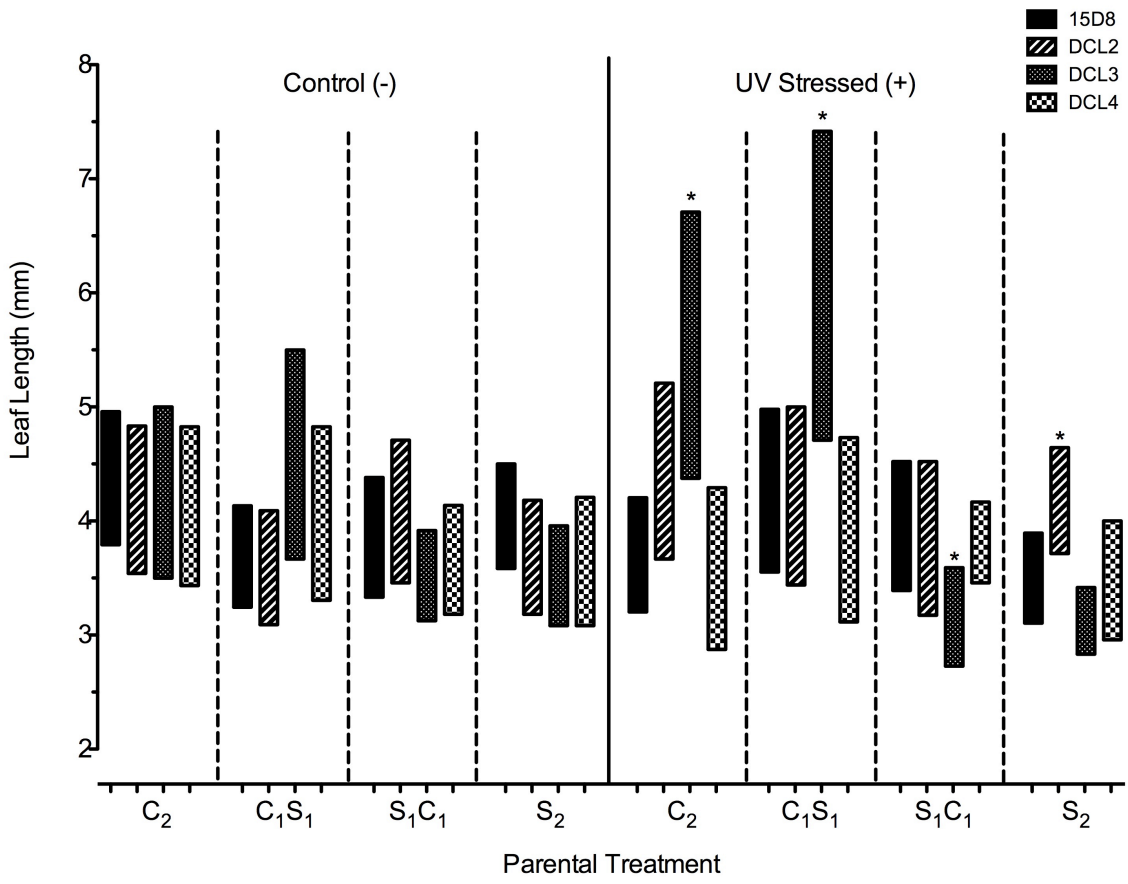

Figure 28. Leaf length in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to UV in F1. "S_1C_1" – the progeny of plants exposed to UV in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to UV in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 29 to observe which responses to stress were statistically significant.

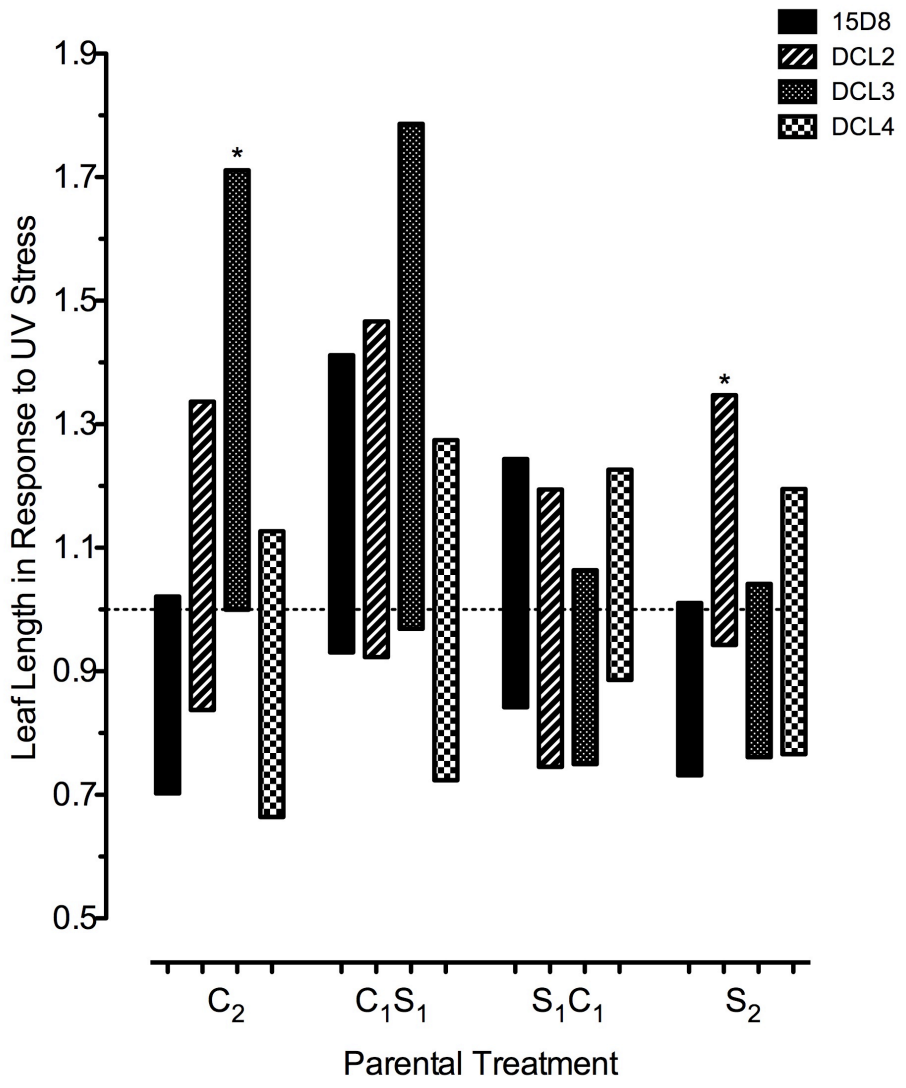

Figure 29. Bars represent ratio of change in leaf length in response to UV-C stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were exposed to 4 minutes of UV-C irradiation with an output of 13.9 W. Ratios which overlap with the value of 1 indicate no significant change under UV stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

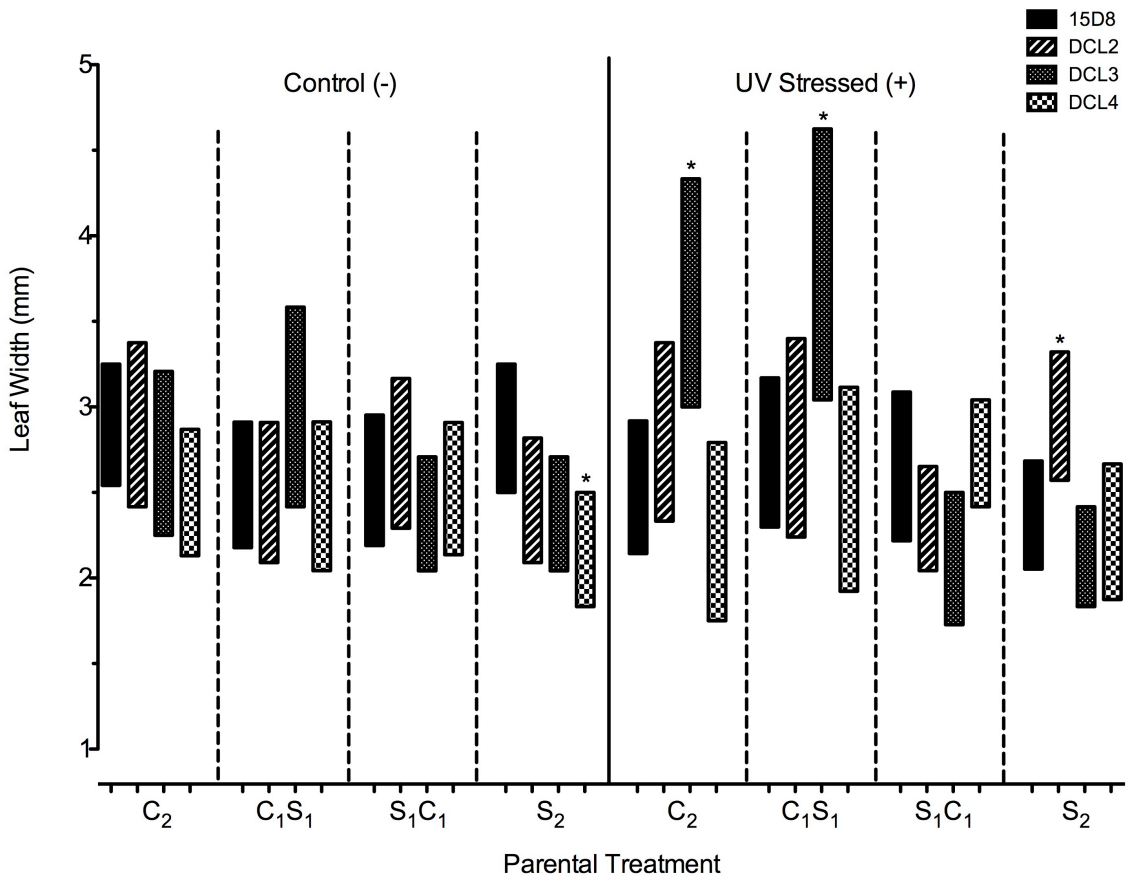

Figure 30. Leaf width in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to UV in F1. "S_1C_1" – the progeny of plants exposed to UV in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to UV in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Values indicate 95% confidence interval, calculated using bootstrap 10,000, as a result of approximately 24 repeats. Legend indicates mutant type. Asterisks (*) indicate a significant difference from wild type, with the same treatment group, as calculated at $p \leq 0.05$. Refer to Figure 31 to observe which responses to stress were statistically significant.

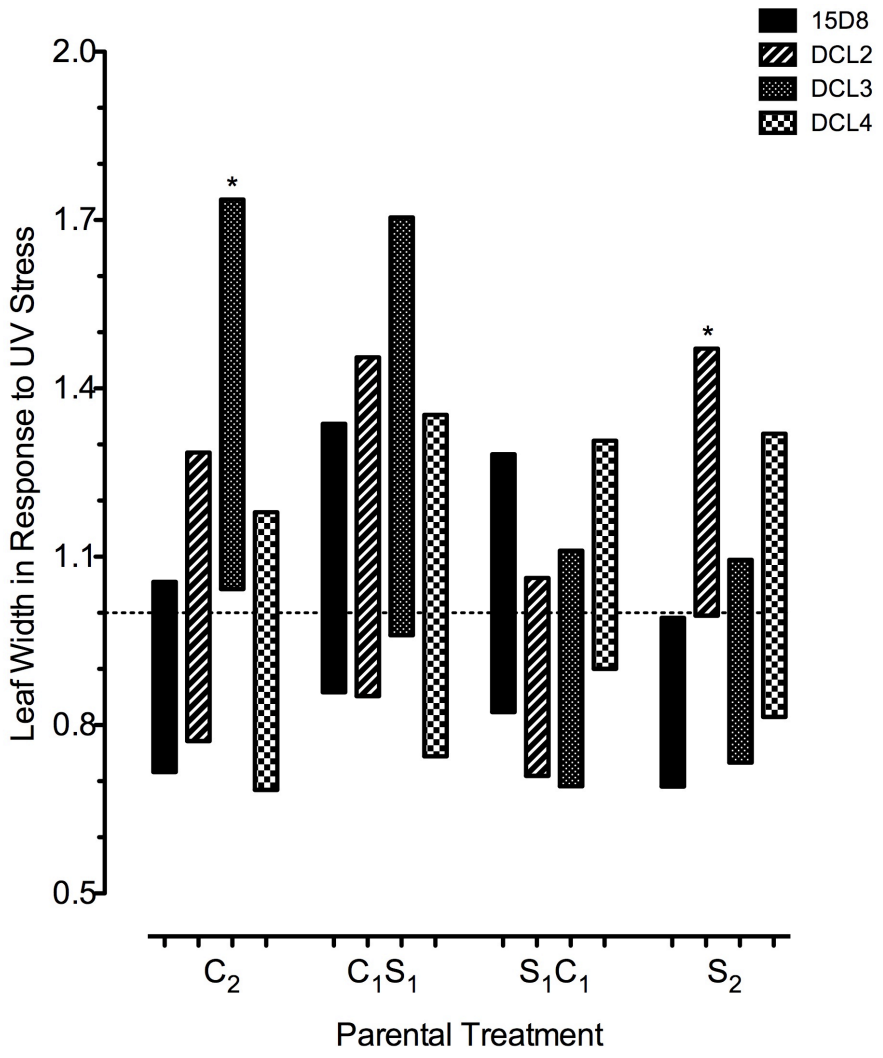

Figure 31. Bars represent ratio of change in leaf width in response to UV-C stress, based on parental treatment. For S_2: S_2+/S_2- for S_1C_1: S_1C_1+/S_1C_1-, for C_1S_1: C_1S_1+/C_1S_1-, and for C_2: C_2+/C_2. Ratios were calculated using 10,000x bootstrap analysis at 95% confidence, p-value of 0.05. Plants were exposed to 4 minutes of UV-C irradiation with an output of 13.9 W. Ratios which overlap with the value of 1 indicate no significant change under UV stress. Asterisks (*) are used to indicate mutants that are significantly different from wild-type (15D8) plants with the same parental treatment. Legend indicates mutant type, and horizontal axis indicates parental treatment.

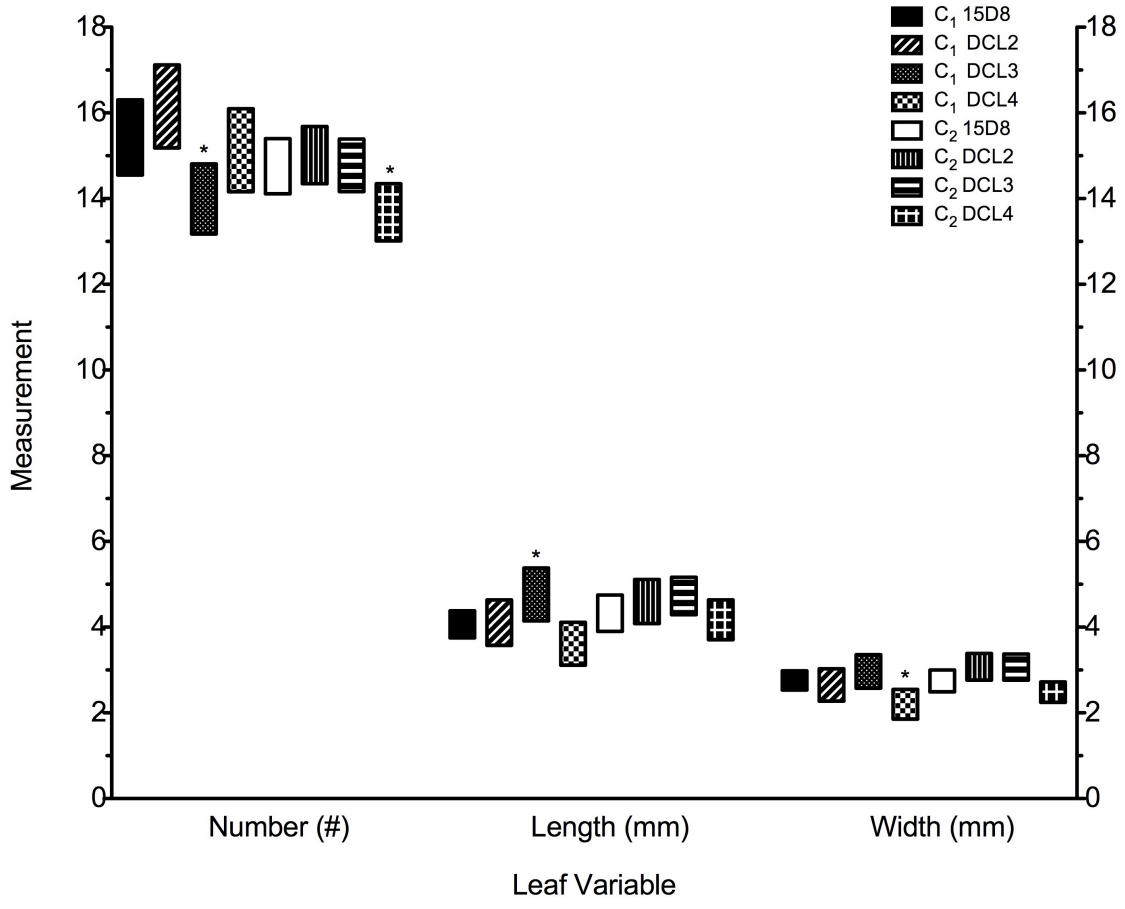

Figure 32. Measurement of natural variation in leaf number and size, within *dcl* mutants and wild-type (15D8) in progeny of plants grown under normal conditions for one (C_1) or two (C_2) generations, when grown under normal conditions. Bar represent the 95% confidence interval (p=0.05) for plant type and variable, calculated using a bootstrap x10,000. Asterisks (*) indicate mutants that vary significantly in comparison to wild-type for the same generation. Approximately 24 plants were sampled from each group. Legend indicates generation and mutant type.

3.1.2. Changes to Seed Phenotype

Our project began with F1 seeds collected from both stressed (S_1) and control (C_1) plants, as described in Figure 33, which were measured and compared. These seeds were germinated, and the plants they produced were grown under both stressed (+) and normal conditions (-). Seeds were collected from both groups of plants, those that had been stressed (S_2 and C_1S_1) and those grown under control conditions (C_2 and S_1 C_1). F2 seed length was also measured and compared.

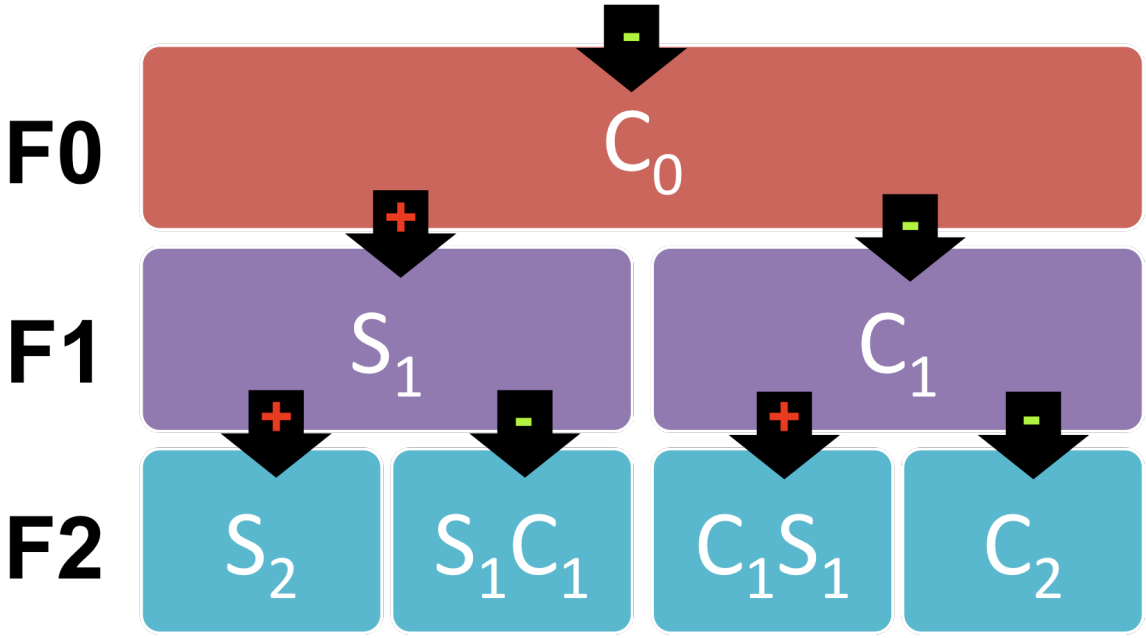

Figure 33. Project began with F1 seeds, which were the progeny of exposed (S0) and control non-exposed (C0) plants. F1 seeds were measured, and then grown under either stress (+) or normal (-) conditions. F2 seeds were then collected and measured. Each step was completed for 15D8, *dcl2*, *dcl3* and *dcl4*. Complete project was repeated for heat, cold, and UV stress.

3.1.2.1. F1 Generation

There was significant variation in the size of seeds produced by both control plants (C_1) and plants stressed with cold, heat or UV (S_1) (Figure 34). *dcl3* seeds produced by stressed plants were the largest, in contrast to C_1 seeds, where *dcl2* was the largest. *dcl2* seeds produced under stress were significantly smaller than those produced by control plants. In particular, heat (S_1) *dcl2* seeds were significantly smaller than those produced by any other mutant type or stress. However, *dcl2* seeds were significantly larger than wild-type in control, cold, and UV conditions. *dcl4* seed size decreased with all abiotic stresses, especially heat, where they were significantly different than 15D8. In response to heat stress, 15D8 and *dcl3* plants produced significantly larger seeds than *dcl2* and *dcl4*. Interestingly, the size variation of 15D8 seeds remained fairly consistent regardless of treatment, with all confidence intervals remaining within approximately 0.43-0.46 mm, in contrast to *dcl* seeds which had a larger fluctuation in size.

F1 seed response to stress was calculated by dividing S_1 seed length by C_1 seed length (Figure 35). 15D8 seeds were not substantially impacted by cold stress, and it was the only mutant type that did not change significantly in response to UV-stress. However, it did increase in response to heat stress. *dcl2* seeds seem to be most impacted, as all three stresses caused a decrease in seed length, as well as a significant decrease in comparison to wild-type under stress. *dcl4* mutants also decreased significantly in response to stress. In contrast, *dcl3* produced larger seeds in response to stress, although only in the case of UV was the change significant.

3.1.2.2. F2 Generation

The most consistency in F2 seed size followed two generations of growth under normal conditions (C_2), although *dcl2* seeds were significantly smaller than wild-type (Figure 36). All 15D8 seeds produced by plants stressed for either one, or two, generations were larger than those produced by C_2 plants, in particular, cold stress during F0 (S_1C_1 and S_2) were significantly larger than those produced by plants grown under normal conditions during F0 ($C_2$and C_1S_1). The largest seeds belonged to 15D8, except for the parental treatment S_1C_1, where they were quite similar to *dcl2* seeds. S_1C_1 *dcl2* seeds were the largest of all *dcl2* seeds, while the smallest were produced following the same F0 treatment, but F1 cold stress (S_2). C_2 and C_1S_1 *dcl2* seeds were similar in size. S_1C_1 15D8, *dcl2* and *dcl4* were significantly larger than other treatment groups. *dcl3* seeds showed the least variation in size, though F0 stress (S_1C_1 and S_2) did result in slightly larger seeds than F0 control conditions (C_2 and C_1S_1).

The response of the F2 generation was calculated by dividing the seed length under stress, S_2 and C_1S_1, by the seed length under normal conditions, S_1C_1, and C_2, respectively. The samples were divided based on if they belonged to either control (C_1) or stressed (S_1) lines.

Response to cold stress remained consistent within C_1 plants and no significant change in seed size in response to a generation of cold stress (Figure 37). However, the S_1 line of seeds was impacted by the second generation of cold stress, with seed size decreasing, except *dcl3*.

15D8 seeds were larger following one generation of heat stress (S_1C_1 or C_1S_1), but two generations (S_2) decreased size (Figure 38). All *dcl* seeds also increased with one

generation of stress in comparison to control (C_2). However, two generations (S_2) did not significantly influence *dcl2* and *dcl4*, resulting in seeds significantly larger than 15D8. S_2 *dcl3* increased in size, resulting in the largest seeds produced.

C_1 *dcl* plants significantly increased in seed length in response to heat (Figure 39). However, wild-type C_1 did not show a significant variation in seed size in response to heat stress. In addition, the responses of *dcl2* and *dcl4* were significantly greater than that of 15D8. The change in seed size in the S_1 line, showed no significant difference in response to heat stress but *dcl3* increased significantly more than 15D8.

Seed size increased under UV stress in comparison to two generations of normal conditions (C_2) (Figure 40). The smallest difference in seed size occurred for C_1S_1 *dcl4*. While C_2 15D8 seeds were significantly larger than all *dcl* ones, after two generations of stress (S_2) there was no longer a significant difference in size. The largest seed size was seen in response to one generation of UV stress, followed by one generation of growth under normal conditions (S_1C_1) for *dcl3* and *dcl4*, which decreased slightly when grown under a second generation of stress (S_2). 15D8 seeds remained the most consistent in size regardless of treatment, with all treatment groups falling within 0.42-0.45 mm.

UV stress resulted in a significant increase in the size of seeds produced by C_1 15D8, *dcl2* and *dcl3* plants (Figure 41). However, the change in seed length for *dcl4* was not significant. Both *dcl2* and *dcl3* also had a significantly higher change in seed length in response to UV than 15D8. The S_1 line showed different changes in response to stress, with 15D8 and *dcl2* seeds not being significantly altered by UV stress. Both *dcl3* and *dcl4* significantly reduced seed length in response to UV stress.

C_1 seeds varied in size, with all three *dcl* mutants having longer seeds than wild-type (Figure 42). *dcl2* and *dcl3* seeds were significantly longer than those of 15D8. However, by the next generation, C_2 the trend had reversed, with all *dcl* mutants showing significant decrease in seed size, while the size of 15D8 remained similar to C_1, resulting in C_2 *dcl* seeds significantly smaller than those of 15D8.

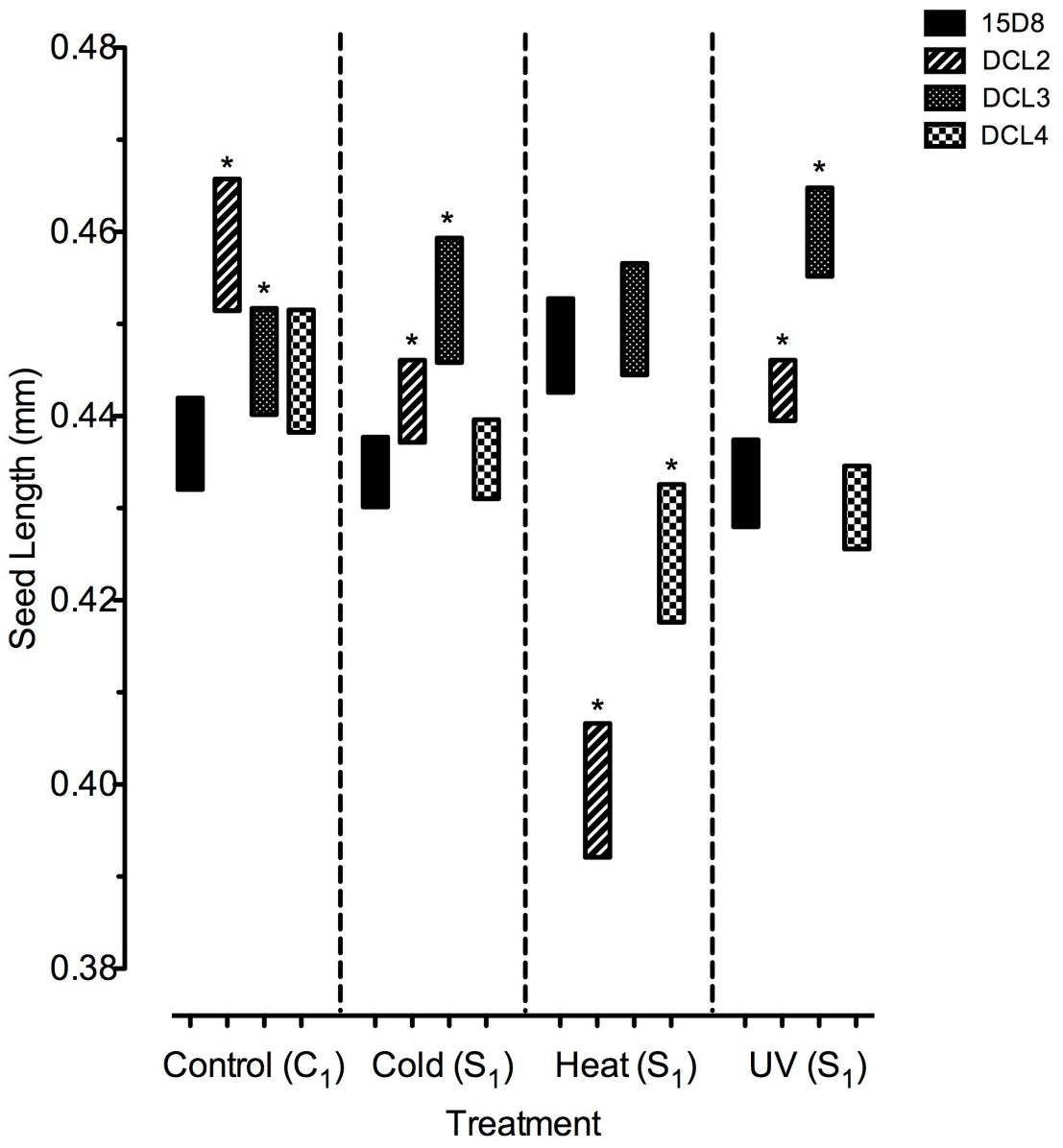

Figure 34. Size of F1 seeds produced by plants grown under either normal conditions (C_1), or abiotic stress (S_1). Approximately 100-200 seeds were measured from each group. Confidence intervals of 95% (p=0.05) were calculated using bootstrap x10,000. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) belonging to the same treatment group. Legend indicates mutant type. Refer to Figure 35 to observe which responses to stress were statistically significant.

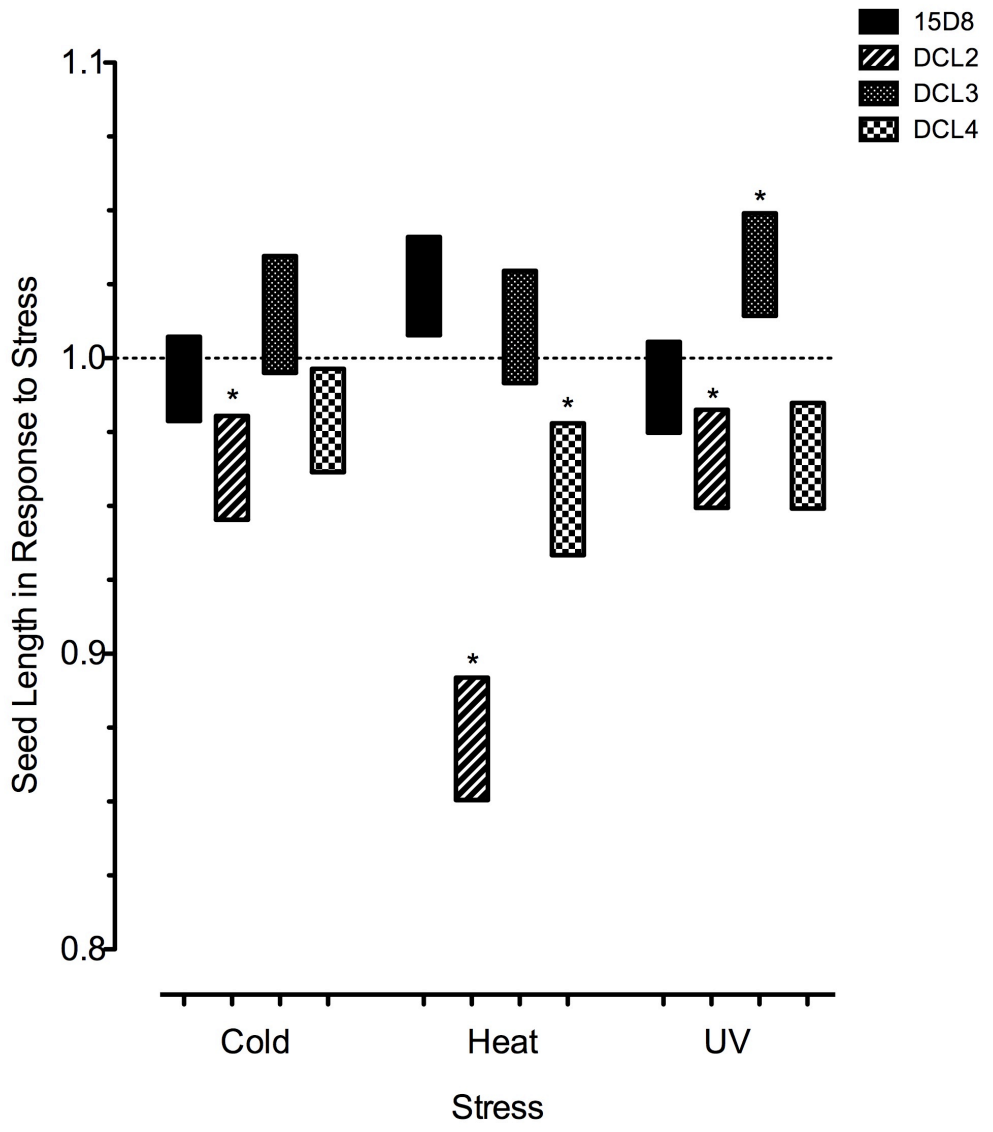

Figure 35. Response to abiotic stress after one generation (F1), as indicated by seed length of seeds produced by stressed plants (S_1), divided by the seeds produced by plants grown under normal conditions (C_1). Approximately 100-200 seeds were measured from each group. Ratios were calculated using bootstrap x10,000 and a confidence interval of 95% (p=0.05). A ratio of 1 indicates no change in size, and CIs overlapping with 1 show no significant change in seed length. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) belonging to the same stress group. Legend indicates mutant type.

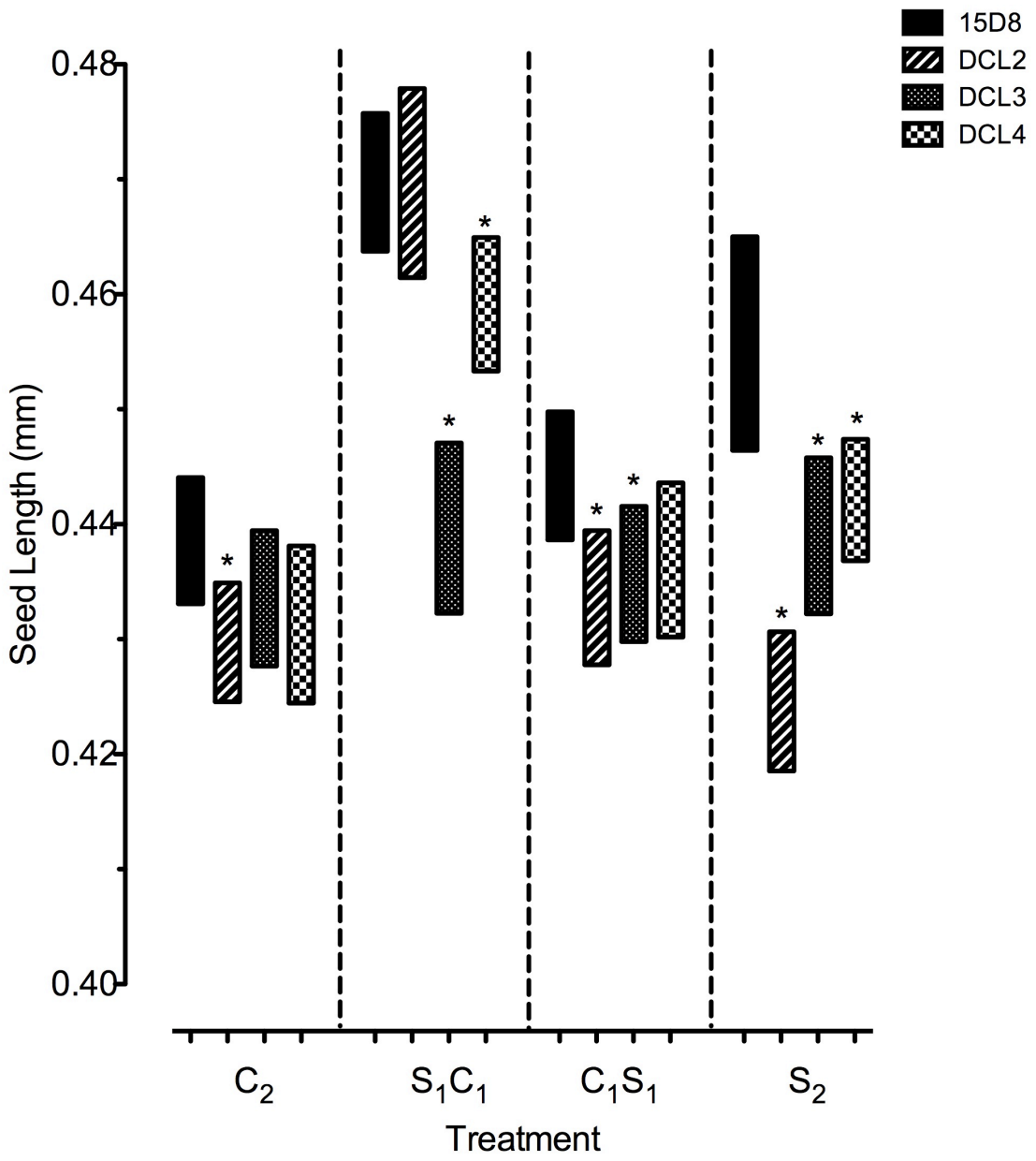

Figure 36. Comparison of F2 seeds produced by the progeny of control plants, grown under normal conditions (C_2), the progeny of cold stressed plants, grown under normal conditions (S_1C_1), the progeny of control plants, grown under cold stress (C_1S_1), or the progeny of cold stressed plants, grown under cold stress (S_2). Approximately 100-200 seeds were measured from each group. Confidence intervals of 95% (p=0.05) were calculated using bootstrap x10,000. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) belonging to the same treatment group. Legend indicates mutant type. Refer to Figure 37 to observe which responses to stress were statistically significant.

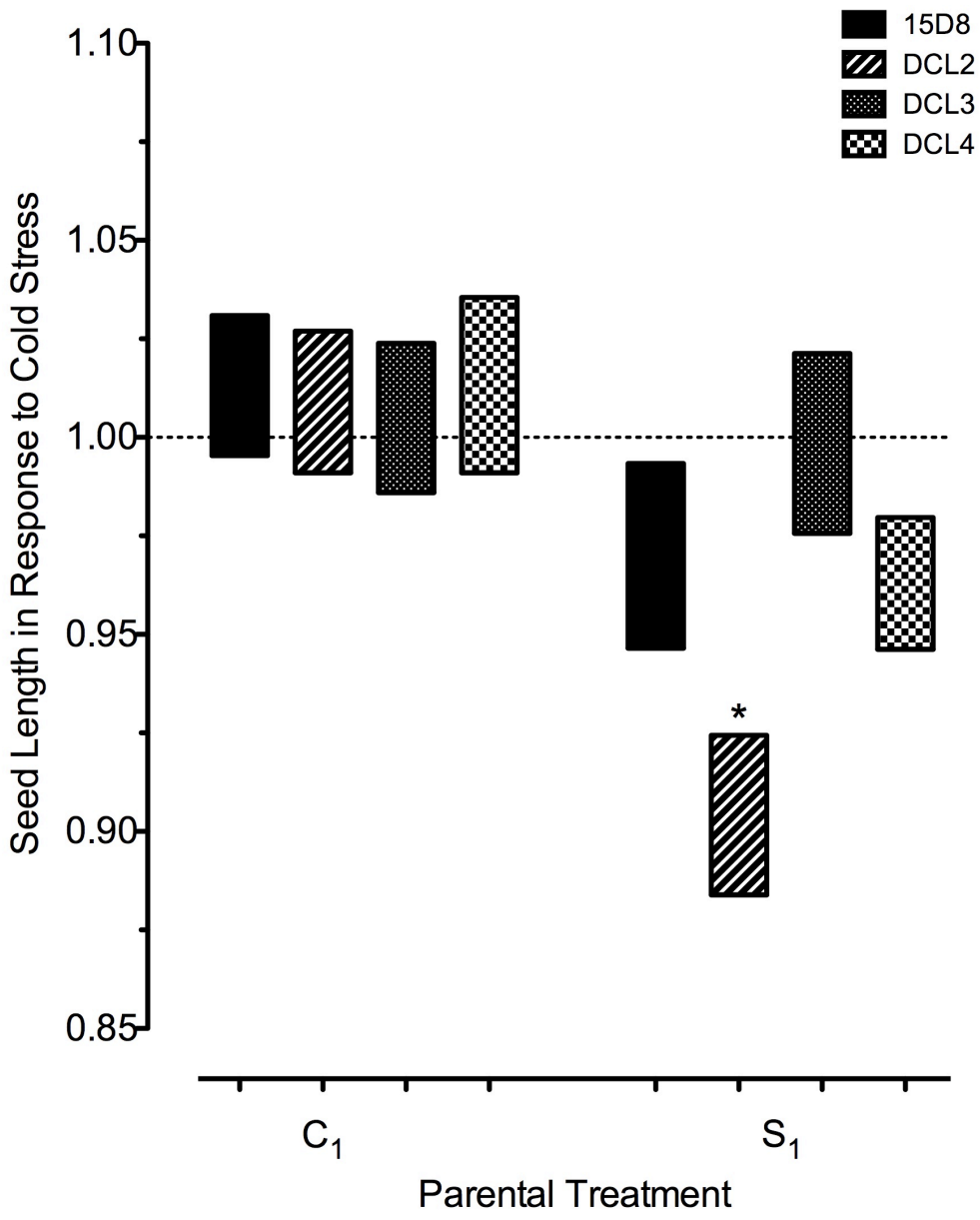

Figure 37. Response of F2 seeds to cold stress, calculated as the seed length of produced by stressed plants, divided by the seed length of seeds produced by non-stressed plants or S_2/S_1C_1 and C_1S_1/C_2. Plants were divided based on F1 parental treatment of either stressed (S_1) or normal growth conditions (C_1). Approximately 100-200 seeds were measured from each group. Ratios were calculated using bootstrap x10,000 and a confidence interval of 95% (p=0.05). A ratio of 1 indicates no change in size, and CIs overlapping with 1 show no significant change in seed length. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) with the same parental treatment. Legend indicates mutant type.

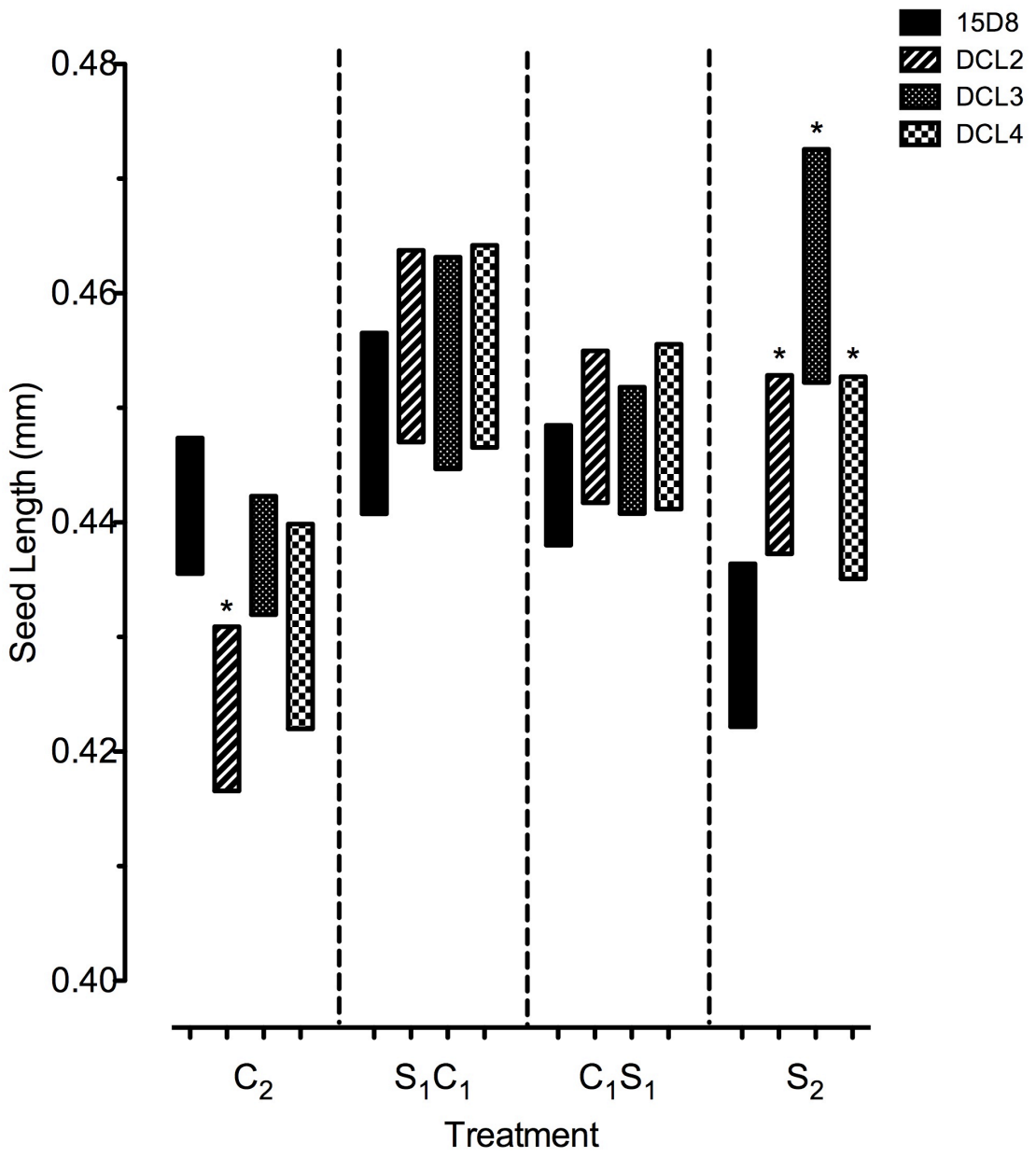

Figure 38. Comparison of F2 seeds produced by the progeny of control plants, grown under normal conditions (C_2), the progeny of het stressed plants, grown under normal conditions (S_1C_1), the progeny of control plants, grown under heat stress (C_1S_1), or the progeny of heat stressed plants, grown under heat stress (S_2). Approximately 100-200 seeds were measured from each group. Confidence intervals of 95% ($p=0.05$) were calculated using bootstrap x10,000. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) belonging to the same treatment group. Legend indicates mutant type. Refer to Figure 39 to observe which responses to stress were statistically significant.

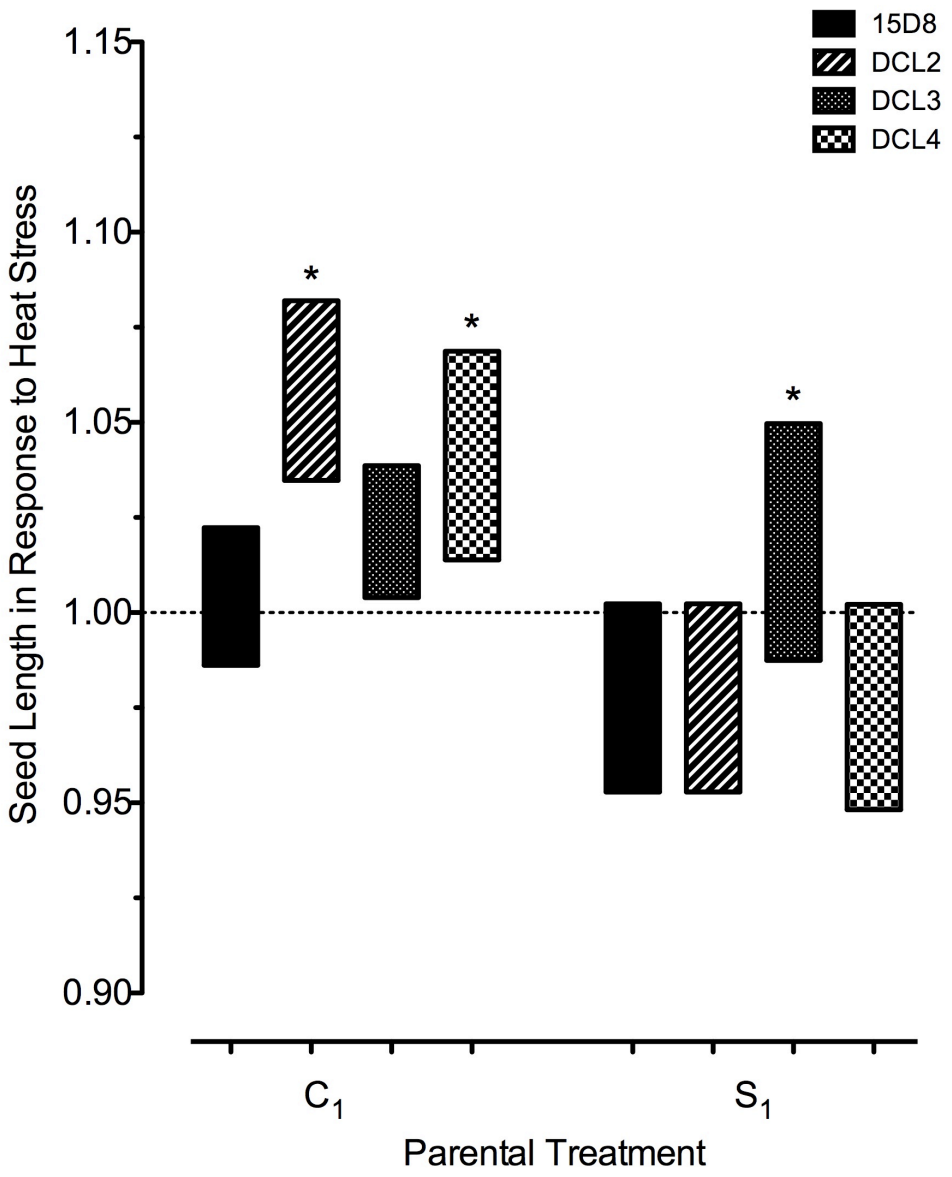

Figure 39. Response of F2 seeds to heat stress, calculated as the seed length of produced by stressed plants, divided by the seed length of seeds produced by non-stressed plants or S_2/S_1C_1 and C_1S_1/C_2. Plants were divided based on F1 parental treatment of either stressed (S_1) or normal growth conditions (C_1). Approximately 100-200 seeds were measured from each group. Ratios were calculated using bootstrap x10,000 and a confidence interval of 95% (p=0.05). A ratio of 1 indicates no change in size, and CIs overlapping with 1 show no significant change in seed length. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) with the same parental treatment. Legend indicates mutant type.

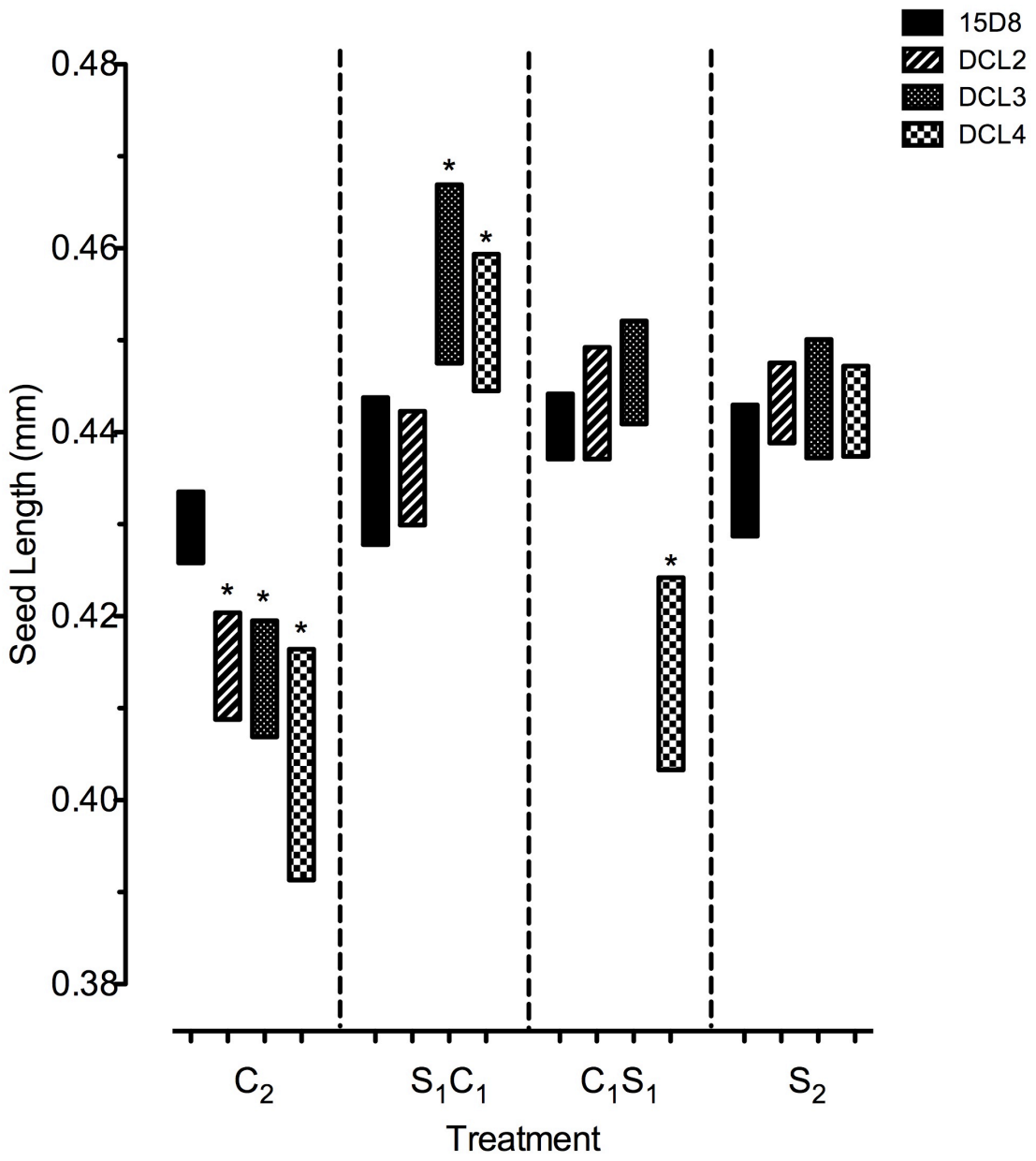

Figure 40. Comparison of F2 seeds produced by the progeny of control plants, grown under normal conditions (C_2), the progeny of UV stressed plants, grown under normal conditions (S_1C_1), the progeny of control plants, grown under UV stress (C_1S_1), or the progeny of UV stressed plants, grown under UV stress (S_2). Approximately 100-200 seeds were measured from each group. Confidence intervals of 95% (p=0.05) were calculated using bootstrap x10,000. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) belonging to the same treatment group. Legend indicates mutant type. Refer to Figure 41 to observe which responses to stress were statistically significant.

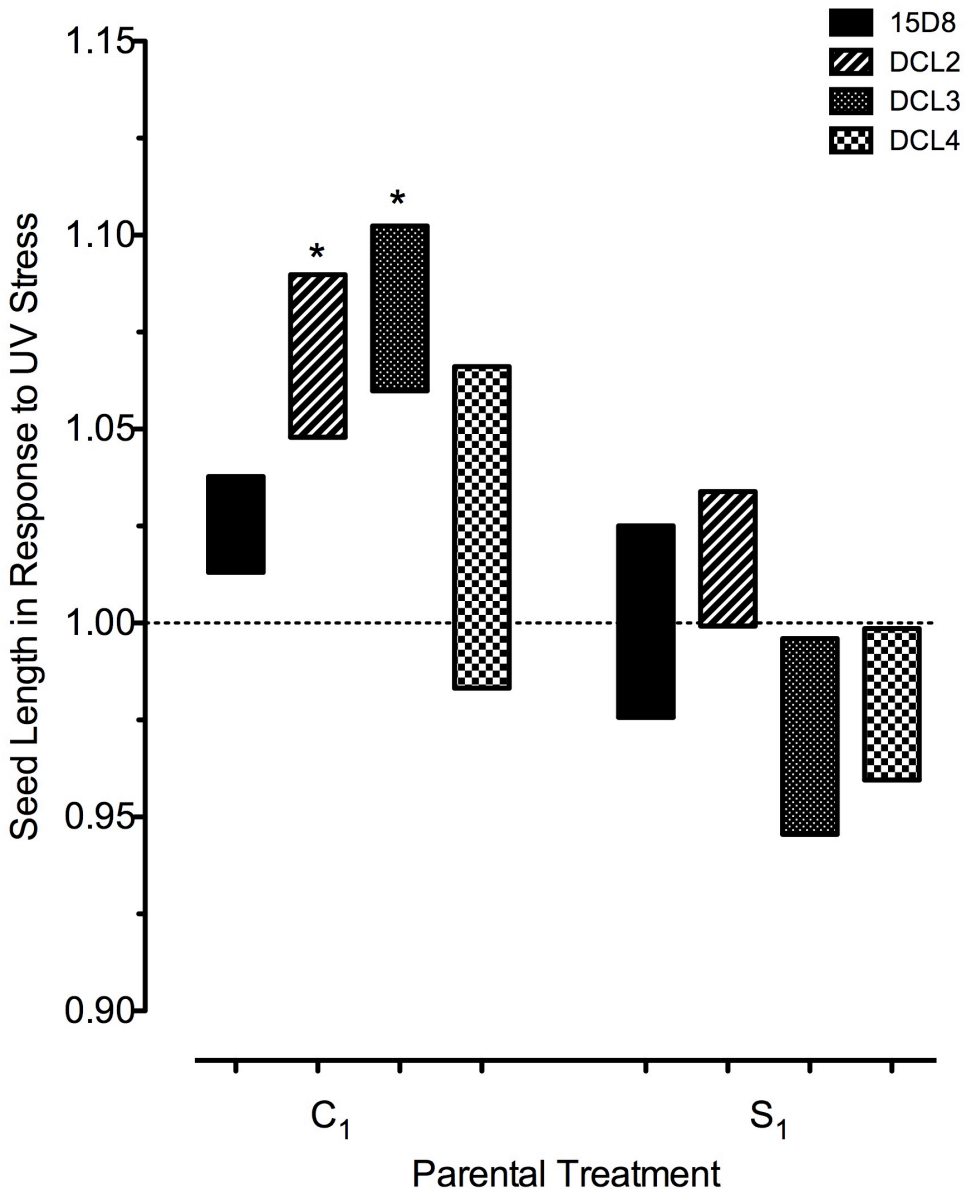

Figure 41. Response of F2 seeds to UV stress, calculated as the seed length of produced by stressed plants, divided by the seed length of seeds produced by non-stressed plants or S_2/S_1C_1 and C_1S_1/C_2. Plants were divided based on F1 parental treatment of either stressed (S_1) or normal growth conditions (C_1). Approximately 100-200 seeds were measured from each group. Ratios were calculated using bootstrap x10,000 and a confidence interval of 95% (p=0.05). A ratio of 1 indicates no change in size, and CIs overlapping with 1 show no significant change in seed length. Asterisks (*) are used to indicate mutants that vary significantly in size from wild-type (15D8) with the same parental treatment. Legend indicates mutant type.

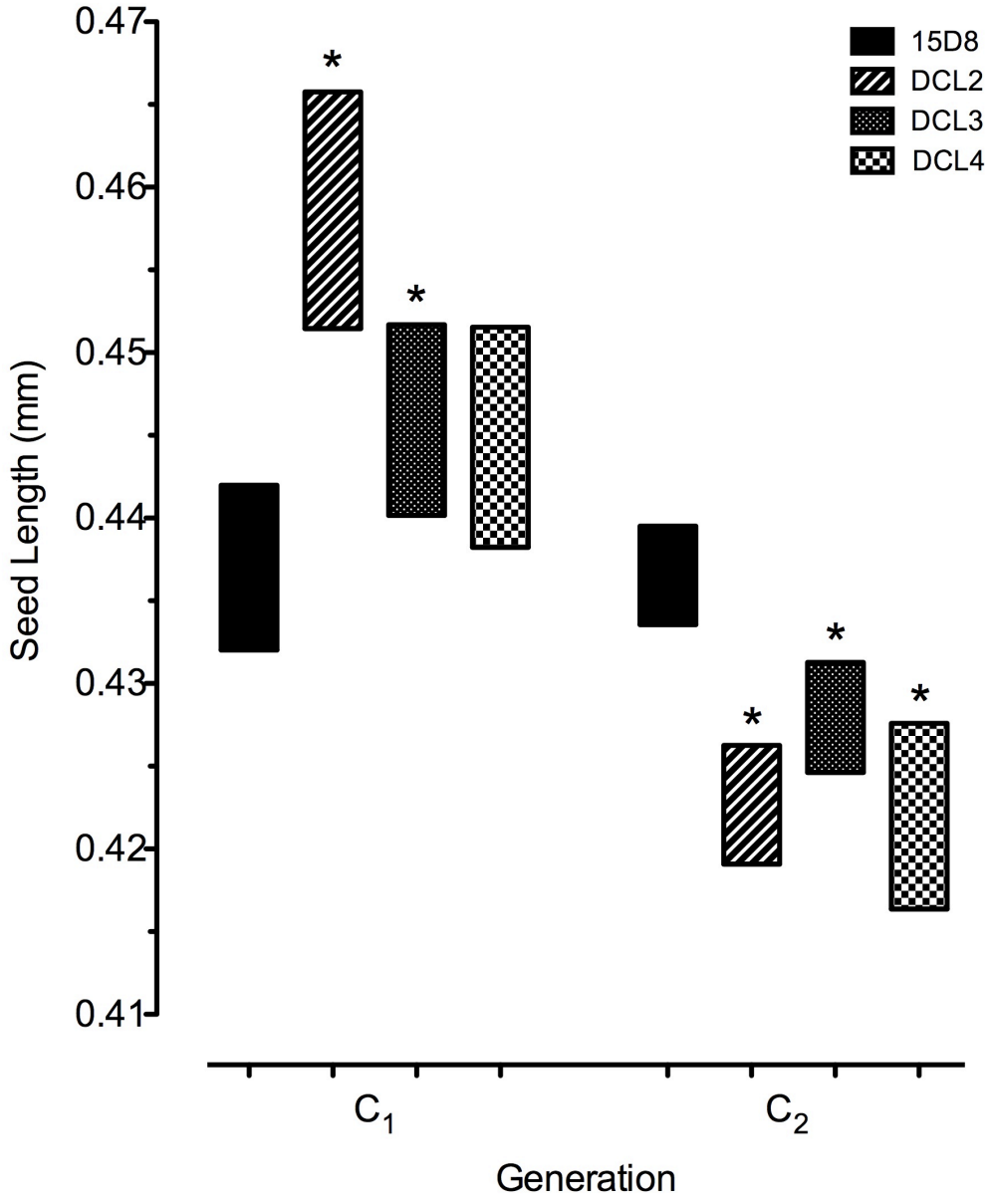

Figure 42. Measurement of natural variation in seed length, within *dcl* mutants and wild-type (15D8) following one (C_1) or two (C_2) generations of growth under normal conditions. Bar represent the 95% confidence interval (p=0.05) for plant type and variable, calculated using a bootstrap x10,000. Asterisks (*) indicate mutants that vary significantly in comparison to wild-type for the same generation. Approximately 100-200 seeds were sampled from each group. Horizontal axis indicates generation. Legend indicates mutant type.

3.1.3. Changes to Bolting Time

3.1.3.1. F1 Generation

In response to cold stress (+), the percentage of plants that bolted by approximately four weeks of age decreased (Figure 43). In contrast, all bolting percentages increased, with the exception of *dcl2* S_1, in response to heat stress. The responses to UV stress were inconsistent, however, 15D8 plants increased bolting in response to UV stress, while *dcl2* and *dcl3* plants decreased. C_1+ *dcl4* increased bolting, but S_1+ *dcl4* showed no variation. UV responses were most similar between 15D8 and *dcl4*, and *dcl2* and *dcl3*.

Parental treatment also impacted the bolting rate of plants under normal growth conditions (Figure 44). The progeny of cold stressed plants had higher bolting under normal growth conditions than the progeny of control (C_1) plants, regardless of mutant type. In contrast, the offspring of UV stressed (S_1) plants had lower bolting rates than the progeny of control plants, when grown under normal conditions. The progeny of plants that were heat stressed, also had higher bolting rates under normal conditions than the progeny of control, with the exception of *dcl4*, which had a lower rate.

3.1.3.2. F2 Generation

F2 plants decreased bolting under cold stress (Figure 45). This change was largest with 15D8 and *dcl2* plants, and less substantial with *dcl3* and *dcl4* plants. In all cases, bolting decreased to nearly zero, and always lower than 20%, in response to cold stress. Parental treatment did not appear to impact bolting rate in response to cold stress.

The impact of heat stress on F2 generation varied with parental treatment (Figure 46). Interestingly, *dcl2* plants appeared to be the least impacted with regards to bolting

percentage change under heat stress, and only one parental treatment (C_1S_1), resulted in a variation in bolting in response to heat. C_2 plants increased bolting, except for *dcl2*. In contrast, C_1S_1 plants decreased bolting under heat stress, except for *dcl4*. S_1C_1 plants did not vary with heat stress, except for an increase for *dcl3*. The progeny of S_2 was inconsistent, with an increase for 15D8 and *dcl4* under stress, a decrease with *dcl3*, and no change for *dcl2*. In general, plants grown under normal conditions in F0 (C_2 and C_1S_1) were more responsive to heat stress than the progeny of S_2 and S_1C_1, with all mutant types. Among F0 control plants, only 1 of 8 did not change in response to heat stress. In contrast, with F0 stress, 4 of the 8 treatment groups did not change.

The response of F2 plants to UV stress was not consistent (Figure 47). The parental treatment S_2 increased bolting under stress, with the exception of *dcl4*, which decreased. S_1C_1 also increased, except for 15D8, which decreased. C_2 showed a lot of variation under UV, as 15D8 increased bolting, while *dcl2* and *dcl4* had no response, and *dcl3* decreased. Finally, for C_1S_1, both 15D8 and *dcl2* decreased, while *dcl3* and *dcl4* increased. Overall, the most dramatic responses to UV were seen with *dcl3* plants, as they had the largest change in bolting under stress, with the exception of the parental treatment S_1C_1, where the change was slightly larger for *dcl4*. Overall, the response to UV fluctuated greatly, but remained fairly consistent within parental treatment groups.

F2 plants grown under normal conditions, showed some interesting trends (Figure 48). For cold, the bolting rate for all 15D8 plants, regardless of parental treatment, was higher than nearly all other mutant types and parental treatments, including all *dcl3* and *dcl4* rates. In almost all cases for 15D8, *dcl2* and *dcl3*, those that had undergone F0 stress showed a decrease in bolting in comparison to those in which F0 was not stressed (S_1C_1

in comparison to C_2, and S_2 in comparison to C_1S_1). However, for *dcl4*, S_1C_2 and S_2 increased bolting in F2. For plants with F0 treatment of normal growth conditions (C_2 and C_1S_1) varying F1 treatment had an inconsistent impact. For example, 15D8 and *dcl2* remained nearly the same, while *dcl3* increased bolting with F1 stress, but *dcl4* decreased. Parental treatments C_2 and C_1S_1 resulted in 15D8 progeny, which had a higher bolting, rate than *dcl* plants. However, with F0 stressing, additional F1 stressing (S_2 in comparison to S_1C_1) resulted in progeny with lower bolting rates for or 15D8 and *dcl3*, did not impact *dcl2*, and increased *dcl4* when compared under normal growth conditions.

In almost every case, F0 heat stress (or S_2 and S_1C_1) increased bolting compared to C_2 and C_1S_1, providing the F1 treatment (S_1C_1 and C_2, S_2 and C_1S_1) was the same (Figure 48). The only exceptions were 15D8 S_2 and C_1S_1, and *dcl3* S_2 and C_1S_1. For the progeny of plants in which F0 was stressed, F1 stress (S_2), in comparison to growth under normal conditions (S_1C_1), decreased bolting in 15D8, *dcl2* and *dcl4*, while *dcl3* increased slightly. For progeny of F0 controls, C_1S_1 increased bolting in comparison to C_2 in 15D8, *dcl3* and *dcl4*, but showed a small decrease in *dcl2*.

For UV, F0 treatment impacted bolting under normal growth conditions (Figure 48). Progeny of F0 controls had higher bolting rates than those which were progeny of F0 stressed plants, providing they had the same F1 treatment (S_1C_1 versus C_2 and S_2 versus C_1S_1). C_1S_1 resulted in offspring with higher bolting than the progeny of those grown under normal conditions for two generations (C_2), with the exception of *dcl3*. S_2 15D8 and *dcl3* had lower bolting rates than S_1C_1, while the bolting remained consistent for *dcl2*, and increased for S_2 *dcl4*.

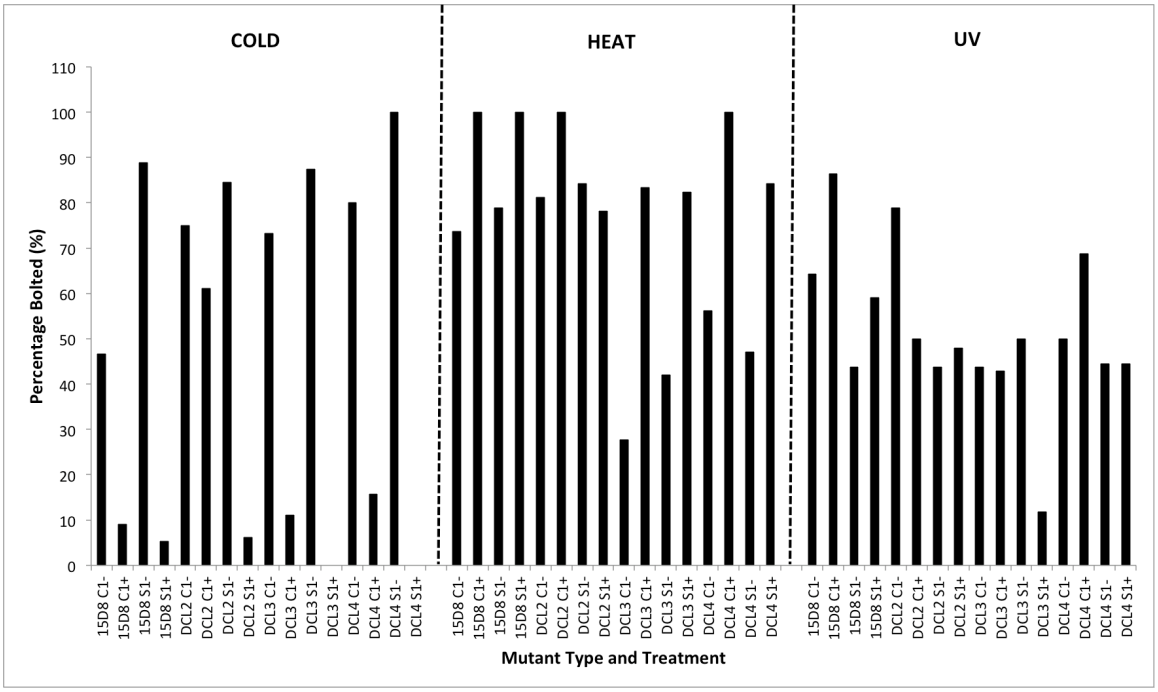

Figure 43. Percentage of F1 plants that had bolted at approximately 4 weeks of age. Plants were grown under either normal (-) or stressed (+) (cold, heat or UV) conditions. Each treatment group is labeled according to treatment and mutant type, on the horizontal axis. Approximately 24 plants were included in each treatment group.

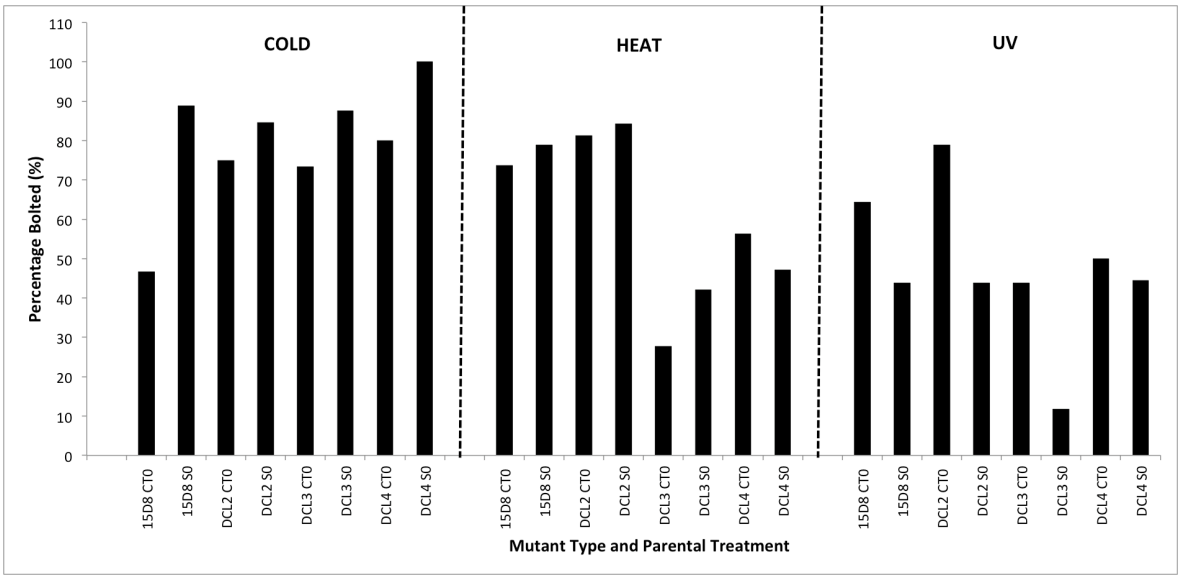

Figure 44. Percentage of F1 plants that had bolted at approximately 4 weeks of age. Plants were grown under normal conditions. Each treatment group is labeled according to parental treatment, and mutant type, on the horizontal axis. Plants are also divided based on parental stress groups of cold, heat and UV. Approximately 24 plants were included in each treatment group.

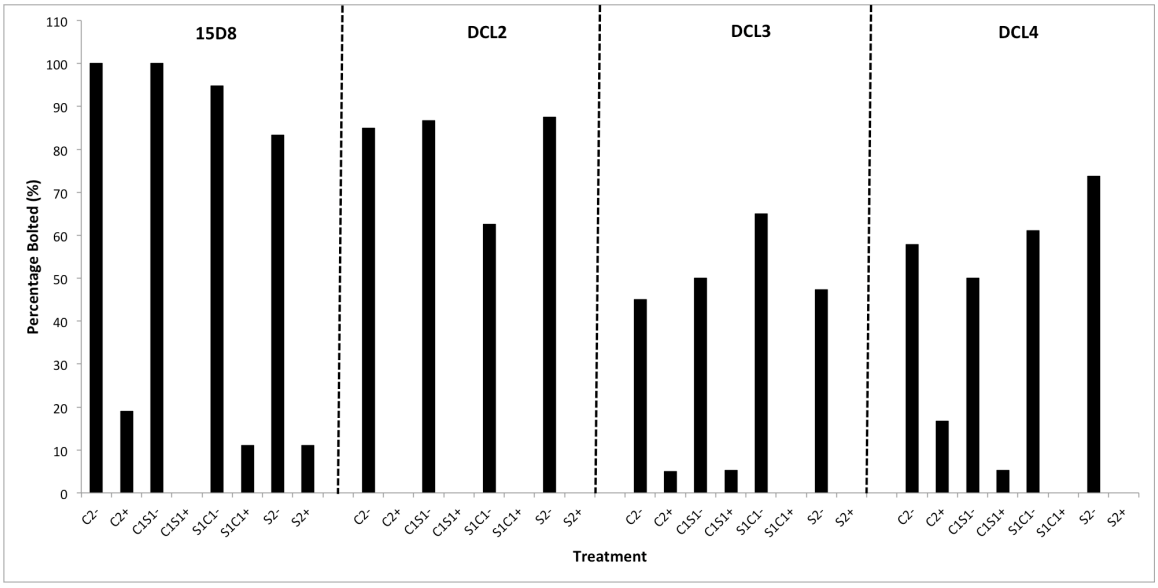

Figure 45. Percentage of F2 plants that had bolted at approximately 4 weeks of age. Plants were grown under normal (-) or cold stress (+) conditions. Each treatment group is labeled according to treatments on the horizontal axis. Plants are also divided based on mutant type. Approximately 24 plants were included in each treatment group.

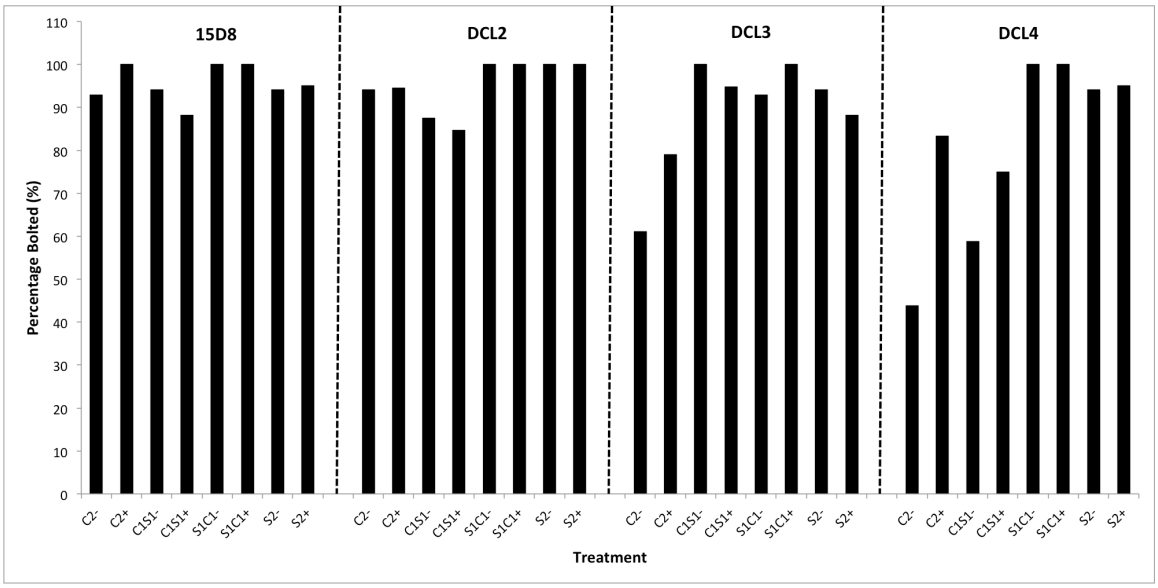

Figure 46. Percentage of F2 plants that had bolted at approximately 4 weeks of age. Plants were grown under normal (-) or heat stress(+) conditions. Each treatment group is labeled according to treatments on the horizontal axis. Plants are also divided based on mutant type. Approximately 24 plants were included in each treatment group.

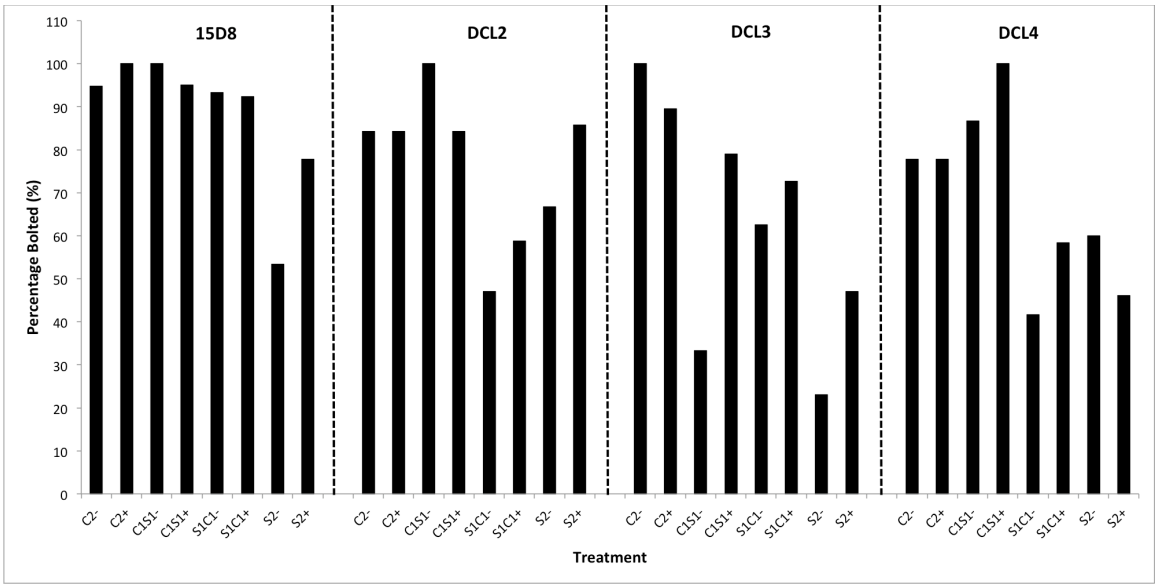

Figure 47. Percentage of F2 plants that had bolted at approximately 4 weeks of age. Plants were grown under normal (-) or UV stress (+) conditions. Each treatment group is labeled according to treatments on the horizontal axis. Plants are also divided based on mutant type. Approximately 24 plants were included in each treatment group.

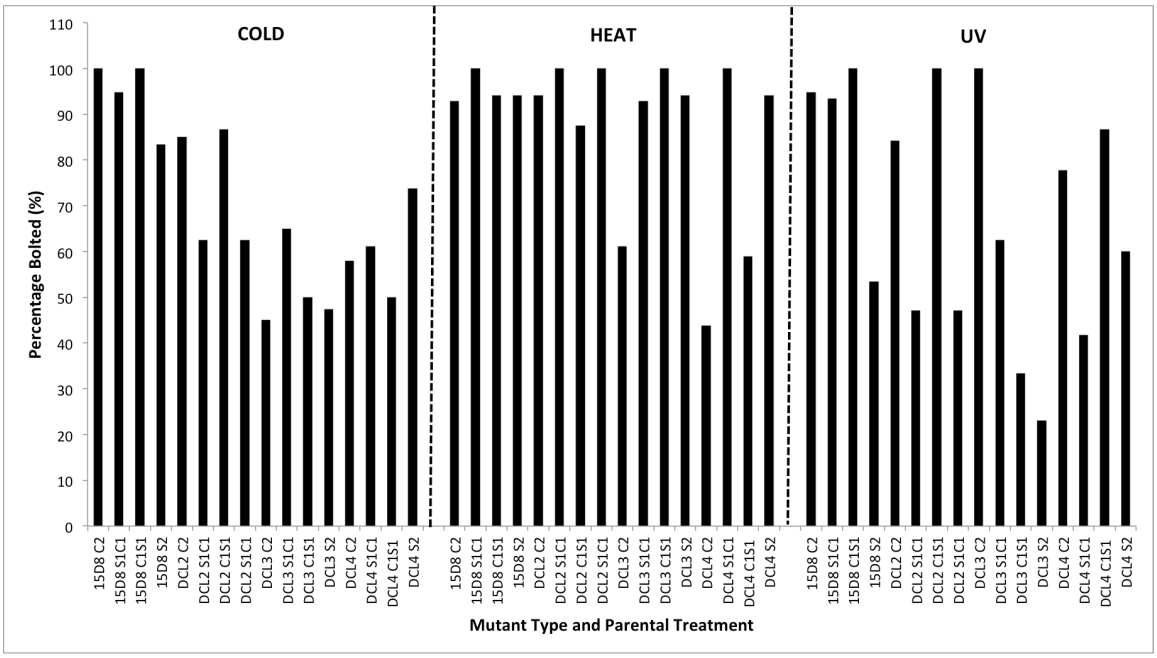

Figure 48. Percentage of F2 plants that had bolted at approximately 4 weeks of age. Plants were grown under normal conditions (-). Each treatment group is labeled according to parental treatments and mutant type on the horizontal axis. Plants are also divided based on parental stress form. Approximately 24 plants were included in each treatment group.

3.2. Molecular Results

3.2.1. Transposon Results

3.2.1.1. F1 Generation

3.2.1.1.1. Cold Stress

Progeny of all control plants (C_1), except *dcl4*, increased ONSEN expression under cold stress (+) (Figure 49). When parental plants were also cold stressed (S_1), stressing offspring did not significantly influence expression of ONSEN for 15D8 and *dcl4*. However, S_1 *dcl2* and *dcl3* saw a decrease in expression under stress.

Cold stressing C_1 15D8 plants did not significantly change TSI expression in comparison to growth under normal conditions, but for *dcl3* it did significantly increase TSI expression (Figure 50). Cold stressing C_1 *dcl2* increased TSI expression, S_1 *dcl2* decreased, S_1 *dcl4* decreased, and C_1 *dcl4* resulted in no significant change.

The progeny of cold stressed plants (S_1) had increased levels of TSI and ONSEN under normal conditions in almost every case (Figure 51). The only exception was TSI in S_1 15D8 plants, where expression was not significantly different than progeny of control plants (C_1). 15D8 had the highest TSI and ONSEN expression among C_1 plants.

Stressing the progeny of control plants (C_1), versus the progeny of stressed plants (S_1) for 15D8 plants, did not significantly influence TSI and ONSEN expression (Figure 52). However, both *dcl2* and *dcl4* significantly decreased in C_1 versus S_1 plants under cold stress. In contrast, *dcl4* increased in expression, especially ONSEN.

3.2.1.1.2. Heat Stress

Expression of ONSEN in the progeny of heat stressed plants (S_1) was significantly higher than progeny of control plants (C_1), regardless of F1 treatment (Figure 53). In most cases, F1 heat stress did not significantly alter expression , especially in the progeny of stressed plants (S_1+ in comparison to S_1-). There was an increase in expression in heat stressed C_1 *dcl4*.

Progeny of heat stressed plants (S_1) usually had higher TSI expression than progeny of control plants (C_1) (Figure 54). While stressing (+) C_1 15D8 increased expression, in most other cases, F1 heat stress did not impact TSI expression.

The progeny of heat stressed plants (S_1) grown under normal conditions had higher TSI and ONSEN expression in than the progeny of control plants (C_1) (Figure 55).

When plants were heat stressed, the progeny of heat stressed plants (S_1+) had a higher level of TSI and ONSEN expression than the progeny of controls (C_1+), though the difference was not significant in most cases (Figure 56).

3.2.1.1.3. UV Stress

15D8, *dcl2* and *dcl4* progeny of controls (C_1), increased expression of ONSEN under UV stress, while *dcl3* decreased (Figure 57). 15D8 and *dcl2* progeny of UV stressed plants (S_1) had no significantly difference in ONSEN expression under stress (+) in comparison to growth under normal conditions (-), but S_1 *dcl3* and *dcl4* increased expression.

15D8 plants increased TSI expression in response to UV stress, while *dcl4* plants decreased TSI expression (Figure 58). C_1 *dcl2* plants increased expression, but C_1 *dcl2*

decreased TSI expression in response to UV stress. While C_1 *dcl3* decreased expression, S_1 *dcl3* did not significantly change in response to UV stress.

15D8, *dcl2*, and *dcl4* plants increased expression of TSI and ONSEN in the progeny of UV stressed plants (S_1) in comparison to the progeny of control plants (C_1), under normal conditions, while *dcl3* decreased expression (Figure 59).

In UV stressed progeny of stressed plants tended to have a higher level of ONSEN and TSI expression than UV stressed progeny of controls (Figure 60). The only exceptions were C_1 in comparison to S_1 15D8 for ONSEN, and C_1 in comparison to S_1 *dcl3* for TSI, neither of which were significantly different.

3.2.1.2. F2 Generation

3.2.1.2.1. Cold Stress

Exposure to cold in F2 plants tended to decrease ONSEN expression (Figure 61). The only exceptions were C_1S_1 for 15D8 and *dcl2*, where ONSEN expression increased with cold stress (+). The progeny of plants cold stressed in F0 (S_1C_1 and S_2) had the highest levels of ONSEN under normal conditions (-). For 15D8 and *dcl2*, the greatest ONSEN expression was in S_2 plants, while for *dcl3* and *dcl4*, it was in S_1C_1. Cold stressing parents in F1 (C_1S_1) was not enough to change ONSEN expression level in *dcl3* and *dcl4*, both of which had C_1S_1 expression similar, or lower than, C_2. However, C_1S_1 15D8 and *dcl2* were higher than C_2 under stress.

Cold stress tended to decrease, or make no significant difference, in TSI expression of F2 plants (Figure 62). The highest expression of TSI for each mutant type included F0 stress (either S_1C_1 or S_2).

The F2 progeny of plants that were cold stressed in F0 (S_2 and S_1C_1), when grown under normal conditions, had higher expression levels of TSI and ONSEN than those grown under normal conditions in F0 (C_2 and C_1S_1), regardless of their parental treatment in F1 (Figure 63). However, a second generation of stress (S_2) tended to further increase expression. The most dramatic difference in transposon expression was observed between C_1S_1 and S_2 plants all plants. C_2 plants had the lowest expression levels of TSI and ONSEN in 15D8 and *dcl3*. However, in *dcl2*, TSI was the lowest in C_2 but ONSEN was the lowest in C_1S_1. In *dcl4* expression levels were not significantly different in C_2 and C_1S_1.

In most cold stressed F2 plants, F0 stress (S_1C_1) or F0 and F1 stress (S_2) resulted in increased expression of TSI and ONSEN in comparison to those that were grown in normal conditions for F0 (C_2), even if they were cold stressed in F1 (C_1S_1) (Figure 64). Exceptions were TSI C_2 15D8 and ONSEN C_1S_1 *dcl2*. In most treatment groups, C_2 had the lowest expression level, or was not significantly different from C_1S_1, which was also low. However, for C_2 15D8, TSI expression was higher than C_1S_1.

3.2.1.2.2. Heat Stress

Heat stressing F2 plants mostly decreased ONSEN expression (Figure 65). However there were a few exceptions: $C_1S_1$15D8, S_1C_1 *dcl3*, and all *dcl* S_2 treatment groups. Thus, heat stressing S_2 plants only resulted in decreased ONSEN expression for 15D8.

In general, expression of TSI in the progeny of heat stressed plants was low (Figure 66). However, in the few cases where expression was elevated, such as C_1S_1-

dcl2 and *dcl4*, it decreased when the treatment groups were stressed (C_1S_1+). Expression of TSI for 15D8 and *dcl3* was consistently low.

The expression of TSI and ONSEN in F2 plants grown under normal conditions varied (Figure 67). Although TSI remained quite low regardless of parental treatment, it did increase in a couple cases, particularly C_1S_1 *dcl2* and *dcl4*, which had the highest expression level both within mutant groups and overall. In contrast, ONSEN showed inconsistent fluctuation in expression, but was highest among 15D8 plants. S_1C_1 plants tended to have a high expression level that was similar among all four mutant types.

In heat stressed F2 plants, the expression of ONSEN was highest in the progeny of stressed plants that were progeny of stressed plants (S_2) in *dcl2*, *dcl3* and *dcl4* (Figure 68). While the value of ONSEN in S_2 15D8 was elevated, it was not significantly different from S_1C_1. Heat stressing of plants in F0, even if F1 was grown under normal conditions, also increased ONSEN expression, as S_1C_1 *dcl2* and *dcl3* had the second highest values for their group, and expression of ONSEN in S_1C_1 *dcl4* was also elevated. The lowest expression values for TSI were seen in C_2 *dcl3* and *dcl4*. However, overall parental stressing did not increase the expression of TSI in F2 heat stressed plants, as the lowest expression value of TSI for 15D8 plants was in C_1S_1 plants.

3.2.1.2.3. UV Stress

UV stressing (+) of F2 plants usually resulted in decreased ONSEN compared to plants grown under normal conditions (-), with the same parental treatment (Figure 69). When ONSEN expression did not decrease, expression was not significantly different from growth under normal conditions, with the exception of C_2 15D8.

Similarly, UV stressing decreased TSI expression, or resulted in expression that was not significantly different than that in plants grown under normal conditions (Figure 70). The only exception was S_2 *dcl2*. *dcl3* had most consistently low TSI expression.

F2 plants grown under normal conditions (-) showed inconsistent variation in expression of TSI and ONSEN (Figure 71). For 15D8, the highest expression level was S_2, and C_2 was the lowest. *dcl2* and *dcl3* had lowest expression of ONSEN and TSI in C_1S_1 plants, while the lowest values for *dcl4* were in S_2. The highest *dcl2* and *dcl3* expression values were for S_1C_1, and for *dcl4* it was for C_1S_1. Thus, the highest transposon expression was found in the progeny that experienced at least one generation of UV stress.

The F2 progeny of plants exposed to UV in F0 (S_1C_1 and S_2) had higher expression levels in nearly all cases than progeny of F0 controls (Figure 72). The only exception was C_2 15D8 ONSEN, which was higher than S_1C_1 ONSEN. The highest levels of TSI and ONSEN occurred in either S_1C_1 or S_2, varying with mutant type, though in most cases the expression levels of the two treatments were not significantly different.

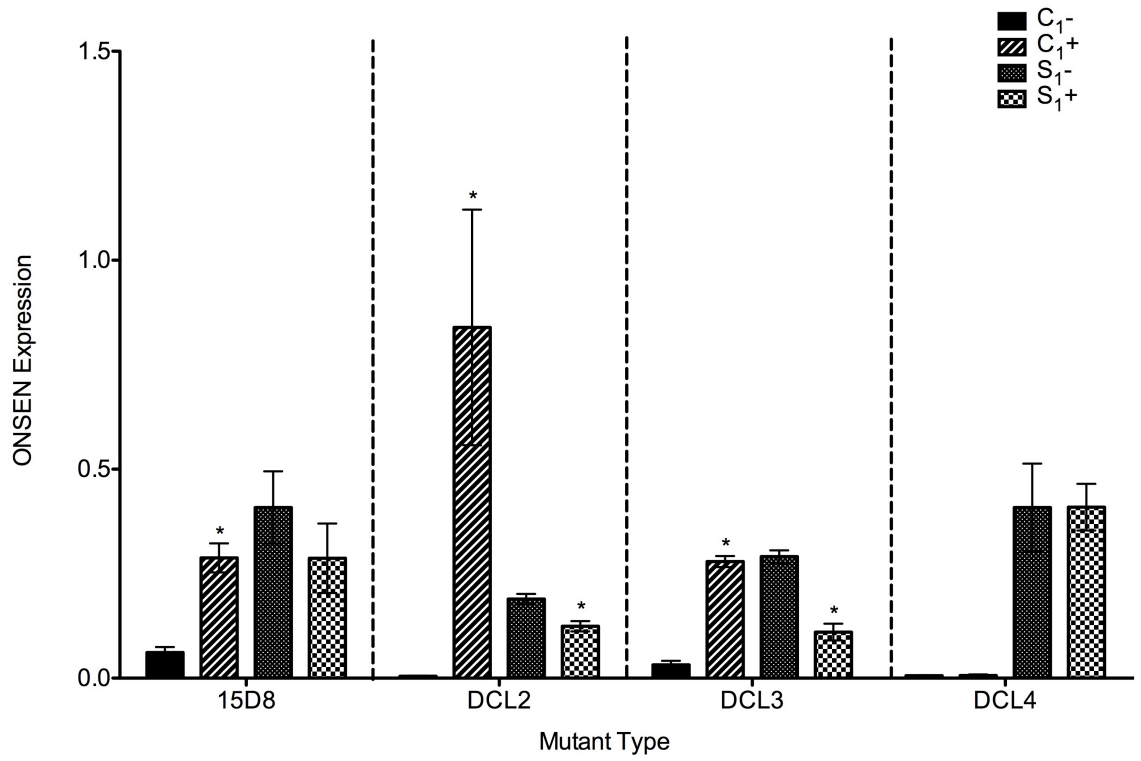

Figure 49. Expression of ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

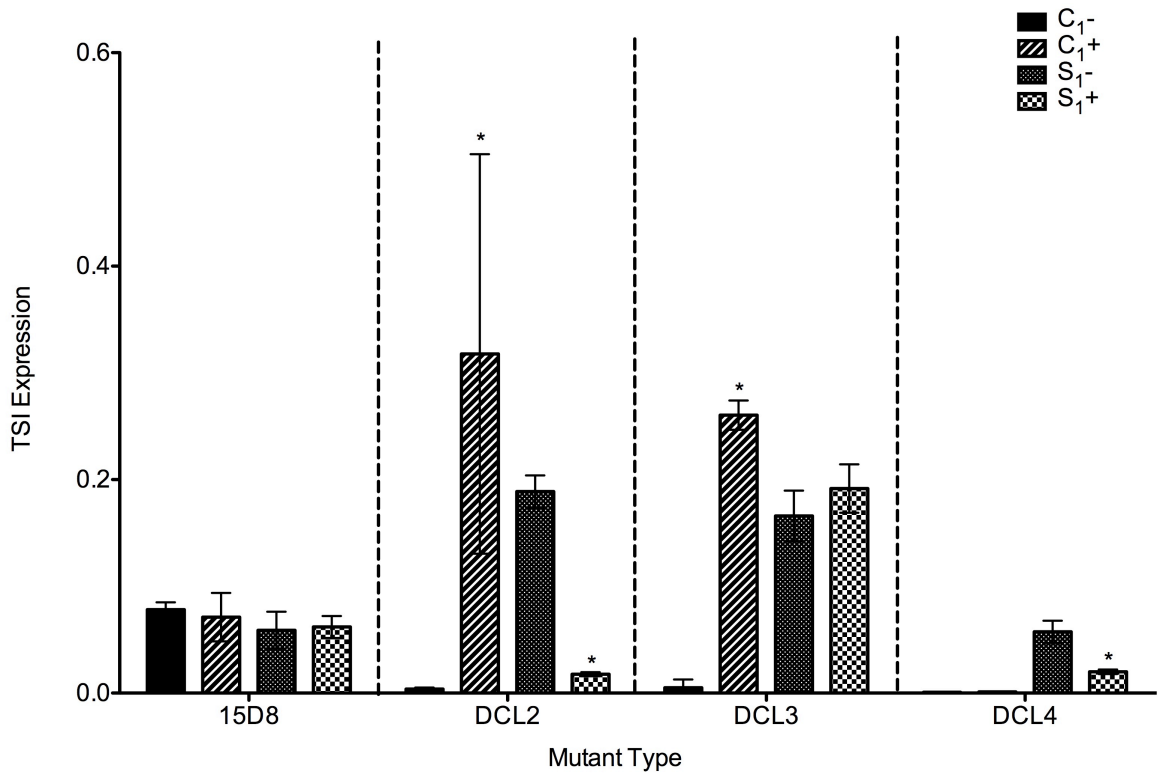

Figure 50. Expression of TSI transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

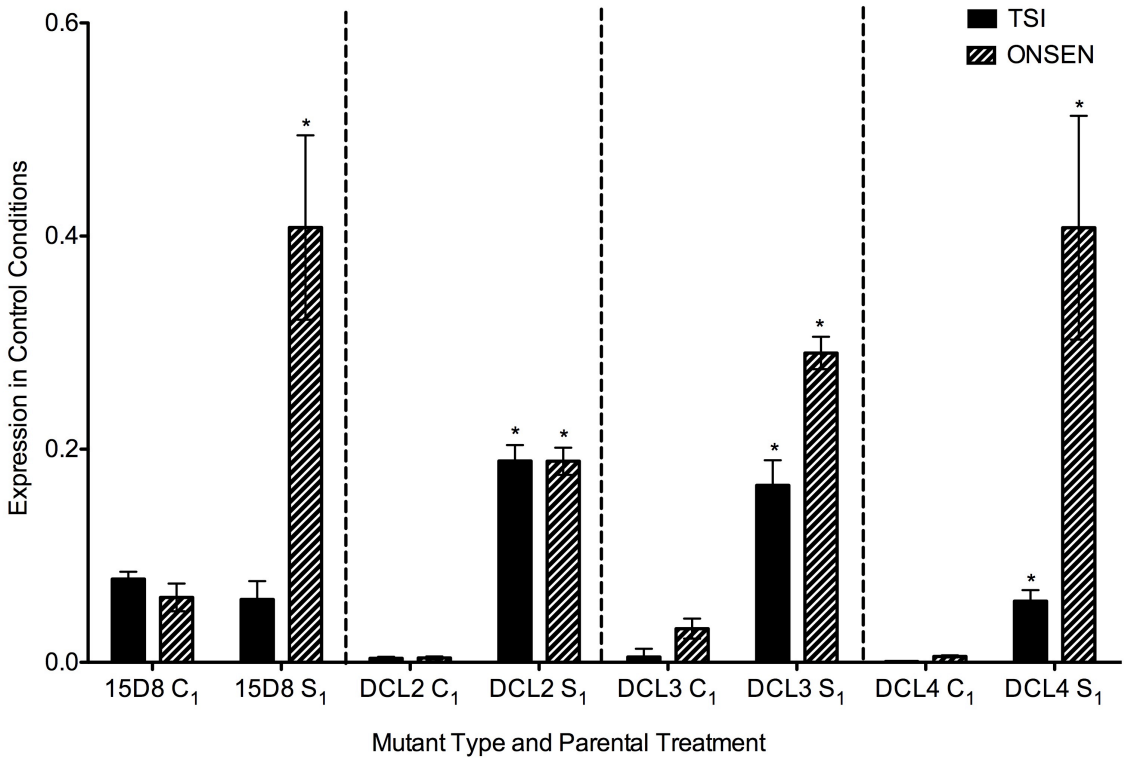

Figure 51. Expression of TSI and ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, grown at normal conditions. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny of control (C_1) and progeny of stressed plants (S_1) for the same gene and mutant type, as calculated using a t-test ($p \leq 0.05$).

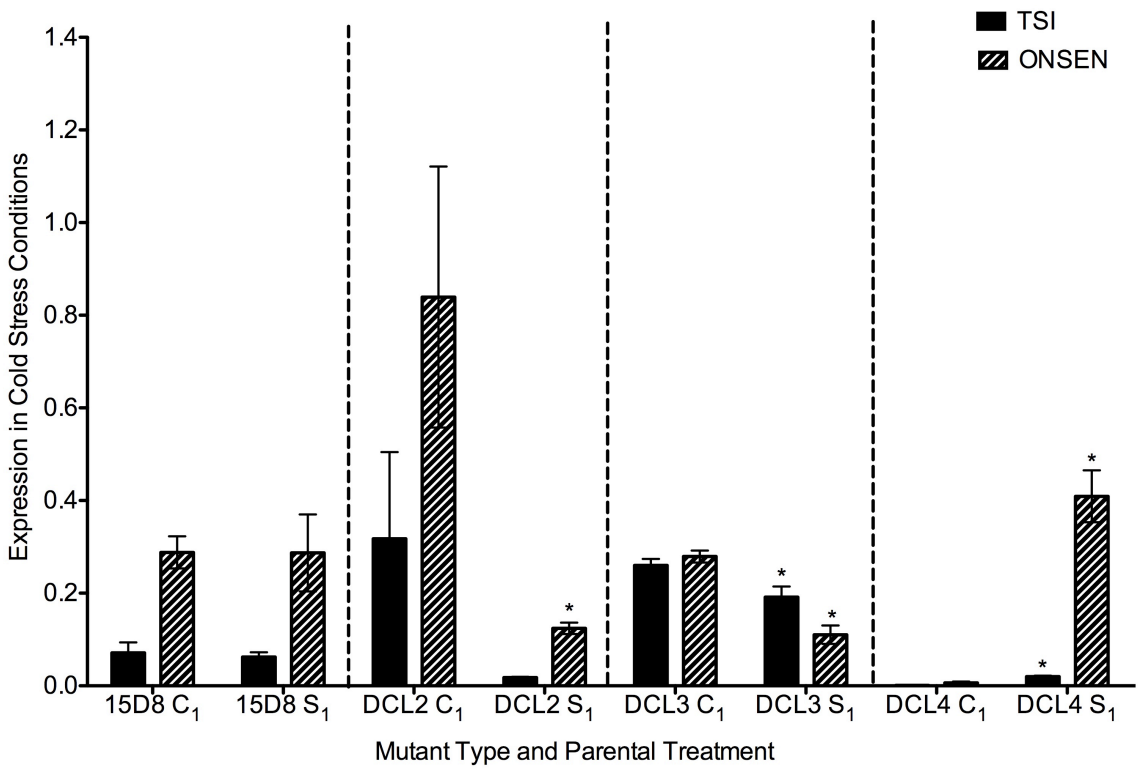

Figure 52. Expression of TSI and ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when exposed to cold stress. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny of control (C_1) and progeny of stressed plants (S_1) for the same gene and mutant type, when exposed to cold, as calculated using a t-test ($p \leq 0.05$).

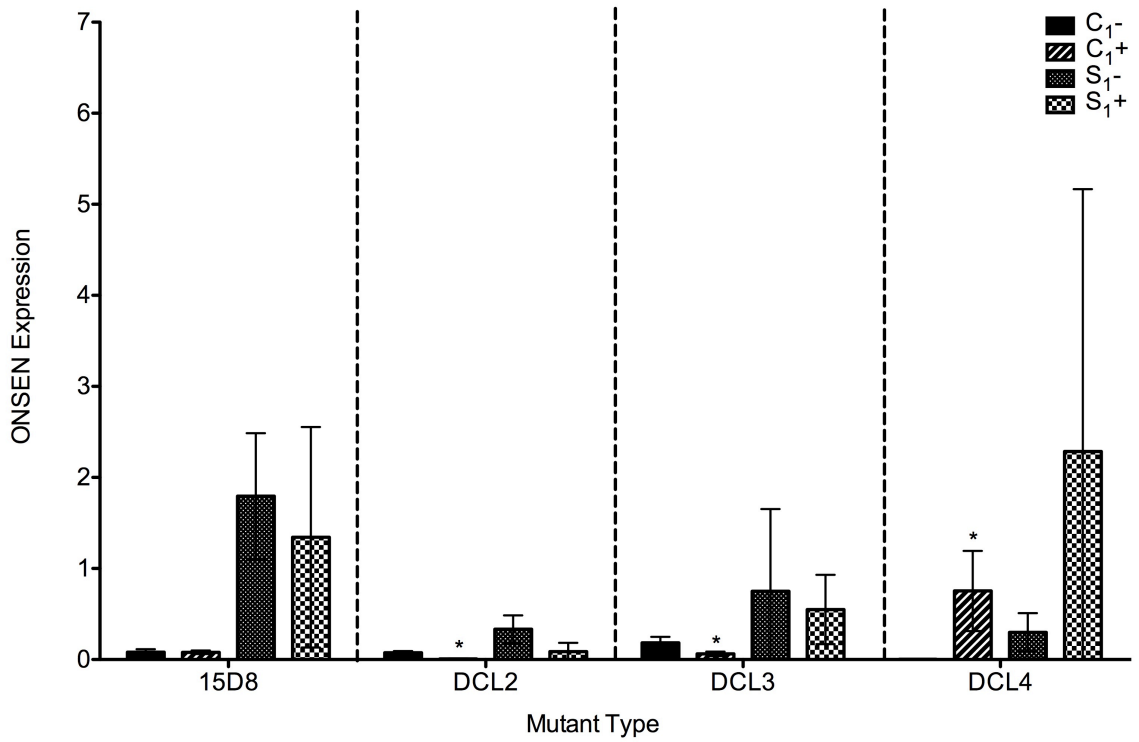

Figure 53. Expression of ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

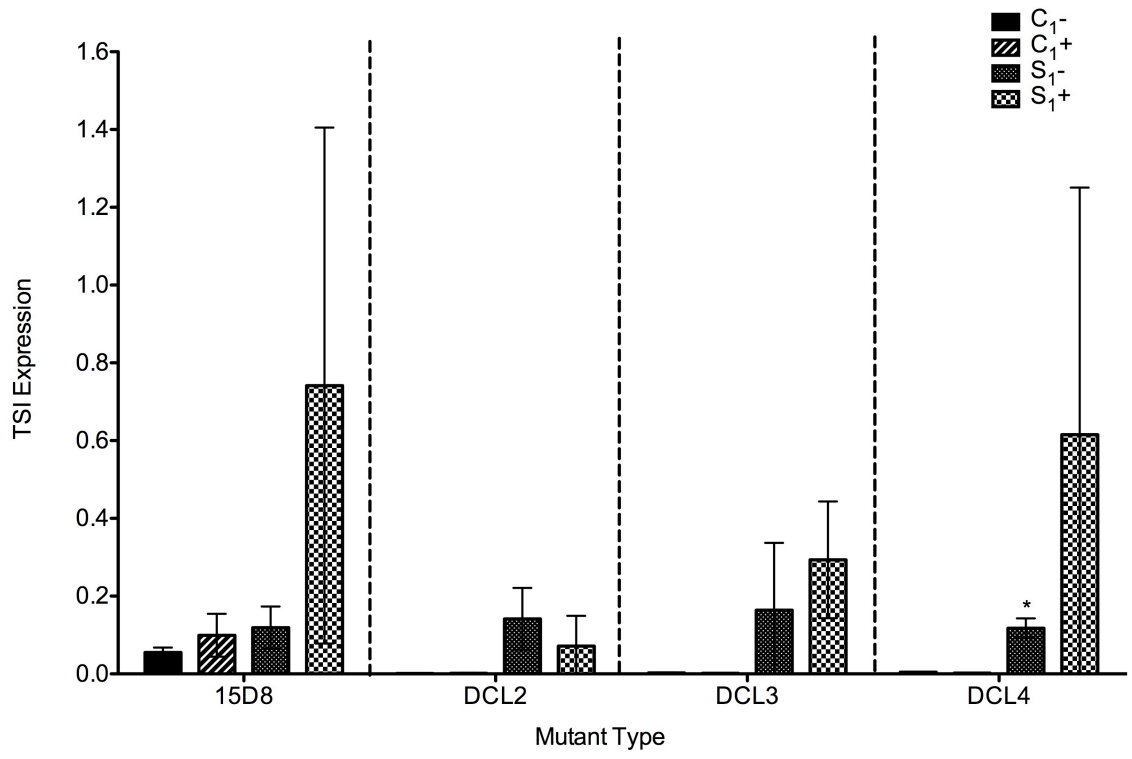

Figure 54. Expression of TSI transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

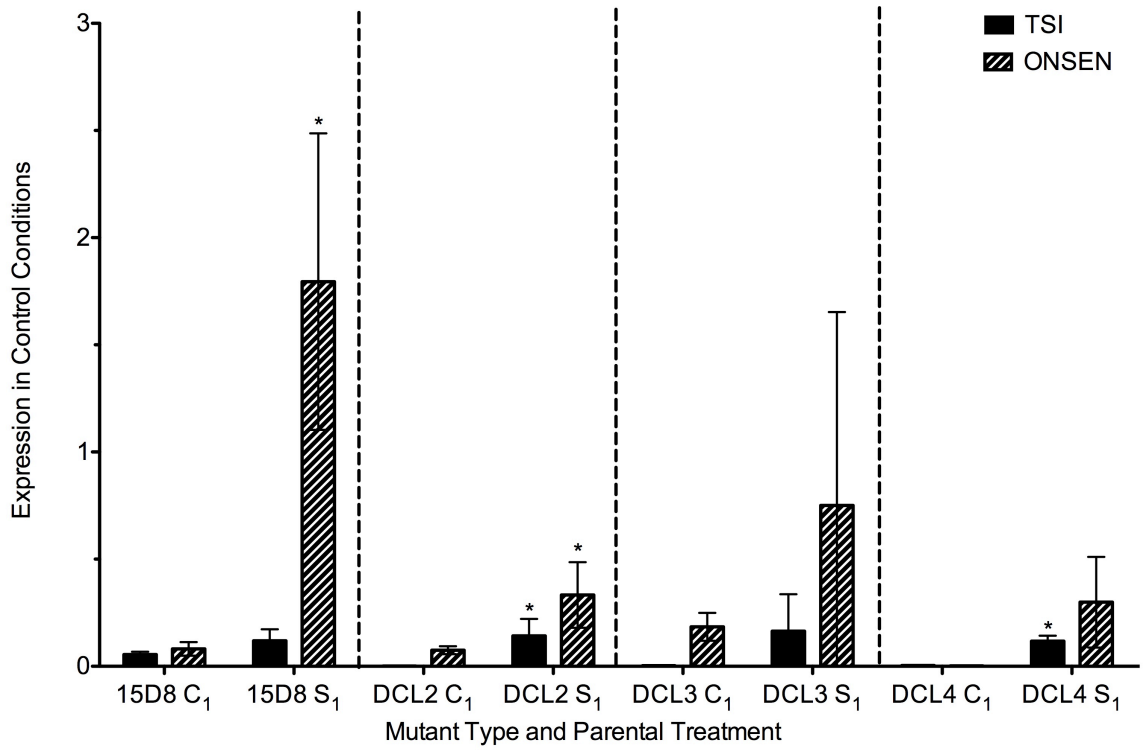

Figure 55. Expression of TSI and ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, grown at normal conditions. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny of control (C_1) and progeny of stressed plants (S_1) for the same gene and mutant type, as calculated using a t-test ($p \leq 0.05$).

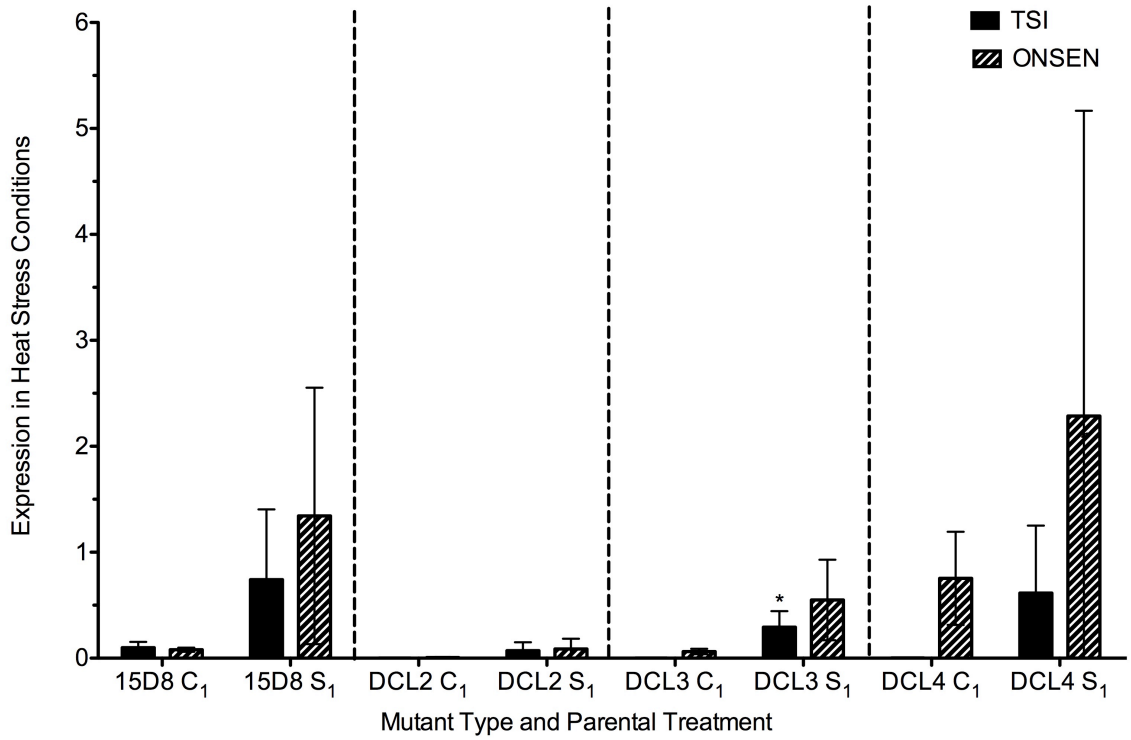

Figure 56. Expression of TSI and ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when exposed to heat stress. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny of control (C_1) and progeny of stressed plants (S_1) for the same gene and mutant type, when exposed to heat, as calculated using a t-test ($p \leq 0.05$).

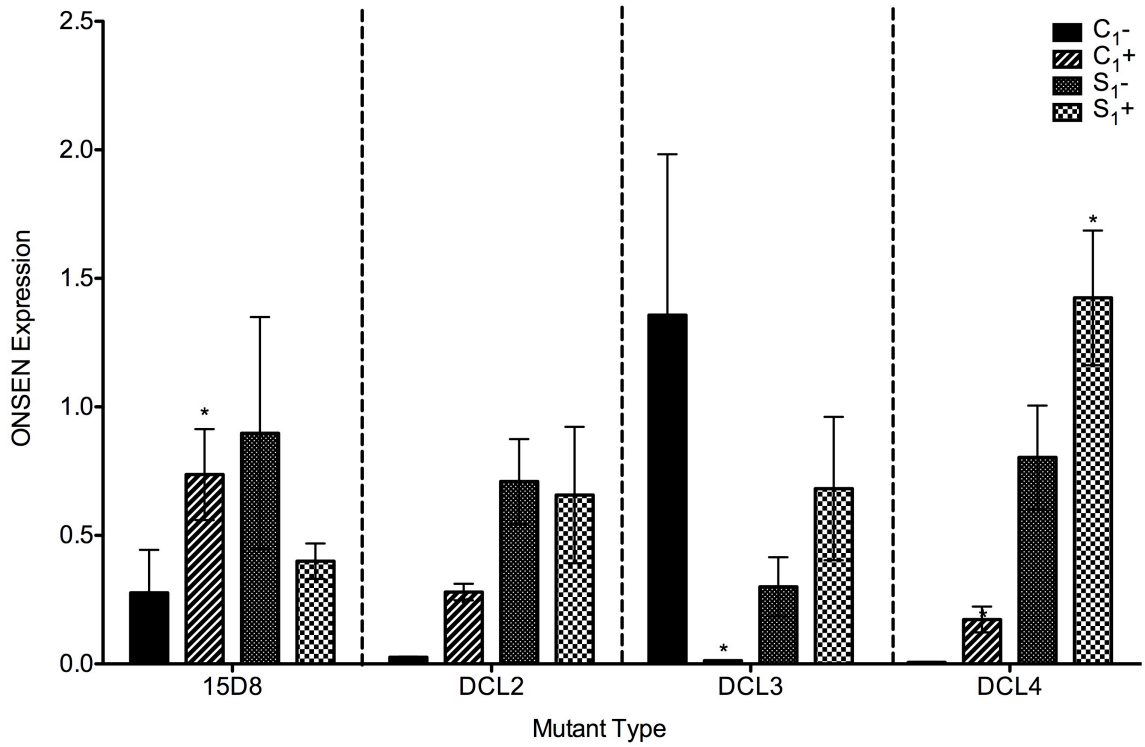

Figure 57. Expression of ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to UV in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

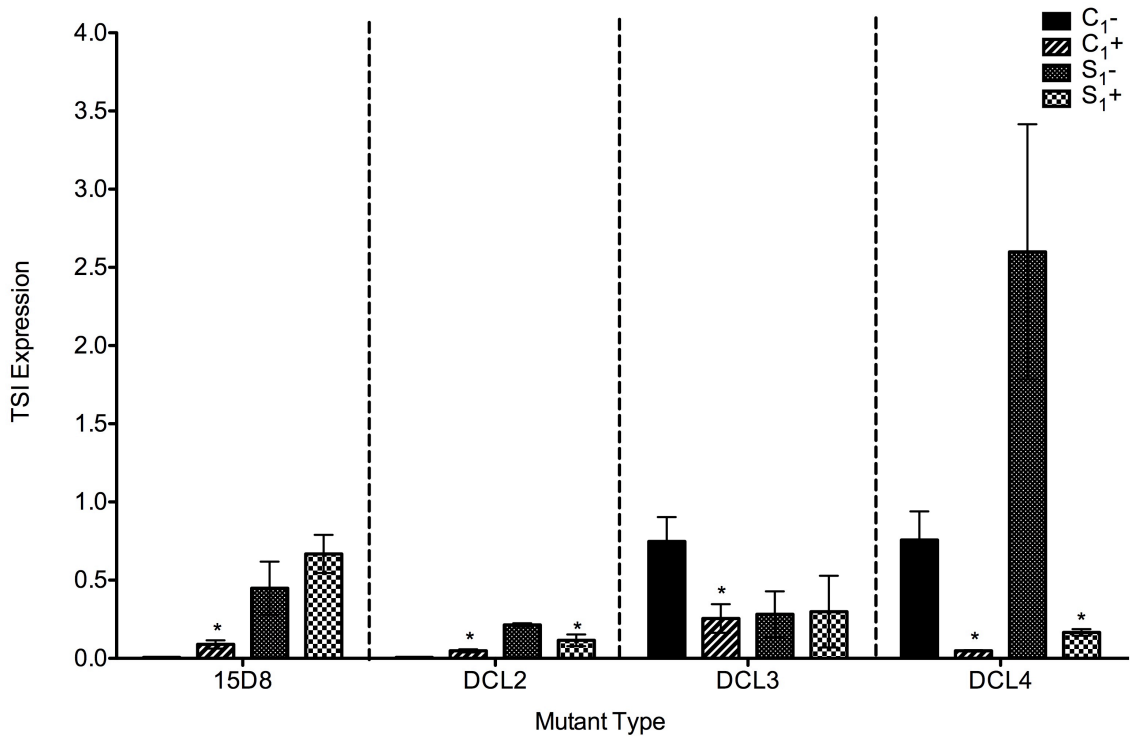

Figure 58. Expression of TSI transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to UV in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

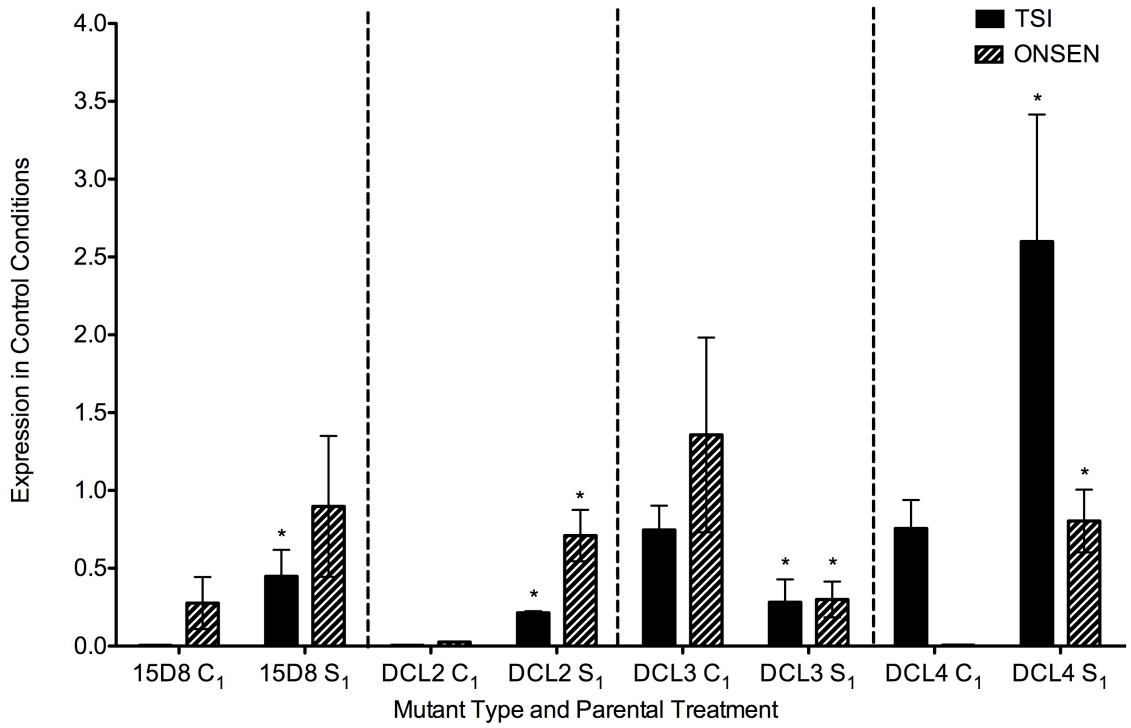

Figure 59. Expression of TSI and ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, grown at normal conditions. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to UV in F0. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny of control (C_1) and progeny of stressed plants (S_1) for the same gene and mutant type, as calculated using a t-test ($p \leq 0.05$).

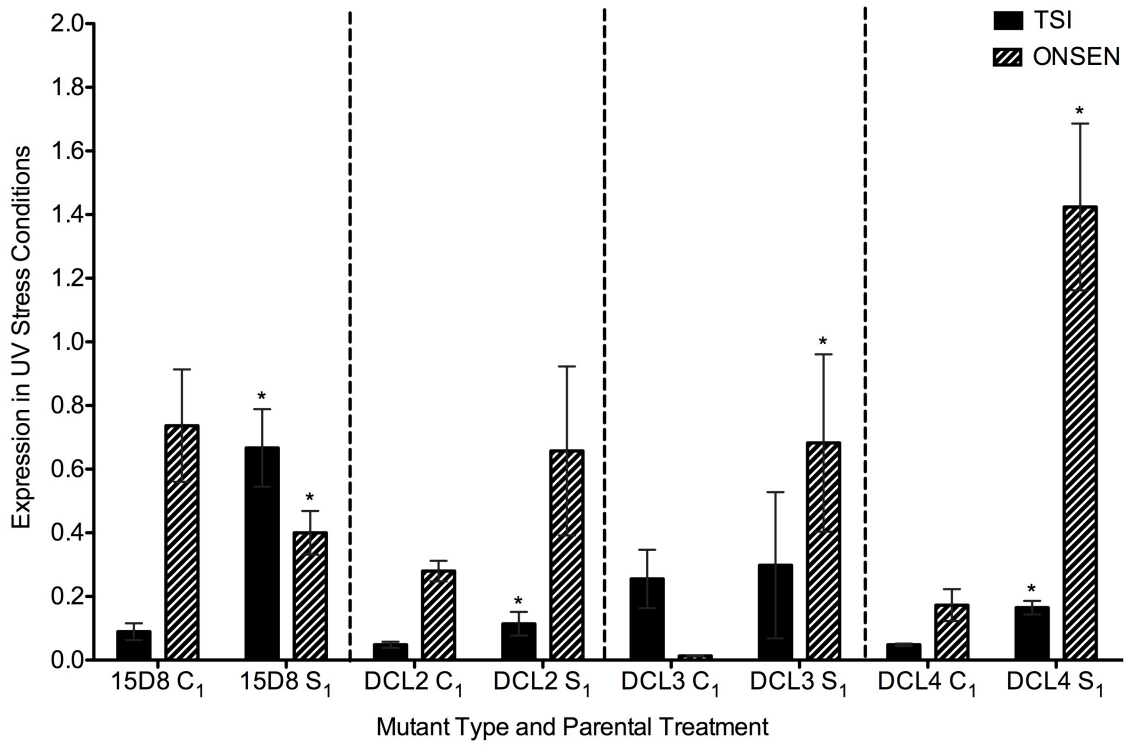

Figure 60. Expression of TSI and ONSEN transposon in F1 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when exposed to UV stress. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to cold in F0. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny of control (C_1) and progeny of stressed plants (S_1) for the same gene and mutant type, when exposed to UV, as calculated using a t-test ($p \leq 0.05$).

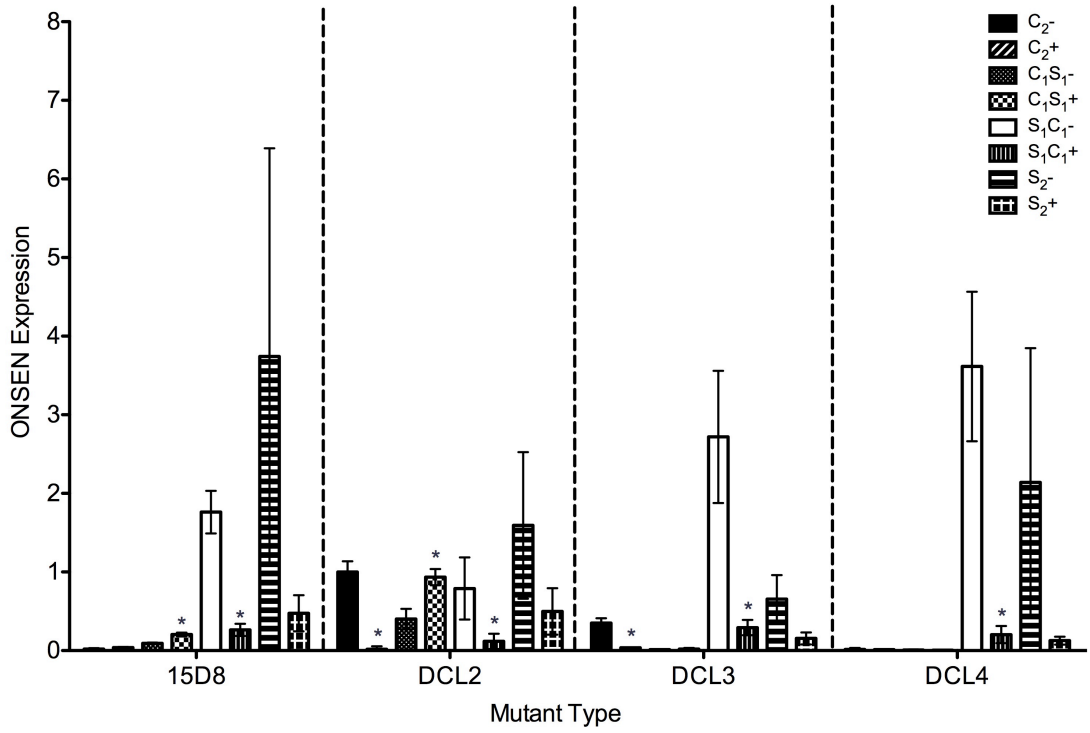

Figure 61. Expression of ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to cold in F1. "S_1C_1" – the progeny of plants exposed to cold in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to cold in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

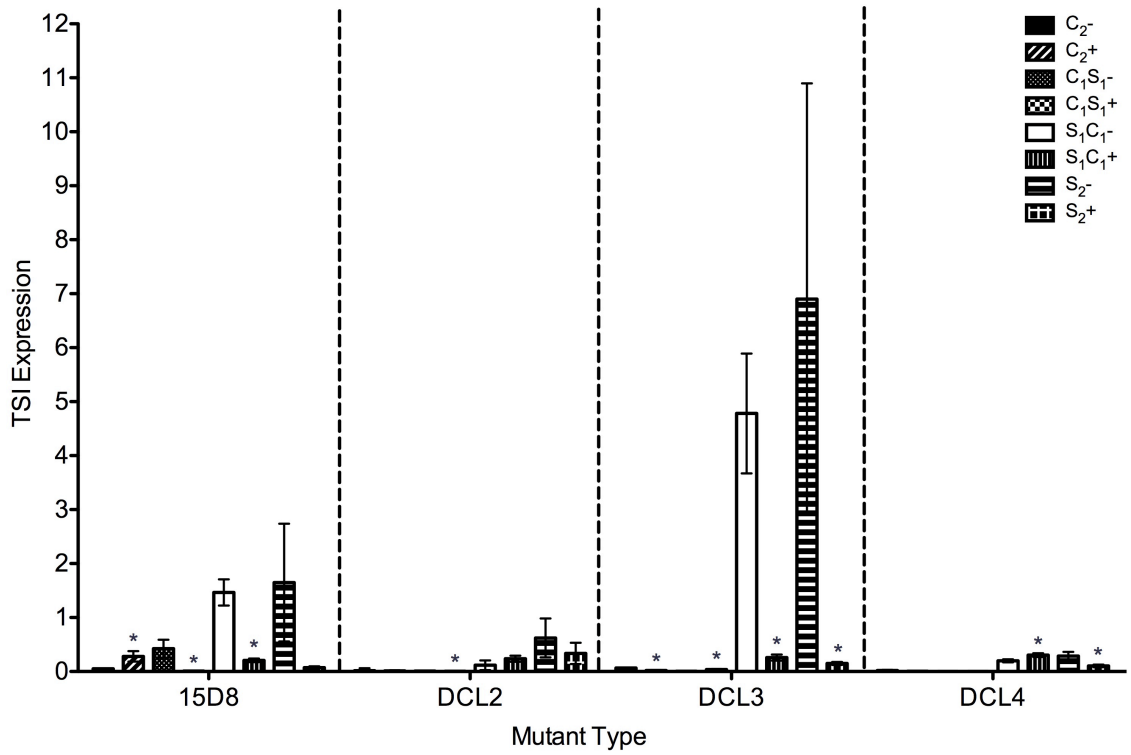

Figure 62. Expression of TSI transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to cold and control plants. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to cold in F1. "S_1C_1" – the progeny of plants exposed to cold in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to cold in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

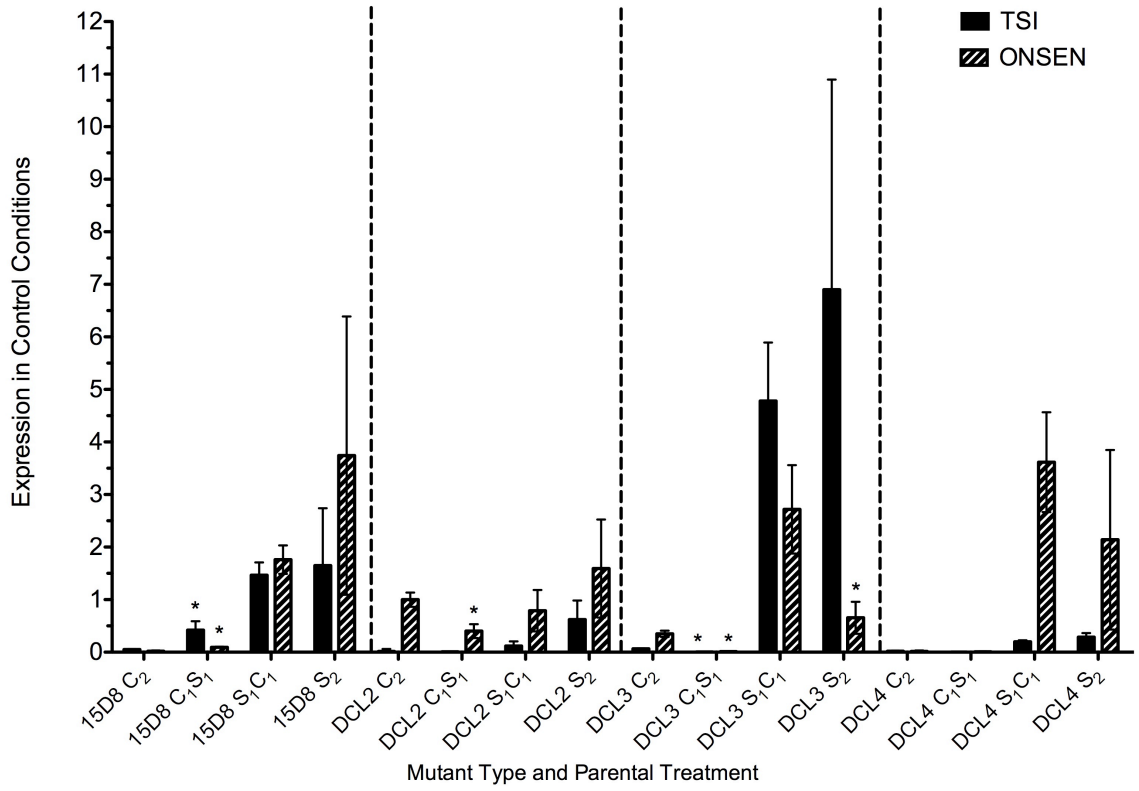

Figure 63. Expression of TSI and ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when grown under normal conditions. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to cold in F1. "S_1C_1" – the progeny of plants exposed to cold in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to cold in F0 and F1. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny with the same F0 treatment but differing F1 conditions (C_1S_1comparison to C_2, and S_2in comparison to S_1C_1), for the same gene and mutant type, when grown under normal conditions, as calculated using a t-test ($p \leq 0.05$).

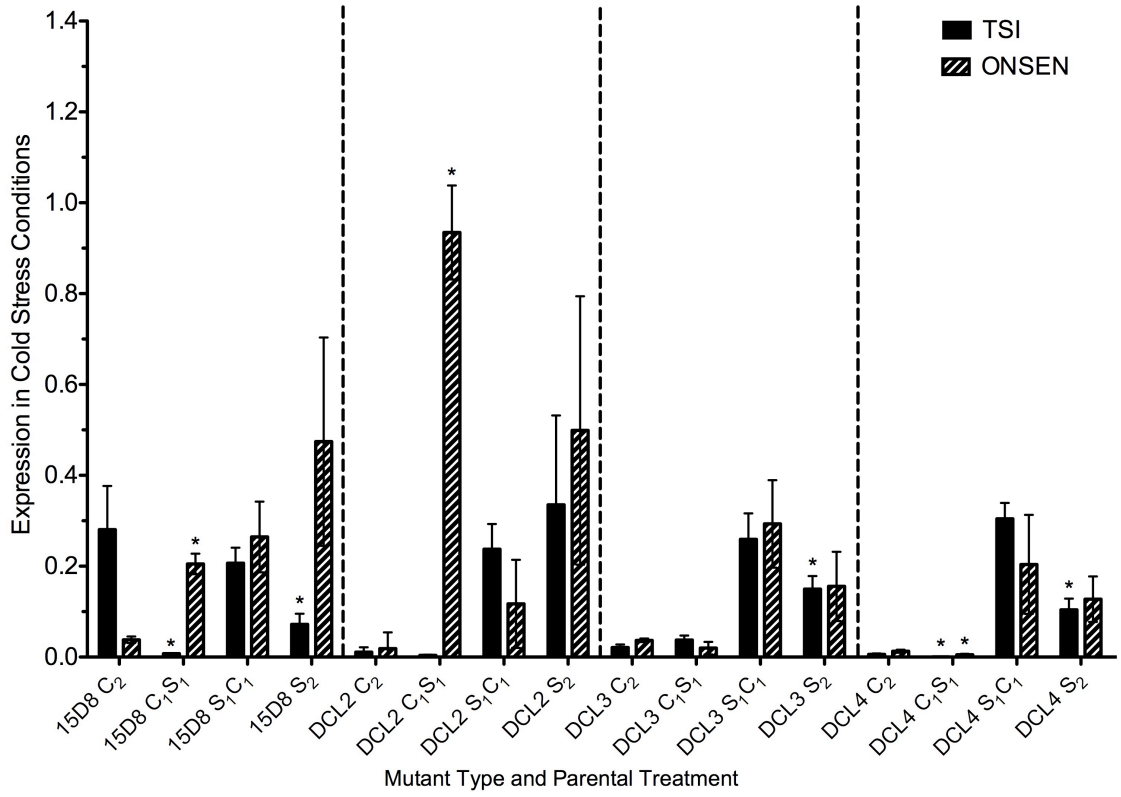

Figure 64. Expression of TSI and ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when exposed to cold stress. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to cold in F1. "S_1C_1" – the progeny of plants exposed to cold in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to cold in F0 and F1. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny with the same F0 treatment but differing F1 conditions (C_1S_1comparison to C_2, and S_2in comparison to S_1C_1), for the same gene and mutant type, when exposed to cold, as calculated using a t-test ($p \leq 0.05$).

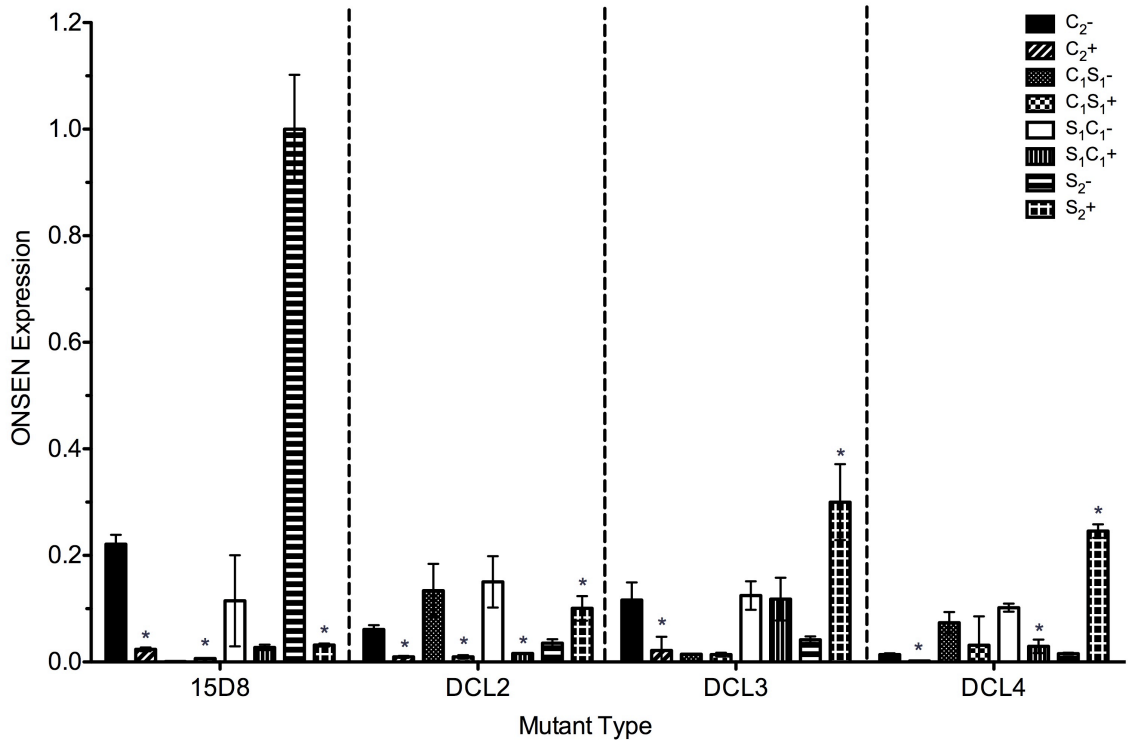

Figure 65. Expression of ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to heat in F1. "S_1C_1" – the progeny of plants exposed to heat in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to heat in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

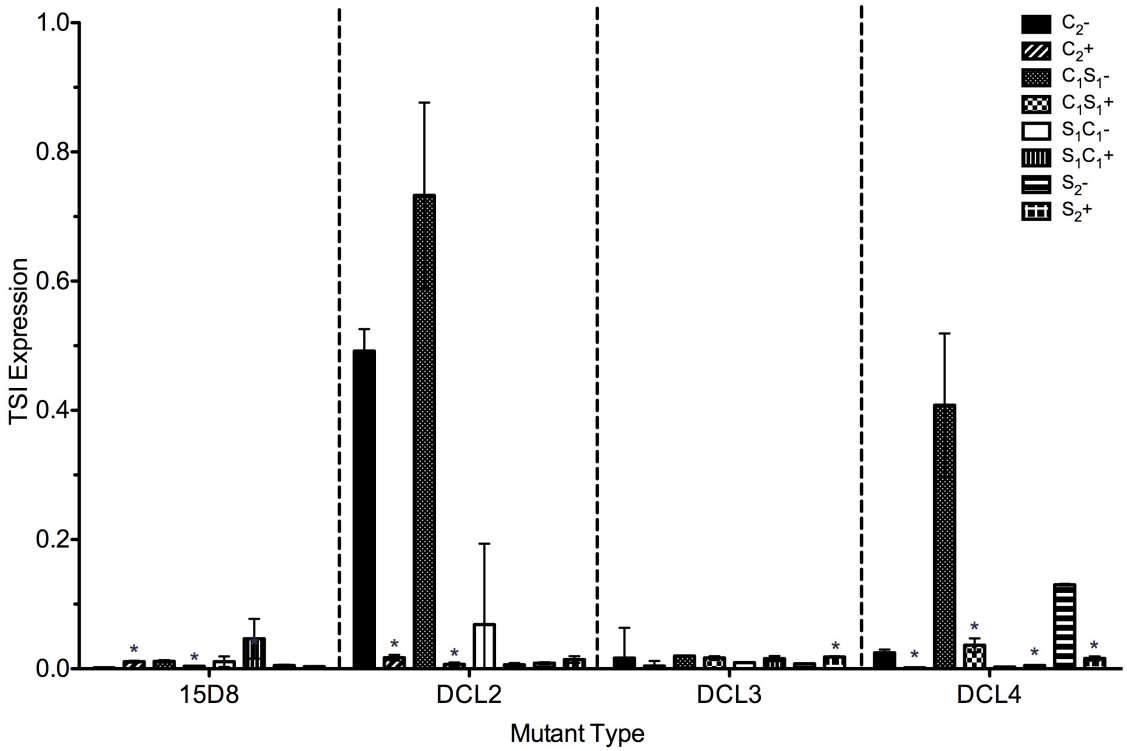

Figure 66. Expression of TSI transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to heat in F1. "S_1C_1" – the progeny of plants exposed to heat in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to heat in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

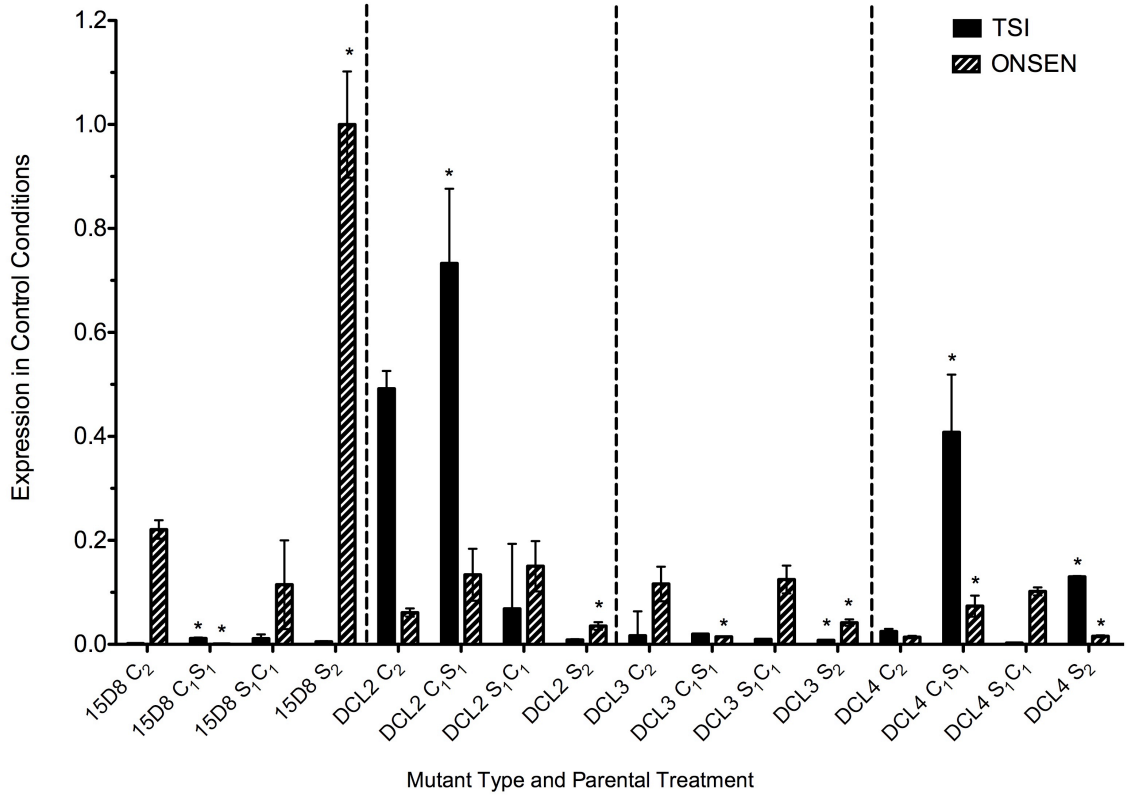

Figure 67. Expression of TSI and ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when grown under normal conditions. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to heat in F1. "S_1C_1" – the progeny of plants exposed to heat in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to heat in F0 and F1. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny with the same F0 treatment but differing F1 conditions (C_1S_1comparison to C_2, and S_2in comparison to S_1C_1), for the same gene and mutant type, when grown under normal conditions, as calculated using a t-test ($p \leq 0.05$).

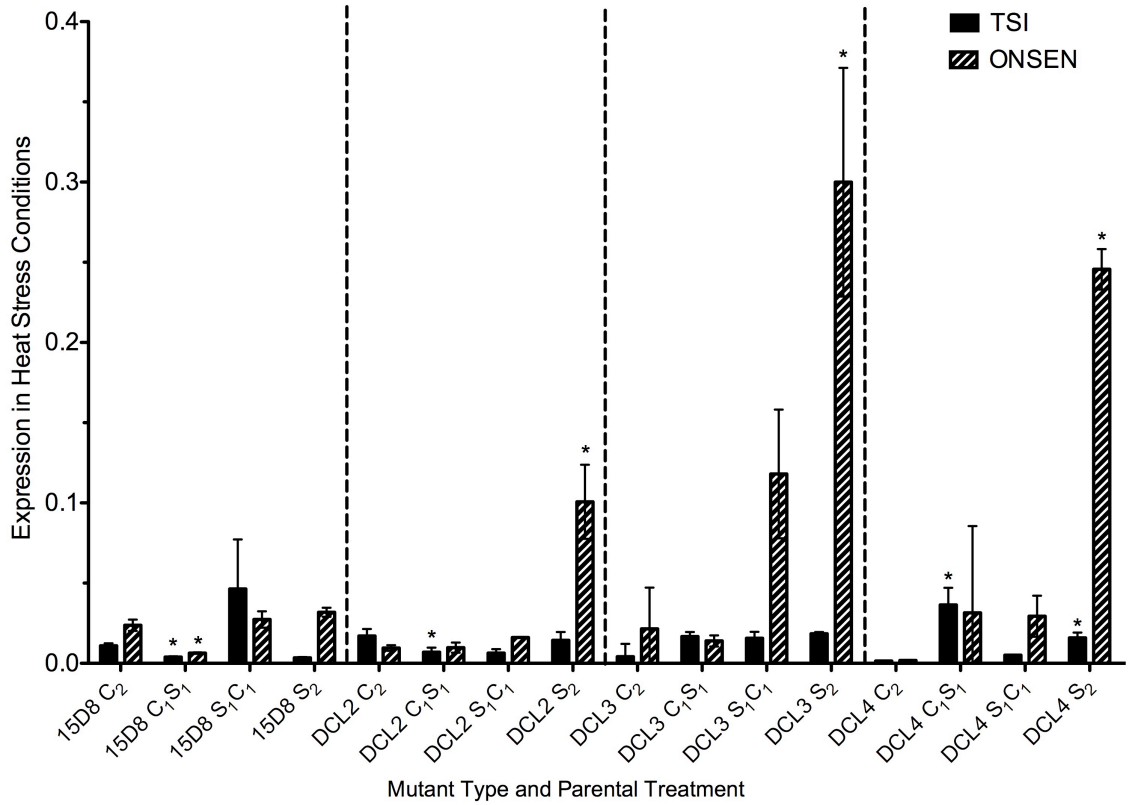

Figure 68. Expression of TSI and ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when exposed to heat stress. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to heat in F1. "S_1C_1" – the progeny of plants exposed to heat in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to heat in F0 and F1. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny with the same F0 treatment but differing F1 conditions (C_1S_1comparison to C_2, and S_2in comparison to S_1C_1), for the same gene and mutant type, when exposed to heat, as calculated using a t-test ($p \leq 0.05$).

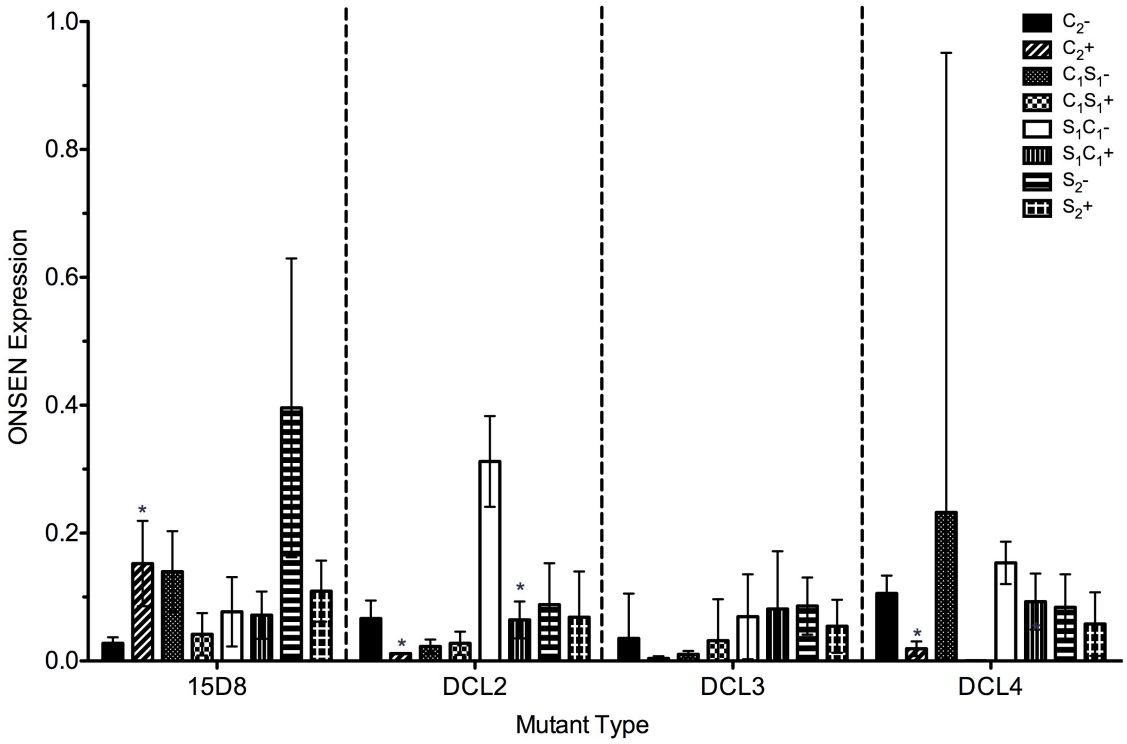

Figure 69. Expression of ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to UV in F1. "S_1C_1" – the progeny of plants exposed to UV in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to UV in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

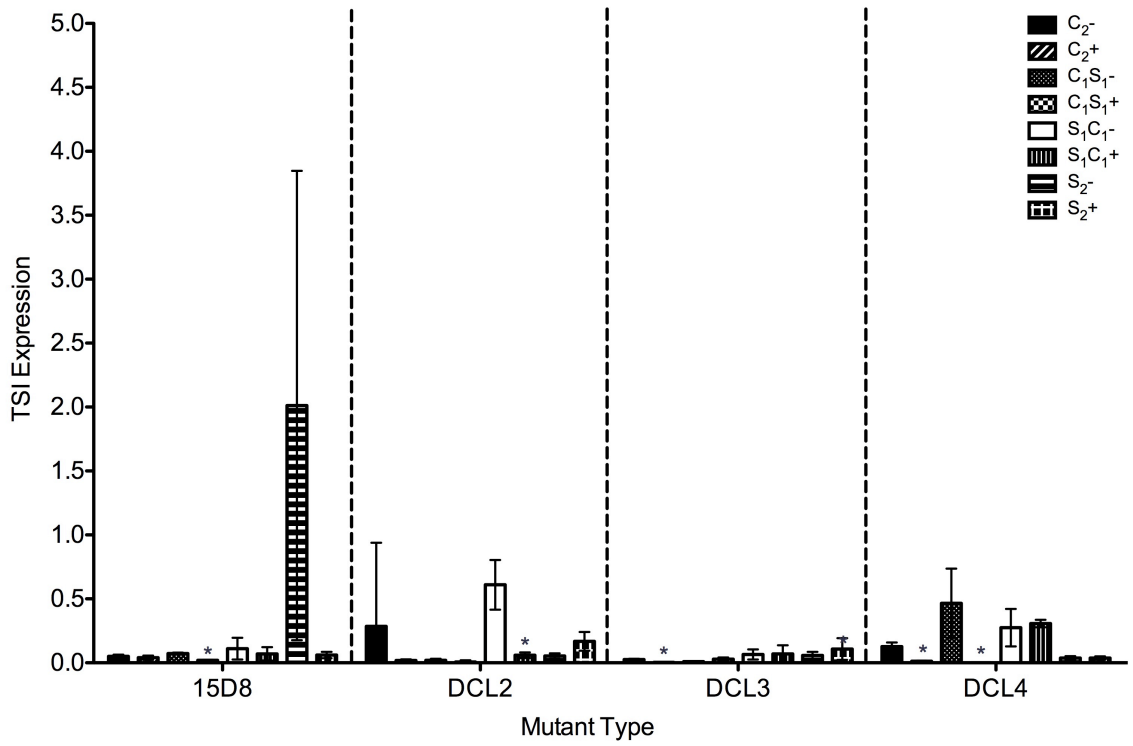

Figure 70. Expression of TSI transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants exposed to UV and control plants. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to UV in F1. "S_1C_1" – the progeny of plants exposed to UV in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to UV in F0 and F1. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

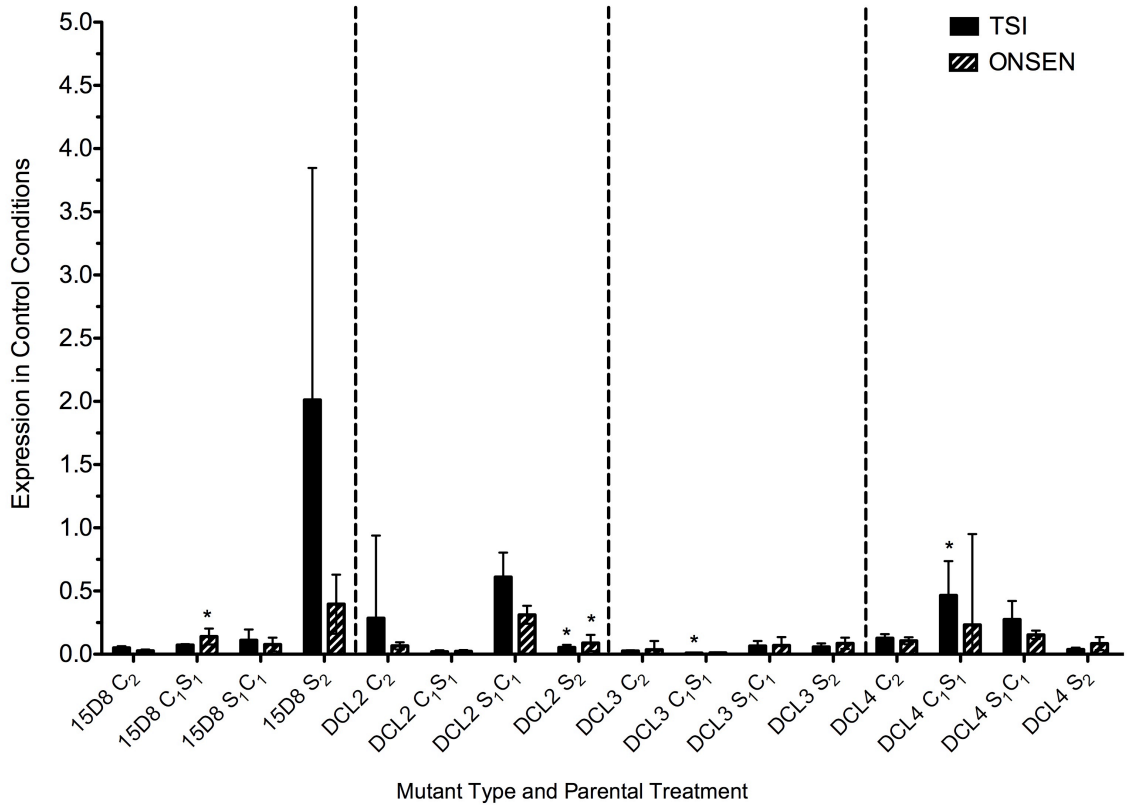

Figure 71. Expression of TSI and ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when grown under normal conditions. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to UV in F1. "S_1C_1" – the progeny of plants exposed to UV in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to UV in F0 and F1. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny with the same F0 treatment but differing F1 conditions (C_1S_1comparison to C_2, and S_2in comparison to S_1C_1), for the same gene and mutant type, when grown under normal conditions, as calculated using a t-test ($p \leq 0.05$).

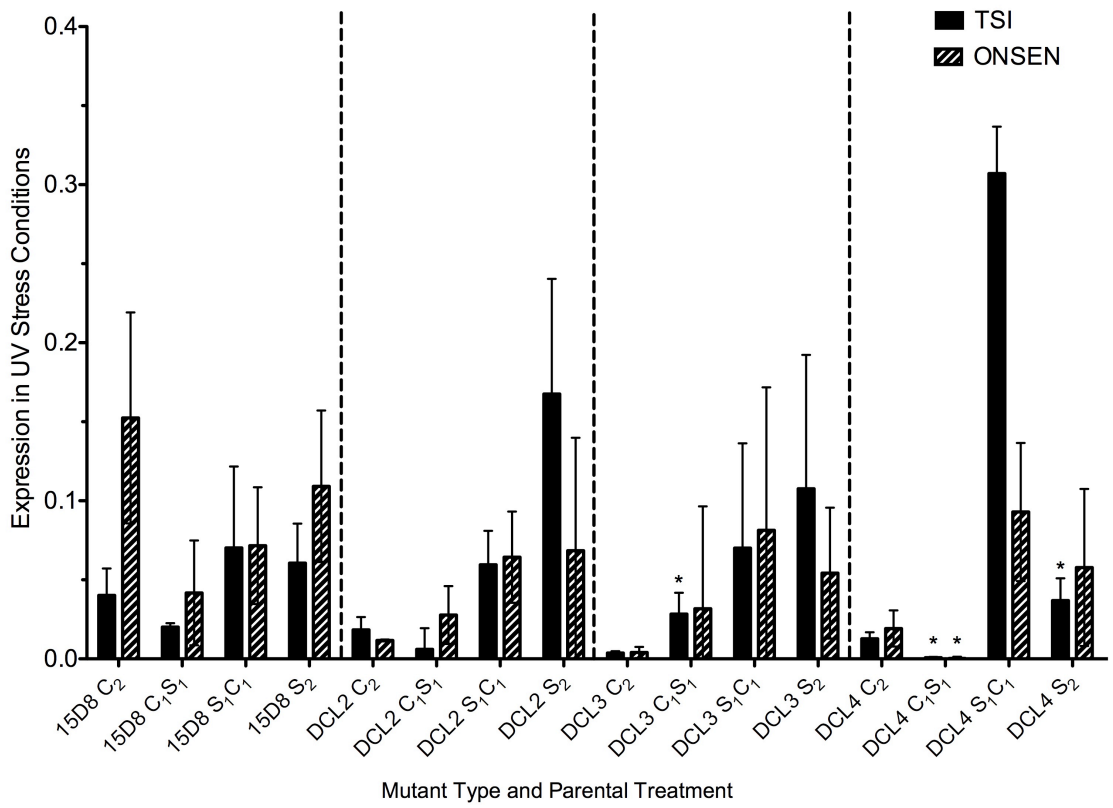

Figure 72. Expression of TSI and ONSEN transposon in F2 progeny of wild type (15D8) and mutant (*dcl2*, *dcl3* and *dcl4*) plants, when exposed to UV stress. Y axis shows arbitrary units of gene expression. "C_2" – the progeny of plants grown at normal conditions in F0 and F1. "C_1S_1" – the progeny of plants grown at normal conditions in F0 and exposed to UV in F1. "S_1C_1" – the progeny of plants exposed to UV in F0 and grown at normal conditions in F1. "S_2" – the progeny of plants exposed to UV in F0 and F1. Bars show standard deviation calculated from three technical repeats. Asterisks (*) indicate a significant difference between progeny with the same F0 treatment but differing F1 conditions (C_1S_1comparison to C_2, and S_2in comparison to S_1C_1), for the same gene and mutant type, when exposed to UV, as calculated using a t-test ($p \leq 0.05$).

3.2.2. Global Genome Methylation Results

A global genome methylation assay was used to determine the level of methylation found in 15D8, *dcl2* and *dcl3* plants that had been grown under both heat stressed (+) and control (-) conditions. The plants used were the progeny of plants grown under either heat stress (S_1) or control (C_1) conditions, thus enabling the ability to determine whether parental treatment played a role in offspring response.

In all cases, methylation decreased in stressed plants of the same generation, for example C_1+ in comparison to C_1-, and S_1+ in comparison to S_1- (Figure 73).This difference was most substantial after a second generation of stress (S_1+) for both *dcl2* and *dcl3* plants, while 15D8 maintained a similar decrease in methylation under stress, regardless of parental treatment. The lowest level of methylation was found in S_1+ *dcl2* and *dcl3*, which also had the most substantial change in methylation under heat stress.

Interestingly, *dcl3* showed the lowest level of methylation after two generations of growth in normal conditions (C_1-), while *dcl2* showed the highest (Figure 74). However, both 15D8 and *dcl3* showed an increase in methylation in the progeny of stressed plants, grown under normal conditions (S_1-) while in the case of *dcl2* methylation actually decreased. The highest level of methylation for plants grown in normal conditions was seen in S_1- *dcl3* and it was also the treatment group with the most substantial difference between non-stressed plants with differing parental treatments (C_1- versus S_1-).

The stressed progeny of stressed plants (S_1+) had lower methylation than the progeny of control (C_1+) for *dcl2* and *dcl3*, as well as the lowest level of methylation among stressed plants (Figure 75). The most methylation was seen in C_1+ *dcl2*.

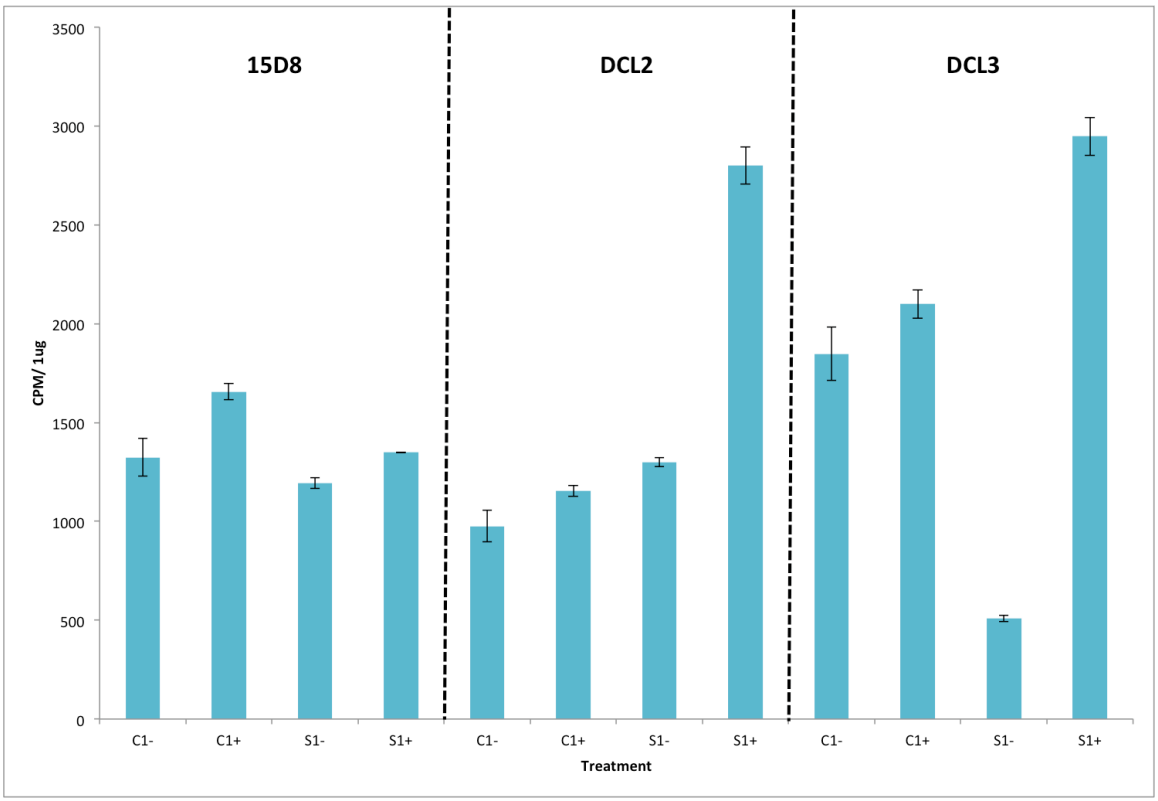

Figure 73. Amount of fluorescence in the F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants following global methylation assay. Higher incorporation indicates a lower degree of methylation. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Error bars show standard deviation calculated from two technical repeats.

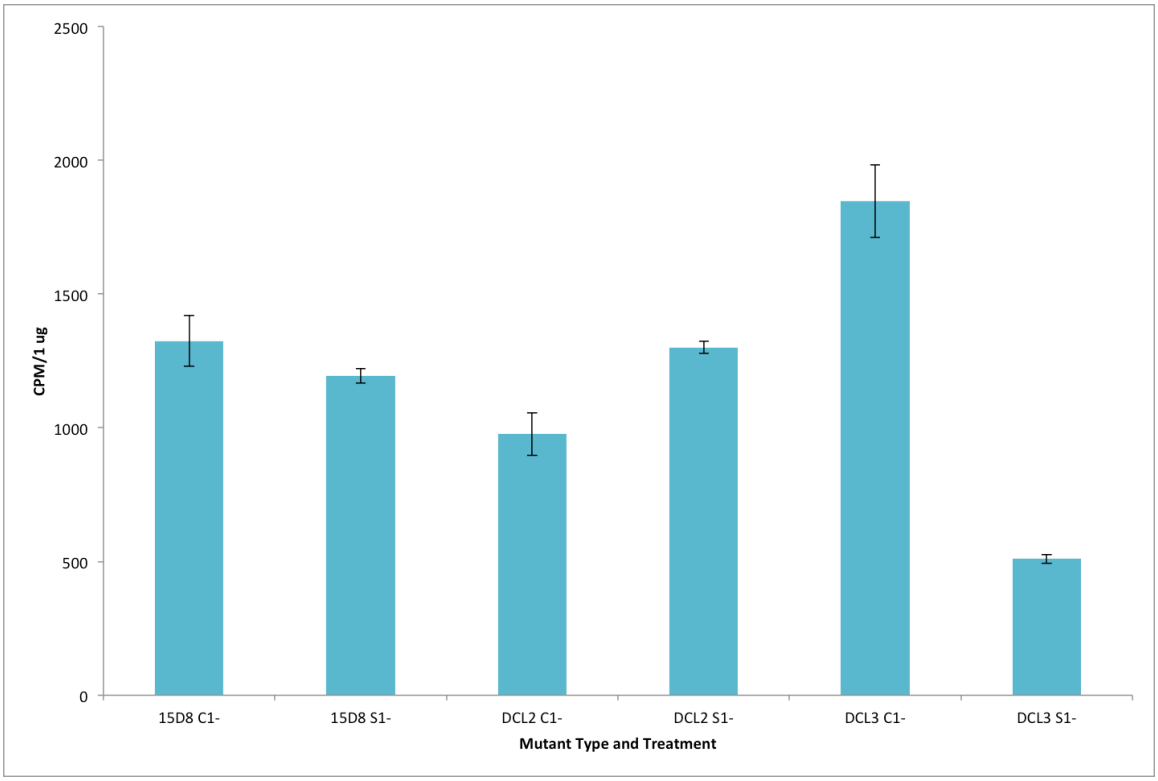

Figure 74. Amount of fluorescence in the F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) control plants following global methylation assay. Higher incorporation indicates a lower degree of methylation. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "-" indicates growth in uninduced conditions, respectively. Error bars show standard deviation calculated from two technical repeats.

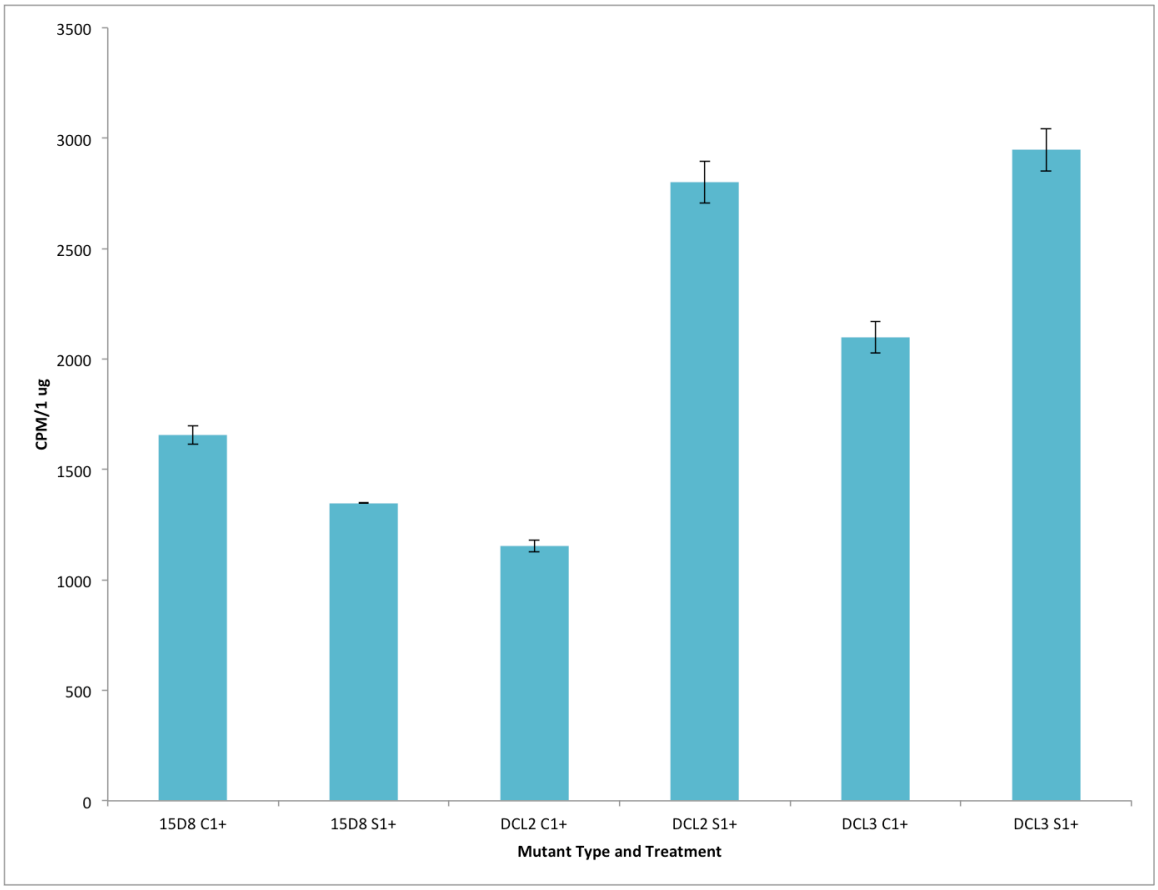

Figure 75. Amount of fluorescence in the F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat, following global methylation assay. Higher incorporation indicates a lower degree of methylation. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" indicate exposure to stress. Error bars show standard deviation calculated from two technical repeats.

3.2.3. mRNA Expression Results

The progeny of heat-stressed plants had a higher level of HSFA2 expression than the progeny of control plants (Figure 76). As such, S_1- and S_1+ were higher than C_1- and C_1+, within mutant type, with the exception of S_1- *dcl2*. 15D8 and *dcl3* had higher HSFA2 expression in the stressed progeny of control plants (C_1+) than under normal conditions (C_1-). Finally, even though the level of HSFA2 in S_1+ 15D8 was higher than the progeny of control plants, expression was significantly higher in its non-stressed counterpart, S_1-, than it was following a second generation of stress (S_1+).

MSH6 expression was significantly lower in stressed offspring of stressed plants (S_1+) than control offspring (C_1+) (Figure 77). In contrast, S_1+ plants had a higher MSH6 expression than progeny of the same stressed plants, grown under normal conditions (S_1-). The expression level in C_1+ *dcl2* plants was the highest. The offspring of control plants (C_1) had a higher level of MSH6 expression than progeny of stressed plants (S_1). Within each mutant type, the lowest level of expression was found in S_1- plants.

Expression of ROS1 was lower in the progeny of stressed plants (S_1) than the progeny of controls (C_1) (Figure 78). Stressing C_1 plants (+) resulted in expression that was even higher than C_1 plants grown under normal conditions (-). In contrast, stressed S_1 plants (+) had lower ROS1 expression than S_1 plants under normal conditions (-).

The progeny of control plants (C_1) had higher SUVH2 expression than the progeny of stressed plants (S_1) (Figure 79). Stressing the progeny of control plants (C_1+) increased SUVH2 expression, especially for *dcl2*. In contrast, stressing the offspring of stressed plants (S_1+) decreased expression compared to normal conditions (S_1-) for 15D8

and *dcl3*, while *dcl2* had a slight increase in S_1+ expression. As such, the lowest level of SUVH2 expression, within mutant type, was seen in S_1+ for 15D8 and *dcl3*, but S_1- for *dcl2*. In all cases, the highest SUVH2 expression level was C_1+.

Among plants grown in normal conditions, SUVH5 expression was significantly lower in progeny of stressed plants (S_1-), than progeny of control plants (C_1-) for 15D8 and *dcl2* (Figure 80). In most cases, stressing plants with the same parental treatment increased SUVH5, in comparison to those grown under normal conditions (S_1+ in comparison to S_1-, and C_1+ in comparison to C_1-). The only exception was C_1 *dcl2*.

The progeny of control plants had higher levels of SUVH6 than the progeny of stressed plants, regardless of F1 treatment (Figure 81). Stressed progeny of control plants (C_1+) had higher levels of expression than the control progeny of control plants (C_1-), resulting C_1+ having the most SUVH6 expression, within mutant type. For C_1 plants, 15D8 expression was significantly lower than the equivalent treatment in *dcl2* and *dcl3*. A second generation of stress (S_1+), further decreased SUVH6 expression in 15D8, but increased it in *dcl2* and *dcl3*, in comparison to the progeny of stressed plants grown under normal conditions (S_1-), but the changes were quite small.

Expression of SUVH8 was significantly higher in C_1+ *dcl2* plants than all other treatment groups (Figure 82). The changes in expression among differing treatment groups were not consistent. In both *dcl2* and *dcl3*, expression was lowest for S_1-, but for 15D8 SUVH8 expression was highest for the same treatment group. Expression of SUVH8 increased in heat stressed C_1, compared to C_1 plants grown under normal conditions.

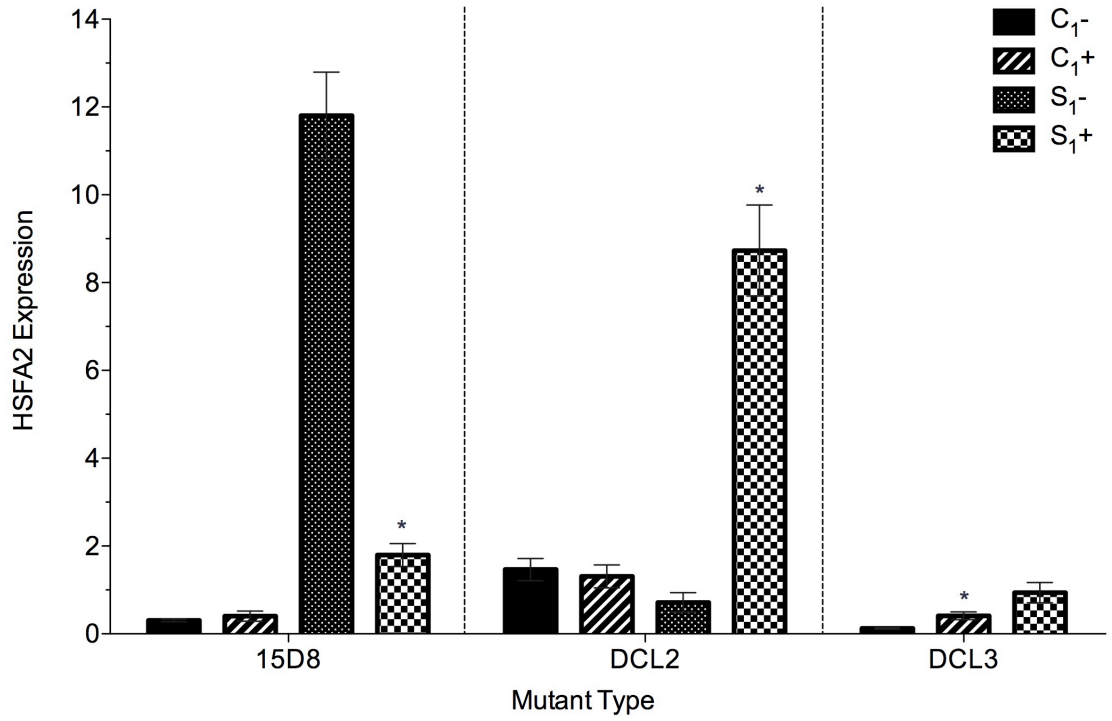

Figure 76. Expression of HSFA2 in F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard error of the mean (SEM) calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

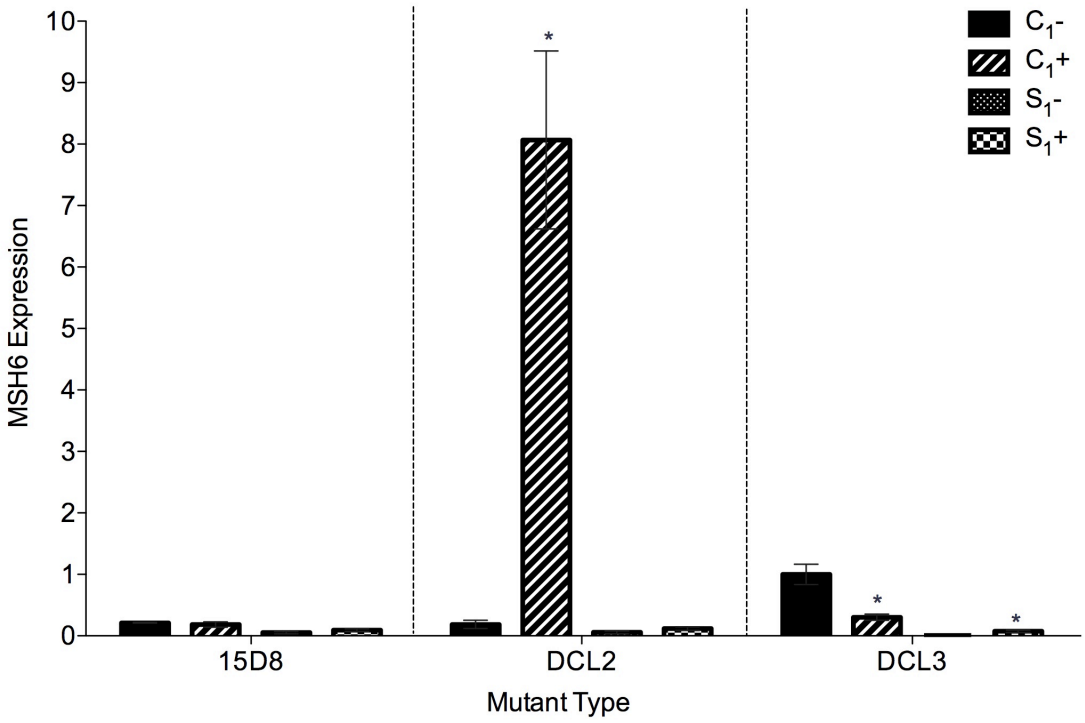

Figure 77. Expression of MSH6 in F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard error of the mean (SEM) calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

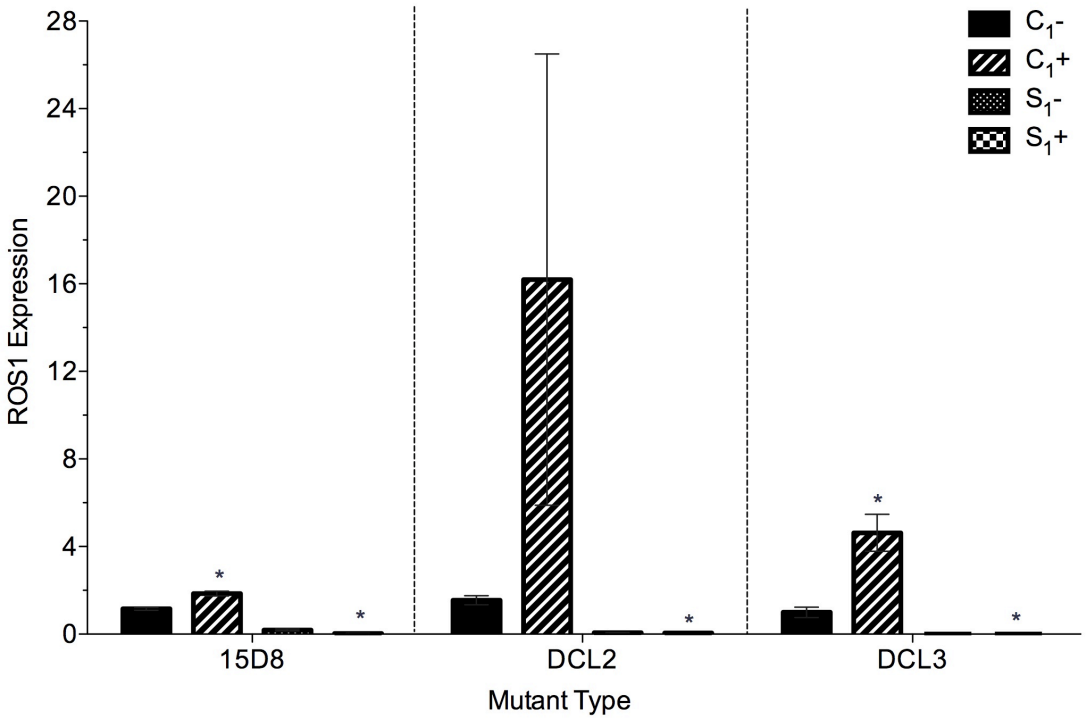

Figure 78. Expression of ROS1 in F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard error of the mean (SEM) calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

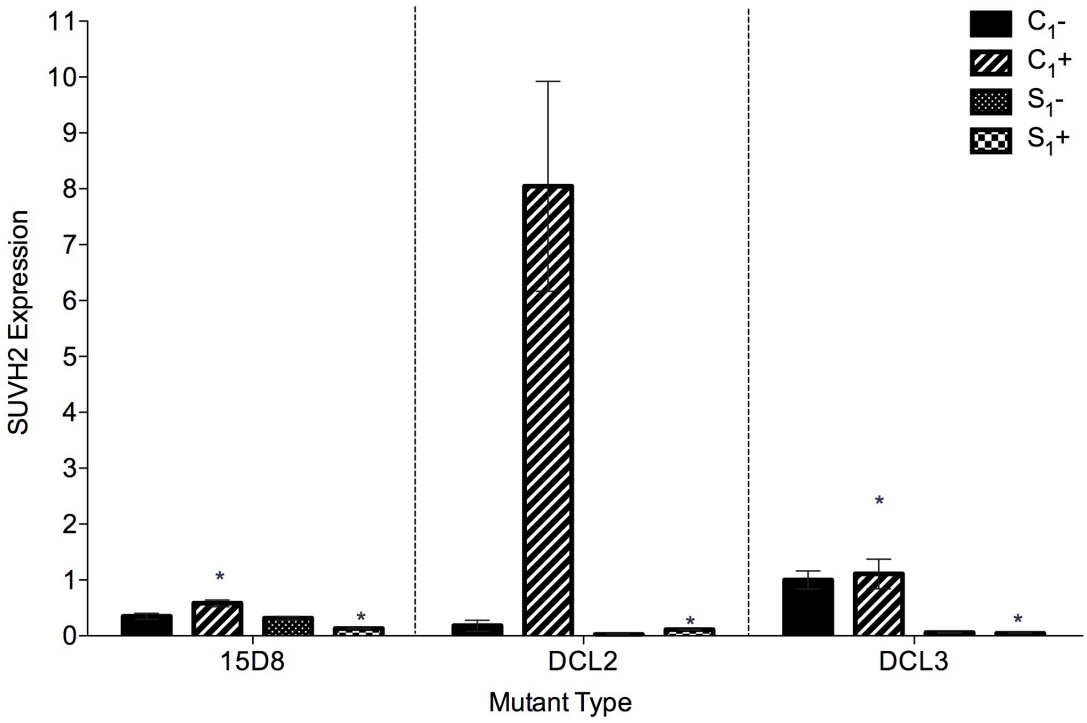

Figure 79. Expression of SUVH2 in F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard error of the mean (SEM) calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

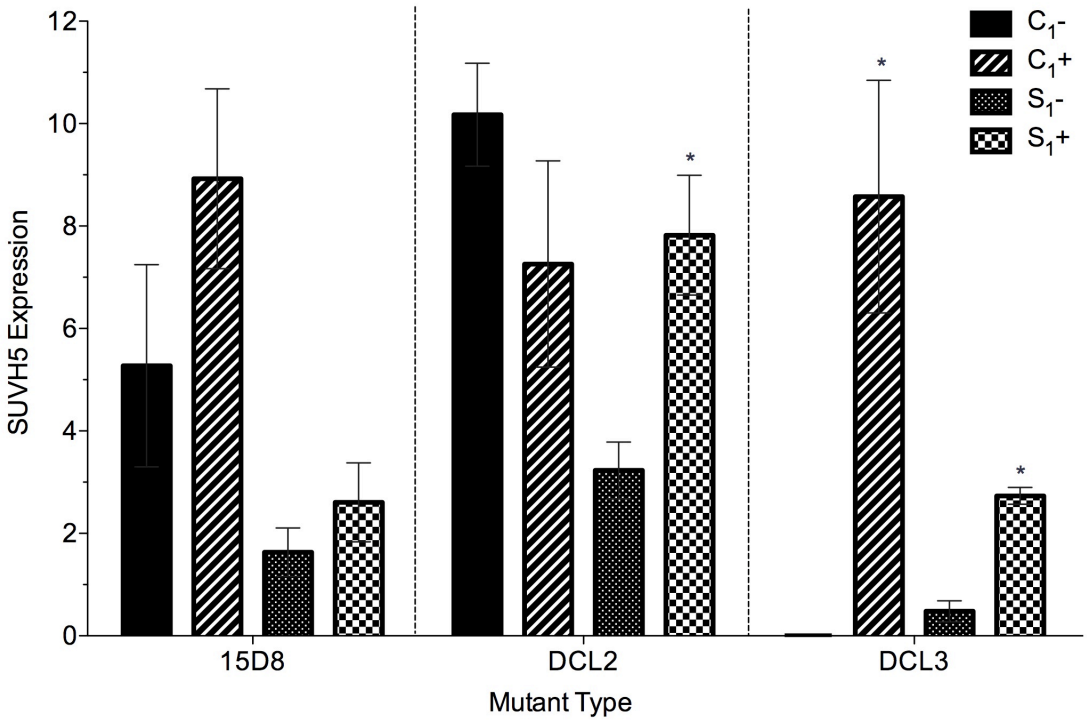

Figure 80. Expression of SUVH5 in F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard error of the mean (SEM) calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

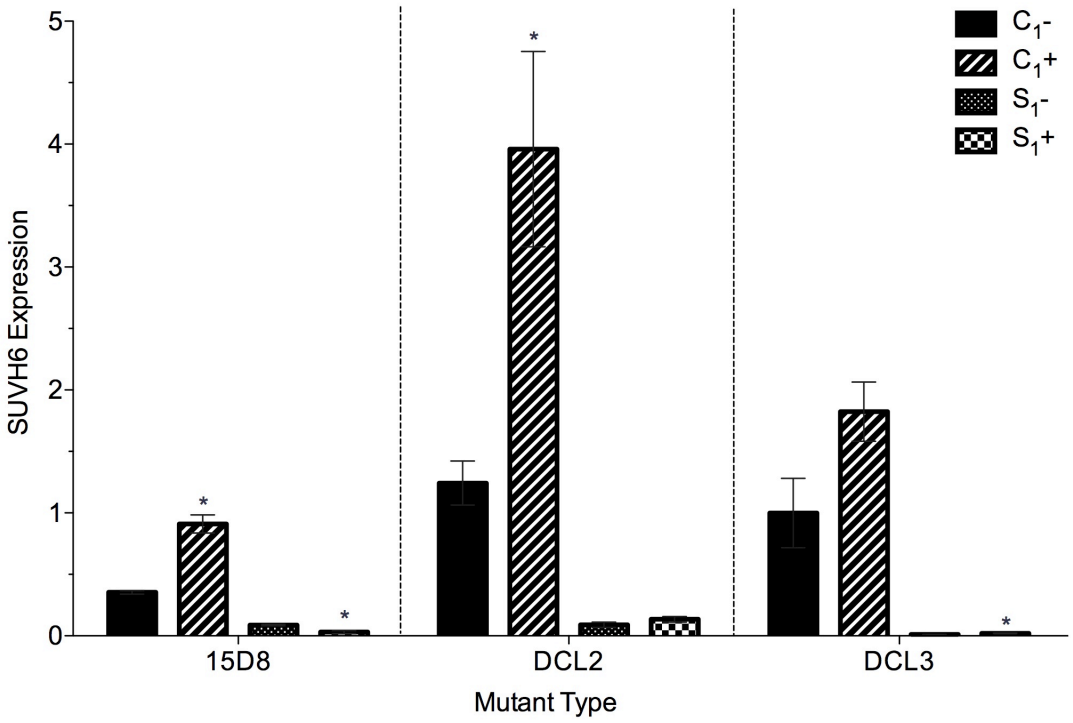

Figure 81. Expression of SUVH6 in F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard error of the mean (SEM) calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

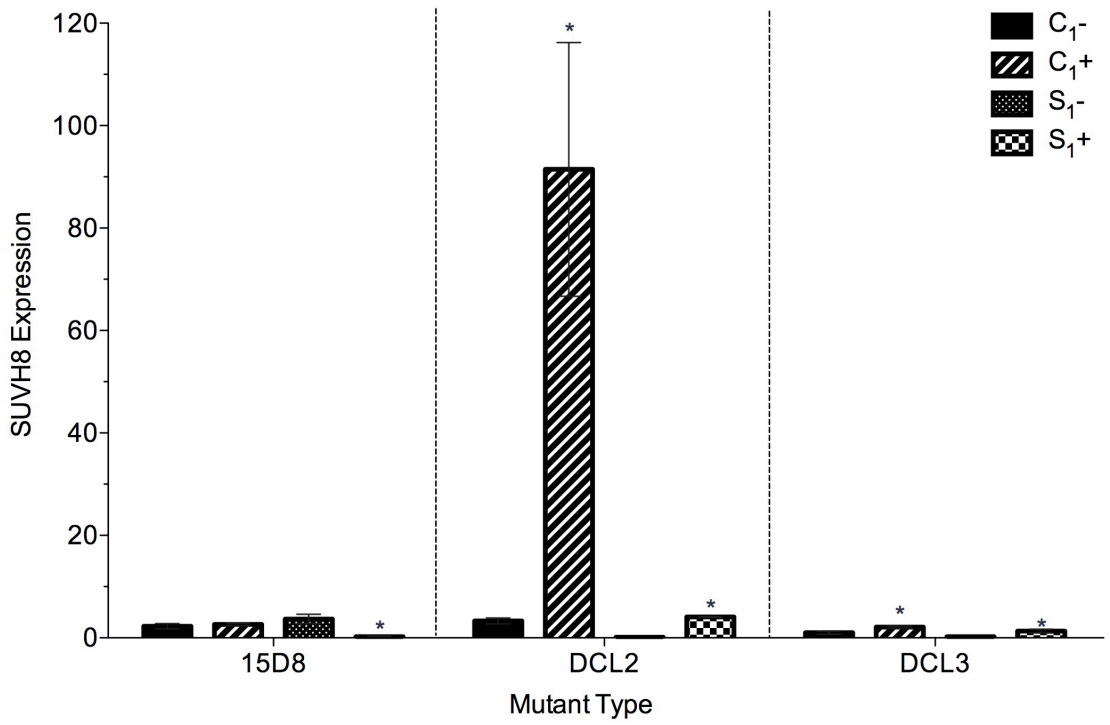

Figure 82. Expression of SUVH8 in F1 progeny of wild type (15D8) and mutant (*dcl2* and *dcl3*) plants exposed to heat and control plants. Y axis shows arbitrary units of gene expression. "C_1" – the progeny of plants grown at normal conditions in F0. "S_1" – the progeny of plants exposed to heat in F0. "+" and "-" indicate exposure to stress or growth in uninduced conditions, respectively. Bars show standard error of the mean (SEM) calculated from three technical repeats. Asterisks (*) indicate a significant difference between control (-) and stress (+) with the same parental treatment, as calculated using a t-test ($p \leq 0.05$).

3.2.4. ChIP Results

ChIP analysis identified acH3K9 and me2H3K9 histone modifications at specific genes of interest in the progeny of heat stressed and control plants. Both acH3K9 and me2H3K9 were enriched in the progeny of 15D8 and *dcl2* heat stressed plants, for heat-shock transcription factor HSFA2, though the changes not significant (Figure 83). The progeny of heat stressed *dcl3* plants showed an enrichment of acH3K9 for HSFA2, but a decrease in me2H3K9.

The promoter region of SUVH2 significantly decreased acH3K9 in the progeny of heat stressed 15D8 plants in comparison to control (Figure 84). acH3K9 also decreased in the progeny of heat stressed *dcl2* plants, but *dcl3* increased. me2H3K9 slightly increased for 15D8 and *dcl2*, and possibly decreased for *dcl3*.

The coding sequence of SUVH2 showed an overall enrichment of the permissive chromatin mark acH3K9 and a decrease in the repressive mark me2H3K9, in the progeny of all heat stressed plants (Figure 85). These changes were not statistically significant.

SUVH5 did not alter chromatin marks for 15D8 progeny of stressed (S_1) plants, in comparison to progeny of control (C_1) (Figure 86). However, *dcl2* and *dcl3* both had a slight, but not significant, increase in acH3K9.

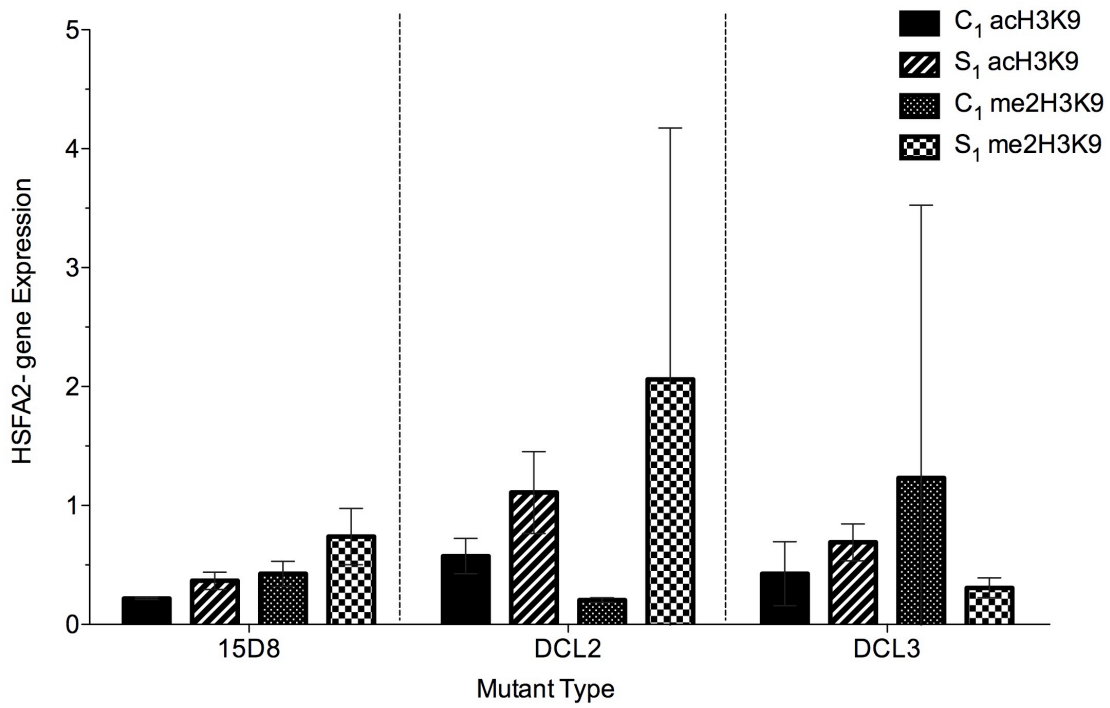

Figure 83. Histone modifications (H3K9me2 and H3K9ac) at the gene body regions of HSFA2 in the progeny of heat-stressed (S_1) and control (C_1) plants. The Y-axis shows the levels of H3K9me2/H3K9ac expression in average arbitrary units (calculated from two technical repeats). The x-axis indicates the mutant type. The asterisks (*) denotes a significant differences between the progeny of control and the progeny of stressed, as determined with a t-test ($p \leq 0.05$).

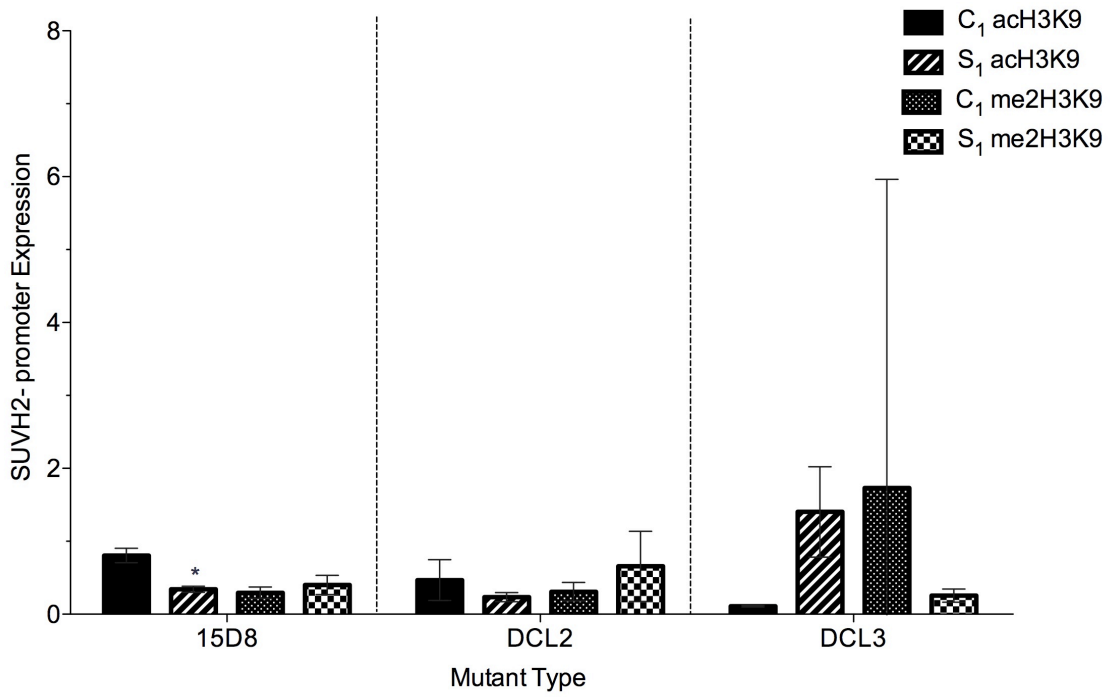

Figure 84. Histone modifications (H3K9me2 and H3K9ac) at the promoter region of SUVH2 in the progeny of heat-stressed (S_1) and control (C_1) plants. The Y-axis shows the levels of H3K9me2/H3K9ac expression in average arbitrary units (calculated from two technical repeats). The x-axis indicates the mutant type. The asterisks (*) denotes a significant differences between the progeny of control and the progeny of stressed, as determined with a t-test ($p \leq 0.05$).

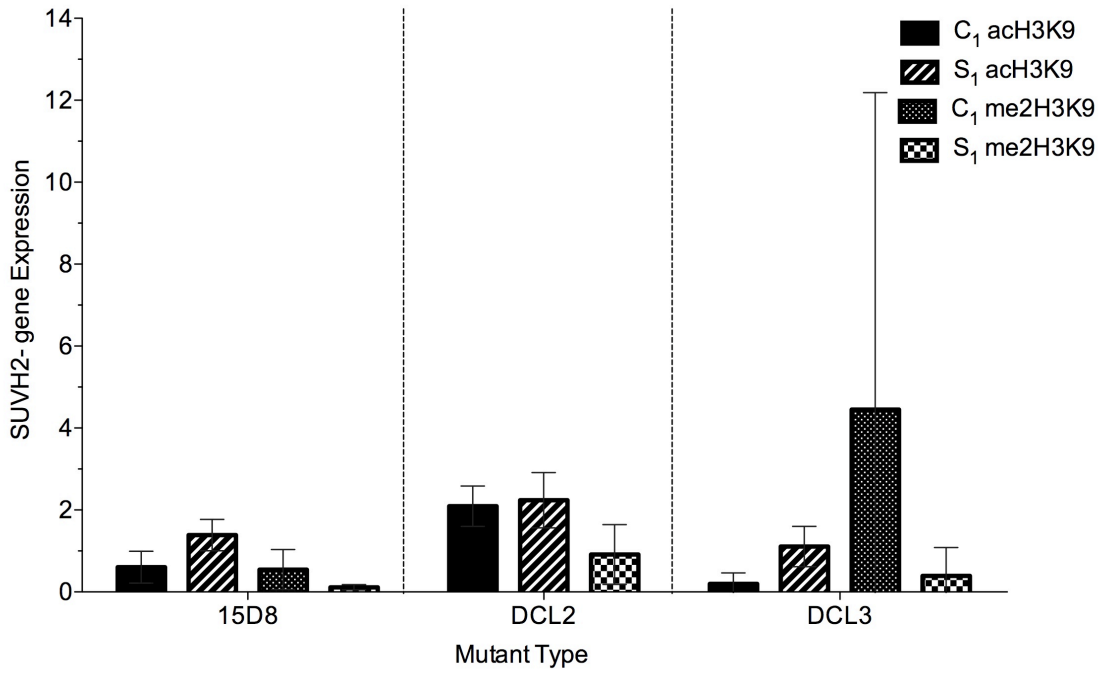

Figure 85. Histone modifications (H3K9me2 and H3K9ac) at the gene body regions of SUVH2 in the progeny of heat-stressed (S_1) and control (C_1) plants. The Y-axis shows the levels of H3K9me2/H3K9ac expression in average arbitrary units (calculated from two technical repeats). The x-axis indicates the mutant type. The asterisks (*) denotes a significant differences between the progeny of control and the progeny of stressed, as determined with a t-test ($p \leq 0.05$).

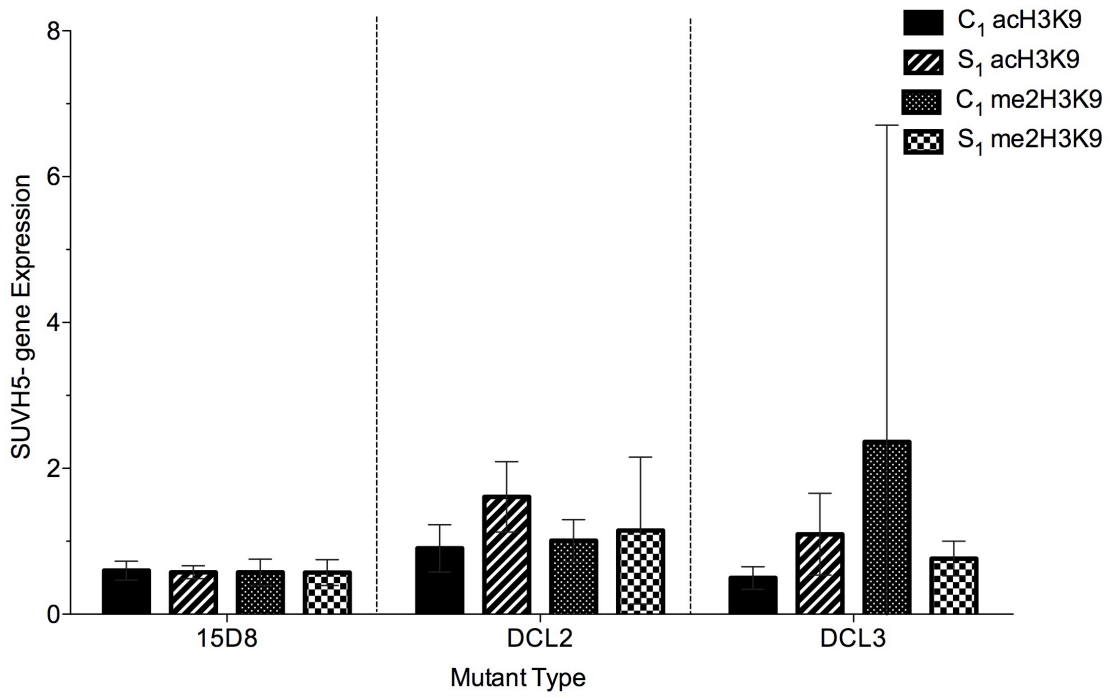

Figure 86. Histone modifications (H3K9me2 and H3K9ac) at the gene body regions of SUVH5 in the progeny of heat-stressed (S_1) and control (C_1) plants. The Y-axis shows the levels of H3K9me2/H3K9ac expression in average arbitrary units (calculated from two technical repeats). The x-axis indicates the mutant type. The asterisks (*) denotes a significant differences between the progeny of control and the progeny of stressed, as determined with a t-test ($p \leq 0.05$).

4. Discussion

4.1. Discussion of Physiological Results

4.1.1. Transgenerational and phenotypic changes in response to heat stress

4.1.1.1. F1 Generation

4.1.1.1.1. Changes to Leaf Phenotype

Changes to leaf number and size were observed in the progeny of heat stressed plants, compared to the progeny of controls. The variation occurred when progeny were grown under stress, or normal conditions, with several differences occurring among wild-type and *dcl* plants deficient in either *dcl2*, *dcl3* or *dcl4*.

Heat stress can shorten the life cycle of a plant, by increasing the rate of plant development, and thereby decreasing the growth period as well as resulting seed and plant yield (Porter, 2005). It can also result in a significant increase in number of leaves (Prasad et al., 2006; Prasad et al., 2008a). Though our study did not observe the response of F0 plants to stress, it did examine the changes in physiology that occurred in the offspring of stressed plants, in comparison to the offspring of plants grown under normal conditions, as well as the variation in their responses to heat stress.

Interestingly, the progeny of heat-stressed plants (S_1) had fewer leaves than the progeny of control plants (C_1), under both normal and heat-stressed conditions. It is possible that the progeny of stressed plants received a form of epigenetic memory that allowed them to retard the growth process, making them better suited to conditions of heat stress, in which development is sped up.

In contrast to leaf number, not only did leaf length generally increase in heat stressed plants, but the progeny of stressed wild-type plants also had longer leaves than the progeny of control, even under normal growth conditions. In conjunction with the aforementioned observation of leaf number, it appears that the progeny of stressed plants are able to increase leaf size, and therefore biomass, without accelerating the developmental process via the growth of new leaves. Interestingly, S_1 *dcl4* leaves were significantly shorter than 15D8, regardless of growth conditions, indicating that DCL4 may play a role in transmitting stress memory.

Leaf width also tended to increase with heat stress but the variation among the progeny of stressed plants and the progeny of controls under heat stress was not significant in most cases. However, the progeny of stressed 15D8 and *dcl4* plants had wider leaves under normal growth conditions than the progeny of control plants, indicating that the stress memory may in fact be more evident under conditions of normal growth, than those of stress.

Previous work has shown that heat can directly impact the appearance of a leaf, increasing both leaf elongation and leaf width (Bos et al., 2000). Though the described study was done in F0 plants, it is possible that some of these characteristics are passed onto progeny of stressed plants, resulting in larger leaves not only in response to high temperature, but also under normal growth conditions as well. In addition, work by Bos et al. (2000), showed that the leaf width is closely associated with specific leaf weight. It is possible that by increasing leaf width, the progeny of heat stressed plants increased plant biomass, providing a positive buffer against the decrease in biomass that is generally associated with heat stress and accelerated development (Porter, 2005).

DCL2 and DCL3, proteins involved in RNA-directed DNA methylation (RdDM), might play a key role in epigenetic modifications that pass on stress memory to progeny. In response to heat stress, *dcl2* and *dcl3* plants often had a different response than 15D8 and *dcl4*. For example, there were significantly fewer leaves in the progeny of stressed (S_1) *dcl2* and *dcl3* plants than 15D8, in response to heat stress. However, changes in leaf length and width were not clearly impacted by parental treatment. Leaf number and size were impacted by heat stress, often becoming significantly larger in response, supporting previous work that heat speeds up development and providing evidence this occurs in the progeny of stressed plants (Porter, 2005).

Some of the physiological changes in the progeny of heat stressed plants may be partially controlled by heat-shock proteins. For example, HSP101 is crucial for resolubilizing proteins following heat stress, but is also present during normal growth (Hong and Vierling, 2001). Altered gene expression, via epigenetic modifications, that are either heat-induced, or increased in response to heat, may have a role in changes to leaf number and morphology, even under normal growth conditions.

4.1.1.1.2. Changes to Seed Phenotype

In our study, wild-type (15D8) seed length increased in response to heat stress (S_1 seeds) but *dcl2* and *dcl4* were both significantly smaller than wild-type, as well as showing a significant decrease in seed size (in comparison to the C_1 seeds produced by control plants). *dcl3* seeds did not undergo a significant change in size in response to heat stress, nor did they vary significantly from 15D8.

Previous work has shown a significant decrease in seed size, taken as a measurement of seed weight, in response to heat stress (Morrison and Stewart, 2002; Prasad et al. 2006). Heat stress decreases seed size and causes lower yields (Sadras, 2007; Prasad et al., 2008b). The reduction in seed size caused by heat stress has also been correlated with decreased germination (Dornbos and Mullen, 1991). Due to the small size of *Arabidopsis* seeds they could not be weighed effectively. Instead, seed length was measured as an indicator of seed size.

It would be particularly interesting to observe variation in F0 seed size, as our study indicates a naturally larger seed (C_1) produced by *dcl* mutants, which shifted substantially towards smaller seeds response to heat stress (S_1) in the case of *dcl2* and *dcl4*. The tendency of seed length to decrease in response to heat stress corresponds with previous work, but the fact that 15D8 seeds increased in size, was unexpected (Krannitz et al., 1991). However, in previous work, larger seeds increased seedling survival under abiotic stress in *Arabidopsis*, providing an important selective advantage in the case of nutrient deprivation (Krannitz et al., 1991). It is possible that 15D8 plants were able to shift the balance towards larger seeds, in response to heat stress. This increase in seed size could potentially result in higher seedling survival of progeny, making them better adapted to withstand stress, a trait *dcl* plants may not have been able to transmit as effectively.

4.1.1.1.3. Changes to Bolting Time

Heat increased bolting time in all cases, regardless of parental treatment. However, under normal conditions the progeny of heat-stressed plants bolted earlier than the progeny of control plants, with the exception of *dcl4*.

A recent study in maize showed that heat stress decreased plant height by nearly 30% in some cases (Cicchino et al., 2010). Heat stressed plants not only decrease height and biomass, but often have fewer flowers, and as a result, fewer seeds and fruit-set. Reduced fruit-set is often a result of fewer pollen grains and decreased pollen viability (Peet et al., 1998; Prasad et al., 2000). As described previously, heat often increases plant growth development, and in this case it likely triggered plants from the rosette stage into bolting at an earlier age. Bolting corresponds to the elongation of reproductive internodes of the leaf zone (Pouteau and Albertini, 2009). It would be interesting to see if a variation in plant height was correlated to bolting age, and if plants that bolted earlier flowered earlier, as well as to determine the exact age of bolting for a more detailed comparison.

Earlier bolting in the progeny of heat-stressed plants (S_1), in comparison to the progeny of control (C_1), under normal growth conditions may be caused by the same phenomenon that triggered bolting earlier in heat-stressed plants. The transgenerational inheritance mechanism is unknown but it is possible changes in the production of plant hormones that regulate the growth cycle may trigger the developmental change earlier, even in unstressed offspring (Kotak et al., 2007). Recent work by Brachi et al. (2012), found selection for early bolting in the progeny of water stressed plants, similar to our results for heat stress. Additionally, inheritance of earlier bolting in response to water stress was more efficient with increased stress severity (Brachi et al., 2012).

4.1.1.2. F2 Generation

4.1.1.2.1. Leaf Phenotype

The most consistent and interesting changes to leaf number and morphology with heat stress occurred in wild-type (15D8) plants. This potentially indicates not only a

decreased fitness with increasing generations of mutant inbreeding in *dcl* plants, but also the possible importance of DCL2 and DCL3 in transmitting an epigenetic stress memory.

15D8 plants with either one (C_1S_1 and S_1C_1), or no (C_2), previous generations of heat stress did not alter their leaf number in response to heat stress significantly. However, the progeny of two generations of heat stress (S_2) did significantly decrease leaf number for 15D8, but not any of the *dcl* plants. Similarly to the response that occurred in F1 plants, it is possible that F2 plants receive a memory that slows down their growth process in response to heat stress, counteracting the usual increased developmental rate that occurs. It is interesting that unlike F1 plants, a single generation of stress did not result in a significant difference in leaf number in F2 plants.

With C_2 and C_1S_1 parental treatments, *dcl3* increased leaf number in response to heat stress, making its response significantly different from wild-type and indicating a more severe response to heat stress, one in which the developmental process is sped up. The decreased response following two generations of parental stress (S_2), which resulted in a significant reduction in comparison to C_2 under heat stress, presents the possibility that a transgenerational memory is transmitted to progeny, resulting in a less severe response to heat. Plants which either decrease, or do not significantly alter, leaf number in response to heat stress are likely benefited by the minimal change to the growth cycle, resulting in greater leaves, biomass, and seeds, after the heat stress has been removed.

C_2 *dcl* plants tended to have longer leaves than wild-type (15D8) plants. However, following two generations of heat stress (S_2), *dcl* plants all had shorter leaves under normal conditions, with the difference being significant for *dcl2* and *dcl3*. Potentially,

15D8 plants are better able to transmit heat stress memory, as DCL2 and DCL3 may be important in its inheritance. Leaf elongation is impacted by heat stress so increased leaf size under normal conditions may benefit plants, especially as it resulted in the plants not significantly altering leaf length in response to heat (Bos et al., 2000). Increased leaf length may be a benefit under heat stress conditions, resulting in less disruption of development, and therefore no significant changes in leaf morphology. In contrast, *dcl2* and *dcl3* plants which were the progeny of those that had been heat stressed in the F0, or F0 and F1, generations tended to increase leaf length in response to heat stress.

The leaf width of wild-type plants also changed in response to heat stress. However, while one, or no, previous generations of heat stress resulted in a significant increase in width, S_2 plants did not significantly change leaf width in response to stress. Similarly to leaf length, S_2 15D8 plants have the widest leaves under normal growth conditions. As heat-stressed plants often increase leaf length and width in response to heat temperatures, it is possible that the progeny of heat-stressed plants receive some of these same characteristics and therefore do not need to alter their morphology in the same way that progeny of control plants do (Bos et al., 2000). The inconsistent fluctuations in leaf width in response to heat stress that occurred in F2 *dcl2* and *dcl3* plants may be partially due to their potential difficulty in passing on a transgenerational memory of heat stress. Interestingly, only C_2 *dcl4* plants significantly altered leaf width in response to heat stress, indicating that even one generation (C_1S_1 and S_1C_1) of heat stress may allow transmission of a stress memory to progeny, allowing them to better cope with heat stress.

4.1.1.2.2. Seed Phenotype

Wild-type seed length increased following one generation of heat stress (S_1C_1 or C_1S_1), but decreased after two generations (S_2). Heat stress has been correlated with decreased seed size, so it is possible that while plants are able to resist one generation of the stress by shifting the balance towards the production of larger seeds, a second generation impacts more severely (Morrison and Stewart, 2002; Prasad et al. 2006).

dcl seeds also increased in size following one generation of stress (C_1S_1) in comparison to control (C_2). Not only did the change in F1 treatment from heat stress to control conditions (C_1S_1 in comparison to C_2) for *dcl2* and *dcl4* resulted in significantly larger seeds, but altering F1 treatment following an F0 generation of stress (S_2 in comparison to S_1C_1) decreased seed size, though not significantly, similar to 15D8. However, the S_2 *dcl2* and *dcl4* seeds were still significantly larger than 15D8. These results provide evidence that while both *dcl2* and *dcl4* mutants may be able to transmit a stress memory, the lack of variation among seeds produced by plants that had been stressed for one or two generations (S_1C_1, C_1S_1 and S_2) indicate that the response, or memory, of these plants is altered in comparison to 15D8.

Interestingly, *dcl3* actually increased seed length following two generations of stress, making S_2 *dcl3* seeds the largest out of all seeds. S_2 *dcl3* seeds were significantly larger than S_1C_1 and C_1S_1 *dcl3* seeds, which were significantly larger than C_2 seeds. In response to stress, the change in seed length of seeds produced by C_1 *dcl3* plants was similar to S_1 *dcl3* plants. It is unclear if these characteristics are a result of an improved stress memory in comparison to the one present in 15D8, or simply a result of higher

germination and seedling survival in larger seeds from the previous generation, which resulted in increased F2 seed size (Dornbos and Mullen, 1991; Krannitz et al., 1991).

4.1.1.2.3. Bolting Time

Changes to bolting time in F2 progeny in response to heat stress varied significantly with mutant type and parental treatment. In general, however, C_2 plants bolted earlier in response to heat stress, while C_1S_1 plants later, and S_1C_1 plants showed no variation in response to heat stress. S_2 response fluctuated, with 15D8 and *dcl4* increasing bolting percentage, *dcl3* decreasing, and no significant change for *dcl2*. These responses varied significantly from F1 generation in which all plants increased bolting under heat stress. The inconsistency in results makes it difficult to conclude if the variation in bolting seen in F2 was due to parental treatment, or potential minute variations in environmental parameters.

Under normal growth conditions, progeny with the same control F0 treatment, but variation in F1, (C_1S_1 in comparison to C_2) resulted in earlier bolting for 15D8, *dcl3* and *dcl4* but a slight decrease for *dcl2*. Similarly, in F1 plants, S_1 had a higher natural bolting rate than C_1. Thus these results provide further evidence that parental heat stress may increase bolting under normal growth conditions, possibly compensating for the decrease in plant height that usually occurs in response to heat stress (Cicchino et al., 2010).

However, if the F0 plants had undergone heat stress, a second generation of stress in F1 (S_2 in comparison to S_1C_1) resulted in either a slight decrease in bolting under normal conditions in F2, or no change. The lack of variation in bolting under normal conditions may be due to slight variation in plant germination age. S_2 seeds decreased in

size, which has been correlated with germination rate in the past (Dornbos and Mullen, 1991). If S_2 plants germinated later than S_1C_1, they may have appeared to have a similar, or lower, bolting rate when measured on the same day– at which point the S_2 plants would have been younger. Based on experimental data collected using plants grown in soil, this hypothesis is impossible to confirm. Further work could examine germination rates as well as the days following seed stratification required for germination, in the progeny of stressed plants, and potentially determine if variation in these rates exists.

4.1.2. Transgenerational and phenotypic changes in response to cold stress

4.1.2.1. F1 Generation

4.1.2.1.1. Changes to Leaf Phenotype

Cold stress tended to decrease leaf number, increase leaf length and increase leaf width in F1 plants. Cold stress inhibits metabolic reactions, as well as inducing internal stresses such as osmotic and oxidative (Chinnusamy et al., 2007). As such, cold may decrease plant yield and survival rate (Chew and Halliday, 2011). In our study, the progeny of cold stressed plants (S_1) had significantly fewer leaves than the progeny of control plants (C_1) under both cold stress and normal growth conditions, for 15D8 and *dcl4* plants. Under normal growth conditions, the progeny of stressed and control plants *dcl2* and *dcl3* were not significantly different. The memory of parental cold stress may slow the growth process, and therefore growth of new leaves in progeny under both stressed and normal conditions. As a result, plants may invest less energy at a time when metabolic processes are slowed and energy is crucially needed. The ability of this memory to be passed on by *dcl2* and *dcl3* plants may be limited.

Additionally, cold stressing parental plants may decrease leaf number in offspring due to tolerance mechanisms that help plants cope with the stress and could influence leaf number. There are many changes that occur in response to cold stress, for example the desaturation of fatty acids in the membrane, and a decrease in cerebrosides content (Bray et al., 2000; Yadav, 2010). Finally, germination may be delayed in the progeny of cold stressed plants, potentially due to decreased seed fitness, resulting in what appears to be fewer leaves (Zinn et al., 2010). It would be interesting to determine if the final leaf number is significantly different in S_1 15D8 and *dcl4* progeny in comparison to C_1.

The progeny of cold stressed plants maintained similar leaf length in response to stress, while the progeny of control plants increased length. However, while the progeny of control and stressed plants had similar leaf widths under normal conditions, the progeny of control plants had wider leaves under cold stress. The exception was 15D8, in which S_1 was slightly less wide than C_1 under normal conditions. Ultimately, all plants increased leaf width in response to cold regardless of parental treatment.

Overall, changes to leaf width and length were very similar among C_1 progeny in response to stress. However, S_1 progeny did show some interesting trends, for example the changes in leaf width and length of *dcl2* and *dcl4* were much greater than that of 15D8 and *dcl3*. The variation in response may be due to differential parental inheritance that could exist in S_1 plants but not C_1.

Finally, differences in leaf morphology responses to cold stress may be an indication of transgenerational inheritance of stress memory in *Arabidopsis*. Leaf measurements were taken on the same leaf on each plant and those that had undergone cold stress also had fewer leaves. Thus, the leaves that plants did possess may have been older, and therefore larger. The same leaf on both stressed and control plants may show variation in size, potentially due to variation in age. Further work could compare germination rates and ages (post seed-stratification), in order to confirm this hypothesis.

4.1.2.1.2. Changes to Seed Phenotype

In response to cold stress, F1 seed length either did not change significantly, as in the case of 15D8 and *dcl3*, or became significantly smaller, such as for *dcl2* and *dcl4*.

Cold-stressed seed length was most similar between 15D8 and *dcl4*, while *dcl2* and *dcl3* were both significantly longer than cold-stressed 15D8.

Cold temperatures may induce pollen sterility, as well as a severe reduction in seed set (Zinn et al., 2010). As a result, cold-stressed plants could potentially have smaller seeds, and certain mutants, such as *dcl2* and *dcl3*, may be more sensitive to the stress than others.

4.1.2.1.3. Changes to Bolting Time

Plants decreased bolting in response to cold stress. Previous work has confirmed that cold stress will usually delay flowering (Zinn et al., 2010). In our study, the progeny of cold stressed plants bolted earlier than the progeny of control plants, under normal conditions. This is likely offsets the natural response that occurs in response to cold. Because cold temperatures delay flowering, seeds will be produced later in the growing season in undesirable temperatures (Zinn et al., 2010). Compensating for the cold-stressed delay in flowering by naturally flowering earlier may be a way for the progeny of stressed plants to be better prepared for facing the same stress, providing further evidence of the strong heritability of bolting time. Previous work found similar results in the progeny of water stressed plants (Brachi et al., 2012).

4.1.2.2. F2 Generation

4.1.2.2.1. Changes to Leaf Phenotype

Under normal conditions, C_2 plants had the most leaves and increasing generations of parental stress decreased leaf number. Cold stress decreased the leaf number, particularly if parental plants were stressed. Thus, either S_1C_1+ or S_2+ plants,

depending on the mutant type, had the fewest leaves. These results indicate transgenerational inheritance, as not only does cold stress decrease leaf number in F0 but plants may pass this characteristic onto progeny (Chew and Halliday, 2011).

In general, leaf length decreased in plants exposed to stress, as expected based on previous work where exposed plants decreased leaf expansion (Yadav, 2010). In *dcl4*, leaf length under normal conditions decreased with increased parental stressing, however length actually increased under stress in these same cases. For 15D8, leaf length was not significantly influenced by stress or parental treatment. *dcl2* and *dcl3* decreased under stress in all cases except S_1C_1. Overall, the most similarities in response to cold, with the same parental treatment, occurred with 15D8 and *dcl4*, as well as *dcl2* and *dcl3*.

Cold stress inhibits leaf growth, and therefore it was not surprising that leaf width tended to be lower in cold-stressed plants (Ben-Haj-Salah and Tardieu, 1995). The exception, as with leaf length, was *dcl4*, where leaf width tended to increase. Decreased leaf size, both length and width, in response to cold stress is distinct from F1 response, where leaf size was more likely to increase. However, both the leaf width and length of 15D8 plants were not impacted by cold stress, regardless of parental treatment.

4.1.2.2.2. Changes to Seed Phenotype

While S_1 plants significantly altered seed size when cold stressed, those produced by C_1 plants did not. In all mutant types except *dcl3*, cold stress decreased the seed size of seeds produced by S_1 plants. Like F1 seeds, *dcl3* did not show a substantial change under cold stress. However, unlike F1, 15D8 seed length decreased significantly under stress, indicating that the response to cold stress may increase in severity with subsequent

generations. This is emphasized by the fact that unlike S_1, the seeds produced by C_1 15D8 plants did not change significantly in response to cold stress.

It would be interesting to compare the total seed set, or approximate number of seeds produced, with the average seed size. It is possible that S_1 plants compensated for reduced seed size by increasing seed yield, and that the opposite is true in C_1 plants (Zinn et al., 2010).

4.1.2.2.3. Changes to Bolting Time

Bolting decreased in plants that were cold stressed, as expected due to the delay in flowering that generally accompanies it (Zinn et al., 2010). The change to bolting was largest with 15D8 and *dcl2* plants, and less substantial with *dcl3* and *dcl4*. However, with all plants the bolting rate decreased to less than 20%, and often close to zero.

After second generation of exposure to stress (S_2 in comparison to S_1C_1), 15D8 and *dcl3* decreased bolting under normal conditions. This potentially indicates parental inheritances, especially considering cold stress tends to reduce bolting.

Interestingly, the bolting rates of all 15D8 plants grown under normal conditions were higher than in nearly all other parental treatments and mutant type. Potentially, the rate of bolting is decreased in *dcl* mutants, although a comparison of F0 plants would be necessary for confirmation.

4.1.3. Transgenerational and phenotypic changes in response to UV-C stress

4.1.3.1. F1 Generation

4.1.3.1.1. Changes to Leaf Phenotype

The progeny of UV-stressed plants tended to have fewer, longer, and wider, leaves than the progeny of control plants under UV-stress and normal growth conditions.

Previous work on UV-C radiation (200-280 nm) has shown it impacts growth responses and biomass, as well as enhancing certain secondary metabolites such as artemisinin and flavonoid (Rai et al., 2011). In our study, the UV-stressed progeny of stressed plants had significantly fewer leaves than the UV-stressed progeny of control plants. The changes to leaf number may be a tolerance mechanism passed on to the progeny of UV-C stressed plants. It is possible that by decreasing leaf number under UV stress, plants are able to better focus their energy on the leaves they already possess.

While leaf number tended to decrease in S_1 plants exposed to UV, leaf length and width tended to increase, though the changes were not significant, with the exception of 15D8. Considering the ability of UV-C stress to reduce photosynthetic capacity, it may be more important for plants to focus on larger leaves, with increased opportunity for photosynthesis, rather than the effort required for the growth of new leaves (Ou et al., 2012). This is especially true considering UV-C radiation induces changes in plant metabolism (Wang et al., 2009). Progeny of UV-stressed 15D8 and *dcl2* plants had fewer leaves under normal conditions, indicating this response may be inherited.

C_1 plants, or the progeny of control plants, are expected to have a similar response to stress as the F0 generation. In this case, C_1 15D8 plants increased leaf number

significantly in response to UV, and decreased length and width. The progeny of most UV stressed plants (15D8, *dcl2* and *dcl4*) also had longer leaves under normal conditions than the progeny of control. Previous work has shown that leaf area and thickness, as well as plant biomass, decrease in response to UV-C (Rai et al., 2011). Therefore, transmitting a memory that increases leaf area, even under normal growth conditions, may be beneficial to progeny that could be faced with UV-C stress.

4.1.3.1.2. Changes to Seed Phenotype

UV-C stress usually resulted in the production of F1 seeds that were smaller than those produced by control plants. This difference was significant for *dcl2* and *dcl4*, while *dcl3* seeds actually increased in seed length significantly in response to UV.

UV-C stress activates the transition to flowering in *Arabidopsis,* through salicylic acid (SA) production (Martinez et al., 2004). If flowering was accelerated in F0 plants as a result of UV-C stress, then accelerated development may have prevented F1 seeds from reaching the same size as those produced under control conditions. Further work could determine if the smaller seeds had decreased germination rates.

Interestingly, C_1 and S_1 *dcl2* and *dcl3* seeds were significantly longer than 15D8, despite the fact that *dcl2* and *dcl3* significantly altered seed length in response to UV stress, while 15D8 did not. Potentially, the *dcl3* plants were not as negatively impacted by the stress, or there was another environmental factor which enabled them to maintain seed size in spite of UV stress.

4.1.3.1.3. Changes to Bolting Time

The progeny of UV-stressed plants had a lower bolting rate than the progeny of control plants at the same age, providing further evidence of the heritability of bolting time (Brachi et al., 2012). Interestingly, UV-C stress has previously been shown to result in shorter plants (Rai et al., 2011). Though the final height of the plants were not measured, it would be interesting to see if decreased bolting corresponded to lower height in the progeny of UV-stress plants, as the parental plants, though not observed in this study, were likely shorter as well as described in previous work (Rai et al., 2011).

UV-C is known to accelerate flowering in *Arabidopsis*, activating it prematurely to enhance the chances that a plant survives the harmful environment and produces progeny, a response triggered by increased SA production. In fact, SA is important even in non-stressed plants, and those that are deficient flower later (Martinez et al., 2004).

As bolting corresponds to the elongation of reproductive internodes in the leaf zone, and therefore occurs before flowering in *Arabidopsis*, UV stress was expected to trigger bolting in F1 plants (Pouteau and Albertini, 2009). However, the response to UV was not universal, nor was it based on parental treatment. The most similarity was seen among 15D8 and *dcl4* plants, which tended to increase bolting in response to UV stress, and *dcl2* and *dcl3* plants, which tended to decrease bolting. Accelerated flowering is only seen in response to stress if SA production and accumulation can occur (Martinez et al., 2004). It is therefore possible that *dcl2* and *dcl3* plants were not entirely successful in the SA production and accumulation process, resulting in a different response to UV-C.

4.1.3.2. F2 Generation

4.1.3.2.1. Changes to Leaf Phenotype

In general, changes to leaf number and size in response to UV-C stress, in F2 plants, were not significant. Leaf number tended to increase in response to the stress, though the change was rarely significant, and this supported with previous work indicating that UV-C accelerated the growth rate of plants (Martinez et al., 2004).

A clear correlation exists between increasing exposure time to UV-C and a reduction in plant growth and biomass (Najeeb et al., 2011). Though the changes were not significant in most cases, it was still unexpected that many of the parental treatment groups actually increased leaf length and width in response to UV-C in our study. The variation and inconsistency in response, as well as the lack of clear inheritance of stress memory, may be in part due to the absence of a distinct UV-C specific response, due to the fact that the ozone layer is able to filter UV-C from reaching the earth's surface and thus plants may have been unable to "learn" a proper response to UV-C (Roy et al., 1998).

4.1.3.2.2. Changes to Seed Phenotype

15D8, *dcl2* and *dcl3* C_1 seeds significantly increased in size in response to UV stress, while *dcl4* C_1 made no significant change. *dcl3* and *dcl4* S_1 seeds significantly decreased in size in response to stress, while 15D8 and *dcl2* did not significantly change. All mutant types that had one, or two, previous generations of UV stress were larger than those grown under control conditions for two generations (C_2). It was unexpected that seed size would actually increase under UV stress, as it resulted in smaller S_1 seeds.

However, S_1 plants began from smaller seeds, and seed size has been linked to seedling survival in abiotic stress conditions, so it is possible that S_1 plants were less able

to withstand UV stress, therefore producing smaller seed than those produced by C_1 plants, which began from larger seeds themselves (Dornbos and Mullen, 1991). This could occur regardless of the potential parental inheritance. C_1 15D8, *dcl2*, and *dcl3* plants significantly increased seed length in response to stress, while S_1 *dcl3* and *dcl4* significantly reduced seed length. Measuring F0 seeds in order to determine the starting seed size would give the best assessment of changes with increasing generations of stress. It would also be interesting to determine if there is a correlation between seed number and seed size, as it is possible that C_1 plants shifted the balance towards fewer, larger, seeds while S_1 plants produced larger quantity of smaller seeds in response to UV stress.

4.1.3.2.3. Changes to Bolting Time

The bolting response of F2 plants to UV stress fluctuated, but in general bolting increased. This was expected based on F1 results, as well as previous work that showed UV-C to accelerate flowering in *Arabidopsis* (Martinez et al., 2004). There were no clear trends with varying parental treatments, potentially because of the lack of a specified response to UV-C and therefore a less specific inheritance pattern (Roy et al., 1998).

4.1.4. Comparison of transgenerational and phenotypic changes in response to abiotic stresses

In general, the progeny of stressed plants showed a larger range of phenotype such as leaf number and size than the progeny of control plants under normal conditions. It is probable that while the progeny of control plants maintained a similar phenotype to parental plants, stressing plants resulted in a variety of molecular changes causing for a larger range in phenotype. Under stressed conditions, however, the progeny of control plants tended to have a large range of phenotype than the progeny of stressed plants. This likely occurs because the epigenetic stress memory inherited by the progeny of stressed plants allows for a more specific response to stress.

4.1.4.1. Comparison of F1 Generation

The progeny of heat, cold and UV-stressed wild-type (15D8) plants had significantly fewer leaves than the progeny of controls, under normal growth conditions. These trends fluctuated within *dcl* mutants, though all three had significantly fewer leaves in the progeny of heat-stressed plants. Decreased growth rates in the progeny of stressed plants may counteract the natural acceleration of plant development, and therefore shortened life cycle, known to occur in response to stress (Porter, 2005). It may also allow the plant to preserve energy in case of future stress.

In general, leaf length did not vary significantly in the progeny of stressed plants grown under normal conditions, compared to progeny of control. However, parental heat and UV stress significantly increased length in 15D8 plants. Cold did not have a significant impact on any mutant types,. Heat significantly decreased leaf length in *dcl2*, and UV significantly increased it. UV stress significantly decreased length of *dcl3* plants.

UV tended to result in wider leaves in the progeny of stressed plants, except *dcl2*, while cold had no significant impact, and heat increased width for 15D8 and *dcl4* but decreased it for *dcl2*. These changes to leaf length and width clearly emphasize that even when a phenotypic change occurs in the progeny of stress plants, it may not be consistent.

The impact of stress on seed length varied. All three stresses decreased seed length in *dcl2*, while both heat and cold decreased it in *dcl4*. UV decreased length for 15D8, but heat increased it, while cold and UV increased *dcl3* seed size. The impact of stress appears to be most severe on *dcl2*, decreasing seed size and thus potentially decreasing germination and seedling survival (Dornbos and Mullen, 1991). These results indicate that DCL2 may play a crucial role in maintaining seed size in response to stress.

Heat and cold led to earlier bolting times in the progeny of stressed plants, except for *dcl4* heat. In contrast, UV actually decreased bolting age in S_1 plants. Heat stress tended to increase leaf number, while cold decreased it, and the impact of UV fluctuated. In a natural environment, plants face temperature extremes, but not UV-C, and thus a specialized response may not exist (Roy et al., 1998).

Cold and heat stresses both increased leaf length, but UV usually did not impact leaf length, especially in S_1 plants. Temperature stress also tended to increase leaf width, however, the progeny of 15D8 stressed plants did not show a significant change under stress. Additionally, UV did not have a significant impact on leaf width.

Generally, bolting time was earlier under heat stress and later under cold. UV tended to increase bolting, although *dcl3* decreased bolting in response to UV. All three stresses decreased the seed length for *dcl2* and *dcl4*. In contrast, 15D8 and *dcl3* were less

impacted, and neither changed significantly under cold stress. 15D8 increased under heat, while *dcl3* increased under UV.

4.1.4.2. Comparison of F2 Generation

Most of the results obtained in the physiological portion for F2 plants were inconclusive. Under normal growth conditions parental heat stress increased leaf number and decreased leaf length, in contrast to F1. Similar to F1, the leaves tended to be wider. Increasing generations of cold stress decreased in leaf number, like F1, but leaf length and width were not significantly impacted. The impact of UV parental stress was inconsistent, though fewest leaves tended to be found following two generations of stress (S_2), which corresponds with F1 results where parental stress decreased leaf number. However, unlike F1, parental UV stress did not significantly impact leaf length or width.

Leaf number was not significantly impacted by heat stress, but generally leaf length and width increased. Cold stress significantly decreased leaf number, but UV tended to increase it. Cold stress decreased leaf length and width but UV had no impact.

F0 cold stress tended to decrease bolting in plants grown at normal conditions, but F1 treatment did not. In contrast, F0 heat stress tended to increase bolting, while a second generation of stress (S_2) resulted in later bolting. UV stress in F0 decreased bolting, but the impact of F1 treatment on plants grown at normal conditions was inconsistent. In F2 plants, cold stress tended to decrease plant bolting, while UV and heat stresses had an inconsistent impact on bolting in plants. Increasing generations of heat and UV stress resulted generally resulted in seeds larger than C_2. The response to cold stress varied, but tended to increase 15D8 seed size.

4.1.4.3. Comparison of Plants Grown in Control Conditions

Following a second generation of growth at normal conditions (C_2) leaf number significantly decreased in *dcl2* and *dcl4* plants, while 15D8 and *dcl3* did not significantly differ from C_1. C_2 leaf length and width did not significantly differ from C_1. While 15D8 seed size remained consistent, C_2 *dcl* seed size significantly decreased. In combination with the decrease in leaf number seen in *dcl2* and *dcl4*, these results may indicate decreased fitness of *dcl* plants with increasing generations. Further work could determine if the seed size correlated with decreased in germination, and if *dcl* seed size and leaf number continued to decrease in subsequent generations (Dornbos and Mullen, 1991).

Table 7. Changes in leaf number, length, width, as well as seed length and bolting time in F1 plants grown under normal conditions. Comparisons were made between the progeny of stressed plants (S_1) and the progeny of control plants (C_1). ↑ indicates S_1 was significantly larger than C_1, → indicates S_1 was not significantly different than C_1, and ↓ indicates S_1 was significantly smaller than C_1.

Stress	Measurement	15D8	*dcl2*	*dcl3*	*dcl4*
Heat	Leaf Number	↓	↓	↓	↓
Cold	Leaf Number	↓	→	→	↓
UV	Leaf Number	↓	↓	↑	→
Heat	Leaf Length	↑	↓	→	→
Cold	Leaf Length	→	→	→	→
UV	Leaf Length	↑	↑	↓	→
Heat	Leaf Width	↑	↓	→	↑
Cold	Leaf Width	→	→	→	→
UV	Leaf Width	↑	↑	→	↑
Heat	Seed Length	↑	↓	→	↓
Cold	Seed Length	→	↓	↑	↓
UV	Seed Length	↓	↓	↑	↑
Heat	Bolting Time	↑	↑	↑	↓
Cold	Bolting Time	↑	↑	↑	↑
UV	Bolting Time	↓	↓	↓	↓

Table 8. Changes in response to F1 stress (heat, cold and UV) as indicated by changes to leaf number, length, width, seed length and bolting time. Plants grown under stress (+) were compared to plants grown under normal conditions (-) with the same parental in order to determine if the change under stress was significant. ↑ indicates F1 plants grown in stressed conditions (+) had a significant increase in comparison to those grown in control conditions (-),→ indicates plants grown in stressed conditions had no significant change from those grown in control conditions, and ↓ indicates grown in stressed conditions had a significant decrease in comparison to those grown in control conditions.

Stress	Measurement	Parental Treatment	15D8	*dcl2*	*dcl3*	*dcl4*
Heat	Leaf Number	Control	↑	→	↑	↑
Heat	Leaf Number	Stressed	↑	→	→	↑
Cold	Leaf Number	Control	↓	↓	↓	↓
Cold	Leaf Number	Stressed	↓	↓	↓	↓
UV	Leaf Number	Control	↑	→	↑	→
UV	Leaf Number	Stressed	→	→	↓	↓
Heat	Leaf Length	Control	↑	→	↑	↑
Heat	Leaf Length	Stressed	↑	↑	↑	↑
Cold	Leaf Length	Control	↑	→	↑	→
Cold	Leaf Length	Stressed	→	↑	↑	↑
UV	Leaf Length	Control	→	↑	↓	→
UV	Leaf Length	Stressed	→	→	→	→
Heat	Leaf Width	Control	↑	→	↑	↑
Heat	Leaf Width	Stressed	→	↑	↑	→
Cold	Leaf Width	Control	↑	→	↑	↑
Cold	Leaf Width	Stressed	→	→	→	↑
UV	Leaf Width	Control	→	↑	↓	→
UV	Leaf Width	Stressed	↓	→	→	→
Heat	Bolting Time	Control	↑	↑	↑	↑
Heat	Bolting Time	Stressed	↑	↓	↑	↑
Cold	Bolting Time	Control	↓	↓	↓	↓
Cold	Bolting Time	Stressed	↓	↓	↓	↓
UV	Bolting Time	Control	↑	↓	↓	↑
UV	Bolting Time	Stressed	↑	↑	↓	→
Heat	Seed Length	Control	↑	↓	→	↓
Cold	Seed Length	Control	→	↓	→	↓
UV	Seed Length	Control	→	↓	↑	↓

4.2. Discussion of Molecular Results

4.2.1. Transposon expression in the progeny of plants exposed to abiotic stress and controls

4.2.1.1. Impact of stress on transposon expression in F1 progeny of stressed and control plants

In general, stressing parental plants with abiotic stresses including cold, heat, and UV-C lead to increased expression of retrotransposons ONSEN and TSI in progeny under normal conditions, in comparison to the progeny of control plants (Table 9).

ONSEN and TSI are just a few of many transposable elements (TEs), which are one of the most important sources of mutation in genomes (He et al., 2012). An important function of epigenetic regulation is to silence these TEs. However, instances of environmental stress may lead to situations where TE expression is increased, and therefore mutation rates are increased (Mirouze and Paszkowski, 2011). By interacting and disrupting other genes, TEs can create new functions that may be beneficial, especially in times of stress which require new coping mechanisms (Civan et al., 2011).

In our study, parental stress never significantly decreased TE expression. Though there were a few instances where the change was not significant, many of these cases did increase, and the values could potentially become significant by increasing sample size (Table 9). It is clear that abiotic stresses transmitted a transgenerational stress memory, resulting in increased TE expression levels in non-exposed offspring. TE proliferation has been associated with biotic and abiotic stress, so it is interesting that this change can be epigenetically inherited (Wessler, 1996; Grandbastien, 1998). Increased TE expression may better prepare progeny for stress.

When exposed to stress, the progeny of stressed and control plants did not usually vary in TE expression levels, especially for 15D8 and *dcl2*. However, there were a few exceptions, for example the cold stressed progeny of cold stressed *dcl3* plants decreased significantly in TSI and ONSEN expression, while the UV-stressed progeny of UV stressed *dcl4* increased in expression (Table 10). This indicates that although the natural level of transposon expression tended to be higher in the progeny of stressed plants, the differences in S_1 and C_1, under stress, were not significantly different. Such a response provides evidence that the expression levels enabled by parental stress are not significantly different than those which would naturally occur in response to the stress.

Although the expression of TSI and ONSEN may not have been significantly different in the progeny of stressed and control plants under stressed conditions, their individual response to stress (+ compared to -) often varied (Table 11). As a result, the progeny of control plants may have increased expression, while the progeny of stressed plants showed no significant change under stress, resulting in expression levels that were not significantly different (Table 10). Some examples of where this occurred include ONSEN cold 15D8 and UV *dcl2*. This emphasizes the need for measurements to be taken in both normal, and stressed conditions.

Table 9. Changes in retrotransposon expression under normal growth conditions indicated by comparing S_1 expression to C_1. ↑ indicates S_1 had a significant increase in expression from C_1,→ indicates S_1 had no significant change from C_1, and ↓ indicates S_1 had a significant reduction in expression in comparison to C_1.

Stress	Retrotransposon	15D8	*dcl2*	*dcl3*	*dcl4*
Cold	ONSEN	↑	↑	↑	↑
Cold	TSI	→	↑	↑	↑
Heat	ONSEN	↑	↑	→	→
Heat	TSI	→	↑	→	↑
UV	ONSEN	→	↑	↑	↑
UV	TSI	↑	↑	↑	↑

Table 10. Changes in retrotransposon expression under stressed growth conditions indicated by comparing S_1 expression to C_1. ↑ indicates S_1 had a significant increase in expression from C_1, → indicates S_1 had no significant change from C_1, and ↓ indicates S_1 had a significant reduction in expression in comparison to C_1.

Stress	Retrotransposon	15D8	*dcl2*	*dcl3*	*dcl4*
Cold	ONSEN	→	↓	↓	↑
Cold	TSI	→	→	↓	↓
Heat	ONSEN	→	→	→	→
Heat	TSI	→	→	↑	→
UV	ONSEN	↓	→	↑	↑
UV	TSI	↑	↑	→	↑

Table 11. Changes in retrotransposon expression under stress in F1 plants, indicated by comparing S_1+ to S_1-, and C_1+ to C_1-, where S_1: indicates progeny of stressed plants. C_1: indicates progeny of control plants. "+" indicates grown under stressed conditions and "-" indicates grown under control conditions. ↑ indicates S_1 or C_1+ had a significant increase in expression from S_1 or C_1-,→ indicates S_1 or C_1+ had no significant change from S_1 or C_1-, and ↓ indicates S_1 or C_1+ had a significant reduction in expression in comparison to S_1 or C_1-.

Stress	Retrotransposon	Parental Treatment	15D8	*dcl2*	*dcl3*	*dcl4*
Cold	ONSEN	Control	↑	↑	↑	→
Cold	ONSEN	Stressed	→	↓	↓	→
Cold	TSI	Control	→	↑	↑	→
Cold	TSI	Stressed	→	↑	→	↓
Heat	ONSEN	Control	→	↓	↓	↑
Heat	ONSEN	Stressed	→	→	→	→
Heat	TSI	Control	→	→	→	→
Heat	TSI	Stressed	→	→	→	↑
UV	ONSEN	Control	↑	↑	↓	↑
UV	ONSEN	Stressed	→	→	→	↑
UV	TSI	Control	↑	↑	↓	↓
UV	TSI	Stressed	→	↓	→	↓

4.2.1.2. Impact of stress on transposon expression in F2 progeny of stressed and control plants

4.2.1.2.1. Impact of cold stress on transposon expression in F2 plants

For 15D8 cold F2 plants grown under normal conditions, those that had been parentally stressed, but grown under control conditions in F0 (C_1S_1) had higher expression rates of TSI and ONSEN than those grown under control conditions for two previous generations (C_2). The change in expression level is likely stress-induced reactivation of transposons, an epigenetic memory that could have been passed onto F2 progeny (Wessler, 1996).

The difference between 15D8 plants that had been stressed for two generations (S_2) and those stressed for one, followed by a generation of normal growth (S_1C_1), was not significant. This indicates that changes in expression produced by cold stress can be transmitted even in the absence of stress, and that a second generation of stress does not significantly alter the response (Table 12).

The majority of parental treatments did not significantly changes TSI and ONSEN expression in F2 *dcl* progeny grown under normal conditions, including all *dcl4* groups, as well as every *dcl2* except for ONSEN C_1S_1 in comparison to C_2. The lack of changes in expression with varying parental treatments may be an indication that the parental memory is not being transmitted as effectively as it was with 15D8 plants, due to the knockout of *dcl* genes that have a recognized involvement in siRNA production and therefore TE silencing (Piriyapongsa and Jordan, 2008; Matsunaga et al., 2011). Interestingly, *dcl3* plants did show changes in expression, including a decrease for ONSEN and TSI C_1S_1 in comparison to C_2, as well as ONSEN S_2 in comparison to S_1C_1.

In most cases, exposure to cold in F2 plants decreased ONSEN expression. However, none of the S_2 plants showed a significant change in ONSEN expression in response to cold stress in F2, potentially indicating that two generations of cold were enough to modify stress response in progeny (Table 14).

4.2.1.2.2. Impact of heat stress on transposon expression in F2 plants

Changes in ONSEN and TSI expression in F2 plants grown under normal conditions, with varying parental treatments, were inconsistent. However, the expression of ONSEN in plants stressed for two generations (S_2) generally decreased as compared to plants stressed for one generation and then propagated at normal conditions (S_1C_1). The exception was 15D8, where there was no significant change, indicating differences in the ability of wild-type and *dcl* plants transmit stress memory (Table 12). The F1 portion of our study indicated increased transposon expression with one generation of stress, so it is possible that the second generation of stress may alter the response.

Heat-stressed F2 plants usually did not vary in TE expression regardless of parental treatment. However, when changes to expression occurred with varying F1 treatments, generally heat stress in F1 lead to increased expression in F2 plants that were heat stressed, in particular with *dcl4*. These results are supported by previous work indicating that environmental stress may lead to the activation of TEs (Wessler, 1996). In contrast, 15D8 plants either showed no significant change with F1 stress, or a decrease in expression in stressed F2 plants (Table 13). It is possible that the stress memory in 15D8 enables a different response than that which occurs in *dcl* plants.

Heat stress decreased expression of ONSEN in most F2 plants. However, among S_2 *dcl* plants, ONSEN expression increased, though it decreased in S_2 15D8. TSI level was generally low in F2 plants. However in the few cases where there was a significant change, expression usually decreased in response to stress (Table 14). These results were unexpected, as previous work indicated that heat stress generally leads to an increase in ONSEN expression, but the work was not done on F2 plants (Ito et al., 2011).

4.2.1.2.3. Impact of UV stress on transposon expression in F2 plants

In general, the impact of F1 UV treatment on F2 plants grown under normal conditions was limited. A generation of normal growth followed by a generation of UV (C_1S_1), in comparison to two generations under control conditions (C_2), did not significantly influence TSI and ONSEN expression. The exceptions were ONSEN 15D8, which significantly increased with F1 stress, as well as TSI *dcl3*, which significantly decreased, and TSI *dcl4*, which significantly increased. In addition, a second generation of stress (S_2) in comparison to one generation of stress followed by one generation of growth in control conditions (S_1C_1) did not significantly change ONSEN and TSI expression, except for ONSEN *dcl2* and TSI *dcl2* (Table 12). These results indicate that the ability of UV stress to lead to substantial TE changes in unstressed progeny is limited.

Nearly all UV-stressed plants did not show a significant difference in expression with varying F1 treatment, including all 15D8 and *dcl2* groups. The major exception was *dcl4*, where expression tended to be significantly higher in the progeny of F1 stressed plants in comparison to plants with the same F0 treatment but F1 control conditions (Table 13). This emphasizes the fact that DCL mutant types may differ in their ability to transmit epigenetic stress memory, and therefore the response of their progeny to stress.

Finally, in most cases UV stressing F2 plants did not significantly change TE expression in comparison to those grown under normal conditions. There were a few fluctuations in expression, generally leading to a decrease under stress (Table 14). It is possible that the severity stress was not significant enough to have an impact on the plants. It is also possible that because UV-C radiation does not reach plants in nature, there was not a specialized response to cause TE expression changes (Roy et al., 1998).

Table 12. Changes in retrotransposon expression in F2 plants under normal growth conditions indicated by comparing variation in F1 treatment: C_1S_1 expression to C_2 (F0 treatment: control) and S_2 to S_1C_1 (F0 treatment: stressed). ↑ indicates C_1S_1 or S_2 had a significant increase in expression from C_2 or S_1C_1, respectively → indicates C_1S_1 or S_2 had not significant change in expression from C_2 or S_1C_1, respectively and ↓ indicates C_1S_1 or S_2 had a significant decrease in expression from C_2 or S_1C_1, respectively.

Stress	Retrotransposon	F0 Treatment	15D8	*dcl2*	*dcl3*	*dcl4*
Cold	ONSEN	Control	↑	↓	↓	→
Cold	ONSEN	Stressed	→	→	↓	→
Cold	TSI	Control	↑	→	↓	→
Cold	TSI	Stressed	→	→	→	→
Heat	ONSEN	Control	↓	→	↓	↑
Heat	ONSEN	Stressed	→	↓	↓	↓
Heat	TSI	Control	↓	↑	→	↑
Heat	TSI	Stressed	↑	→	↓	↑
UV	ONSEN	Control	↑	→	→	→
UV	ONSEN	Stressed	→	↓	→	→
UV	TSI	Control	→	→	↓	↑
UV	TSI	Stressed	→	↓	→	→

Table 13. Changes in retrotransposon expression in F2 plants under stressed growth conditions indicated by comparing variation in F1 treatment: C_1S_1 expression to C_2 (F0 treatment: control) and S_2 to S_1C_1 (F0 treatment: stressed). ↑ indicates C_1S_1 or S_2 had a significant increase in expression from C_2 or S_1C_1, respectively → indicates C_1S_1 or S_2 had not significant change in expression from C_2 or S_1C_1, respectively and ↓ indicates C_1S_1 or S_2 had a significant decrease in expression from C_2 or S_1C_1, respectively.

Stress	Retrotransposon	F0 Treatment	15D8	*dcl2*	*dcl3*	*dcl4*
Cold	ONSEN	Control	↑	↑	→	↓
Cold	ONSEN	Stressed	→	→	→	→
Cold	TSI	Control	↓	→	→	↓
Cold	TSI	Stressed	↓	→	↓	↓
Heat	ONSEN	Control	↓	→	→	→
Heat	ONSEN	Stressed	→	↑	↑	↑
Heat	TSI	Control	↓	↓	→	↑
Heat	TSI	Stressed	→	→	→	↑
UV	ONSEN	Control	→	→	→	↑
UV	ONSEN	Stressed	→	→	→	→
UV	TSI	Control	→	→	↑	↑
UV	TSI	Stressed	→	→	→	↑

Table 14. Changes in retrotransposon expression under stress in F2 plants indicated by comparing the same parental treatment with stress (+) to plants grown under control conditions (-). C_2: indicates two previous generations of control growth. C_1S_1: indicates a generation of growth under control conditions followed by a generation under stress. S_1C_1: indicates a generation of growth under stressed conditions followed by a generation under control conditions. S_2: indicates two previous generations of stressed growth. ↑ indicates F2 plants grown in stressed conditions (+) had a significant increase in expression from those grown in control conditions (-),→ indicates grown in stressed conditions had no significant change from those grown in control conditions, and ↓ indicates grown in stressed conditions had a significant reduction in expression in comparison to those grown in control conditions.

Stress	Retrotransposon	Parental Treatment	15D8	*dcl2*	*dcl3*	*dcl4*
Cold	ONSEN	C_2	→	↓	↓	→
Cold	ONSEN	C_1S_1	↑	↑	→	→
Cold	ONSEN	S_1C_1	↓	↓	↓	↓
Cold	ONSEN	S_2	→	→	→	→
Cold	TSI	C_2	↑	→	↓	→
Cold	TSI	C_1S_1	↓	↓	↑	→
Cold	TSI	S_1C_1	↓	→	↓	↑
Cold	TSI	S_2	→	→	↓	↓
Heat	ONSEN	C_2	↓	↓	↓	↓
Heat	ONSEN	C_1S_1	↑	↓	→	→
Heat	ONSEN	S_1C_1	→	↓	→	↓
Heat	ONSEN	S_2	↓	↑	↑	↑
Heat	TSI	C_2	↑	↓	→	↓
Heat	TSI	C_1S_1	↓	↓	→	↓
Heat	TSI	S_1C_1	→	→	→	↑
Heat	TSI	S_2	→	→	↑	↓
UV	ONSEN	C_2	↑	↓	→	↓
UV	ONSEN	C_1S_1	→	→	→	→
UV	ONSEN	S_1C_1	→	↓	→	→
UV	ONSEN	S_2	→	→	→	→
UV	TSI	C_2	→	→	↓	↓
UV	TSI	C_1S_1	↓	→	→	↓
UV	TSI	S_1C_1	→	↓	→	→
UV	TSI	S_2	→	→	→	→

4.2.2. Global genome methylation in the progeny of heat-stressed plants and controls

dcl3 and 15D8 plants increased methylation in the progeny of heat stressed plants, in comparison to the progeny of control plants, when grown at normal conditions. These results are supported by previous work showing transgenerational inheritance of abiotic stress memory, via DNA hypermethylation, in progeny of salt-stressed plants (Boyko et al., 2010).

In F1 plants that were heat stressed, methylation decreased in comparison to plants with the same parental treatment, but grown under normal conditions. Previous work has showed that abiotic and biotic stresses induced the accumulation of transcripts associated with active demethylation in plants. Examples include exposure of tobacco to aluminum, heavy metal stress in hemp and clover and drought in pea (Labra et al., 2002; Aina et al., 2004; Wada et al., 2004).

The most substantial decrease in methylation was seen in the stressed progeny of stressed plants for *dcl2* and *dcl3*, which resulted in the lowest overall levels of methylation. However, the response to heat stress did not significantly change with differing parental treatments for 15D8, indicating the inheritance of epigenetic stress memory in wild-type may differ from *dcl* plants.

The importance of DCL2 in the production of small RNAs needed for hypermethylation may explain why *dcl2* plants did not show and increase in DNA methylation in the progeny of heat stressed plants. Previous work by Boyko et al. (2010) also found *dcl2* to be impaired in transgenerational changes in DNA methylation in the progeny of heat-stressed plants. However, while the previous study found *dcl3* to be

impaired in transgenerational inheritance, the progeny of heat stressed *dcl3* showed a similar response to the progeny of heat stressed wild-type plants in our study (Boyko et al., 2010). Potentially, additional sampling and replicates could provide evidence of partial deficiency in transgenerational inheritance in *dcl3*.

4.2.3. mRNA expression in the progeny of heat-stressed plants and controls

In general, parental heat stress resulted in decreased expression of the genes examined, in comparison to the progeny of control plants. The only exception was the gene coding for heat stress transcription factor *HSFA2*, which had a higher transcript level in the progeny of stressed plants than the progeny of controls (Table 15).

Progeny of heat-stressed plants (S_1) had higher levels of *HSFA2* expression than progeny of control plants (C_1). HSFA2 is a dominant HSF in *Arabidopsis* due to its high activator potential for transcription of HSP genes and its continued accumulation during repeated cycles of heat stress and recovery (Schramm et al., 2006). These results indicate that a mechanism for transgenerational inheritance of increased HSFA2 expression in the progeny of heat-stressed plants exists.

In contrast to HSFA2, all other genes decreased expression in S_1 plants, compared to C_1, within the same mutant type. The genes examined included MSH6, ROS1, SUVH2, SUVH5 (except for S_1+ *dcl2*), SUVH6, and SUVH8 (except for C_1- 15D8).

Previous work has shown that the progeny of salt-stressed plants had hypermethylation of many genes involved in the regulation of chromatin structure, results consist with the decrease in expression seen in our study (Bilichak et al., 2012). All of the genes examined in our study that decreased expression in the progeny of heat stressed plants, were hypermethylated at the promoters or transcribed regions in the progeny of salt stressed plants (Bilichak et al., 2012). These results indicate that some responses to stress are common among the progeny of both salt and heat stressed plants.

MSH6 is a DNA mismatch repair protein that, along with MSH2, is involved in the initial recognition of DNA errors (Culligan and Hays, 2000; Lario et al., 2011). Reduction in MSH6 expression in the progeny of stressed plants potentially allows for more point mutation, as well as other genomic changes, as has been observed in the progeny of stressed plants (Boyko and Kovalchuk, 2010; Yao and Kovalchuk, 2011).

ROS1 encodes a DEMETER DNA glycosylase that catalyzes the excision of methylated cytosines, thereby triggering increased activity of DNA methyltransferases (Gong et al., 2002). Decreased ROS1 expression may lead to hypermethylation, including increased methylation of transposons in the progeny of stressed plants, as has been observed previously with salt stress (Boyko et al., 2010). Changes to the expression level of transposons were also observed in our study, and are described elsewhere.

SUVH proteins in *Arabidopsis*, including SUVH2, SUVH5 and SUVH6, are involved in H3K9 methylation and link it to DNA methylation as well as the transposon repression (Thorstensen et al., 2011). These genes, as well as another SET-domain protein also involved in epigenetic regulation of gene expression, SUVH8, previously showed decreased mRNA expression in the progeny of salt-stressed plants, as well as the progeny of heat-stressed plants in our study (Baumbusch et al., 2001; Bilichak et al., 2012). Potentially, decreased expression of these homologs may protect the progeny of stressed plants from genome-wide hypermethylation and contribute to TE activation.

Table 15. Significant differences in expression in comparison to the progeny of heat-stressed plants (S_1) to the progeny of control plants (C_1). Provides an overall summary of general trends in 15D8, *dcl2*, and *dcl3* mutants.

Gene	S_1 expression in comparison to C_1 expression
HSFA2	↑
MSH6	↓
ROS1	↓
SUVH2	↓
SUVH5	↓
SUVH6	↓
SUVH8	↓

4.2.4. ChIP Discussion

acH3K9 and me2H3K9 increased in the progeny of heat stressed 15D8 and *dcl2* plants. acH3K9 increase but me2H3K9 decreased in *dcl3*. These results are supported by mRNA data that showed a higher level of HSFA2 expression in S_1 than C_1 plants.

SUVH2 promoter decreased acH3K9 in 15D8 and *dcl2*, but increased in *dcl3*. me2H3k9 showed a small increase in 15D8 and *dcl2*. Similarly, SUVH2 promoter region had an increased in methylation and a decreased acetylation in the progeny of salt stressed plants, corresponding to decreased mRNA expression (Bilichak et al., 2012). These results are supported by our work with the progeny of heat-stressed plants.

However, SUVH2 was also found to be hypermethylated at the transcribed region of the progeny of salt-stressed plants, while the progeny of heat stressed plants had an enrichment of acH3K9 and a decrease in me2H3K9, refuting previous results (Bilichak et al., 2012). Despite the increase in permissive marks, transcription of the gene decreased resulting in lower SUVH2 expression, as discussed previously.

While change to SUVH5 occurred in 15D8, there was a slight increase in acH3K9 in S_1 *dcl2* and *dcl3* plants. This was unexpected, as SUVH5 mRNA expression decreased in S_1 plants. It is possible that like SUVH2, the changes in histone marks were present in the promoter region, but not the gene body. This hypothesis is supported by previous work in the progeny of salt-stressed plants, which showed an increase in methylation in the promoter region of SUVH5. However, previous work also showed a decrease in acH3K9 in the gene body, but no significant change in me2H3K9 (Bilichak et al., 2012). It is possible that a larger sample size could reveal the same trends.

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