MULTIGENERATIONAL UV STRESS EFFECTS IN *ARABIDOPSIS THALIANA* OFFSPRING

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DEDICATION

To my family, who are my greatest support, inspiration, and happiness in life. To Becky, who has been the best life companion and given me endless love—but above all, to Pascal the Dog and Kevin the Cat—thank you for being the chaos in my life, reminding me there are things even more challenging than writing a thesis.

ABSTRACT

Continuous exposure to environmental stress contributes to species diversity and drives microevolutionary processes. However, whether epigenetic alterations, such as differential DNA methylation, are prerequisites for speciation events remains unclear. We hypothesized that prolonged stress exposure would increase epigenetic diversity more than genetic diversity. In this study, we analysed the effects of 25 consecutive generations of UV-C stress on the genome and epigenome of Arabidopsis thaliana. Our results showed that multigenerational UV-C exposure led to significant genetic and epigenetic changes in the progeny. Genomic analysis of the UV-stressed progeny (F25UV) revealed a higher frequency of genetic variations compared to controls (F25C, F2C), with deletions being more prevalent than insertions, probably due to the mechanisms of DNA damage and repair processes triggered by UV radiation. F25UV also exhibited a significantly higher proportion of nonsense mutations, with C-to-T transitions being the most common, likely due to deamination of methylated cytosines. Epigenomic analysis showed that F25UV plants had more differentially methylated cytosines (DMCs) across all contexts than the F2C group. F25UV also had more DMCs than the F25C group in the CHG and CHH contexts, with the most striking difference in the CHH context, where F25UV had over 10-fold more DMCs than the F25C group. Despite these genetic and epigenetic changes, no differences in progeny resilience under stress were observed, based on root analysis, reactive oxygen species levels, and overall growth at both the seedling and mature stages. Our findings suggest that UV radiation stress can induce genetic and epigenetic changes that are capable of being inherited in transgenerational manner. Although these changes did not result in more resilient plants, their non-random nature suggests an evolutionary and adaptive direction.

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Argonaute (AGO)

Ataxia telangiectasia mutated (ATM)

Base excision repair (BER)

BES1-INTERACTINGMYC-LIKE 1 (BIM1)

BRASSINOSTEROID INSENSITIVE 1 (BRI1)

Brassinosteroids (BRs)

BRI1-EMS-SUPPRESSOR1 (BES1)

Chalcone synthase (CHS)

CHROMOMETHYLASE 3 (CMT3)/CMT2, DRM2/CMT2

CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1)

Cryptochrome (CRY)

Cyclobutane-type pyrimidine dimers (CPDs)

Dicer-like protein (DCL)

Differentially methylated positions or cytosines (DMPs/DMCs)

Differentially methylated regions (DMRs)

DNA damage response (DDR)

DNA double-strand breaks (DSBs)

DNA methyltransferases (MTases)

DNA single-strand breaks (SSBs)

Domains Rearranged Methyltransferase 2 (DRM2)

ELONGATED HYPOCOTYL 5 (HY5)

Filial generation “n” under control conditions (F1/2/25 C)

Filial generation “n” under UV stress (F1/2/25 UV)

Gene body methylation (gbM)

Genome-wide association studies (GWAS)

Helix-loop-helix (bHLH)

Histone H3 lysine 27 (H3K27)

Histone H3 lysine 4 trimethylation (H3K4me3)

Holliday junction (HJ)

Homologous recombination (HR)

Homologous recombination frequency (HRF)

HUA ENHANCER 1 (HEN1)

INDOLE-3-ACETIC ACID INDUCIBLE 19 (IAA19)

Insertions/deletions (InDels)

Kilobase (kb)

Lipid peroxidation (LPO)

METHYLTRANSFERASE 1 (MET1)

microRNA (miRNA)

Next generation sequencing (NGS)

Non-homologous end-joining (NHEJ)

Ozone (O_3)

Phosphatidylinositol 3-kinase-like protein kinases (PIKKs)

Phototropin 1 (PHOT1)

Phytochrome interacting factors (PIFs)

Plant secondary metabolites (PSMs)

Polyunsaturated fatty acids (PUFAs)

Posttranscriptional gene silencing (PTGS)

Pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs)

Rad3-related (ATR)

Reactive oxygen species (ROS)

Replication protein A (RPA)

REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 / 2 (RUP1/2)

RNA induced silencing complex (RISC)

RNA Polymerase (RNA Pol)

RNA-directed DNA methylation (RdDM)

RNA-directed RNA polymerase 2 (RDR2)

SET and RING- ASSOCIATED (SRA)

Simple sequence repeats (SSRs)

Single nucleotide polymorphisms (SNPs)

Single-stranded DNA (ssDNA)

SMALL AUXIN UPREGULATED RNA (SAUR)

small interfering RNAs (siRNAs)

Small RNAs (sRNAs)

Suppressor of gamma response 1 (SOG1)

SUPPRESSOR OF PHYA-105 (SPA)

Synthesis-dependent strand annealing (SDSA)

Transcription factors (TFs)

Transcription start sites (TSSs)

Transposable element (TEs)

Ultraviolet (UV)

UV RESISTANCE LOCUS 8 (UVR8)

v-Myb myeloblastosis viral oncogene homolog (MYB)

Whole genome sequencing (WGS)

Whole-genome association studies (WGAS)

CHAPTER 1: INTRODUCTION

All living organisms are exposed to environmental stressors, to which they are capable to adapt and survive. Plants, as sessile organisms, proper regulation of gene expression is crucial for their response to stress factors (Yaish, 2017). Plants exemplify the intricate interplay between genetic variations within populations and environmental stimuli, culminating in the manifestation of complex adaptive traits. (Geber & Griffen, 2003). Complex trait variation in natural populations is influenced by multiple factors, including the environments in which they originally evolved. This variation is shaped by ecological adaptation to both biotic and abiotic environmental factors over extended periods of time (Reed et al., 2011). Additionally, native species in undisturbed habitats may retain heritable variation shaped by mutation, genetic drift, demography, and adaptation over thousands of generations (Anderson et al., 2011). However, due to a slow rate of mutation accumulation and randomness of such mutations, genetic change is often not a solution for plants to adapt to changing environment (Adams & Wendel, 2005).

Plants as sessile photosynthetic organisms need sunlight and thus are inevitably exposed to sun. Solar radiation is a complex mixture of ultraviolet (UV), visible light and infrared wavelengths ultraviolet (UV) radiation (Verdaguer et al., 2017). UV radiation constitutes three categories based on wavelength band ranges: UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (100–280 nm), although only wavelengths greater than 290 nm can reach the surface of the planet because of ozone layer (Gupta et al., 2017). Approximately 95% of near-UV photons are ultraviolet-A (UV-A, 320–400 nm), while the remainder are ultraviolet-B (UV-B 290–320 nm), and both have been shown to elicit physiological responses in a variety of organisms (White & Jahnke, 2002). While most of the UV-B light is absorbed by the ozone layer, some can penetrate through it into the troposphere, which has been increased due to ozone layer thinning, unlike UV-A which is not affected by changes in the ozone layer (Gupta et al., 2017). Thus, several plants biomacromolecules (DNA, RNA, lipids, and proteins) absorb in this region of the UV spectrum; also, UV-B photons have the highest energy of all wavelengths in sunlight and, hence, the potential to cause cellular damage (Ballaré, 2011). It is therefore that UV-B radiation is a common abiotic stress type for plants, due to their sessile nature and physiological requirements (Nawkar et al., 2013). Finally, UV-C is completely absorbed by the ozone layer. However, it is lethal for animals and plants. Artificial UV-C can cause severe damage to exposed tissues (Gupta et al., 2017).

The levels of UV-A, as well as those of UV-B, vary with latitude, altitude, solar zenith angle, cloud-cover, season, and time of the day (Rafieepour et al., 2015). However, in a natural environment, plants are exposed approximately to 10–100 times more UV-A photons than they are to UV-B photons (Rai et al., 2021). UV-B stress can be divided according to the dose (high or low) and exposure time, going from acute (seconds to hours) to chronic (hours to days) (Hideg et al., 2013). Also, while the daily solar UV-B flux is restricted to the hours around solar noon, the daily UV-A flux is present during a larger part of the day (Rafieepour et al., 2015). Distinct effects resulting from UV-A radiation were found when multiple genotypes were compared under similar radiation conditions. Using *A. thaliana* ecotypes response to UV-A exposure, biomass accumulation was reduced in four out of the eight ecotypes studied, in some cases by up to 30% and, in three other ecotypes substantial (but not significant) increases in total biomass were observed (Verdaguer et al., 2017). In some species (like tomato and radish), higher biomass accumulation in response to UV-A radiation was related to higher leaf chlorophyll content and photosynthetic activity (Verdaguer et al., 2017). UV-A also has decisive morphological effects, especially on leaf size and rosette diameter. A study found that supplemental UV-A increased rosette diameter substantially (30%–150%) in eight distinct accessions of *A. thaliana* grown indoors under low PAR conditions (Biswas & Jansen, 2012). On the other hand, UV-A can damage the catalytic Mn cluster of the water-oxidizing complex of PSII; therefore, affections in this function result in a decrease in the maximum quantum efficiency of PSII photochemistry, electron transport rate, and photosynthesis (Gupta et al., 2017). However, there is limited information on the specific genetic components associated with UV-A signaling in plants (Verdaguer et al., 2017).

Previous studies suggest that plant adaptation can occur through mechanisms other than genetic changes, primarily through the modulation of gene expression, which can be inherited, enabling the progeny of stressed plants to better survive under challenging conditions (Richards et al., 2010). This process, known as "epigenetics," refers to acquired traits that can be passed down across generations, often through stable chromatin modifications such as DNA methylation (Eichten et al., 2014). As a result, both genetic and epigenetic alterations in stressed plants can create a form of transgenerational memory that is inherited by their progeny (Miryeganeh & Saze, 2020). These phenotypic changes are influenced not only by genetic factors and environmental conditions but also by the phenotype or environmental context of the parents (Badyaev & Uller, 2009). Thus, both epigenetic and genetic inheritance contribute to transgenerational adaptation. While evidence for parental effects is well-established, recent studies suggest that environmental responses can persist across multiple generations (Groot et al., 2016). However, challenges such as the inconsistency of transgenerational effects remain (Uller et al., 2013). Understanding these plant stress responses is crucial for developing biotechnological strategies, such as improving sustainable agricultural production under unpredictable environmental conditions (Mozgova et al., 2018).

Since the discovery of ozone layer depletion and the consequent increase in UV-B radiation penetrating the atmosphere, alongside its undeniable contribution to global warming, the effects of UV radiation on plants have been extensively studied as a current main environmental stressor. Plants use sunlight as an energy source for synthesizing carbon compounds and an environmental signal, triggering various systemic responses related to photosynthesis, phototropism, photoperiodism, and photomorphogenesis (Robson et al., 2014). The abnormal incidence of UV radiation in the atmosphere can disrupt these processes; thus, its impact has been a focus of research in recent decades.

1.1 Effects of UV Radiation in Plants

UV radiation is classified into three categories based on wavelength, being from least to most energetic as follows: UV-A (315-400 nm), UV-B (280-315 nm), and UV-C (100-280 nm) (Gupta et al., 2017). Although the most energetic, UV-C is mostly absorbed by the atmosphere due to its interaction with molecular oxygen and ozone, preventing them to reach the planet's surface. In contrast, UV-A and UV-B radiation penetrate the atmosphere and impact terrestrial plants (Heijde & Ulm, 2012; Hideg et al., 2013). Among these, UV-B radiation has garnered considerable attention due to its profound biological effects, ranging from DNA damage to changes in plant morphology and secondary metabolism (Robson et al., 2014; Jenkins, 2017). At sea level, around 7% of the solar radiation comprises UV, which is divided based on wavelength band ranges into: UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (100–280 nm) (Gupta et al., 2017).

However, all UV-C and UV-B radiation no less than 290 nm is absorbed in the atmosphere, allowing 95% of the UV photon irradiance reaching the Earth's surface falls within the UV-A (Rai et al., 2021). Although UV-B represents less than 1% of the total solar spectrum, it has a major effect on plant growth and development (Liu et al., 2015).

As mentioned above, ozone layer thinning has resulted in an increase of UV radiation, particularly UV-B on the Earth's surface, which has been recognized as one of the serious global environmental problems in the next few decades (Chen, 2012). This suggest that sunlight stress may represents a challenge not only for plant ecology but for crops production. However, the type of stress response induced by light is determined by the fluence rate, exposure time, and whether plants have been acclimated by prior exposure to light (Müller-Xing et al., 2014). For this reason, it is important to define the conditions of exposition to understand the role and effects of UV on plants.

As mentioned previously, natural UV-C radiation is absorbed in the atmosphere by ozone (O_3), water vapour, oxygen, and carbon dioxide. Therefore, UV-C light is not biologically significant to plants due to O_3 layer was fully formed around 1500 MY ago, obstructing the interaction between living beings and UV-C radiation since then (Robson et al., 2019). However, UV-C light has been identified as a harmful factor for cells. In Arabidopsis, the effects of exposure to UV-C radiation include the differential expression of genes involved in signaling pathways, and production and scavenging of reactive oxygen species (ROS) in defense-response to genotoxic stress conditions (Molinier et al., 2005). In *Allium*, it was observed growth inhibition, cytogenotoxicity, oxidative stress, and meristematic cell damages caused by this type of radiation (Çavuşoğlu et al., 2022). To date, no reports on a UV-C specific photoreceptor exist (Vanhaelewyn et al., 2020). However, some studies have speculated about the role of UV RESISTANCE LOCUS 8 (UVR8) (Christie et al., 2012) or phototropin1 (PHOT1) (Magerøy et al., 2010) in the perception and response of UV-C radiation.

1.1.1 Mechanisms of Plant Perception and Response to UV Stress

The effect of UV-B on plants is the most widely studied among UV radiation types, owing to their strong wavelength compared to UV-A. Some of the natural responses that UV-B induces in plants include photomorphogenesis (Gruber et al., 2010), acclimation responses and production of secondary metabolites (Casati & Walbot, 2003), and modulation in gene expression or protein accumulation (Tilbrook et al., 2013). UV-B perception is crucial for the regulatory response in plants. UVR8 is commonly described as a UV-B photoreceptor (Rizzini et al., 2011). UVR8 signaling is mainly nuclear and affects gene expression, triggering many different physiological responses. This protein is composed of 440 amino acids with seven bladed β-propeller domains that, in the absence of UV-B, exists as a homodimer held together by interactions between charged amino acids, arranged to form a ring structure (Ulm & Jenkins, 2015; Shi & Liu, 2021). UVR8 is formed by 14 tryptophan residues (6 residues are part of the β-propeller structure, 7 are located at the dimer interface, and 1 is in the C-terminal extension) of which W285 and W233 have a function as the chromophore for perception (Jenkins, 2014). In the presence of UV-B, UVR8 absorbs UV-B by using its tryptophans and undergoes into the active monomers by structural changes (Rizzini et al., 2011). Interestingly, UVR8 is mainly active in the nucleus of plant cells, although in its non-active form UVR8 localizes predominantly in the cytosol (Kaiserli & Jenkins, 2007). Indeed, UV-B activation results in the nuclear accumulation of UVR8, but their presence in the cytoplasm may suggests functions that remain unknown. Thus, the conserved function of UVR8 from green algae to angiosperms, highlights the key role of this protein in photoprotection for plants (Fernández et al., 2016). At the present, two different mechanisms of UVR8 signaling are known as: 1) The UVR8–COP1 pathway and 2) UVR8-interaction with specific transcription factors (TFs) (Podolec et al., 2021).

The UVR8-COP1 pathway involves different key regulatory factors. CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) is a negative regulator of plant photomorphogenesis in the dark and in visible light. Under those conditions, COP1 forms stable complexes with the four partially redundant SUPPRESSOR OF PHYA-105 (SPA) protein family members (Ordoñez-Herrera et al., 2015). COP1/SPA serves as an E3 ubiquitin ligase which ubiquitinates positive regulators of photomorphogenesis for degradation through 26S proteasome, mainly TFs, for example, the basic leucine-zipper ELONGATED HYPOCOTYL 5 (HY5) (Ponnu & Hoecker, 2021). In UV-B conditions, COP1 protects HY5 from proteasome degradation. This is achieved due to active UVR8 monomers which directly interact with COP1, allowing the conformation of a complex that enables COP1 to act as a positive regulator in the UV-B, thus facilitating the stability and activity of HY5 (Ulm & Jenkins, 2015). Back to no UV-B presence, REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and RUP2, interact with UVR8 and disrupt the physical contact of UVR8-COP1 complex, and redimerizing UVR8 into their inactive form (Heijde & Ulm, 2013).

The second mechanism involves the direct binding of UVR8 to specific TFs. Nuclear accumulation of UVR8 associates its function with the regulation of gene expression through modulation of TFs (Kaiserli & Jenkins, 2007). At the present, several proteins which interact with UVR8, moreover

COP1 or RUP1/RUP2, have been identified. Among these, the most well-studied are WRKY36 (Yang et al., 2018), v-Myb myeloblastosis viral oncogene homolog MYB13 (MYB) (Qian et al., 2020), MYB73/MYB77 (Yang et al., 2019), BRI1-EMS-SUPPRESSOR1/BES1-INTERACTINGMYC-LIKE 1 (BES1 and BIM1, respectively) (Liang et al., 2018) and PIF4/PIF5 (Tavridou et al., 2020).

WRKY transcription factor

WRKY proteins have highly conserved domains containing ≈60 amino acid long four-stranded β-sheet WRKY DNA binding domains and Zinc-finger motifs (Apoorva et al., 2021). WRKY DBD is mostly conserved, and it interacts mainly with W-box and other cis motif, providing binding specificity to WRKY TFs under different conditions (Phukan, Jeena and Shukla, 2016). The function of WRKY in plants are widely recognized to be involved in regulatory roles, controlling a broad range of biological functions such as abiotic-biotic stress responses, senescence, embryogenesis, seed development, hormonal regulation, among others. (Chen et al., 2012). In UV-B radiation, as described by Yang et al. (2018), the WRKY DNA-BINDING PROTEIN 36 (WRKY36) has a main role as TF. WRKY36 binds to the W-box of the HY5 promoter, repressing their expression in continuous visible light while, in response to UVB light, UVR8 physically interacts with WRKY36 in the nucleus and represses their binding to the HY5 promoter to promote HY5 transcription. UVR8 physically interacts with WRKY36 in the nucleus, to inhibit WRKY36's DNA-binding activity. Thus, WRKY36 acts as a transcription repressor of HY5, while UVB light induces the nuclear localization of UVR8.

MYB transcription factor

The v-Myb myeloblastosis viral oncogene homolog (MYB) is a large family of TF proteins. Most MYB proteins have different numbers of MYB domain repeats which confer their ability to bind DNA (Ambawat et al., 2013). MYB domain at the N-terminus and is an evolutionarily conserved present in nearly all eukaryotes, while in plants their functions have been related to the biosynthesis of secondary metabolites, DNA binding and interaction with other transcription factors (Cao et al., 2020). On the other hand, the variable C-terminal region is responsible for modulating the regulatory activity of the protein (Roy, 2016). Therefore, the MYB TFs are classified into four subfamilies: 1R-MYB, 2R-MYB, 3R-MYB, and 4R-MYB, based on the number of MYB domains (Dubos et al., 2010). In recent years, a couple of MYB proteins have been discovered that are related to UV-B perception.

Yang et al. (2019) demonstrated the role in plants of MYB73/MYB77 under UV-B light conditions. UVR8 is localized in both the shoot and root and is responsible for morphogenesis and root development. In the absence of UV-B light, MYB73/MYB77 positively regulates the expression of auxin-responsive genes in these tissues and promotes hypocotyl elongation and lateral root development/growth. However, in the presence of UV-B light, the monomerization of UVR8 is triggered, as mentioned previously. UVR8 monomers interact with MYB73/MYB77 in the nuclei, resulting in the inhibition of the DNA-binding activity of MYB73/MYB77, thus repressing the expression of auxin-responsive genes like INDOLE-3-ACETIC ACID INDUCIBLE 19 (IAA19). This UV-B light-dependent UVR8–MYB73/MYB77 interaction enables UV-B light and auxin signaling to coordinately regulate shoot and lateral root development in the plant.

In a more recent study conducted by Qian et al. (2020), MYB13 was identified as a positive regulator in UV-B-induced conditions. MYB13 directly interacts with photoactivated UVR8, which induces the expression of both auxin-responsive genes and flavonoid biosynthetic genes, via direct binding with their promoters, to positively regulate UV-B-induced cotyledon expansion. It is interesting to mention that UVR8 does not possess a DNA-binding domain, so UVR8 sequesters MYB13 from SMALL AUXIN UPREGULATED RNA (SAUR) promoters and represses SAUR expression, which enables UVR8 to be directly involved in transcriptional regulation. As exposure to UV-B is extended, MYB13 is activated and more MYB13 proteins probably accumulate free from UVR8 sequestration to activate SAURs in the cotyledons but not in the hypocotyls, leading to distinct developmental programs of these two organs. Although remains unclear, MYB13 and its targets may be present within a large protein–DNA complex together with UVR8, while UVR8-MYB13 can function as a UV-B-specific regulatory mechanism that facilitates plant growth and stress acclimation.

BES1/BIM1

Brassinosteroids (BRs) are a class of steroidal hormones that have a regulatory function in plant growth, development, photomorphogenesis, flowering, and abiotic and biotic stress responses (Nolan et al., 2020). These hormones are related to abiotic environmental conditions, for example, UV-B inhibits plant growth by repressing plant BR-promoted plant growth. UVR8 physically interacts with the functional dephosphorylated BES1 and BIM1 TFs, which mediate BR-regulated gene expression and plant growth, to inhibit their activities (Liang et al., 2018). BES1 is activated by a signaling cascade, initiated by the perception of BRs receptors including BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Tang et al., 2010). Subsequently BRI1 interacts with BSU1 phosphatase, inactivating the GSK3-like kinase BIN2 (Tang et al., 2008). Inactivation of BIN2 allows the accumulation of unphosphorylated transcription factors BES1 and BZR1 in the nucleus, which at the same time regulates BR target genes (He et al., 2005). Thus, UV-B-activated UVR8 inhibits the DNA-binding activities of BES1/BIM1, mediating BR signaling, to repress their DNA-binding activities and thus affect growth and photomorphogenesis (Liang et al., 2018).

PIF4/PIF5

The basic helix-loop-helix (bHLH) protein family is one of the largest transcription factor gene families in Arabidopsis. Named after its highly conserved alkaline/helix-loop-helix domain, composed of approximately 60 conserved amino acid residues and consists of two conserved motifs, is the second largest transcription factor family and is widely present in eukaryotes (Hao et al., 2021). Phytochrome interacting factors (PIFs) is a group of bHLH proteins that regulate external environmental and internal signals, for example, seed germination, high-temperature response, shade avoidance and the circadian clock (Sun et al., 2013). PIF4 and PIF5 have a key role in elongation growth, directly regulating the expression of several elongation-associated genes. It has been shown that UV-B antagonizes shade avoidance responses through the degradation of PIF4 and PIF5 (Hayes et al., 2017). Thus, UVR8 repress repression of several hypocotyl elongation-related genes is largely associated with UVR8-dependent degradation of PIF4 and PIF5, a process that consequently diminishes PIF4/5 target promoter occupancy (Tavridou et al., 2020). However, the mechanism by which UVR8 triggers their degradation remains unknown.

1.1.2 Plant Defense Against UV Radiation

UV radiation is a highly energetic fraction of sunlight. Among the subtypes of UV radiation, UV-B plays a crucial role in photomorphogenesis processes, influencing various biological responses, including the rate of photosynthesis, hypocotyl elongation, alterations in flowering time, reductions in petal and leaf size, and the induction of hormones and secondary metabolites. (Meyer et al., 2021). In plant sciences the term “stress factor” refers to any external condition that may cause changes in the biology of organisms, either biotic or abiotic. The exposure of plants to a stressor can lead to two different stress types: eustress (mild and usually temporal stress event, which helps the organism to acclimate or response to it) and distress (unfavorable environmental conditions, typically after acute eustress conditions, leading to metabolic damage or death of the whole organism) (Hideg et al., 2013). According to recent research, UV radiation is considered as a regulator of growth and development of plants, more than a stress factor (Jenkins, 2017). In some cases, responses to UV- B increase plant adaptation and, in addition, increase resistance to biotic stresses (Escobar-Bravo et al., 2017). In general terms, UV-B radiation can initiate a range of defense mechanisms in plants, including, but not limited to photomorphogenesis (light-mediated development), DNA repair mechanisms, MAPK kinase signaling, ROS scavenging systems (enzymatic, and non-enzymatic), photoprotective agents (phenolics and flavonoids compounds) and synthesis of stress-related hormones (salicylic acid, ethylene, etc.) (Meyer et al., 2021; Yeshi et al., 2022).

UV-C radiation can influence the biosynthesis of secondary metabolites, leading to an increase in these compounds, which act as scavengers for free radicals and help protect cells from radiation-induced damage (Schreiner et al., 2014). The production of secondary metabolites is interconnected, resulting in some compounds increasing while others may decrease in response to abiotic stress (Sharma et al., 2022). Many of these metabolites possess strong ROS scavenging abilities, offering protection against UV radiation. High doses of UV-C radiation negatively impact plant growth and development through several mechanisms: Increasing ROS levels and inducing oxidative stress; causing protein denaturation; reducing cell viability; and leading to cell death (Nawkar et al., 2013). Prolonged exposure to UV-C radiation (exceeding 30 minutes) causes irreversible physiological and morphological damage, ultimately resulting in plant death. However, low doses of UV-C exposure (up to 30 minutes) allow plants to partially recover their normal physiological functions and activate adaptive responses, such as enhancing both enzymatic and non-enzymatic defense systems (Katerova et al., 2008; Rai et al., 2011; Castronuovo et al., 2014). According to Jamwal et al. (2018), secondary metabolites can be classified into three primary groups: phenolic compounds, which are synthesized via the shikimate pathway; terpenes, produced through the mevalonate pathway; and nitrogen-containing compounds, generated within the tricarboxylic acid cycle pathway.

Plant phenolics including simple phenols, phenolic acids, flavonoids, coumarins, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins are the most abundant secondary metabolites, produced mainly through the shikimate pathway from L-phenylalanine and L-tyrosine, and containing one or more hydroxyl groups attached directly to aromatic ring (Kumar & Goel, 2019). Flavonoids, phenolic acids, stilbenes, coumarins, and tannins are the primary compounds used by plants for UV protection.

Plants produce phenolic compounds that act as a protective screen within the epidermal layer of cells, shielding tissues from harmful radiation and regulating antioxidant mechanisms at both the cellular and organismal levels. This process helps prevent mutations and cellular damage caused by thymine dimerization in DNA and the potential photodegradation of coenzymes like NAD and NADP. Flavonoids are particularly effective as UV screens due to their strong absorbance in the 250–270 nm and 335–360 nm ranges. Both flavonoids and phenolic compounds play a crucial role in UV protection. As described by Ferreyra et al. (2021), light or UV radiation are the main cause that change the amount of flavonoids compound in plant leaves, by activating the genes responsible for flavonoid biosynthesis.

Phenolics, specifically anthocyanin. flavonoids (kaempferol derivatives particularly), isoflavonoids or phenolic acid esters, and psoralens aggregate UV-B from entering the mesophyll. Flavonols and flavones, the major types of flavonoids present in the flowers, aggregate in the leaves, stems epidermal layers, and grab light intensely in the zone of UV-B without interfering with wavelengths of observable PAR, to protect cells from severe radiation of UV-B (Dehghanian et al., 2022).

Terpenes are secondary metabolites that plants naturally produce, categorized into monoterpenes, sesquiterpenes, diterpenes, triterpenes, and carotenes based on their isoprene structures (Ninkuu et al., 2021). The functions of only a few terpenoids are well understood; for example, isoprene has been shown to protect leaves from rapid heating caused by UV-B radiation (Tiiva et al. 2007). The biosynthesis of terpenes can be affected by environmental factors such as temperature, rainfall, and sunlight exposure, which often interact under natural field conditions (De Oliveira & Nieddu, 2016). UV-B radiation can activate plant defense mechanisms and stimulate the production of secondary metabolites, including terpenes and phenolics, while UV-A and visible light (400–700 nm) may work together to boost the production of UV-B-absorbing compounds, thereby enhancing the plant's resistance to UV-B radiation (Gil et al., 2012; Gregan et al., 2012; Liu et al., 2017; Miao et al., 2020).

In Arabidopsis, Badmus et al. (2022) reported how UV radiation affects the carotenoid profile of *Arabidopsis thaliana* plants over a ten-day period in a controlled environment. The findings showed that UV exposure leads to specific changes in carotenoid levels, such as increased antheraxanthin, neoxanthin, violaxanthin, and lutein in the leaves, with the extent of these changes depending on the duration of exposure. However, no specific UVR8 or UV-A (cryptochrome or phototropin) photoreceptor was identified as mediating these changes. Interestingly, the accumulation of violaxanthin was not linked to protecting the photosynthetic machinery from UV damage, suggesting the response's functional relevance may lie elsewhere. However, it is proposed that plants might use UV radiation as a signal for other environmental stressors, implying that the UV-induced changes in carotenoid profiles could be more about protecting against stressors like high-intensity visible light, which often accompanies UV radiation, rather than providing direct UV protection.

1.1.3 Changes at Morphological and Physiological Level

Plants have acquired different protective strategies to avoid the adverse effects of UV: the shielding by flavonoids and phenolic compounds, and the DNA repair such as photoreactivation and dark repair (Kimura et al., 2004). Photomorphogenesis (light-regulated plant development) under UV light has been extensively researched (Podolec et al., 2021). At the same time, these phenomena influence the phenotype and physiological structure of plants. Many species alter their organ development in response to UV light; for instance, stems generally shorten, the number of axillary buds increases, roots become longer and more numerous, decreasing of leaf area, abnormal thickening, or rolling (Robson et al., 2019).

Phenolic compounds, a large and structurally diverse class of plant secondary metabolites (PSMs), are characterized by the presence of one or more hydroxyl groups and are primarily synthesized by plants for stress protection. UV-C irradiation enhances the accumulation of these phenolic compounds, along with their associated antioxidant properties. This induction of phenolic compounds has been observed in lettuce plants (*Lactuca sativa* L.); however, leading to a significant reduction in growth (Lee et al., 2014). Similar findings were reported in *Silene littorea* (Del Valle et al., 2020).

Numerous studies have demonstrated that excessive sunlight and UV radiation affect the flavonoid content in plant leaves (Ferreyra et al., 2021). Researchers have identified polyphenols as crucial protective agents against UV radiation, acting both as physical shields and key biological components (Reboredo & Lidon, 2012). Larcher (1995) notes that phenolics, particularly anthocyanins which accumulate in the epidermis, function as a filter to darken and protect the mesophyll from intense radiation. Under stress, flavonoids (especially kaempferol derivatives), isoflavonoids, phenolic acid esters, and psoralens prevent UV-B from penetrating the mesophyll (Tamrat Alemu & Roro, 2020). Flavonols and flavones, which are predominant in flowers and accumulate in the epidermal layers of leaves and stems, absorb UV-B light while allowing visible PAR wavelengths to pass through, thereby safeguarding cells from excessive UV-B radiation

(Ferreyra et al., 2021). Exposure to UV-B has been shown to increase flavonol levels in *Picea abies* (Norway spruce) (Hammerbacher et al., 2020). Additionally, UV light has been found to transcriptionally activate the expression of chalcone synthase (CHS), a key enzyme in the flavonoid biosynthesis pathway (Loyall, 2000).

1.1.4 Damage to Molecules and DNA Repair Systems

Biological membranes consist primarily of phospholipids, and the main component of the plasma membrane undergoes degradation when exposed to ionizing radiation, leading to oxidative damage to the membrane. The ROS generated by IR can disrupt the membrane bilayer and cause damage to enzymes, nucleic acids, and proteins, resulting in lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFAs) (Juan et al., 2021). IR can also negatively impact chromatin lipids, lipid-rich membrane structures, and lipoproteins. A key stage in LPO is the formation of peroxyl radicals, which contributes to cellular dysfunction and the cross-linking of lipids (Ayala et al., 2014). The accumulation of polar products due to LPO affects ion transport across the membrane, and LPO-derived products play a role in the development of oxidative stress-related diseases and radiation sickness (Van Der Paal et al., 2016).

O_3 is the important component absorbing UV-B radiation in atmosphere. Enhanced use of environmental pollutants, such as chlorofluorocarbon gases, has led to significant ozone layer depletion. Consequently, increased levels of UV-B radiation now reach the Earth's surface, exerting harmful effects on plants, including genomic alterations, lipid peroxidation, oxidative stress, disruptions in biochemical pathways, and stunted growth (Inostroza-Blancheteau et al., 2016). At the molecular level, UV-B radiation induces damage to lipids, proteins, and nucleic acids. Notably, exposure to UV-B radiation generates multiple DNA photoproducts, which can alter DNA sequences and lead to mutations during replication (Wang et al., 2021). The most prevalent forms of DNA damage include the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs). Additionally, UV-B radiation can result in base pair deletions and insertions, DNA-protein crosslinks, and DNA strand breaks (Azarafshan et al., 2020).

Proteins within cells are vital targets for oxidative damage and can be directly or indirectly affected by IR through various mechanisms, such as oxidation, cleavage, carbonylation, protein cross-linking, amino acid conversion, and ROS-induced redox reactions (Xie et al., 2019). One example of protein oxidation induced by IR is the formation of mixed disulfide bonds between protein thiols and small molecules like glutathione, caused by the oxidation of cysteine side chains (Vašková et al., 2023). Cleavage of peptide bonds, such as through α-amidation or diamide formation, can result in the breakdown of protein derivatives. Carbonyl groups in oxidized proteins can lead to both inter- and intra-protein cross-linking. Exposure of proteins to IR leads to their degradation and functional loss (Kehm et al., 2021).

Under conditions of intense and prolonged stress, cell death can occur in plant tissues. Oxidative stress creates an imbalance in the cellular redox state, damaging biomolecules like proteins and lipids and causing mitochondrial depolarization, which ultimately triggers cell death through a series of reactions, as reviewed by Dowlath et al. (2021). Mitochondrial depolarization results in the release of cytochrome C into the cytoplasm through channels formed by proapoptotic proteins such as BAX and BAK. Cytochrome C then binds to APAF-1 proteins, forming a complex known as the apoptosome, which activates the caspase cascade (Düssmann et al., 2017). This activation leads to the fragmentation of DNA by DNase and the cleavage of cytosolic and nuclear proteins, ultimately causing cell death. Severe oxidative stress or IR-induced cellular damage can also trigger an alternative apoptosis pathway mediated by the p53 protein (Sueiro-Benavides et al., 2020). Further regulation of pro-apoptotic proteins, such as PUMA, activates BAX in the cytosol. The activated BAX then integrates into the mitochondrial membrane, opening the voltage-dependent anion channel, which releases cytochrome C. The release of cytochrome C initiates a cascade of proteins, leading to cell death (Rahmanian et al., 2016)

DNA is the most susceptible molecule to ionizing radiation. Damage induced by UV may damages genetic material, causing genotoxicity and hereditary effects (Dowlath et al., 2021). As reviewed in Nawkar et al. (2013), high UV-B radiation fluence rates can have detrimental effects on plants, such as ROS generation and DNA damage; whilst low fluence rates, may help plants to utilize UV-B as a signal for recovery of DNA damage and photomorphogenesis. Ionizing radiation causes damage to DNA either directly or indirectly. Directly, UV-B and UV-C radiation are strongly absorbed by DNA in the nucleus, mitochondria and chloroplast, causing several mutations (Vanhaelewyn et al., 2020). UV radiation produce photolesions such as pyrimidine dimers (occurring in TT, CC, TC, and CT contexts) such as CPDs, and 6–4 PP (Yagura et al., 2011). Indirectly, UV induces water radiolysis in the cells, producing hydroxyl radicals, a type of ROS, which at the same time can induce DNA-damage (Gill et al., 2015). Different genomic lesions can be produced by presence of ROS, for example oxidized purines and pyrimidines, point mutations, DNA single-strand breaks (SSBs), DNA double-strand breaks (DSBs), tandem base damage, and DNA–DNA/DNA–protein crosslinks (Kryston et al., 2011; Molinier, 2017). Radiation can also induce apoptosis or trigger a DNA repair mechanism. SSBs are often repaired by cellular mechanisms and hence have a limited chance of cell death or mutagenesis.

Two main DNA repair pathways exist in plant cells: homologous recombination (HR) and non-homologous end-joining (NHEJ). While these pathways are highly conserved across eukaryotes, their roles can vary depending on cell type and the cell cycle stage (Shen & Li, 2022). HR operates by restoring the genomic sequence using a template from the sister chromatid, or when unavailable, the homologous chromosome or nearby homologous repeats, enabling highly accurate repair (Siebert & Puchta, 2002). This makes HR an error-free mechanism. In contrast, NHEJ rejoins broken chromosomes without requiring sequence homology, which often introduces nucleotide changes, leading to deletions or, less frequently, insertions at the break site (Gorbunova & Levy, 1997). While unicellular eukaryotes tend to rely on HR due to their smaller genomes, NHEJ is more prevalent in higher eukaryotes, such as mammals and plants, which possess large, repetitive sequences. In the somatic cells of higher plants, NHEJ predominates, but when homologous sequences are accessible, either from sister chromatids or regions near the break, HR is employed for DSB repair (Steinert et al., 2016).

Prior to detailing repair mechanisms, it is essential to understand the cellular response to DSBs. The DNA damage response (DDR) involves a signaling process that activates metabolic pathways to repair the DNA. This response can alter cellular activity by inducing cell-cycle arrest, triggering DNA repair, or, if the damage is extensive, leading to senescence and apoptosis (Spampinato, 2017). The phosphatidylinositol 3-kinase-like protein kinases (PIKKs) family plays a key role in the DDR, controlling the signaling cascade through the recruitment and phosphorylation of substrates (Block et al., 2004). Although DDR has been extensively studied in animal models, significant differences exist in plant orthologs, influencing their mechanisms of action. Shen & Li (2022) noted that while many key DDR pathway components have been identified in plants, the overall response remains less well understood. This gap may be attributed to the evolutionary history of plants, marked by gene duplications and their sessile nature, which necessitates a more refined DNA repair system.

In both plants and animals, the primary DNA damage signaling is mediated by Ataxia Telangiectasia Mutated (ATM), and ATM and Rad3-related (ATR). ATM responds mainly to DSBs, while ATR is activated by single-stranded DNA damage and replication fork issues (Nisa et al., 2019). In DSBs, the MNR complex activates ATM signaling, while ATR is recruited to single-stranded DNA via replication protein A (RPA) proteins (Maréchal & Zou, 2013). ATM and ATR phosphorylate suppressor of gamma response 1 (SOG1), the central regulator in the DDR (Yoshiyama et al., 2009). Once activated, SOG1 governs the expression of genes involved in DSB response, leading to cell-cycle arrest or programmed cell death, or activating DNA repair (Bourbousse et al., 2018). The choice between HR and NHEJ depends on the cell cycle phase and the extent of DNA end resection (Ceccaldi et al., 2016).

HR is noted for its precision, maintaining genome stability by facilitating error-free repair of DSBs, interstrand crosslinks, and DNA gaps (Heyer et al., 2010). This pathway occurs predominantly during the S/G2 phases of the cell cycle when sister chromatids are available as homologous templates (Karanam et al., 2012). In both animals and plants, HR activity decreases in G2/M and is nearly absent in G1 (Mao et al., 2008). HR involves four key steps: DNA end resection and nucleoprotein filament formation, homology search and strand invasion, extension of the DNA heteroduplex, and resolution of the D-loop through synthesis-dependent strand annealing (SDSA) or Holliday junction (HJ) resolution (Spampinato, 2017). The key feature of HR is the use of homologous sequences as templates, with Rad51 playing a crucial role in homology search and strand invasion by forming nucleoprotein filaments on single-stranded DNA (ssDNA).

Initially, DSBs are processed to generate ssDNA overhangs through 5' to 3' end resection involving the Exo1 and MRN complex (Zhao et al., 2020). These ssDNA overhangs are coated with RPA to prevent degradation and the formation of secondary structures, allowing Rad51 filament formation (Chen et al., 2013). Rad51 facilitates homology search and strand invasion, creating a D-loop structure. Rad54 assists in this process by promoting strand invasion and homology search, while DNA synthesis is initiated by DNA polymerase δ, PCNA, and replication factor C (Tavares et al., 2019). In plants, HR may proceed through SDSA, involving helicase-mediated strand displacement, or DSBR, characterized by the capture of the second DSB end, forming a double HJ structure that is resolved by specialized resolvases (Spampinato, 2017).

In contrast, NHEJ is the primary DSB repair mechanism in higher plants, functioning efficiently due to repetitive sequences in their genomes. NHEJ operates throughout the cell cycle, particularly during G1 and G2, and does not require a homologous sequence for DNA repair (Manova & Gruszka, 2015). However, it is prone to errors, often resulting in deletions, mutations, and, less frequently, insertions (Lieber, 2010). The NHEJ pathway is subdivided into canonical (cNHEJ) and alternative NHEJ sub-pathways (Manova & Gruszka, 2015). In plants, cNHEJ begins with Ku70/Ku80 heterodimer binding to the DSB ends, protecting them from resection. This promotes the recruitment of DNA-PKcs, Artemis, PNKP, Pol μ, Pol λ, and the MRN complex (Mannuss et al., 2012). These factors enable end processing and final ligation by the XRCC4-Ligase 4 complex (Rulten et al., 2011; West et al., 2000).

The a-NHEJ pathway involves 3' end resection using HR end-resection machinery, leading to microhomology-mediated junctions between single-stranded overhangs (Lieber, 2010). Genetic information may be lost at the junction site due to trimming and rejoining of the DNA ends. Although the molecular details of a-NHEJ initiation are not fully understood, the MRN complex and PARP1 are key players. PARP1, which is highly expressed in seeds, likely plays a role in DNA damage response during dormancy and germination (Manova & Gruszka, 2015; Deriano & Roth, 2013). Furthermore, PARP1 has a well-established role in base excision repair (BER) and SSBR repair, recruiting XRCC1-Ligase 3 to stimulate repair and competing with Ku proteins and ATM for binding to DNA ends (Haince et al., 2008; Aguilar-Quesada et al., 2007). Without Ku70/Ku80 protection, DNA ends are resected by Mre11 and CtIP, with PARP1 serving as a platform for recruiting DNA polymerase θ for microhomology-based repair. Ultimately, XRCC1 and Ligase I (since plants lack a Ligase 3 homolog) contribute to DSB ligation (Bétermier et al., 2014).

In the context of UV stress, as reported by our research group (Boyko et al., 2006a) while strand breaks are not the primary form of DNA damage caused by UVB, the repair of dimers can lead to the temporary formation of strand break. These breaks, which occur during early developmental stages, are more likely to be repaired through HR. Furthermore, younger plants tend to rely more heavily on HR repair mechanisms compared to older plants. Alternatively, plants in their early developmental stages may be more sensitive to UVB radiation due to lower levels of protective pigments, such as anthocyanins, and/or thinner secondary cell walls in their epidermal cells. In fact, several studies suggest that younger plants possess less effective defenses against various types of stress (Boyko et al., 2006b).

Photoreactivation is another strategy widely conserved among plants to avoid the adverse damage of UV, which some organisms, such as placental mammals have lost through evolution (Kimura et al., 2004). Photoreactivation is a light-dependent DNA repair mechanism in plants that helps reverse UV-induced damage, particularly CPDs and 6-4PPs. This process is catalyzed by specific enzymes known as photolyases (Strzałka et al., 2020). When plants are exposed to UV radiation, CPDs and 6-4PPs form in their DNA, which can disrupt replication and transcription. During photoreactivation, photolyases bind to these damaged sites and, upon absorbing light (usually in the blue and UV-A range; 320–480 nm), they use the energy to break the abnormal bonds between pyrimidine bases (Britt et al., 1996). This restores the DNA to its original, undamaged state. Photoreactivation is a crucial repair mechanism in plants, especially those exposed to high levels of sunlight, as it enables them to efficiently reverse UV-induced DNA lesions and maintain genomic stability. As reviewed by Vechtomovan et al. (2021), these photolyases belong to the DNA photolyase/cryptochrome family. Cryptochromes (CRYs), which are protein receptors sensitive to near-UV and blue light, are related to photolyases phylogenetically but have distinct roles. Unlike photolyases, CRYs regulate the transcription of various genes through light-dependent mechanisms. Proteins in the CPF family share a similar structure and each contains two non-covalently bound chromophores in a fixed ratio. These are monomeric, globular proteins with two distinct domains: an N-terminal α/β domain and a C-terminal α-helical domain. The C-terminal α-helical domain across all family members includes: (1) a conserved amino acid sequence, (2) a binding site for the flavin chromophore, and (3) a substrate binding site (for DNA photoproducts in DNA photolyases and ATP in cryptochromes).

The reaction mechanism of DNA photolyase involves classical enzymatic catalysis with a light-dependent stage (Vechtomovan et al., 2021). In the initial phase, the enzyme attaches to the damaged DNA and inserts it into the active site, forming a stable enzyme-substrate complex, which does not require light. The binding site is located in a pocket on the surface of the α-helical domain. At the base of this pocket, FAD is present in a unique U-shaped conformation. This conformation is complementary to the damaged site, allowing for strong binding of CPD (with a binding constant of 10^{-9} M) and facilitating the extrusion of the damaged sites from the DNA double helix. In the second stage, an antenna molecule absorbs a photon of UVA or blue light and transfers the excitation energy to $FADH^-$. This energy enables $FADH^-$ to donate an electron to the CPD or 6-4PP, thereby repairing the DNA. Following the repair, the electron returns to $FADH^-$, regenerating its original form. This entire photocatalysis process takes place in approximately 1.2 nanoseconds. In the final stage, the repaired DNA, now with reduced affinity for the photolyase binding site, dissociates from the enzyme.

Furthermore, a recent study by Nakamura et al. (2021) reported the development of a supF-based system for the detection of a broad spectrum of mutations in the chromosomal DNA of Arabidopsis. In their study, supF-transgenic plants subjected to UV-C radiation (500 and 1000 J/m^2) led to a significant rise in mutation frequencies, significantly increased mutation frequencies (26- and 45-fold, respectively). G:C to A:T transitions (43–67% of base substitutions) dominated in the mutation spectrum and were distributed throughout single, tandem, and multiple base substitutions. However, most of these mutations disappeared after UV-irradiated plants were exposed to white light for photoreactivation.

These results indicated that not only G:C to A:T single base substitutions, but also tandem and multiple base substitutions were caused by two major UV-induced photoproducts, CPDs and pyrimidine (6-4) pyrimidone photoproducts (6-4 PPs). In contrast, a high proportion of A:T to T:A transversions (56% of base substitutions) was a characteristic feature of the mutation spectrum obtained from photoreactivated plants. CPDs are the predominant UV-induced DNA lesions in plants, accounting for approximately 75% to 90% (Dany et al., 2001). Both CPDs and 6-4PPs can obstruct transcription and replication processes and lead to fork collapse, which can in turn cause DSBs (Gill et al., 2015). Furthermore, UV radiation can trigger the production of ROS, leading to oxidative damage, as well as DNA–DNA and DNA–protein crosslinks (Garinis et al., 2005). These results indicate the presence of UV-induced mutation characteristics and offer insights into the DNA repair mechanisms in plants, and it establishes a significant reference framework for further studies investigating the impact on genomic stability in plants.

1.2 Genetic Variations in DNA and Their Role in Plant Adaptation: SNPs and InDels

Single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) are two primary forms of genetic variation that significantly impact plant genetic diversity and adaptation to environmental stress. SNPs are variations at a single nucleotide position within a DNA sequence. These variations are the most prevalent form of genetic diversity across plant genomes and are key in differentiating individual plants within a species. SNPs can affect a broad range of traits, including morphological characteristics, physiological processes, and stress tolerance (Huq et al., 2016). For example, SNPs may influence the expression or function of stress-responsive genes, thereby modulating physiological and biochemical reactions to stress. Due to their abundance and stability, SNPs are valuable for constructing genetic maps, identifying quantitative trait loci, and conducting association studies to uncover genes associated with specific traits (Wang et al., 2019). The identification and characterization of SNPs provide valuable insights into the genetic mechanisms underlying stress tolerance. By analyzing SNPs, researchers can uncover genetic markers linked to stress resistance traits, which can then be utilized in marker-assisted selection to breed stress-resilient varieties. Furthermore, the study of SNPs contributes to our understanding of the evolutionary processes that drive adaptation to changing environments.

InDels involve the addition or loss of nucleotide sequences in the genome. These variations can range from 1-20 bp, but up to 1000 bp. InDels can disrupt coding sequences, potentially leading to frameshift mutations, altered protein products, or changes in regulatory regions (Lin et al., 2017). In plants, SNP polymorphisms are present at a high density throughout the genome (Ching et al. 2002). For instance, in the Nipponbare rice genome, an average of 0.64 SNPs per kilobase (kb) have been detected (Jeong et al. 2013), while in the tomato genome, an average of 6.1 SNPs per kb was observed (Kim et al. 2014). The presence of InDels can affect gene dosage and contribute to phenotypic diversity by influencing traits such as growth, development, and stress tolerance (Alonge et al., 2020). Both SNPs and InDels play crucial roles in plant adaptation to environmental stress. SNPs represent the most prevalent type of genetic variation. Due to their high frequency, SNPs are the most frequently genotyped polymorphisms in crop breeding and comparative studies (Mammadov et al., 2012). In contrast, InDels are also common forms of genetic variation; however, because INDEL events can overlap each other, it becomes increasingly challenging to discern the precise boundaries of individual INDEL occurrences over evolutionary timescales (Graham et al, 2020).

While SNPs have numerous applications, including genetic mapping, genotyping, and serving as potential genetic markers, for purposes of this study's objective it is relevant to focus on their role in evolutionary studies. In this context, as reviewed by Huq et al. (2016), SNPs can be valuable tools in evolutionary genome studies, providing insights into population history, and the impact of breeding systems and selection on genetic variation. SNPs are commonly used to examine sequence variation across species, and since such variations occur throughout evolutionary processes, they can shed light on how modern genomes have evolved. Traditional markers for evolutionary studies, like simple sequence repeats (SSRs) and mitochondrial DNA, can sometimes lead to misinterpretation due to homoplasy (Morin et al. 2004). This issue can be avoided by using SNP markers, which represent single-base nucleotide substitutions (Vignal et al. 2002). Numerous successful studies have utilized SNPs to explore gene evolution, such as WAG-2 in wheat (Wei et al. 2011). In Arabidopsis, recent research has found over 3 million markers from 2029 accessions and over 3 million markers. Moreover, using these imputed datasets, Arouisse et al. (2020) reanalyzed genome-wide association studies (GWAS) on five stress-related traits, leading to the identification of new candidate genes associated with stress tolerance.

Several studies have reported the adaptative role of genetic variations in Arabidopsis in a number of stress types: drought (Lasky et al., 2014; Exposito-Alonso et al., 2018; Monroe et al., 2018), heat (Lu et al., 2021; Yadav et al., 2022), cold (Lasky et al., 2014; Rahman et al., 2024), salinity

(Deolu-Ajayi et al., 2019; Casellas et al., 2022), submergence tolerance (Vashisht et al., 2011), herbivory (Kerwin et al., 2015), and ionizing radiation (Belfield et al., 2012; Piofczyk et al., 2015; Schulz et al., 2021).

SNP/INDEL represent a valuable source of genetic variability as drivers of new genes or allelic variants, which might be selected by natural or artificial ways when resultant phenotypes exhibit advantageous traits (Muñoz-Espinoza et al., 2020). SNP is a variation at a single position in DNA sequence among individuals of same species. SNPs are the most common DNA polymorphisms in genome sequences of human, animals, and plants and they are thought to play a major role in the induction of phenotypic variations (Morgil et al., 2020). Among all DNA markers, SNPs are the most abundant and available for multiple assay options using different technology platforms to meet the demand for genetic studies and molecular breeding in crop plants (Govindaraj et al., 2015). SNPs also can identify the genomic diversity of species to demonstrate the speciation and evolution, and associate genomic variations with phenotypic traits (Govindaraj et al., 2015). SNPs can be used for evolutionary studies of genome that can reveal about population history.

Generally SNP is used for study of sequence variation among species and provide an understanding of how modern genome has evolved, specially across adaptative events, through whole genome sequencing (WGS) (Huq et al., 2016). The commonly used markers for evolutionary studies are SSRs (simple sequence repeats) and mitochondrial DNA which may be misinterpreted due to homoplasy (Wheeler et al., 2014). It is possible to avoid this problem by using SNP markers that represent single base nucleotide substitutions (Kumar et al., 2012). Many successful reports are already published about the use of SNPs to study the evolution of genes such as WAG-2 (wheat AG-2) in wheat (Wei et al., 2011). The developments in the sequencing technologies have resulted in decreasing cost along with rapid progress in next-generation sequencing (NGS) and related bioinformatic computing resources. These developments accelerated the whole-genome association studies (WGAS) and the identification of many new SNPs in model and non-model plants. In the post-genomic era, SNPs became the commonly used marker systems in many plants with several advantages such as stability, ease of use, considerably low mutation rates, and high-throughput genotyping (Morgil et al., 2020).

1.3 Epigenetic Regulation in Plants

Epigenetics is defined by three fundamental characteristics: it does not entail changes to the DNA sequence, it is heritable, and reversible (Cao & Chen, 2024). These changes are either mitotically long-term stable or may be heritable changes in gene activity and expression, meiotically heritable, for several future generations despite the fact that they do not cause any change in the core DNA sequences of an organism (Bonasio et al., 2010; Sudan et al., 2018). In general, changes to the epigenome result in transcriptional activation or repression, thereby altering gene expression patterns, (Molinier, 2017). In plants, most epigenetic studies are related to the adaptation of plants to biotic/abiotic stress, for example, heat, cold, drought, salinity, UV radiation and predators (Kinoshita & Seki, 2014; Chang et al., 2019). However, these changes do not occur only under stress, and they can continue over generations as creating a memory (Boyko and Kovalchuk, 2008). Epigenetic stress memory can be divided in three classes: 1) “short-term stress memory” but persistent changes in histone variants, histone modification, non-heritable and/or DNA methylation that influence future transcription responses; 2) “long-term stress memory,” mitotically heritable changes; and 3) “transgenerational stress memory,” mitotically and meiotically heritable changes (Müller-Xing et al., 2014). Epigenetic processes generally involve DNA methylation, histone modifications, and RNA-mediated gene silencing, which together constitute the epigenome (Bernstein et al., 2007).

Building on this understanding, epigenetic modifications represent a flexible and dynamic means through which plants respond to environmental challenges. Furthermore, epigenetic modifications are particularly significant in agronomy, as they may provide crops with a mechanism to adapt to environmental stresses such as drought, salinity, and pathogen attacks. Understanding the epigenome adds a "second layer of regulation" beyond classical genetics, offering the potential to significantly expand our knowledge of plant genetics and development. Three primary mechanisms drive these epigenetic processes in plants, which will be discussed in detail in the following sections: DNA methylation, histone modification, and RNA-directed DNA methylation (RdDM).

1.3.1 DNA Methylation in Plants

Ultraviolet radiation has been shown to induce significant changes in the DNA methylation patterns of plants, with varying effects depending on the wavelength and intensity of exposure. DNA methylation is an epigenetic modification involving the addition of a methyl group to the cytosine base of DNA, typically at CpG, CHG, and CHH sequences (where H represents A, T, or C), which can regulate gene expression by altering chromatin structure and accessibility (Lucibelli et al., 2022).

Experimental evidence has suggested that plants have evolved specific mechanisms to counteract the negative effects of UV radiation on DNA methylation. For example, the increased expression of DNA repair genes and antioxidant enzymes under UV stress conditions helps mitigate DNA damage and maintain methylation patterns (Chatterjee & Walker, 2017). These protective mechanisms are critical for plant survival and adaptation in environments with varying UV radiation levels.

UV-C radiation, although does not typically affect plants under natural conditions, in controlled environments, UV-C exposure has been found to cause substantial DNA damage and subsequent changes in methylation. A study by Melamed-Bessudo & Levy (2012) indicated that UV-C radiation resulted in extensive genome-wide hypomethylation in maize, disrupting normal gene expression and cellular functions. Overall, the differential effects of UV-A, UV-B, and UV-C on DNA methylation in plants underscore the complexity of plant responses to UV radiation. While UV-B appears to have the most significant and direct impact on methylation patterns, UV-A influences methylation through oxidative stress, and UV-C, under artificial conditions, causes widespread hypomethylation.

DNA methylation refers to the addition of a methyl group to the fifth carbon (5´) of the cytosine ring (Liu & Lang, 2020). This phenomenon is important to many biological processes and are crucial for organism development (Zhang et al., 2018). In plants, DNA methylation plays a role in silencing genes and transposons, and in developmental regulation by silencing genes, such as those that control flowering time, fertility, and leaf morphology, and also regulating imprinting (Ito, 2014; Satyaki & Gehring, 2017).

While cytosine methylation is mostly limited to CG dinucleotide sequence contexts in mammalian genomes, in plants, cytosines are methylated in two ways symmetrical (CG or CHG) or asymmetrical (CHH, where H is A, T, or C) throughout their genomes (Cokus et al., 2008). According to Cokus et al. (2008) DNA methylation levels in plants of approximately 24%, 6.7% and 1.7% are observed for CG, CHG, and CHH contexts, respectively. Is important to mention that, unlike in mammals, DNA methylation in plants predominantly occurs on transposons and other repetitive elements (Zhang et al., 2006). This chemical change contributes to the epigenetic regulation of nuclear gene expression and to genome stability. According to Ghanbari Moheb Seraj et al. (2019), DNA methylation is catalyzed by a large family of enzymes called DNA methyltransferases (MTases). There are two main types of methylation mechanisms in plants: *de novo* methylases and maintenance methylases.

In plants, *de novo* DNA methylation is mediated through the RNA-directed DNA methylation (RdDM) pathway, which involves small interfering RNAs (siRNAs) and scaffold RNAs in addition to an array of proteins (Zhang et al., 2018). RdDM plays a key role in silencing DNA TEs in plants by adding DNA methylation over new TE insertions and constantly reinforcing DNA methylation over existing TEs, inhibiting transposition, and maintaining long-term genome stability (Sigman & Slotkin, 2015). RdDM is also the only pathway capable of adding DNA methylation de novo to previously unmethylated regions in plants (Matzke & Mosher, 2014) Continuous activity by RdDM opposes the spread of active chromatin, maintaining a silent, repressive heterochromatic state over TEs in these otherwise euchromatic regions (Erdmann & Picard, 2020). However, excessive RdDM activity can also cause the silent of nearby genes and repress them, with potentially harmful consequences for the organism. Therefore, RdDM activity must be punctually regulated to maintain a balance between repressing TEs and allowing expression of nearby genes (Sigman & Slotkin, 2015).

In this context, according to the understanding of these mechanisms, literature distinguished between two process of RdDM: the canonical and non-canonical pathway (Zhang et al., 2018). The canonical RdDM pathway is the most well-characterized and makes up the majority of RdDM activity in a cell (Cuerda-gil & Slotkin, 2016). Canonical RdDM preferentially recruits to regions that are already DNA-methylated, thus acts to reinforce existing DNA methylation patterns, forming a positive feedback loop (Cuerda-gil & Slotkin, 2016; Matzke & Mosher, 2014). This pathway can be split up into two main processes, which ultimately lead to DNA methylation: 1) production of sRNAs and 2) recruitment of DNA methylation machinery by those sRNAs to specific target loci in the DNA. These two activities together comprise RdDM, and ultimately lead to DNA methylation being added to cytosines at specific target loci. (Erdmann & Picard, 2020).

The process for canonical RdDM are well described in Wambui Mbichi et al. (2020) and Erdmann and Picard (2020). For the first step, sRNAs are generated in a process directed by RNA Polymerase IV (Pol IV), which is recruited to silent heterochromatin via its interaction with CLASSY (CLSY) proteins and SAWADEE homeodomain homolog 1 (Law & Jacobsen, 2010). These binds to histone H3K9me and unmethylated H3K4 and transcribes single stranded RNA at the target (Matzke & Mosher, 2014). RNA Pol IV transcribes these regions to produce short single-stranded RNAs (ssRNAs) roughly 30 to 45 nucleotides in length, each of which is the precursor for a single sRNA. These ssRNAs are converted into double-stranded RNAs (dsRNAs) co-transcriptionally by RNA-directed RNA polymerase 2 (RDR2), which physically associates with

RNA Pol IV (Blevins et al., 2015). Then DCL3 cleaves the double stranded RNA into 24 nucleotide siRNA which are made stable by HUA ENHANCER 1 (HEN1) (Haag et al., 2012); Matzke & Mosher, 2014). SUVH2 and SUVH9 are SET and RING- ASSOCIATED (SRA) domain proteins and members of the SU (VAR) 3-9 histone methyltransferase family involved in recruiting Pol V via their SRA domains which bind methylated DNA (Johnson et al., 2004).

In the second part of the pathway, the RdDM DNA methylation machinery is guided to DNA sequences complementary to the sRNAs generated in the first part of the pathway. One strand from each 24 nt double-stranded sRNA is loaded into AGO proteins AGO4, AGO6, or AGO9 (Matzke & Mosher, 2014). AGO3 may also be able to function in this pathway (Zhang et al., 2016). Once formed, the AGO-sRNA duplex finds and binds complementary sequences along an RNA 'scaffold' produced by the plant-specific RNA Polymerase V (Pol V), with the help of interactions with Suppressor of Ty insertion 5-like (SPT5L), the Involved in de novo 2—IDN2 Paralog (IDN2-IDP) complex, and the Pol V subunit NRPE1 (Wierzbicki et. al, 2008). This leads to the recruitment of the DNA methyltransferase enzyme Domains Rearranged Methyltransferase 2 (DRM2), which methylates nearby DNA (Cuerda-gil & Slotkin, 2016) . The mechanism by which the AGO-sRNA duplex recruits DRM2 is not yet well understood (Gallego-Bartolomé et al., 2019).

At the same time, several types of non-canonical RdDM pathways have been reported, including Pol II-DCL3 RdDM pathway, RDR6 RdDM pathway, RDR6-DCL3 RdDM pathway, Pol IV-NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD) RdDM pathway, and dicer-independent RdDM pathway (Liu & He, 2020). The primary difference between the canonical and non-canonical RdDM pathways lies in the origin and biogenesis of the sRNAs involved. These pathways utilize DCL2, RDR2, AGO4, Pol IV and Pol V and 21–22 nt siRNA and the non-canonical RdDM pathways involve 21–22 nt sRNAs from a variety of sources, allowing *de novo* DNA methylation to be initiated at many different types of loci (Marí-ordóñez et al., 2013). Unlike canonical RdDM, the non-canonical pathways are generally involved in establishing initial DNA methylation at new target loci, like novel TE insertions, rather than maintaining existing heterochromatin. Some pathways of RdDM have emerged different from the canonical RdDM pathway which was discovered in relation to silencing virus infections too. However, since the non-canonical pathways are not well characterized yet, there likely remain additional sources of sRNAs used for RdDM that have not yet been uncovered (Erdmann & Picard, 2020).

On the other hand, the maintenance mechanism of methylation depends on the cytosine sequence context and is catalysed by DNA methyltransferases, as mentioned previously (Zhang et al., 2018). In plants, DNA methylation is maintained in three different contexts by three different pathways. CG, CHG and asymmetric CHH methylation are maintained by METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3)/CMT2, DRM2/CMT2, respectively (Liu & He, 2020). CG methylation is the most predominant, present both in heterochromatin and genic regions, unlike CHG and CHH methylation, which are predominantly present in heterochromatic regions (Wambui Mbichi et al., 2020). Maintenance of asymmetric methylation may also be affected by MET1 and CMT3 because MET1-dependent methylation can be recognized by SUVH2 and SUVH9 for recruitment of POL V at some RdDM loci. Furthermore, CMT3-dependent CHG methylation facilitates CMT2-catalysed non-CG methylation (Stroud et al., 2013). Although CHH cytosines can be methylated only by DRM2 and CMT2, these two enzymes can also methylate cytosines in other context (Zhang et al., 2018).

The genome-wide DNA methylation changes induced by abiotic stresses differ according to dose and exposure time to stress, the developmental stages, sampled tissues, genotypes and species (Liu & He, 2020). In molecular diversity context, is important to detect positions or regions that are differentially methylated due to treatment or different environments, and multiple samples are required since differentially methylated positions (DMPs) are called using statistical approaches (Can et al., 2021). Also, differentially methylated regions (DMRs), are genomic regions where multiple adjacent positions reveal differential methylation (Tam et al., 2019). Some studies have highlighted the role of DMPs/DMRs in plant adaptation. A study in cucumber, cold stress imposes a substantial and global impact on TE-related RdDM, leading to the demethylation of ^{m}CHH. Besides, cold-induced DMRs may be involved in the regulation of genes in ethylene biosynthesis and signaling (Lai et al., 2017). In rice, drought stress induces differential 5mC methylation alterations in drought-tolerant variety and drought-sensitive variety (Wang et al., 2016). Also, the DMR-associated genes in drought-tolerant line DK151 are mainly involved in stress response, programmed cell death, and nutrient reservoir activity, which may contribute to the constitutive drought tolerance. According to Zheng et al. (2017), a high proportion of multi-generational drought-induced alteration in DNA methylation is maintained in across generations. This may be explained by the offspring improved drought adaptability in rice. However, inheritance of DMRs induced by abiotic stress and their contribution to the transgenerational inheritance requires further investigation. In this context, it is important to map causal DMRs underlying specific phenotypes or stress tolerance in plants.

1.3.2 Histone Modifications

Histone modifications play an essential epigenetic role in plant evolution, regulating gene expression and maintaining genome stability. UV-A radiation has been shown to induce significant changes in histone methylation patterns in plants. In *A. thaliana*, UV-A exposure has been observed to cause an increase in histone H3 lysine 4 trimethylation (H3K4me3), a marker associated with active transcription (Zhang et al., 2009). This modification was particularly noted in genes involved in stress responses and DNA repair, suggesting a role for H3K4me3 in facilitating rapid transcriptional activation under UV-A stress conditions. Furthermore, UV-A exposure has been found to enhance the trimethylation of H3K4me3, promoting the expression of antioxidant defense genes (Jansen et al., 2012). However, in rice, UV-A radiation has been observed to reduce the levels of repressive histone marks such as H3K27me3, leading to the de-repression of certain stress-responsive genes (Chen et al., 2019).

In maize, UV-A radiation causes genome-wide alterations in histone methylation patterns, including both H3K4me3 and H3K27me3, affecting a broad range of genes involved in growth and development (Zhao et al., 2020). These findings align with the observations in Arabidopsis and rice, highlighting the conserved role of histone methylation in mediating UV-A responses across different plant species. In contrast, research on rice (*Oryza sativa*) revealed that UV-A radiation can also result in the demethylation of histone H3 lysine 27 (H3K27me3), a repressive mark, leading to the de-repression of certain stress-responsive genes (Cheng et al., 2018). This indicates a complex regulatory mechanism where UV-A exposure can both activate and repress gene expression through histone methylation changes. The differential effects observed in

Arabidopsis and rice underlines the species-specific responses to UV-A-induced histone methylation.

Further studies on maize (*Zea mays*) demonstrated the effect UV-A radiation on genome-wide alterations in histone methylation patterns, including both H3K4me3 and H3K27me3, affecting a broad range of genes involved in growth and development (Zhao et al., 2020). These findings align with the observations in Arabidopsis and rice, highlighting the conserved role of histone methylation in mediating UV-A responses across different plant species. However, inconsistencies exist in the specific patterns and extents of histone methylation changes induced by UV-A radiation. For instance, while Arabidopsis and maize show increased H3K4me3 levels, rice exhibits a reduction in H3K27me3, suggesting that the balance between activating and repressive histone marks can vary significantly between species. This variation may be attributed to differences in the expression and activity of histone methyltransferases and demethylases, as well as the distinct regulatory networks governing stress responses in different plants.

In eukaryotes genomic DNA is enveloped by a complex known as chromatin. Chromatin is a structure that regulates the accessibility of transcription factors in all DNA-templated process. The basic unit of chromatin is the nucleosome, which is composed of approximately 146 base pairs of DNA wrapped on a histone octamer (containing two copies of each of the four histone proteins H2A, H2B, H3, and H4) (Liu et al., 2010). Histones are basic proteins,

rich in lysine and arginine residues, which facilitate inter- action with the negatively charged DNA through electrostatic interactions (Probst et al., 2020). As a dynamic platform, chromatin integrates signaling information from the cellular environment; stores and perpetuates heritable epigenetic information and influences accessibility of the cellular machinery to DNA during transcription, replication, recombination, and repair (Probst et al., 2020). Nucleosomes are connected to others via linker DNA of variable length, which can be bound by the linker histone H1 (Fyodorov et al., 2018).

According to the requirements of a dynamic stage for epigenetic information, chromatin structure is modified at different levels. In this context, histone modifications are also highly important to regulate the expression and structure of DNA chromatin. Histone acetylation and methylation are recognized as two types of important epigenetic marks in gene expression (Xu et al., 2016). Thus, histones are subject to several post-translational modifications including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation. Histone acetylation and methylation are now recognized as two types of important and ubiquitous epigenetic marks in gene expression (Xu et al., 2016).

Histone methylation is one of the most important epigenetic modifications for histones. This requires a set of enzymes for adding or removing and recognize the covalent methylation marks; this enzymes are called “writers”, “readers” and “erasers” (Liu et al., 2010). The writers are responsible for stablished the methyl groups in histone proteins. As one of the most complex modifications, it occurs at different residues like lysine and arginine, distinct sites and even differs in the number of methyl groups added (Liu et al., 2010). In *Arabidopsis*, histone lysine methylation occurs mainly at Lys4 (K4), Lys9 (K9), Lys27 (K27), and Lys36 (K36) of histone H3 (Kouzarides, 2007). These modifications are written by different histone lysine methyltransferases (HKMTs) (Johnson et al., 2004). Most of the histone lysine methylation is catalyzed by a family of SET domain-containing proteins in *Arabidopsis* and rice (Liu & Paroo, 2010). There are 49 SET domain-containing proteins in *Arabidopsis*, known as the SET DOMAIN GROUP (SDG) proteins, which can be classified into five groups based on their activity and domain architecture (Batra et al., 2020).

Readers have been identified as proteins that can recognize specific lysine methylation and translate the epigenetic information to direct downstream functions. For example, in *Arabidopsis*, a few readers, such as LHP1/TFL2, ORC1, AtING, AL, and WDR5a, have been shown to bind methylated histone *in vivo* or *in vitro* (Zhang et al., 2006). At the present, these proteins are classified into three main groups: 1) The royal family domain group which share a conserved structural core and are readers of methylated histones, 2) the plant homeodomain (PHD) finger group proteins and 3) WD40 repeat-containing WDR5 (Duan et al., 2018). Histone demethylation is catalyzed by two types of erasers (demethylases) with different mechanisms, amine oxidation by lysine-specific demethylase 1 (LSD1) family proteins and hydroxylation by JumonjiC (JmjC) domain-containing proteins (JMJs); LSD1 family demethylases act on di- and mono-methylation, whereas JMJ demethylases act on all three kinds of lysine methylation (Duan et al., 2018; Liu & Paroo, 2010).

The second most important histone modification is via acetylation. Acetylation is detected at many lysine residues of H2A, H2B, H3 and H4 (Probst et al., 2020). Histone acetylation consists of addition of acetyl group (CH3COO-), to the ε-amine group of N-terminal lysine residues. Histone lysine acetylation neutralizes the positive charge of the amine group, and enhances the hydrophobicity and increases the size of the lysine side chain, and causes the weakening of the DNA-histone interaction by acetyl groups (Hu et al., 2019). This results in the switching from repressive (closed chromatin) to expressive state (open chromatin) of chromatin, allowing the binding of RNA polymerase and transcription factors to the promoter region of the target genes for initiating their transcription platforms (Kumar et al., 2021). Generally, histone acetyltransferases (HATs) and cause gene activation, on the other hand, histone deacetylases (HDACs) restore the histones to its basal state removing acetyl groups, which mostly cause gene repression (Li et al., 2021). However, in rare cases, acetylation, and deacetylation, may cause repression and activation of genes, respectively (Tasset et al., 2018).

1.4 Stress Memory in Plants

Epigenetic mechanisms play an essential role in gene expression regulation in response to environmental stresses in plants (Boyko & Kovalchuk, 2008). DNA methylation levels in plants are different according to the species and occurs predominantly by the addition of a methyl group to the fifth position of the pyrimidine ring of cytosine bases (5mC) or the sixth position of the purine ring of adenine bases (6mA) (Liang et al., 2018). In Arabidopsis, whole-genome bisulfite sequencing reveals that genome-wide levels of 24% CG, 6.7% CHG and 1.7% CHH contexts are methylated, which predominantly occurs on transposons and other repetitive DNA elements (Cokus et al., 2008). This epigenetic 5mC plays important roles in defending the genome against selfish DNA elements and regulating gene expression, which are essential for normal plant growth, development, and reproduction as well as appropriate biotic and abiotic stress responses (Zhang et al., 2018). Evolutionary conserved DNA repair pathways involve chromatin modifications and remodeling to correct various DNA lesions including UV-induced damage (Schmidt and Jackson, 2013), which might link UV and epigenetic memory.

As one of the most important epigenetic modifications, DNA methylation plays important roles in stress responses in diverse plant species. However, the roles and mechanisms of DNA methylation in plant abiotic stress responses remain largely scattered and fragmented (Liu & He, 2020). For example, some studies have revealed the effect of different abiotic stress type and their relationship with methylation. Zhong et al., (2014) and (Liu et al., 2019) found that *A. thaliana* exposed to heat (30°C for 14 days) caused PTGS release, early flowering, and attenuated immunity, due to H3K27me3 demethylation and siRNAs production. For their part, Wibowo et al. (2016) described that DNA methylation machinery helps *Arabidopsis* plants to adapt during 4 weeks of salt stress. Studies in other plants like rice (*Oryza sativa*) revealed that DNA methylation helped to improve resistance to heavy metal stress via gene expression modulation (Hg^{2+}(50 µM/L) for 7 days) (Cong et al., 2019), and improved resistance to drought (successive generations of drought stress) (Zheng et al., 2017).

As result of secondary metabolites produced by light-response in plants, many studies have found a subsequent improvement in the plant defense system. Roeber et al. (2021) summarize how changes in light quality and quantity have an impact in the outcome of diverse abiotic and biotic stresses, for example, enhancing temperature and drought stress tolerance, as well as the plant responses to pathogens or herbivores.

UV-B has been found as an effective elicitor to improve plant defenses by increasing secondary metabolites production. Several studies have found an increase in total phenolics compounds and flavonoids concentration in different plants' species under UV-B radiation (Piccini et al., 2020;

Martínez-Zamora et al., 2021; Del Valle et al., 2020; Santin et al., 2020; Yildirim, 2020; Wang et al., 2017; Zhang et al., 2017; Nascimento et al., 2015; Usano-Alemany & Panjai, 2015; Pandey & Pandey-Rai, 2014; Eichholz et al., 2011). Flavonoids have great potential to inhibit the generation of ROS and reduce their levels, thus biosynthesis of effective antioxidant flavonoids is enhanced when plants growing under strong light and serve as a photo-protective molecule from oxidative damage (Agati et al., 2012). Alkaloids and glucosinolates have been found to be UV-B responsive in plants. A study in *Catharanthus roseus* suggests that binary stress caused by UV-B and dark enhanced the production of alkaloids, by upregulation of genes like *tdc*, *g10h*, *10-hgo* and ORCA3, which encode the key enzymes related to alkaloid biosynthesis (Zhu et al., 2015); whilst other study in the same species found that UV-B stressed plants can increased N-containing compounds for biosynthesis of alkaloids under high nitrate condition (Takshak & Agrawal et al., 2019). In case of glucosinolates, was found that UV-A or UV-B light exposure enhanced their levels in 8-day-old broccoli sprouts, showing an increase up to ~36% increase of total glucosinolates after 24 h exposure (Moreira-Rodríguez et al., 2017).

Although less studied than UV-B, UV-A and UV-C can act as elicitors for plants SM. UV-A can improve the antioxidant content and induce the accumulation of phytochemicals with antioxidant properties, such as flavonoid, polyphenol, vitamin C, and total anthocyanin contents of the two lettuce cultivars (*Lactuca sativa* L., 'Yanzhi' and 'Red butter') (Li et al., 2021). In tomato plants, UV-A stimulus the polyphenols pathway, through the upregulation of *chalcone synthase* and *flavonol synthase* genes (Mariz-Ponte et al., 2018). On the other hand, UV-C can be effective in disease control as well, by induction of defense systems in hosts (Vanhaelewyn et al., 2020). In fact, a study by Sheng et al. (2018) found that UV-C radiation (3.6 kJ m^{-2}) enhanced the production of total phenolic compounds, total flavonoid, total flavanol, and total anthocyanin content and antioxidant activities of grapes, at higher levels compared to same dose of UV-B. However, the same applied dose of UV-B or UV-C irradiation have different impact on gene expression and phenolic metabolites synthesis, being UV-C a more effective stimulating for gene expression of the phenolic compounds biosynthesis, such as *PAL*, *CHS*, *F3H*. Similar results were found in tomato fruit irradiated with UV-C (Liu et al., 2018).

The response of plants to harmful organisms can be influenced by UV radiation, is widely more studied than abiotic stress. In this context, plants are exposed to biotic stress from pathogens and herbivores. In order to cope with their predator, plants possessed a defense system response, or Innate Immunity, which involves physical barriers such as waxy cuticles, rigid cell walls, and antimicrobial secondary metabolites (Nishad et al., 2020). When a pathogen breaks these barriers, cause the triggering of the plant immune system throughout two responsive-strategies. The first mechanism utilizes the complex recognition of the pathogen or damage-associated molecular patterns (MAMP/PAMP/DAMP) through plant cell surface-anchored pattern recognition receptors, recognize as pattern−triggered immunity (Saijo et al., 2018; Shamrai, 2014). The second mechanism involves the recognition and counteract of the pathogen virulence factors through resistance (R) proteins, activating a cascade of complex signaling events, leading to effector-triggered immunity (Jones & Dangl, 2006). Once previously challenged or exposed to pathogens, plants exhibit a greater resistance against subsequent challenges, established mostly by ROS, which enable plants to show a greater defense to harmful agents. This is known as Systemic Acquired Resistance (SAR) (Nishad et al., 2020).

As mentioned before, both UV- B and UV-C light are useful in horticulture to increase SM production, required to prevent disease outbreaks or herbivores predation. UV-B receptor, UVR8, was found to has a role in the defense response of the fungi *A. thaliana* against *Botrytis cinerea* by increasing the levels of flavonoids and sinapates in leaf tissue (Demkura & Ballaré, 2012). Similar results were obtained in tomato (*Solanum lycopersicum* L.) where, after exposure to a single dose of UV-C light, significantly increased plant defenses against *B. cinerea*, mainly associated with increased phenylalanine lyase activity and the amount of bound phenolics (Vàsquez et al., 2020). On the other hand, Aarrouf & Urban (2020) determined that UV-C doses are linked to the plant response before infections. Exposing leaves, from different plant species (tomato, lettuce, pepper, and grapevine) to UV-C for one second resulted in a better response against specific fungal infections (48 hours after the last dose). This suggests that mechanisms of light perception and metabolic pathways may be different when UV-C light is supplied as flashes, instead of prolonged irradiations (Aarrouf & Urban, 2020; Urban et al., 2018). Furthermore, UV-C has been found to improve shelf life and/or defensive responses in post-harvest fruits as well, which increases the horticultural value of UV radiation as elicitor.

Besides pathogens and infections, depredation by herbivores is a limiting factor for plants growing, usually being insects the most common pest. However, light can be used as a tool to increase plant resistance against herbivorous arthropods, allowing eventually the plant yield (Escobar-Bravo et al., 2017). In a study with *Brassica oleracea* var. *italica* (broccoli) was found that moderate UV-B (1 kJ m^{-2} d^{-1}) doses induced specific UV-B-mediated glucosinolates, especially of 4-methylsulfinylbutyl and 4-methoxy-indol-3-ylmethyl glucosinolates (Mewis et al., 2012). Pre-exposed plants to moderate UV-B doses, showed a negative effect on the performance or population growth of the caterpillar *Pieris brassicae* (L.) and the aphid *Myzus persicae*.

1.4.1 Priming and Imprinting

Plants' capacity to adapt their response mechanisms to a continually changing environment significantly influences their future fitness and enables their survival in highly diverse habitats (Schneider, 2022). In response to stress, plants adjust their epigenetic, physiological, and metabolic processes, which modifies their reactions to similar stressors either within the same generation (somatic) or across subsequent generations (intergenerational and transgenerational stress memory (Kambona et al., 2023). Regardless of future conditions, the initial exposure to stress will leave a lasting imprint on the plant, which will affect its response to subsequent stressors. This phenomenon is known as priming and can be defined as a mechanism which leads to a physiological state induced by a temporally limited environmental stimulus, that enables plants to respond more efficiently after exposure to a recurring biotic or abiotic stress (Lämke and Bäurle, 2017; Aranega-Bou et al., 2014). Priming event is directly followed by a period of stress memory, which involves changes in gene expression or a modified transcriptional response but acting only at the phenotypic level and does not introduce changes in DNA sequence and is thus eventually (Conrath et al., 2015). Current research on stress memory in plants has advanced the understanding of priming by elucidating the epigenetic, transcriptional, proteomic, and physiological changes that create imprints, thereby establishing stress memory in plants (Schwachtje et al., 2019; Liu et al., 2021).

UV radiation as a priming agent has gained much attention, especially for priming of seed and seedlings of important agricultural crops (Thomas & Puthur, 2019). In fact, a series of experiments conducted on rice (*Oryza sativa* L.) showed the role of UV-B in plant priming to cope with abiotic stress. The UV-B radiation (6 kJ m^{-2}) primed both seeds and rice seedlings, showing an enhanced salinity stress tolerance potential, even greater than UV-B and drought stress tolerance potential, through the accumulation of nonenzymatic (ascorbate and glutathione) and enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, HSP and LEA) antioxidants (Sen et al., 2021; Thomas et al., 2020; Thomas & Puthur, 2019; Thomas et al., 2019). Moreover, a recent study suggests that priming memory can be transgenerational retained in the seeds and transferred to one offspring generation without any loss (Sen et al., 2022).

1.4.2 Inter- and Transgenerational Memory

Different categories have been developed to describe various types of stress memory, typically based on the developmental stage of the plant during priming and the mode of inheritance. As mentioned previously, stress memory that is limited to one generation in duration is referred to as somatic stress memory, lasts for a short period of time, and its memory imprints are inherited mitotically. (Lämke and Bäurle 2017). On the other hand, as described by Heard & Martienssen (2014), when parental generation is subjected to drought stress during its reproductive phase, this stress also affects its reproductive cells and the resulting seeds. Consequently, stress memory in the offspring generation may be influenced by the environmental cues introduced into the seed or embryo by the parent plant. This type of stress memory, known as intergenerational, involves the direct exposure of both the parental generation and its immediate progeny to the stressor through the developing germ cells. In contrast, transgenerational transmission occurs when the effects of ancestral exposure to an environment during the reproductive stage are evident in descendants that have not been directly exposed to the stress (Binder et al., 2024). Therefore, if the grandparental generation experienced stress during reproduction, true transgenerational inheritance is observed in the progeny generation, provided that the immediate parental generation was not exposed to the stress and had a recovery period (Skinner et al., 2011).

Plants are subjected to constant environmental challenges. Whereas some stresses occur occasionally, generating a temporal stress response in the plant, many of the abiotic changes occur as daily or seasonal fluctuations (Turgut-Kara et al., 2020). Recurrent stresses can therefore induce a cellular memory that poses or primes the plant for a more efficient response upon repeated stress exposure (Bruce et al., 2007). This stress memory is also known as priming. As is reviewed in Alvarez-Venegas et al. (2019), the priming of plants implies a series of steps: (1) the action of a first stress condition that induce a stress response in the plant and may trigger the formation of a molecular memory, (2) the end of this first stress condition, (3) a period during which the memory can perdure in the absence of the original condition, and (4) the occurrence of a second stress that will activate the recovery of the stress memory to induce a new enhanced response. Furthermore, an additional step entails resetting the memory or maintaining it for transfer to the next generation(s). This process is called inter/transgenerational memory.

In this context, some stress responses can be only transmitted to the direct progeny, which is termed as intergenerational stress memory, while some stress responses can be memorized for at least two subsequent stress-free generations, which is known as transgenerational stress memory (Lämke & Bäurle, 2017). The intergenerational stress memory can be triggered by multiple biotic and abiotic stresses, such as flagellin (an elicitor of plant defense), ultraviolet-C, salt, cold, heat and drought stress, β-aminobutyric acid, methyl jasmonate and the bacteria (for example, *Pseudomonas syringae*) (Liu & He, 2020). Some abiotic stress can induce transgenerational phenotypic changes along with chromatin alterations, which can be detectable until at least one non-stressed generation. In *Arabidopsis*, short-wavelength radiation (ultraviolet-C, UV- C) or flagellin treatment increases the frequency of somatic homologous recombination of a transgenic reporter, which persists in the next four untreated generations (Molinier et al., 2006). It is known that prior mild exposure may prime a plant against future stress or promote an acclimated state that may persist until a subsequent exposure. This phenomenon in plants is usually described as memory (Crisp et al., 2016).

Plant memory is often characterized by heightened molecular responses upon exposure to a subsequent stress, which can be composed of an enhanced response. It is important to remark that transgenerational epigenetic inheritance requires that epigenetic marks can be transmitted to the progeny (Hauser et al., 2011). According to Crisp et al. (2016), two possible mechanism of memory formation are proposed: 1) sustained alterations in levels of key signaling metabolites or transcription factors and 2) alterations to chromatin states, such as histone tail modifications, DNA methylation, or paused RNA polymerase II (RNA Pol II), which could play a further role in the coordinated changes in the patterns of gene expression that underpin memory responses. More recent studies empathize that epigenetic modifications provide heritable changes through small RNA, expression of miRNA or siRNA for post-transcriptional gene silencing, DNA methylation, and histone changes (Sun et al., 2021). The variation in methylation, the most well studied epigenetic modification, of the same gene between different plants is denominated epialleles. Epialleles differ in the number or distribution of methylated nucleotides at specific genes, so that different epialleles can result in different phenotypes which are heritable in a new generation (Thiebaut et al., 2019).

Transgenerational inheritance has been broadly studied in Arabidopsis. For example, Whittle et al. (2009) investigated stress memory to determine if Arabidopsis plants adaptively responded to environmental conditions experienced by their ancestors. They studied plants subjected to mild heat or cold environments across the parental and F1 generations and found that prior exposure to elevated temperatures led to a more than fivefold increase in fitness in the F3 generation. Their research also revealed that the benefits of heat memory persisted in the F3 generation, even when the heat-exposed parental and F1 plants were grown under normal temperature conditions in the F2 generation. Similarly, Boyko et al. (2010) reported that transgenic Arabidopsis offspring from salt-stressed parents exhibited increased salt tolerance and higher rates of recombination. Zhong et al. (2012) interestingly demonstrated that raising the growth temperature from 22°C to 30°C effectively prevents transgene-induced posttranscriptional gene silencing (PTGS) in Arabidopsis. Notably, this temperature-induced release of PTGS exhibited transgenerational epigenetic inheritance. Our findings reveal that the release of PTGS triggered by warmth occurs during a crucial step in the formation of dsRNA, which is necessary to produce siRNAs.

UV radiation has been identified as a relevant stressor capable to induce transgenerational inheritance. Rahavi and Kovalchuk (2013) found that exposing plants to stress early in development, specifically at 7 days post-germination (dpg), leads to beneficial effects on seed size and enhances stress responses in the offspring. Overall, positive transgenerational changes were observed when plants experience mild stress early in development. Conversely, when plants are subjected to severe stress later in development, negative transgenerational effects manifest as reduced stress tolerance in the progeny. Strikingly, Willing et al. (2016) reported that the reversal of pyrimidine dimers by UVR2 photolyase is a key mechanism for preserving plant genome stability across generations under UV-B exposure. Besides its extensive somatic expression, UVR2 exhibits germline-specific activity during late flower development, which is crucial for maintaining low mutation rates in both male and female cell lineages.

Migicovsky and Kovalchuk (2014) analyzed seed size, leaf size, bolting time and transposon expression during 2 consecutive generations of Arabidopsis plants (*dcl2, dcl3,* and *dcl4* mutants alongside wild-type plants) exposed to moderate UV-C stress. It was found that the number of leaves decreased, and bolting was delayed in the progeny of plants subjected to UV-C stress. Additionally, transposons were reactivated in the stressed progenies. The changes observed in the *dcl* mutants were less significant compared to the wild-type plants. DCL2 and DCL3 were identified as more crucial for transgenerational stress memory than DCL4, as the transgenerational effects were less pronounced in the *dcl2* and *dcl3* mutants.

1.5 Hypothesis

Taking into consideration our previous research and the literature reviewed, we hypothesized that multigenerational exposure to UV-C in Arabidopsis plants is expected to induce genetic and epigenetic changes that will enhance the resistance of plants to this stress, due to favorable phenotypic changes. Specifically, our objectives were:

1. To obtain 25 generations of plants induced by UV-C.
2. Conduct whole-genome sequencing and whole-genome bisulfite sequencing to detect changes between the plant generation under stress and controls.

3. Analyze the potential resistance of the plants obtained after 25 generations to UV conditions, through root growth, germination, and the presence of reactive oxygen species.

To achieve these objectives and test our hypothesis, we conducted a comprehensive analysis of both genomic and epigenomic sequencing data to pinpoint specific alterations. For the genomic aspect, we focused on identifying SNPs, and InDels, as well as characterizing the nature of these changes, such as whether they represented synonymous and non-synonymous mutations, missense and nonsense mutations, and distinguishing between transitions and transversions. On the epigenomic front, differentially methylated cytosines (DMCs) were assessed across three methylation contexts: CG, CHG, and CHH. Enrichment analyses, hierarchical clustering, and gene ontology were employed to verify the adaptive direction of these changes across different molecular levels. To evaluate stress resistance, we subjected the plants from our study to UV-C irradiation at different developmental stages. ROS were quantified using well-established protocols with DAB and NBT staining, allowing us to confirm the plants' physiological response to UV stress. Statistical analyses were conducted in accordance with each of the experiments performed.

CHAPTER 2: METHODOLOGY

2.1 Parental Generation and Progeny Plants

The entire process of seed generation and treatment application across 25 generations was conducted by members of the research group led by Dr. Igor Kovalchuk. Arabidopsis seeds used as starting material representing the parental generation (P) were obtained from single homozygous Arabidopsis thaliana (Columbia ecotype, Col-0) plant transgenic for the luciferase (LUC) recombination reporter gene consisting of a copy of a direct repeat of the luciferase recombinant transgene. All parental plants were grown to flower, and seeds were collected and pooled from approximately 16-20 plants per each tested experimental line. The seeds were stored under dry conditions at room temperature. All subsequent progeny plants were obtained from these seeds; specifically, ~ 100 seeds were sown, and ~ 20 randomly selected plants formed the next generation. Two groups of plants were obtained, those propagated without stress – control group (C), and those propagated under UV-C stress – stress group (UV). While control plants were propagated in normal growth conditions for 25 generations, plants representing the 'UV' group were exposed to UV-C irradiation (G30T8) of 30.5 W and 99 V, and UV output of 13.9 W for 2 min daily (at 10 am) for four consecutive days in each of 25 generations. There were two lineages of control and three lineages of stressed plants propagated in a similar manner, representing two experimental repeats of control and three experimental repeats of stressed plants.

2.2 Plant Growth Conditions

The seeds were kept at 4°C for seven days to initiate stratification, and then sown in all-purpose potting soil prepared with water containing a generic fertilizer (Miracle-Gro, Scotts Canada). The moist soil containing seeds was further stratified for 48 hours at 4°C. Plants were grown in a growth chamber (BioChambers, Manitoba, Canada) at 22°C under the extended day conditions of 16 hours light (120 μmol photons m^{-2} s^{-1}) and 8 hours dark. Approximately three to five days post germination (dpg), plant seedlings were transplanted into individual pots containing a 9:1 ratio of soil to vermiculite composition prepared with a fertilizer. Pots containing plants were placed in trays and watered from below.

2.3 Lineages Used for UV Stress Phenotyping

The seeds of F0 (parental) and F1 generations were no longer viable, hence F2C seeds were used as parental control. Overall, the lineages F2C (parental control), F25UV (the 25th generation of stressed progeny) and F25C (the 25th generation of parallel control progeny) were used for this study.

2.4 Phenotypic Analysis Under UV Stress Treatment

To test the UV tolerance in the progeny, the F25UV plants were exposed to UV-C stress at 5 days post germination and pictures of recovery were taken a week later. Plants were exposed to UV-C irradiation (G30T8) of 30.5 W and 99 V, and UV output of 13.9 for 5, 10, 20, 30, 45, 60 or 90 min. Three replicates of stressed progeny and two replicates of control progeny were used per each MS plate and five sets were used for each treatment.

2.5 Primary Root Length Measurements

The seeds underwent a sterilization process using a 1% sodium hypochlorite solution for 7 minutes, followed by three washes with sterile water, each lasting 3 minutes. Seedlings were then grown on MS-agar plates under sterile conditions and subsequently transplanted in groups of five onto plates containing the same agar medium for final analysis. Plates were placed in a growth chamber at

22°C under the extended light conditions of 16 h light and 8 h dark. At approximately 3 days after germination the seedling were transplanted to their final plate to conduct the experiment and grown vertically. At approximately 5 days after germination, the experiments were conducted with the following treatments and conditions: Both F25UV and F25C were irradiated with UV-C germicidal lamp located 30 cm above horizontally placed plates with no lid. Depending on the treatment, full seedlings or only shoots and leaves were irradiated (roots remained covered with a white cardboard paper), either for 4 or 30 minutes. Plates were then kept under normal growth conditions in absence of UV-C for 4 more days. Plates were photographed before the treatment and every 24 hours during the next 4 consecutive days. 3 plates containing 5 plants each were used per treatment. Primary root length was measured using the ImageJ version 1.54g software. Different letters indicate statistically significant differences using a Two-way ANOVA ($P < 0.05$).

2.6 Detection of Reactive Oxygen Species

Stress treatment of plants was conducted using UV irradiation, employing the same germicidal lamp as described previously. The UV-radiation source was positioned approximately 30 cm above pots containing 21-day-old Arabidopsis plants (grown under normal conditions as other treatments) from the F25UV and F25CT lines. The plants were exposed to UV radiation for 30 minutes to induce reactive oxygen species (ROS) accumulation. At the end of the treatment, at least three leaves from five plants of both UV-stressed and control plants leaves were sampled immediately.

Hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) were used as indicators of stress due to UV exposure. For detection, 3,3′-Diaminobenzidine (DAB) and Nitro Blue Tetrazolium Chloride

(NBT) were used as chromogenic substrates, respectively. The staining protocol followed was as described by Daudi and O'Brien (2012) for DAB and by Kumar et al. (2014) for NBT. Images of sampled leaves were analyzed using ImageJ version 1.54g.

2.7 Lineages Used for Genomic and Epigenomic Study

The parental plants (F2C) and the progeny of 25th generation (F25UV and F25C) plants were grown at normal conditions for 21 days and the rosette leaves tissues were harvested from individual plants and snap frozen in liquid nitrogen and stored at -80°C for DNA extraction for sequence analysis. From each generation, five individual plants were sequenced, representing all lineages (3 from stressed group and 2 from control group), resulting in a total of fifteen samples for WGS and WGBS analysis.

2.8 Whole Genome Sequencing (WGS) and Whole Genomic Bisulfite Sequencing (WGBS)

DNA extraction and subsequent bioinformatic analyses were performed by Dr. Narendra Singh Yadav, a member of Dr. Kovalchuk's Research Group. The total genomic DNA was extracted from approximately 100 mg of leaf tissue homogenized in liquid nitrogen using a CTAB protocol. The isolated genomic DNA was used for both whole genome sequencing (WGS) and whole genome bisulfite sequencing (WGBS) to assist in identifying the genomic and epigenomic variations. The WGS and WGBS libraries construction and sequencing have been carried out at the Centre d’expertise et de service Génome Québec, Montreal, Canada.

2.8.1 WGS and WGBS Libraries Construction and Sequencing

For whole genome sequencing (WGS), gDNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). Libraries were generated using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs) as per the manufacturer's recommendations. Adapters and PCR primers were purchased from IDT. Size selection of libraries contained the desired insert size has been performed using SparQ beads (Qiagen). Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument.

gDNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). Libraries were generated with the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs) using 250 ng of input genomic DNA. Adapters were purchased from NEB. Size selection of libraries containing the desired insert size have been performed using sparQ PureMag Beads (Quantabio). For whole genome bisilfite sequencing (WGBS), bisulfite conversion had been carried out with the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA, USA). Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems) and average size fragment was determined using a LabChip GX (PerkinElmer) instrument.

The libraries were normalized and pooled and then denatured in 0.05N NaOH and neutralized using HT1 buffer. ExAMP was added to the mix following the manufacturer's instructions. The pool was loaded at 200pM on an Illumina cBot and the flowcell was ran on a HiSeq X for 2x151 cycles (paired-end mode). A phiX library was used as a control and mixed with libraries at 1% level. The Illumina control software was HCS HD 3.4.0.38, the real-time analysis program was

RTA v. 2.7.7. Program bcl2fastq2 v2.20 was then used to demultiplex samples and generate fastq reads. Bisulfite conversion rate was 99.7 and the spiked-in unmethylated lambda phage DNA was used to estimate the bisulfite conversion rate.

2.9 The Computation and Analysis of Genome Sequence Data

Raw sequencing reads were quality controlled and trimmed using Trim Galore software (version 0.4.4). The trimmed reads were aligned to the TAIR10 reference genome Col-0, and the duplicates were marked using Picard tool. Local realignments around SNPs and InDels were performed using GATK (genome analysis toolkit) which accounts for genome aligners and mapping errors and identifies the consistent regions that contain SNPs and InDels. The resulting reads were quality controlled with Haplotype scores and variant sample sites were called individually and jointly using the HaplotypeCaller with GATK. The sites marked as those that had a low-quality score by GATK were filtered out. The effects of variants in the genome sequences were classified using the SnpEff program (Cingolani et al. 2012). According to the SnpEff program (Cingolani et al. 2012) utilized in this study, upstream was defined as 5 kb region upstream of the distal transcription start site, and downstream was defined 5 kb region downstream of the most distal polyA addition site. Variants affecting the non-coding regions were expounded, and biotypes were identified with the available information after comparing with the TAIR 10 reference Arabidopsis genome.

The genomation tools were used to obtain a biological understanding of genomic intervals over the pre-defined functional regions such as promoters, exons, and introns and the Functional Classification SuperViewer to create gene association profiles and show the difference between samples. The genes nearest to the non-overlapping SNP and INDEL sites were annotated.

Binomial tests were applied and used to determine the observed methylation frequency against the bisulfite conversion reaction, and the percentage of methylation levels were calculated at each base (Schultz et al. 2012). The WGBS raw sequencing data were analysed using tools found in the methylKit package. Raw sequencing reads were quality controlled and trimmed using Trim Galore software (version 0.4.4). The trimmed reads were then aligned to the TAIR10 reference genome using the bisulfite mapping tool Bismark (Krueger and Andrews 2011). The methylated cytosines (mCs) were extracted from the aligned reads with the Bismark methylation extractor on default parameters followed by the computation of methylation frequency using the R package software, methylKit. The percentage of methylation was calculated by counting the frequency ratio of Cs divided by reads with C or a T at each base and computed at bases with coverage ≥ 10 (Akalin et al. 2012).

The percentage (%) of methylation = {Frequency of C ÷ readcoverage} × 100 $\boldsymbol{Frequency\ of\ C \div read\ coverage} \times \mathbf{100}$

Common bases covered across all samples were extracted and compared, and the differential hyper- and hypo- methylated cytosines were extracted. The differentially methylated cytosines (DMCs) overlapping with genomic regions were assessed (in the preference of promotor > exon > intron), and the average percentage of methylation of DMCs around genes with the distances of DMCs to the nearest transcription start sites (TSSs) were also calculated.

The annotation analysis was performed with genomation package within methylKit to obtain a biological understanding of genomic intervals over the pre-defined functional regions such as promoters, exons, and introns (Akalin et al. 2014). The functional commentary of the generated gene methylation profiles was performed using the SuperViewer tool with Bootstrap to show the difference between samples (Provart and Zhu 2003). Hierarchical clustering of samples was used to analyse similarities and detect sample outliers based on percentage methylation scores. Principal Component Analysis was utilized for variations and any biological relevant clustering of samples. Scatterplots and bar plots showing the percentage of hyper-/hypo- methylated bases and heatmaps were used to visualize similarities and differences between DNA methylation profiles.

2.10 The Differentially Methylated Regions (DMRS)

A comparison of differential DNA methylation levels between samples reveals the locations of significant differential changes in the epigenome. The obtained information of DMRs was investigated over the predefined regions in all contexts: CG, CHG, and CHH for 100 bp and 1,000 bp tiles across the genome to identify both stochastic and treatment associated DMRs (Akalin et al. 2012). The differential hyper-/hypo- methylated regions were extracted and compared across the samples. By default, DMRs were extracted with q-values < 0.01 and the percentage of methylation difference > 25%. The differential methylation patterns between the sample groups were also extracted. The methylation profiles of the sample groups used were F25UV versus F2C, F25UV versus F25C, and F25C versus F2C. In summary, the sliding windows of 100 bp and 1,000 bp were considered for both DMRs and DMCs, and extractions were made based on at least 25% and 50% differences (q-values < 0.01) to assess the significant differences among samples.

2.11 Statistical Analysis and Quality Control Values

The mapped reads were obtained with a quality score of <30, the differential hyper- and hypomethylated bases were extracted with q-values < 0.01 and the percentage of methylation difference larger than 25% in methylKit. The Heatmaps of differentially methylated bases were quantified at q-values < 0.01, and the percentage of methylation difference was more significant than 50%. The distances of DMCs to the nearest TSSs obtained from genomation at both >25% and >50% of methylation change. The distance between TSSs and DMCs was extracted within +/- 1,000 bp and annotated at DMCs >50% methylation difference. DNA methylation profiles obtained from the melthylKit used the obtained pairwise correlation coefficients of methylation levels (in %) and the 1-Pearson's correlation coefficients for hierarchical clustering of samples. Logistic regression and Fisher's exact test were used for the determination of differential methylation with calculations of q-values and the Benjamini-Hochberg procedure for the correction of p-values. The t-test for mean difference between groups was calculated with p-values < 0.05. The results of global genome methylation were analysed by one-way ANOVA with the Tukey's multiple comparisons test using GraphPad Prism version 8.4.2 for Windows. The data are shown as the average percentage (with ±SD) of methylated cytosines from five individual methylomes in each of the progenies. The asterisks show a significant difference between the stressed progeny and the parental control progeny (*$p < 0.1$, ** $p < 0.05$). The phenotypic data were analysed by the unpaired t-test with Welch's correction using GraphPad Prism version 9.1.1 (255) for Windows. The data were shown as mean ±SD. A p-value less than 0.05 ($p \leq 0.05$) was considered statistically significant.

CHAPTER 3: RESULTS

3.1 The Progeny of UV-C-Stressed Plants did not Exhibit UV-Tolerant Phenotype

To analyse the effect of UV on the progeny of *A. thaliana* plants, we propagated them on UV for 25 generations; plants grown at normal conditions served as controls (Figure 1A). To test whether F25UV plants acquired UV tolerance, we exposed them to UV for different duration of time. The experiments showed that F25UV plants were not different from F25C plants in their response to UV-C (Figure 1B and Figure S1).

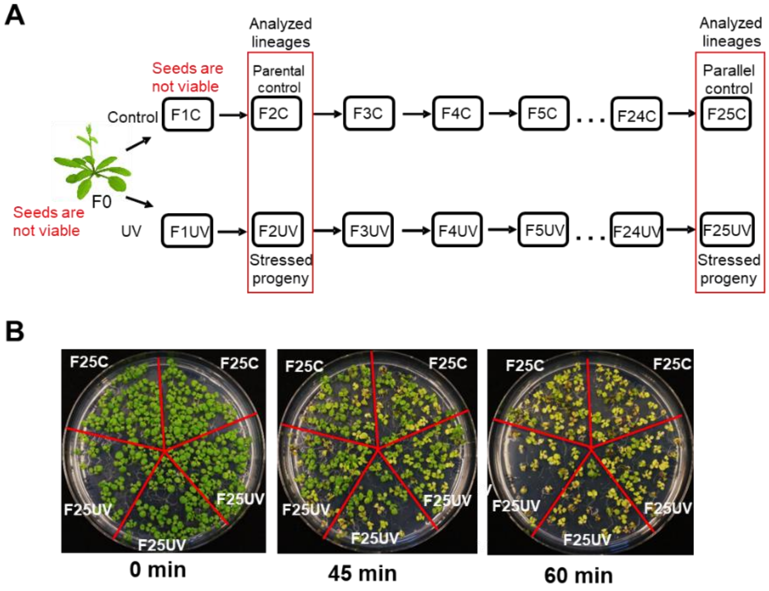


Figure 3.1. The progeny of UV-stressed plants did not show any change in tolerance to UV stress. *(**A**) Diagram of propagation of 25 generations of Arabidopsis thaliana (Col-0, 15d8 line) plants grown under normal conditions (C group) or exposed to UV-C (UV group). Seeds from the second-generation F2C plants were used as parental controls since F0 and F1C seeds were no longer viable. Lineages used in this study are indicated in the red box. Plants were grown in a controlled growth chamber at 22°C with a 16-h light/8-h dark photoperiod. (**B**) Phenotype of F25C and F25UV plants after 0-, 45-, and 60-min UV-C exposure. Each condition was tested using a*

single plate containing multiple seeds, and their growth under UV stress was assessed visually. Images show two individual control lineages and three individual stress lineages.

3.2 Primary root elongation is inhibited by UV-C irradiation, regardless of transgenerational inheritance

To determine root growth, the F25C and F25UV generations were sown on vertically positioned plates. Whole seedlings or only their leaves were irradiated with UV-C radiation for periods of 4 or 30 minutes. Root length was measured before and during the subsequent 4 days. The results suggest that UV-C radiation significantly reduced root growth in all cases. Plants subjected to 4 minutes of UV exposure showed a notable decrease in root length compared to the control under normal conditions. Generally, in plants exposed only on their leaves, root length was slightly greater compared to whole seedling exposure. However, no significant difference in root development was detected between F25C and F25UV under either control or UV treatment. Under 30 minutes of exposure, similar results were found, although root growth during the subsequent days appeared stagnant compared to a slight increase when only the aerial parts of the plant were exposed.

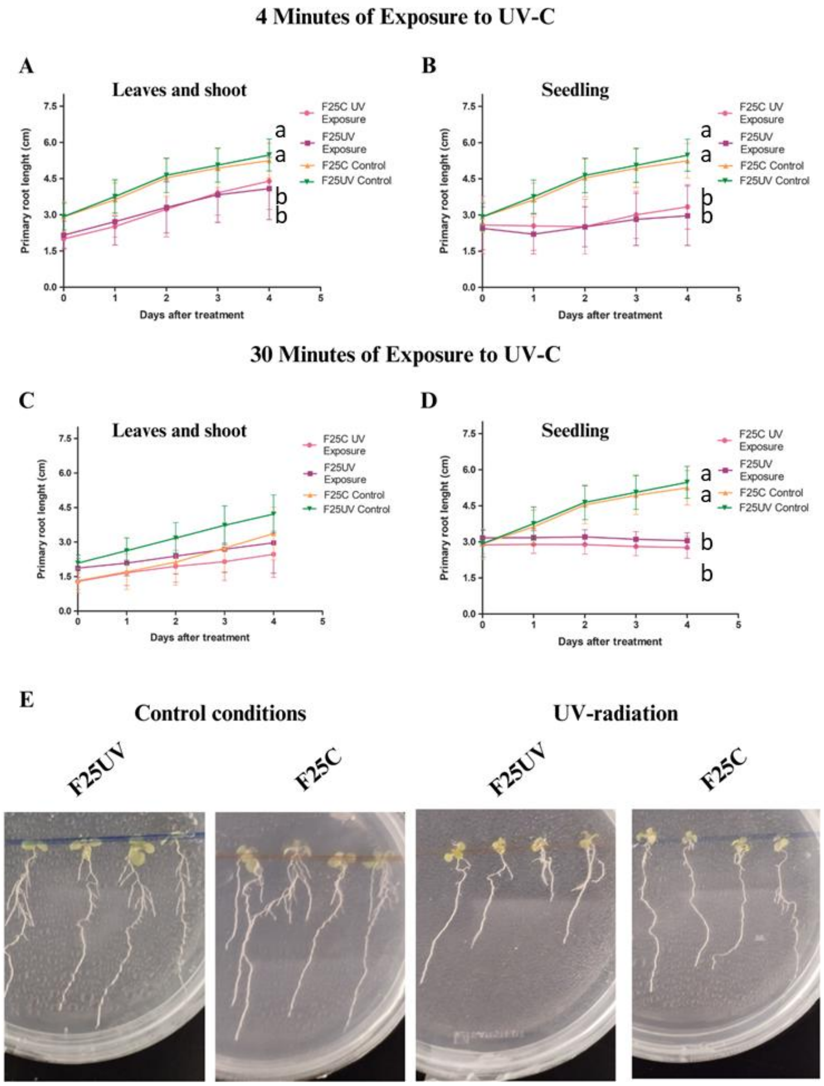
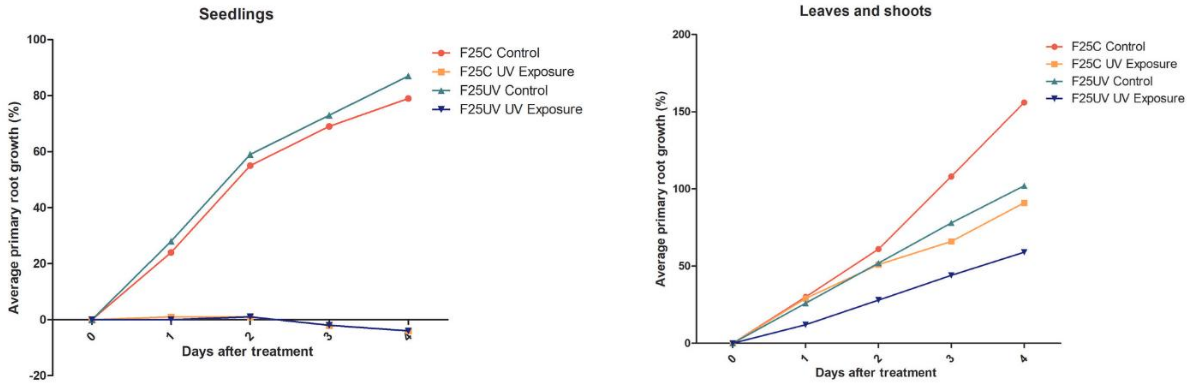


Figure 3.2. Primary root growth inhibition assays in F25UV and F25C seedlings under different UV-C exposure conditions. *The average primary root lengths of Arabidopsis thaliana (15d8 line) seedlings from F25Ct and F25UV groups were measured after growth in the absence of UV-C (control) or following UV-C irradiation. Germicidal lamps were positioned approximately 30 cm above the plates, and root length measurements were taken daily from 0 to 4 days after treatment. (A-D) Experimental conditions: (**A**) Leaves and shoots irradiated for 4 min, (B) full seedlings irradiated for 4 min, (**C**) leaves and shoots irradiated for 30 min, and (**D**) full seedlings irradiated for 30 min. (**E**) Representative images showing primary roots under the conditions described in*

*(**C**). For each UV stress treatment, three plates containing five seedlings each were used (n = 15), while for controls, two plates with five seedlings each were used (n = 10). Root lengths were measured using image analysis software, and data are presented as means ± standard deviation. Different letters represent significant differences determined by Two-way ANOVA followed by Tukey's post hoc test (P < 0.05).*

4 Minutes of Exposure to UV-C



30 Minutes of Exposure to UV-C

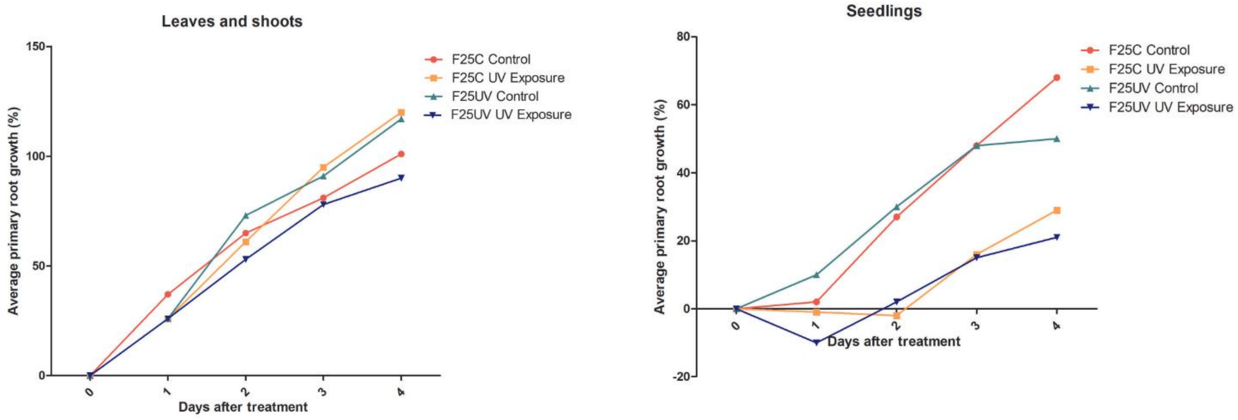


Figure 3.3. Percentage of primary root growth relative to initial size after UV irradiation.

The graph values were calculated based on the root measurements from Figure 3.2. The percentages indicate the relative difference in root growth compared to day 0, before UV radiation *exposure. Negative percentages indicate a decrease in root length compared to the initial root size prior to exposure. The groups shown correspond to seedlings where either the whole plant or only the leaves were exposed to UV radiation. The lines used were F25UV and F25C, either under the effect of UV or not.*

3.3 Superoxide radical staining and Hydrogen Peroxide Staining

DAB (3,3'-diaminobenzidine) and NBT (nitroblue tetrazolium) staining revealed distinct patterns of ROS accumulation in treated plants. DAB staining, which detects hydrogen peroxide (H_2O_2), showed localized dark brown deposits, indicating elevated H_2O_2 levels in specific tissues. In contrast, NBT staining, which detects superoxide radicals (O_2^-), resulted in deep blue precipitates, primarily concentrated in areas of high oxidative stress. Together, these results suggest a differential accumulation of ROS, with H_2O_2 and superoxide playing distinct roles in the plant's stress response. Visually, we were unable to detect any difference in ROS concentration between the two treatments (Figure S2). The low prevalence of ROS in the control group without UV exposure is clearly discernible; however, there does not appear to be a notable difference between the leaves of F25C and F25UV after radiation exposure, suggesting that both plant lines respond similarly to these stress conditions.

3.4 F25UV Plants Showed Higher Levels of InDels and Unique SNPs

To determine SNPs and InDels, we mapped sequencing reads to TAIR 10 reference genome. The total number of SNPs and InDels was significantly higher in F25UV group as compared to two other groups ($p<0.05$) (Figure 3.4A, B). The difference in InDels was attributed to deletions, rather than to insertions; there was no difference among groups in the number of insertions (Figure 3.4C, D). F25C group was not different from F2C group.

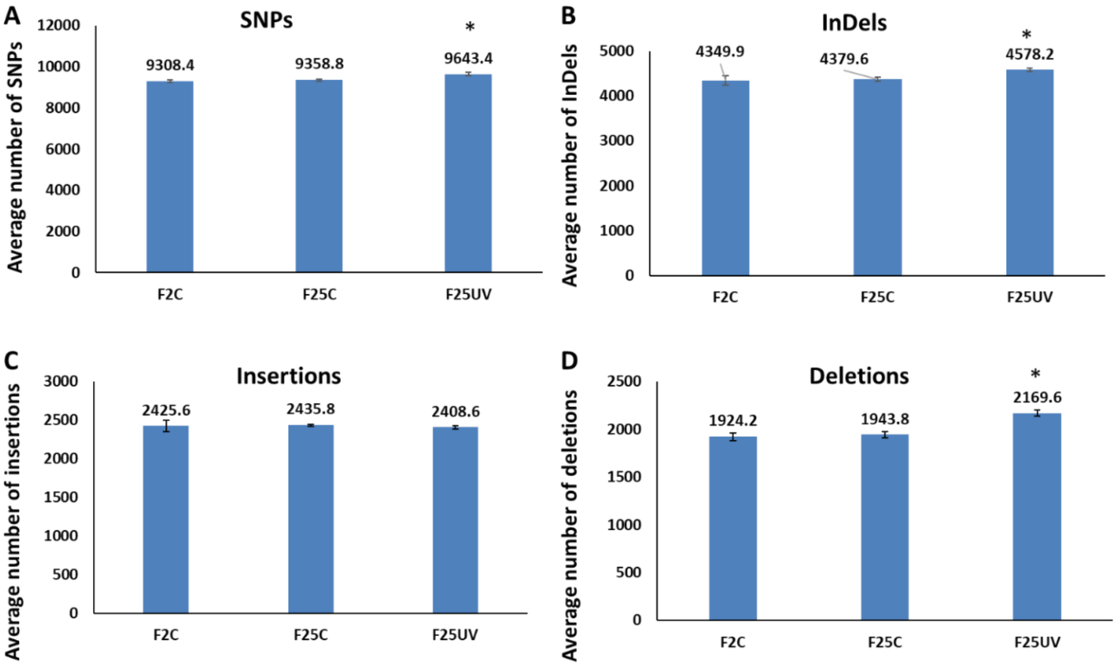


***Figure 3.4**. **The UV-stressed progeny showed higher number of genetic variations.** The total number of SNPs (**A**) InDels (**B**), insertions (**C**) and deletions (**D**) in the genomes of stressed and control progeny and parental control when all samples were jointly considered. Y axis shows the average (with SD), calculated from five independent biological repeats. The asterisk above (*) shows a significant difference between the F25UV and parental or parallel control generations (t-test, two-sample assuming unequal variances; $p < 0.05$).*

When unique sequence variations were considered, F25UV was found to have 674 unique SNPs accumulated, compared to 410 for F25C and 454 for F2C (Figure 3.5A). As far as InDels were concerned, F25UV had 131 unique InDels, while F25C and F2C had 81 and 133, respectively (Figure 3.5A).

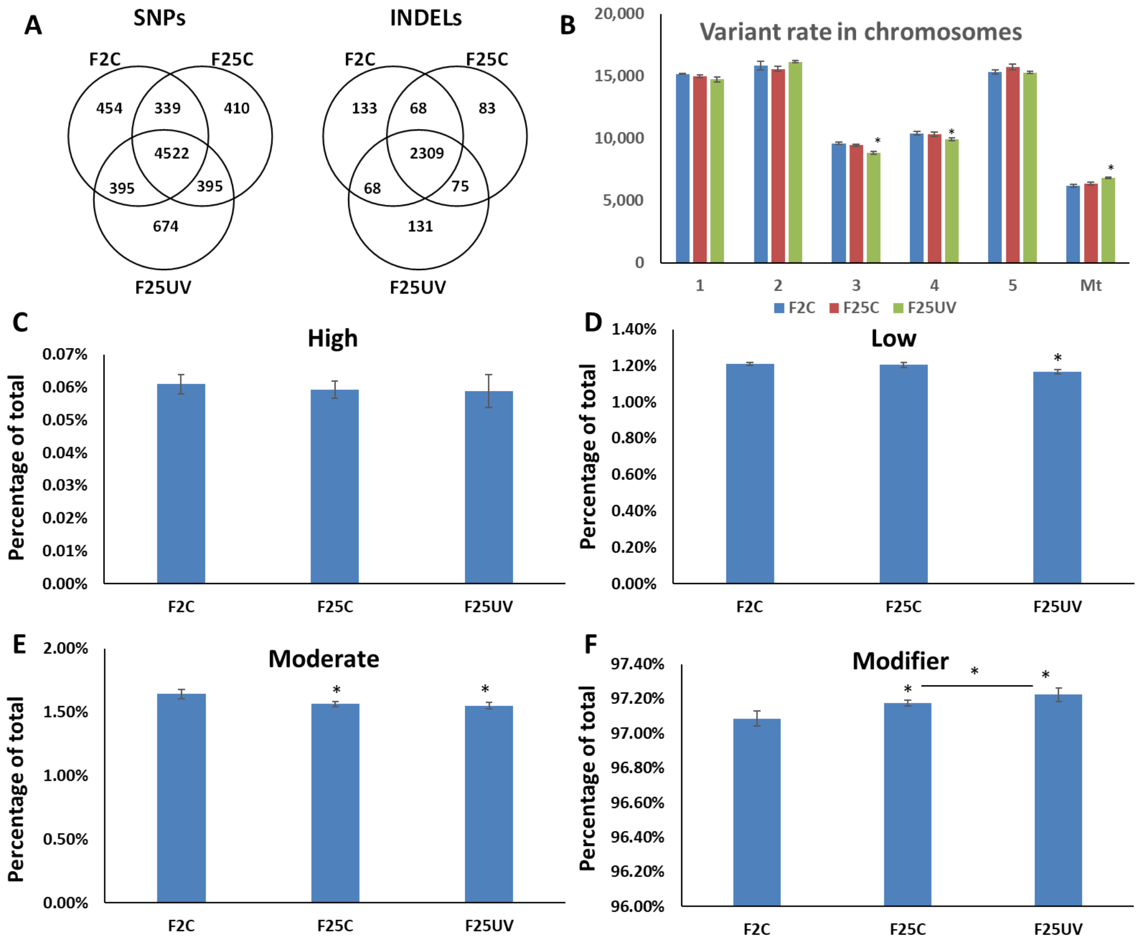


Figure 3.5. Analysis of SNPs uniqueness. *(**A**), SNPs distribution between chromosomes (**B**) and potential SNPs impact (C-F). **A.** Venn diagrams of jointly called SNP and INDEL variants. The non-overlapping area of Venn indicates the number of unique SNPs and InDels in each sample group. Sample variants were jointly called by the "GATK haplotype Caller" using five samples together. **B**. Variant rate in different chromosomes shown as number of nucleotides per one SNP in each chromosome; Mt – mitochondrial genome. **C-F.** Demonstrates the percentage of specific type of SNPs by their impact on gene expression, where "high" (**C**) has the highest impact on gene expression, protein composition or/and protein function, while "low" (**D**) and "moderate" (**E**) have less of an effect. "Modifier" (F) has the least effect on gene expression. All terms are from SnpEff. Data are averaged (with SE) from five individual plants. Asterisks over F25C or F25UV*

show significant difference to FC (t-test, two-sample assuming unequal variances; p<0.05). Asterisk over the horizontal bar indicates significant difference between F25UV and F25C.

SNPs may have different effects on the genome, from neutral to deleterious. We used SnpEff for classifying SNPs to modifiers, with the lowest impact on gene expression, low, moderate, and high impact, that have progressively stronger effect on gene expression (Figure 3.5B). There was no change in “high” category, while there was a decrease in “moderate” and “low” category in F25UV compared to F2C (Figure 3.5C-E). Finally, there was an increase in “modifier” category of SNPs in F25C and F25UV, which was significantly higher in the latter (Figure 3.5F).

We also analyzed SNPs in categories of synonymous and non-synonymous mutations, with further categorization of non-synonymous mutations into missense and nonsense mutations. There was a significantly higher percentage of synonymous and a significantly lower percentage of non-synonymous mutations in F25C as compared to F2C ($p<0.05$) (Figure 3.6A, B). F25C group had lower percentage of missense mutations as compared F2C, while F25UV group had significantly higher percentage of nonsense mutations than either F2C or F25C groups (Figure 3.6C, D).

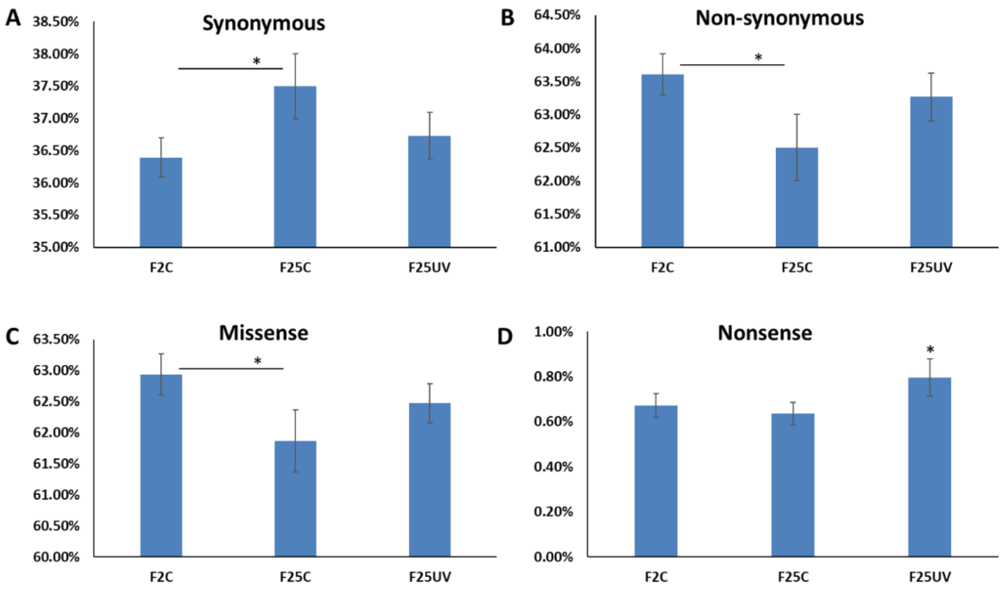


Figure 3.6. Percentage of synonymous. (A), non-synonymous (B), missense (C) and nonsense (D) mutations in F2, F25C and F25UV plants. *The Y axis shows the percentage of different kinds of mutations, and the X axis shows the plant group. Data are shown as an average ± SE, calculated from five individual samples. Asterisks over the horizontal bar show significance (t-test, two-sample assuming unequal variances; p<0.05) between groups, while asterisk over F25UV groups shows the difference between this group and either F2C or F25C, p<0.05.*

Point mutations in the form of nucleotide change are not equally distributed; some mutations are more frequent than others. We analysed mutations by a nucleotide change and found C to T and G to A mutations to be most frequent among all other mutations (Figure 3.7 and Figure S3). F25UV group also had significantly ($p<0.05$) higher percentage of C to G and C to A mutations than either F25C or F2C group (Figure 3.7). F25UV group also had lower percentage of A to C and A to G mutations ($p<0.05$) (Figure 3.7). C to T transitions appeared to be the most common type of mutations associated with deamination of methylated cytosines (Weng et al., 2019). Also, the percentage of all mutations at cytosines (C/T, C/A and C/G) was significantly higher in F25UV group. The analysis of mutations of cytosines at CpG context or at other cytosine locations (CHG and CHH contexts) showed that there was no significant difference between the percentage of C/T, C/A or C/G mutations among F2C, F25C or F25UV. It can thus be suggested that higher frequency of mutations at cytosines occurs at CHG and CHH sites.

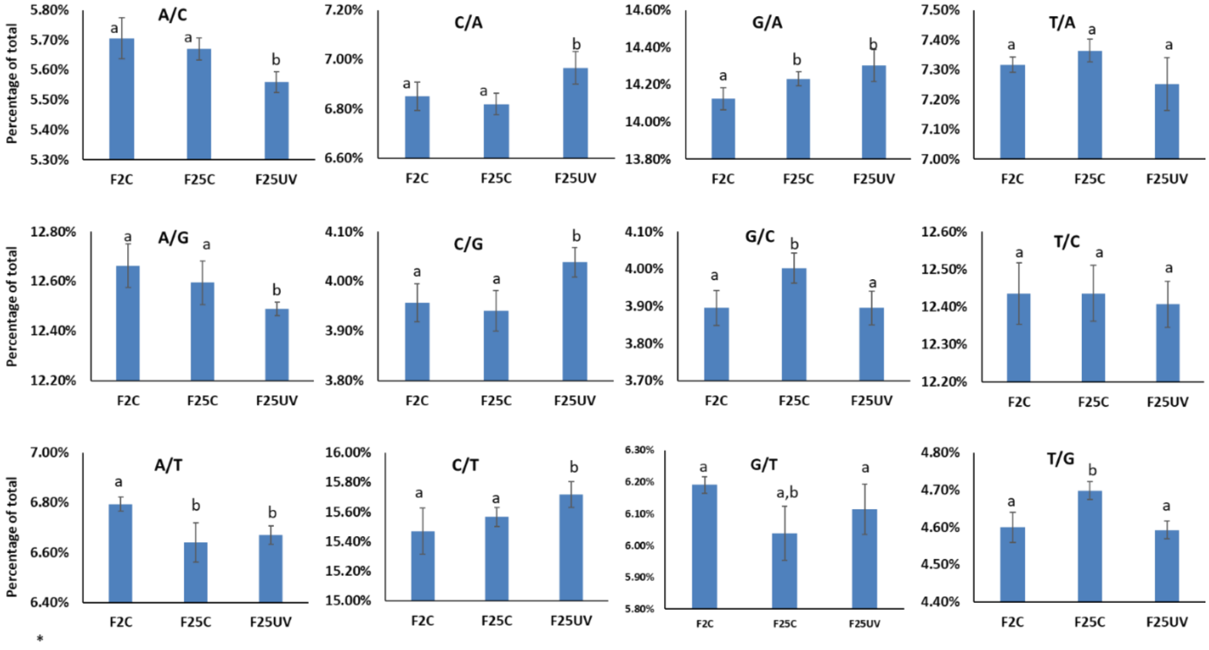


Figure 3.7*. *Type of single nucleotide substitutions in F2, F25C and F25UV groups. *Y axis demonstrates the percentage of mutations among all mutations, while X axis shows the three tested groups. Data are shown as an average from five samples ± SE. Letters (a,b) show significant differences (t-test, two-sample assuming unequal variances; p<0.05). A – adenine, C – cytosine, G – guanine, T – thymine.*

SNPs are often unevenly distributed in the genome. We only found significant difference among the groups in the intergenic regions – higher percentage of SNPs was in F25UV as compared to other groups (Figure S4). Mutations of purine to purine and pyrimidine to pyrimidine are called transitions (Ti), while purine to pyrimidine and vice versa – transversions (Ts). Ti mutations commonly are more frequent than Ts in most organism, and there is also evidence that Ti/Ts ratio changes with stress. We found the rate of transitions to be similar among all three groups (Figure 3.8A), while the rate of transversions to be significantly ($p<0.05$) lower in F25UV group than in the other two groups (Figure 3.8B). We also found that the Ti/Tv ratio was significantly higher in F25UV and F25C groups as compared to F2C group (Figure 3.8C); even though Ti/Ts ratio was larger in F25UV as compared to F25C, the difference was not significant. We found no significant difference in Ti/Tv ratios in different genomic locations among groups (Figure 3.8D).

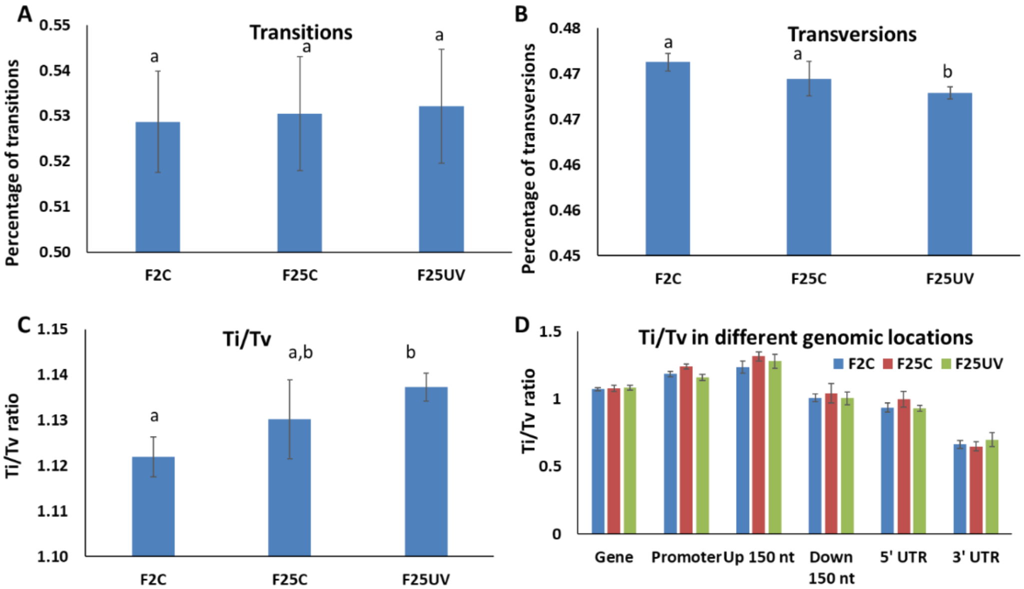


Figure 3.8. Transitions and transversions in F2C, F25C and F25UV groups. *Percentage of transitions (**A**), transversions (**B**) and ratio of transition to transversion (Ti/Tv) (**C**) as well as the ratio of Ti/Tv in various genic regions (**D**). Y axis shows either the percentage of Ti or Tv (**A, B**) or Ti/Tv ratios (**C, D**), while X axis shows the specific group. Data are shown as an average calculated from five different samples, with SE. Letters over the bars show the significant difference (t-test, two-sample assuming unequal variances;* $p<0.05$*) among the groups.*

3.5 Genetic Variants in F25UV Were Enriched in Several Components

Enrichment analysis of InDels revealed that cellular components “cell wall” and “extracellular” were significantly enriched in F25UV group, but not in the other groups (Figure 3.9A). Molecular functions “kinase activity” and “nucleotide binding” and biological processes “cell organization and biogenesis”, “response to stress”, “response to abiotic and biotic stimulus” and “other biological processes” were also enriched in F25UV as compared to other groups. In SNPs, molecular function “other binding” was enriched in F25UV group (Figure 3.9B).

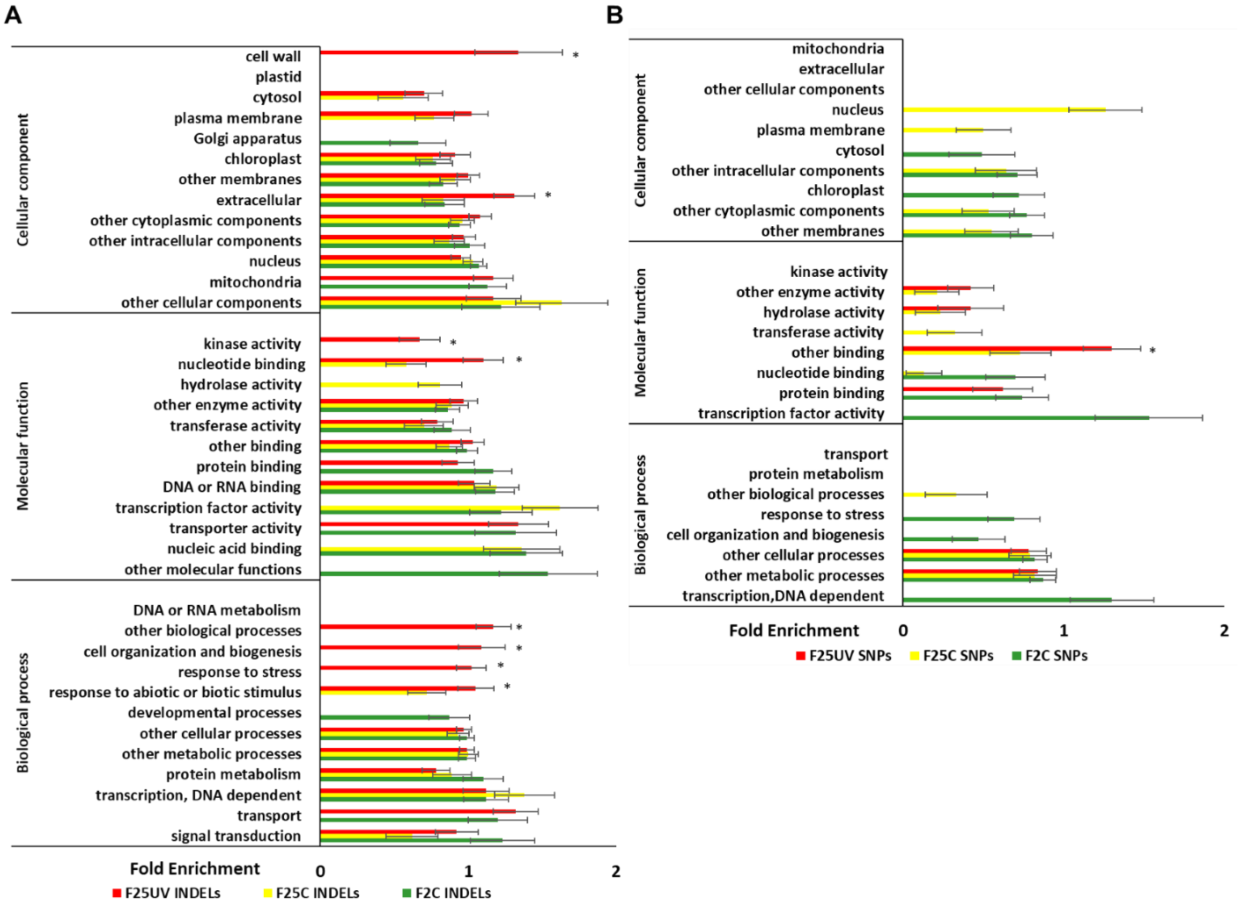


Figure 3.9. Enrichment of cellular components, molecular function and biological processes in INDELS (A) and SNPs (B) in F2C, F25C and F25UV groups. *Y-axis shows normalized Class*

Score with binomial coefficients as calculated by SuperViewer. To calculate enrichment, p-value of < 0.05, ±bootstrap SD were used. X-axis shows the groups.

3.6 Analysis of Epigenetic Variations

Analysis of DNA methylation showed that global genome methylation was significantly higher in F25HC plants as compared to F25C and F2C plants at the CG context, while was similar at CHG and CHH sites, although there was a trend to the increased methylation at CHG sites (Figure 3.10A). Overall, multigenerational exposure to UV-C increases the global genome methylation at the CpG cites.

3.7 F25UV Group Shows Higher Number of DMCs and DMRs in Comparison to F2C

Analysis of DMCs showed that F25UV plants had more differentially methylated cytosines than F2C group at all contexts; it also had more DMCs in comparison to F25C groups at CHG and CHH contexts, while F25C in comparison to F2C group had the lowest number of DMCs (Figure 3.10C). The most striking difference was observed for CHH context, where F25UV comparison to F2C revealed over 10-fold more DMCs at CHH position as compared to the control group comparison F25C to F2C. Our data suggest that in terms of methylation, F25UV group is substantially more divergent from F2C group, than F25C; at the same time F25UV group is more similar to F25C group than to F2C group. Furthermore, our data suggest that multigenerational UV-C stress results in the increased number of hypermethylated cytosines at CG sites, equally increased the number of hyper- and hypomethylated cytosines at CHG sites and drastically increased the number of differentially methylated cytosines at CHH sites, with hypomethylated cytosines prevailing. Analysis of DMRs revealed a very similar picture, except that at symmetrical CG and CHG contexts, F25UV group was different from F2C group, predominantly due to hypermethylated cytosines (Figure 3.10D).

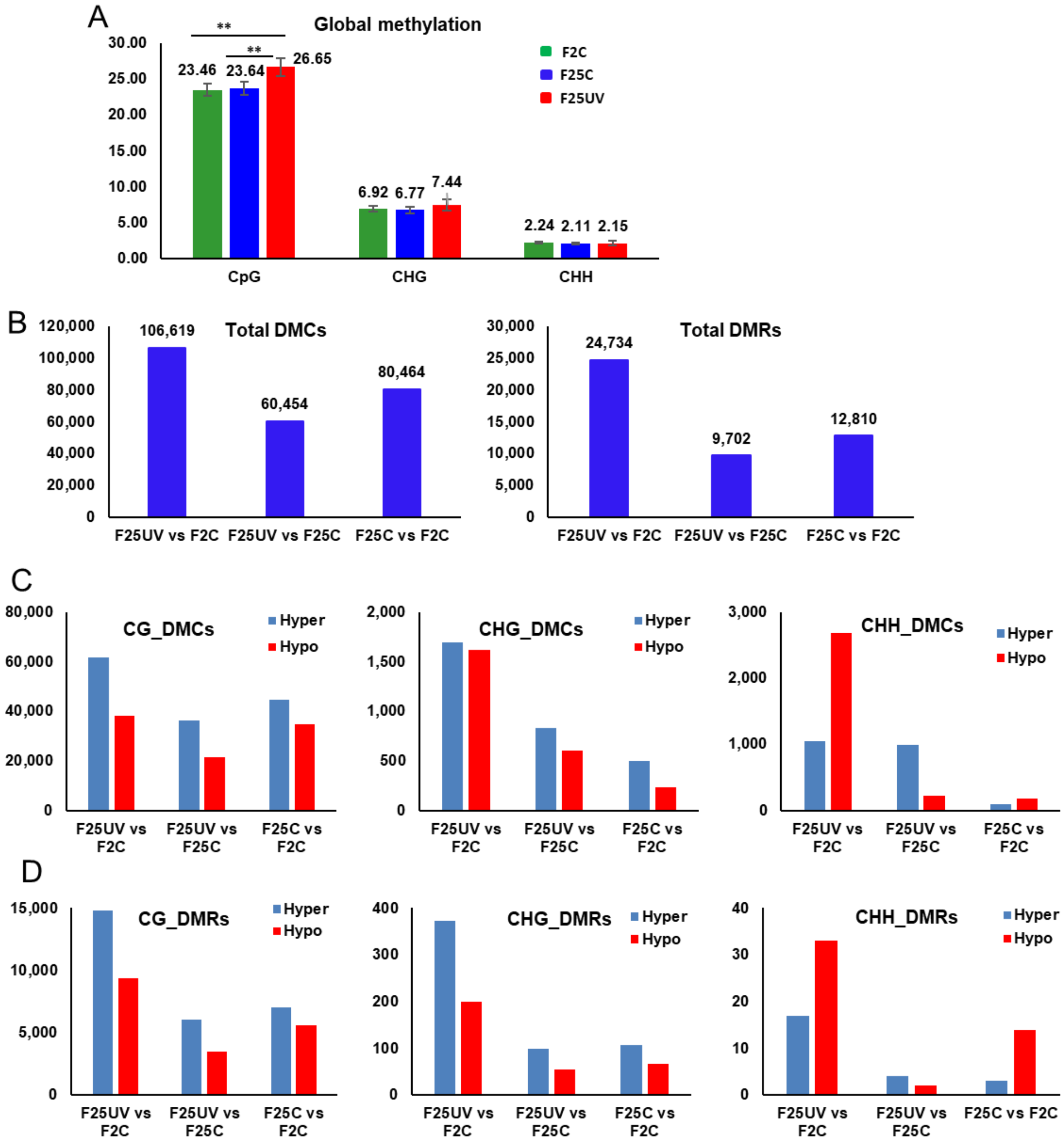


Figure 3.10. Global methylation changes, differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) in F25UV, F25C and F2C plants. *(A) The average percentage of methylated cytosines in F2C, F25C, and F25UV in the CG, CHG, and CHH*

*sequence contexts (H=A, T, C). Methylation levels were determined from reads with the minimum coverage ≥ 10 mapped to the TAIR 10 reference genome by using Bismark software. The data were analysed by one-way ANOVA with the Tukey's multiple comparisons test using GraphPad Prism version 8.4.2 for Windows. The data are shown as the average percentage (with ±SD) of methylated cytosines from five individual methylomes in each of the progenies. The asterisk shows a significant difference between the stressed progeny and the parental control progeny (** $p < 0.05$). (**B**) The total number of DMCs and DMRs in F25UV vs. F2C, F25UV vs. F25C and F25C vs F2C comparison groups. (**C**) The total number of hyper- and hypomethylated DMCs in the CG, CHG and CHH contexts in F25UV vs. F2C, F25H vs. F25C and F25C vs F2C. (**D**) The total number of DMRs in CG, CHG and CHH contexts in F25UV vs. F2C, F25UV vs. F25C and F25C vs F2C comparison groups.*

3.8 Plants Belonging to F25UV and F2C Groups Cluster Separately in All Cytosine Contexts

To analyze whether individual plants in F25UV and F2C groups are different from each other, we performed clustering analysis. For DMCs, F25UV and F2C clustered completely separately at symmetrical CG and CHG, while at non-symmetrical CHH, F25UV plants clustered together, while F2C plants were split into two groups (Figure 3.11A). For DMRs, F25UV and F2C groups clustered separately in all sequence contexts (Figure 3.11B). Clustering of F25UV and F25C groups did not reveal complete separation for CG and CHG content but showed it for CHH content (Figure 3.11). Finally, clustering of F25C and F2C demonstrated rather random positioning of individual samples (Figure 3.11; Figure S5, and S6). We continued our analysis of relatedness of samples using the hierarchical clustering heatmap analysis (Figure 3.12). The heatmap analysis of DMCs in all three contexts revealed a clear separation of five samples of F25UV from five samples of the F2C or samples of the F25C groups, while comparison of F25C to F2C had less clear separation, with several F25C samples clustering together with F2C samples (Figure 3.12). Similar picture was observed for DMRs at CG context. This analysis revealed that while F25UV group was different from F2C and F25C groups, F25C and F2C appeared to be similar.

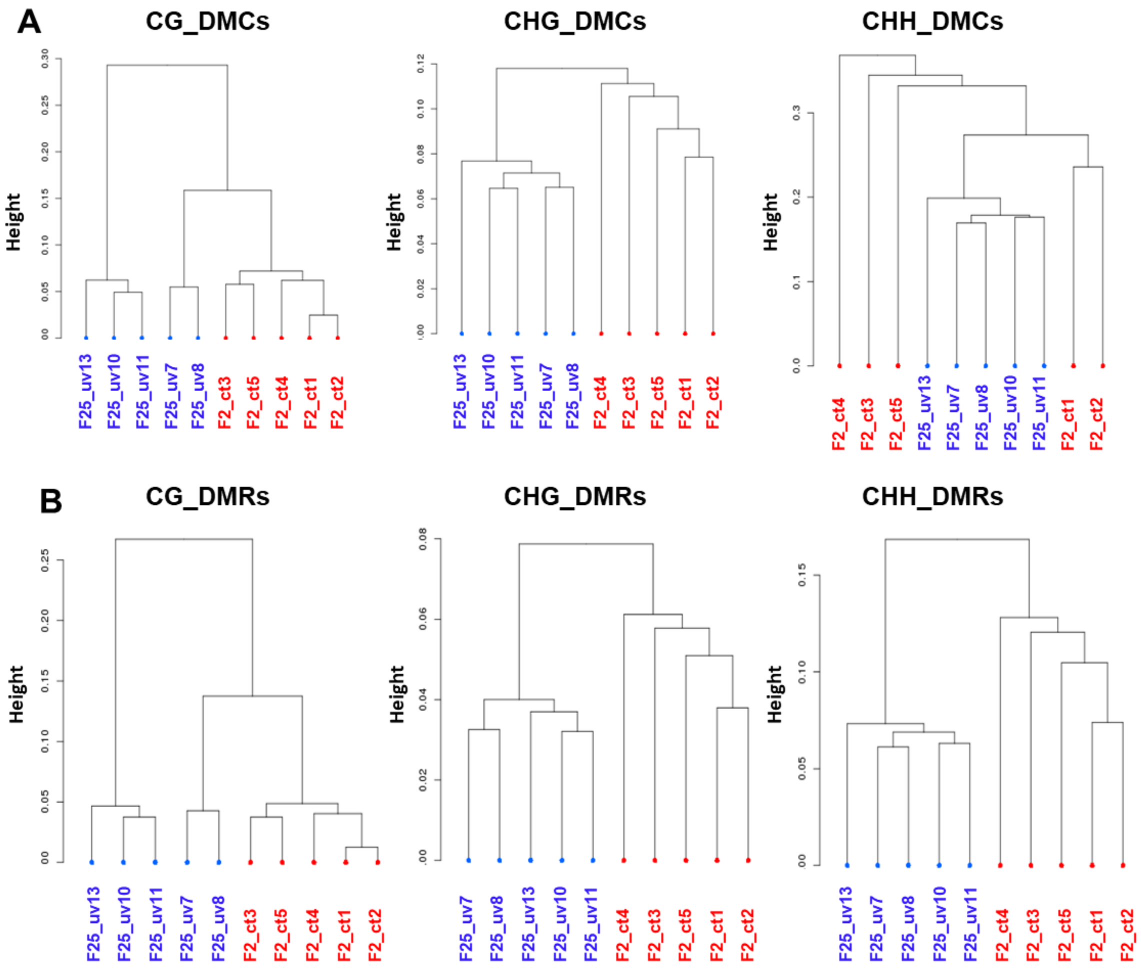


Figure 3.11. Global methylation clustering of DMCs (A) and DMRs (B) at CG, CHG and CHH context in F25UV vs F2 comparison. *Hierarchical clustering of F25UV vs. F2C using 1-Pearson's correlation distance. "Height" indicates the distance of split.*

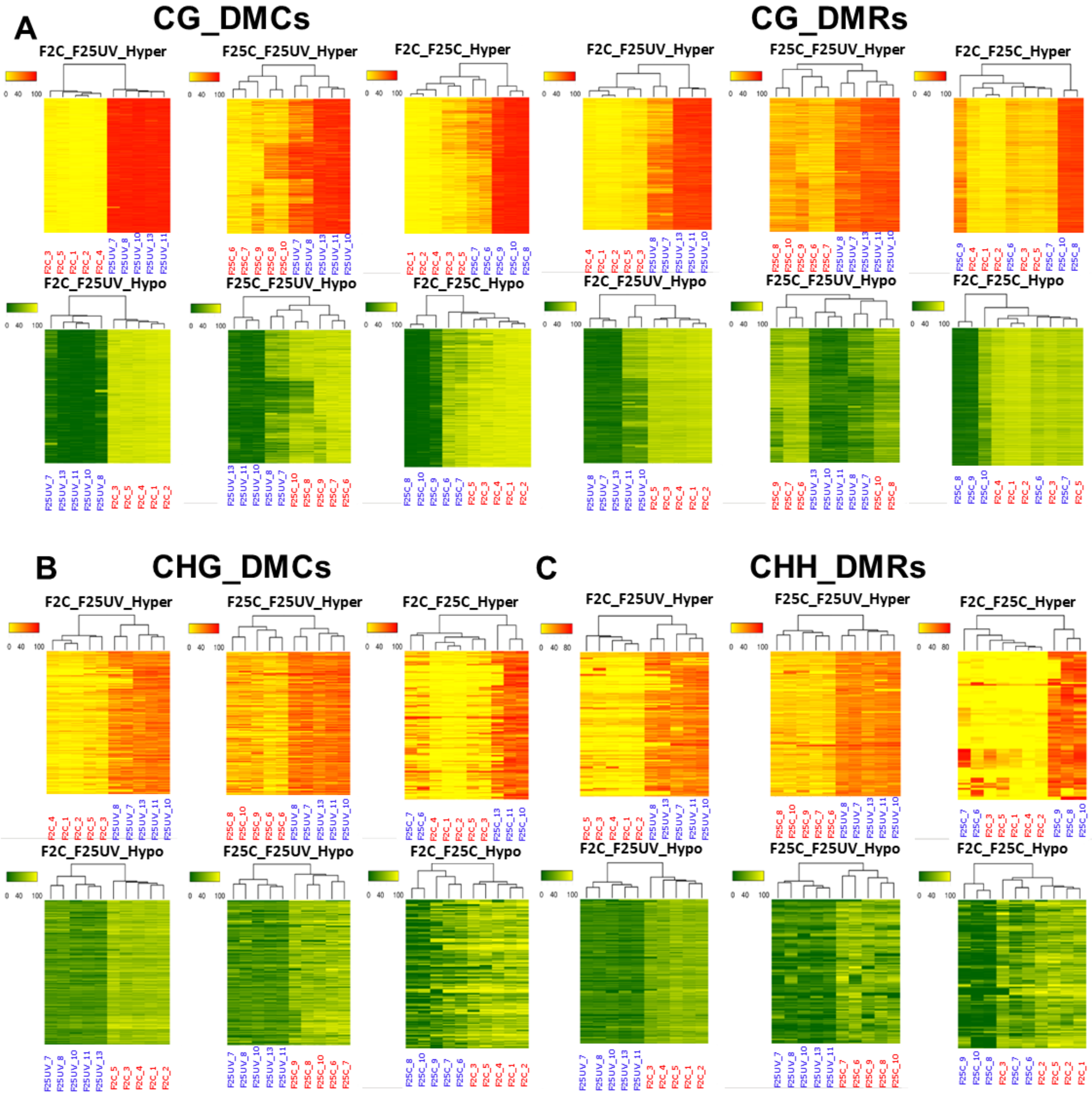


Figure 3.12. A hierarchical clustering heatmap analysis. *Heat maps of DMCs and DMRs for hypermethylated cytosines (the upper panel) and hypomethylated cytosines (the lower panel) in CG (**A**), CHG (**B**) and CHH (**C**) contexts in F25UV vs. F2C, F25UV vs F25C and F25C vs F2C comparison groups. Differentially methylated cytosines in the genome with differences of > 50% in F25H vs. F2C. In 'the upper panel', the red section indicates the larger percentage of methylation, and the yellow section indicates the lower percentage, and in 'the lower panel', the*

green section indicates the larger percentage of methylation, and the yellow one indicates the lower percentage, q-value <0.01.

3.9 Analysis of Methylation in Genic and Intergenic Regions

DMCs and DMRs were then characterized related to their genic and intergenic position. The location of hypo- or hyper- methylated DMCs and DMRs was compared to the annotated Arabidopsis genes using *genomation* Bioconductor package (Akalin et al., 2014). Both hyper- and hypo-methylated DMCs at the CG context were predominantly located in the genic regions, with higher fraction found in the gene body, and specifically in the exons (Figure 3.13A). No substantial differences between comparison groups were found, however, we noticed that F25UV group in comparison to F2C or F25C groups had lower percentage of hypermethylated DMCs in the exon region and higher percentage in the promoter region as compared to F25C vs F2C group comparison (Figure 3.13A). When similar analysis was performed in CHG and CHH contexts, we found large fraction of DMCs in promoter or intergenic regions. For hypomethylated CHG, drastic difference was found for DMCs in the intergenic regions; there were over ~20% more DMCs in F25UV comparison groups as compared to F25C vs F2C comparison group. Opposite was observed for promoter regions – F25UV comparison groups had lower percentage of hypomethylated DMCs as compared to F25C vs F2C group (Figure 3.13D). In the CHH context, there was the decrease in hypermethylated and hypomethylated cytosines in the promoter region in F25UV as compared to F25C and F2C groups, and the increase in the intergenic region (Figure 3.13E).

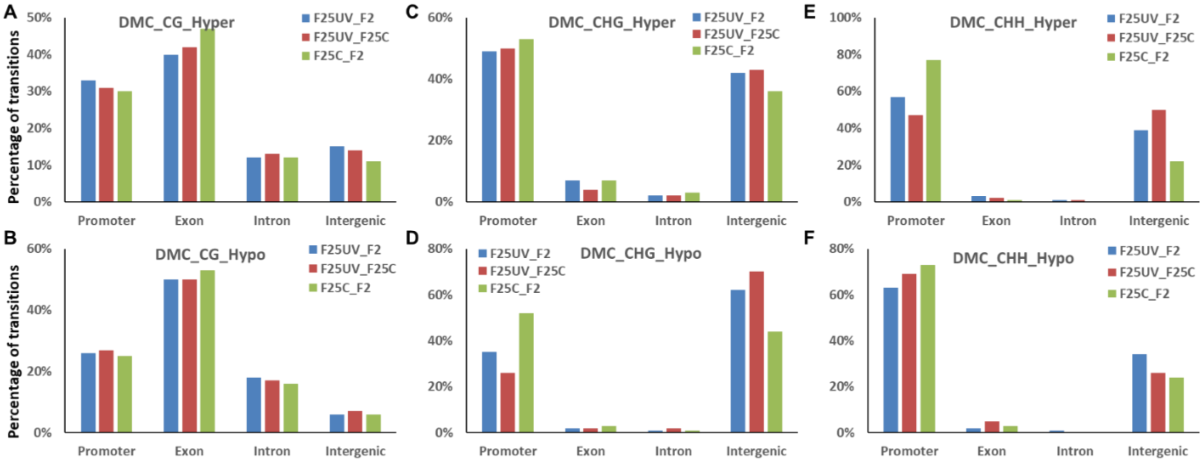


***Figure 3.13. The distribution of hyper-and hypo-methylated DMCs in the genic and intergenic regions in F25UV vs. F2C, F25UV vs F25C and F25C vs. F2C comparison groups**. (**A**) Distribution of CG DMCs and DMRs. (**B**) Distribution of CHG DMCs and DMRs. (**C**) Distribution of CHH DMCs and DMRs.*

In DMRs, no substantial difference was observed in CG contexts (Figure S7A, B). In the CHG context, changes in DMRs were similar to DMCs - over ~20% more hypomethylated DMRs were in F25UV comparison groups as compared to F25C vs F2C comparison group in the intergenic region, and opposite was observed in the promoter regions (Figure S7C, D). In the CHH context, there was the increase in the hypermethylated cytosines in the promoter region in F25UV as compared to F25C and F2C groups, and the decrease in the intergenic region (Figure S7E, F). Opposite was found for hypomethylated DMRs in CHH context – decrease in the promoter regions and increase in the intergenic regions (Figure S7F).

3.10 Pathway Enrichment for Epimutation-Associated Genes

Gene Ontology (GO) analysis of DMRs in the hyper- and hypomethylated CG contexts did not show any substantial differences in the enrichment of biological processes; “unknown biological processes" were enriched in F25UV comparison groups but were absent in F25C vs F2C group (Figure 3.14A,B). There were differences in "DNA and RNA metabolism", but they were not significant. In contrast, when the same was analysed in the CHG context, we found enrichment in biological process "DNA or RNA metabolism" in the F25UV hypomethylated group, and underrepresentation in "response to stress", "response to biotic and abiotic stimulus", "signal transduction" and "other biological processes" in the F25UV hypermethylated group (Figure 3.14C).

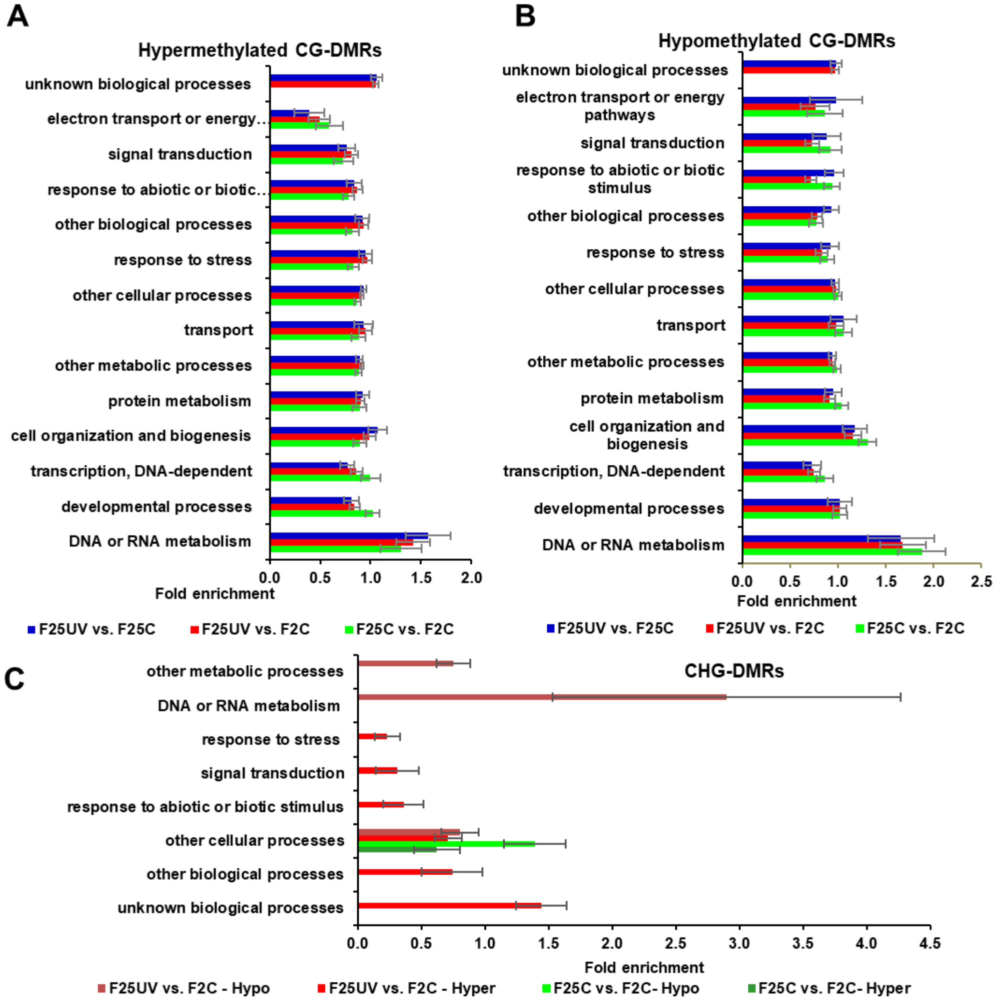


***Figure 3.14*. Enrichment analysis of hypermethylated DMRs on CG sites and their classification based on biological processes**. *Y-axis shows normalized Class Score with binomial coefficients as*

calculated by SuperViewer. To calculate enrichment, p-value of < 0.05, ±bootstrap StdDev were used.

CHAPTER 4: DISCUSSION

Phenotypic variations and biological significance. To evaluate the resistance of F25UV plants to UV-C, two different experiments were performed: in one, plants were irradiated and tested visually, while in another, plants were irradiated, and root elongation was measured. In neither case, we observed any difference in the performance of the F25UV group from the F25C group, indicating no increase, or even a decrease in the UV-C sensitivity in the F25UV group. Consistent with previous studies, our irradiated plants showed a significant reduction in root length in response to UV-C (Sheridan et al. (2022). The suppression of growth was noticeable in response to both UV-C doses, with a more acute drop in the root length when the full seedling was exposed rather than when only leaves were exposed. The absence of significant differences between the F25C and F25UV plants suggests the absence of phenotypic resilience to UV-C, at least in our settings.

Our previous research identified transgenerational phenotypic changes induced by the UV-C radiation, such as decreased leaf number in the progeny of UV-C stressed plants, alterations in bolting time, and larger seed size observed in the F1 progeny of stressed plants (Migicovsky & Kovalchuk, 2014; Rahavi & Kovalchuk, 2013). These findings suggest that it is feasible to expect further development of phenotypic resilience with continuous exposure to UV-C. It is possible that such an adaptive response to UV-C specifically is transient in nature since UV-C is not a stress that plants are commonly exposed to. It could be that such continuous exposure results in an adaptive response that is not distinguishable from the response of naïve plants; indeed, we did not observe any difference in the response of the F25UV and F25C plants to UV-C. A similar situation was reported by Zhang et al. (2024); rice plants were subjected to UV-C for four generations; analysis of G1 and G2 showed that plants exhibited a significant decrease in the grain length, grain width, spike weight, and thousand-grain weight, along with an increase in empty grain percentage and proanthocyanidin content compared to natural light conditions, demonstrating a negative response to UV-C. However, in generation G3, these parameters returned to control levels. This supports our observations; in our case, we noted phenotypic changes in the first and second generations of UV-C-exposed plants but observed responses similar to control in F25. (Migicovsky & Kovalchuk, 2014; Rahavi & Kovalchuk, 2013).

Some studies have suggested that transgenerational effects may not be a general response to abiotic stress in Arabidopsis (Pecinka et al., 2009). This indicates that the transmission of stress memory through epigenetic mechanisms may not always result in increased adaptability to stressors in plants and could be influenced by specific conditions or genetic factors. As seen in other cases, recurring biotic stress can induce transgenerational inheritance, although it does not necessarily imply the adaptability of an organism to a particular condition (Shahmohamadloo et al., 2024; Yun et al., 2022). In another study, Arabidopsis was subjected to a mild drought in a multi-generational experiment. Although plastic responses were observed, the descendants of stressed and non-stressed plants were phenotypically indistinguishable after being propagated without stress, regardless of whether they were grown under control conditions or a water deficit (Van Dooren et al., 2020).

In contrast to our findings, in other studies from our research group across 25 generations under heat and cold stress, enhanced stress resilience was found (Yadav et al., 2022; Rahman et al., 2024). Their work on Arabidopsis exposed to abiotic stress demonstrated transgenerational epigenetic inheritance that improved resistance to both cold and heat. In our study, we found that *A. thaliana* F25UV did not develop enhanced UV tolerance compared to control plants grown under normal conditions F25C. Despite the prolonged UV exposure across generations, F25UV plants exhibited a similar response to UV-C irradiation as the F25C plants, indicating that UV-C tolerance was neither acquired nor passed down through generations. The divergence in outcomes might be attributed to differences in the type of stress, with biotic factors perhaps eliciting more robust adaptive responses compared to abiotic stressors like UV-C radiation. Also, since UV-C is not naturally present on Earth's surface, no specific receptor to this type of radiation or responsive mechanism have been found on plants so far. Consequently, the stress induced by UV-C may lack significant biological relevance for plants in the short term from an evolutionary perspective.

When we examined the impact of UV-C radiation on root growth, we observed that UV-C exposure significantly inhibited primary root elongation in both F25C and F25UV plants. In our experiments, root length was measured after subjecting whole seedlings or just their leaves to 4 or 30 minutes of UV-C radiation. We found that a brief 4-minute exposure to UV-C caused a noticeable decrease in root length compared to control conditions. Interestingly, when only the leaves were exposed, root elongation was slightly greater than when the entire seedling was irradiated, although no significant difference was observed between F25C and F25UV plants.

With prolonged 30-minute UV-C exposure, we observed a further reduction in root growth, with minimal recovery over the following days. Root growth was largely stagnant in plants exposed to whole-seedling irradiation, underscoring the detrimental effects of UV-C on root development. However, similar to the shorter exposure duration, we did not find any significant differences in root growth between F25C and F25UV plants under both control and UV-treated conditions.

Similar results were found by Sheridan et al. (2022), where they reported the impact of UV-B radiation on primary root elongation in *A. thaliana seedlings.* In their work, focused on the role of cell proliferation and elongation in different root zones, UV-B exposure significantly inhibited root elongation by reducing cell proliferation in the meristematic zone, despite an increase in cell elongation, which partially compensated for the reduced proliferation. However, this compensation was insufficient to maintain normal meristem size, leading to smaller meristems in irradiated roots. Interestingly, while direct UV-B exposure to the root was necessary for complete inhibition, irradiation of leaves and shoots also resulted in a partial decrease in root length, suggesting the involvement of systemic signals transmitted from irradiated tissues to the roots. This supports the idea that UV-B-induced DNA damage triggers a broader stress response, impacting cell division and growth in both exposed and shielded tissues.

According to the series of research of Zhang et al. (2020), UV-B radiation inhibits primary root growth in *Arabidopsis thaliana* by inducing cell death in root tips, which disrupts cell division, elongation, and auxin flow. This cell death leads to reduced auxin accumulation in roots, further impeding root growth. Supplementing with auxin (α-naphthylacetic acid) alleviated the UV-B-induced growth inhibition, highlighting auxin's role in this process. Additionally, UV-B radiation downregulated genes related to cell wall structure, wax, cutin, and suberin biosynthesis, as well as photosynthesis-related genes in roots, collectively repressing root system growth and development. This study suggests that UV-B radiation impacts root growth by causing root tip cell death and altering metabolic processes in roots. (Wan et al., 2018a; 2018b).

In the context of UV-C exposure, as previously reviewed, extended exposure beyond 30 minutes leads to permanent physiological and morphological damage, eventually causing plant death. However, lower duration of exposure to UV-C (up to 30 minutes) enables plants to partially restore normal physiological functions and trigger adaptive responses, including the activation of both enzymatic and non-enzymatic defense mechanisms (Katerova et al., 2008; Rai et al., 2011;

Castronuovo et al., 2014). In our previous reports, Boyko et al. (2010) showed that exposure of Arabidopsis plants to different stresses, including UV-C (plantlets were exposed to 5,000 erg UV-C every day for 4 days starting at 7 dpg), resulted in a higher homologous recombination frequency (HRF), increased global genome methylation, and higher tolerance to stress in the untreated progeny. However, this transgenerational effect did not persist in successive generations. The results showed that whereas changes in HRF usually persisted in the S2 generation plants (plants that were exposed to stress continuously for two generations), the S1C1 (plants that experienced stress in the first generation but not in the second) plants usually exhibited HRF values comparable to control. Thus, except for a slight but significant effect in UV-C-treated plants, the transgenerational effect does not appear to be persistent in successive generations of untreated plants.

In more recent research, our group analyzed seed size, leaf size, bolting time and transposon expression in 2 consecutive generations of *A. thaliana* plants *dcl2*, *dcl3* and *dcl4* mutants in parallel with wild-type plants exposed to moderate UV-C stress. At approximately one-week post germination, plants were exposed to 4 minutes of UV-C irradiation (G30T8) of 30.5 W and 99 V, and UV output of 13.9 W and measurements were taken seven days post-stress (Migicovsky & Kovalchuk, 2014). The physiological changes observed in the progeny of UV-C-stressed *A. thaliana* highlight significant transgenerational effects on leaf morphology, seed size, and bolting time. In the F1 generation, the progeny exhibited fewer but larger leaves, with this effect being less pronounced or absent in *dcl2*, *dcl3*, and *dcl4* mutants, suggesting a critical role for Dicer-like proteins in mediating these transgenerational responses. Interestingly, seeds from UV-C-stressed plants were smaller, particularly in *dcl2* and *dcl4* mutants, whereas *dcl3* seeds displayed an increase in length, indicating variability in stress responses depending on genetic background. The delayed bolting observed in F1, coupled with accelerated flowering due to salicylic acid (SA) accumulation, aligns with previous findings that UV-C stress triggers early reproductive development to enhance survival. However, the F2 generation showed a different pattern, with fewer leaves following two generations of stress, and an unexpected increase in seed size, potentially as a compensatory mechanism. Furthermore, bolting time in F2 decreased, suggesting an adaptive transgenerational shift opposite to the immediate F1 response. These findings underscore the complex interplay between stress exposure duration, transgenerational memory, and genetic regulation in shaping plant physiological adaptation to environmental stressors.

Overall, our findings suggest that even after 25 generations of UV exposure, *A. thaliana* progeny did not develop a UV-tolerant phenotype. This indicates that the stress induced by UV-C does not result in heritable changes that enhance tolerance in subsequent generations.

Genetic changes. InDels and SNPs play critical roles in transgenerational inheritance and evolutionary processes, particularly under stress conditions. InDels can introduce frameshift mutations or disrupt regulatory elements, leading to changes in gene expression that may be inherited by future generations if they confer adaptive advantages. Stress conditions may increase the frequency of InDels through replication or repair errors, potentially contributing to long-term adaptation. Similarly, SNPs, the most common form of genetic variation, can affect protein-coding sequences or regulatory regions, influencing phenotypic plasticity and enhancing an organism's ability to cope with environmental stress. Both InDels and SNPs provide the genetic diversity required for natural selection, driving evolutionary changes as beneficial variants are passed on to successive generations, promoting adaptation and survival in changing environments.

UV radiation is known to cause various types of DNA damage, including the formation of pyrimidine dimers, which can lead to errors during DNA replication. This often results in an increased mutation rate, which includes both SNPs and InDels.

The increase in genetic variations through SNPs and InDels in the F25UV group might indicate an adaptive response to the environmental stress imposed by UV radiation. Plants exposed to UV light may accumulate mutations that could potentially confer resistance or tolerance to UV-induced damage. Deletions are more likely to occur under stress conditions, such as UV exposure, due to error-prone repair mechanisms like NHEJ. UV-induced DNA damage often results in double-strand breaks, which are more prone to being inaccurately repaired, leading to deletions. The lack of a significant difference in the number of insertions suggests that the mechanisms leading to insertions might not be as influenced by UV exposure or that they are counterbalanced by other repair mechanisms.

Epigenetic changes. The results of our study revealed that multigenerational UV-C exposure in Arabidopsis thaliana induced significant genetic and epigenetic changes but did not result in increased UV tolerance, challenging previous assumptions about the potential adaptive value of such mutations. Despite the accumulation of unique SNPs and DMCs, no phenotypic resilience to UV stress was observed in the F25UV generation. In accordance with our findings, earlier studies demonstrated that stress-induced epigenetic modifications do not always confer direct fitness advantages. For example, Ferreira et al. (2015) observed no improved salt tolerance in multigenerational stressed rice despite an increase in epigenetic variability. This supports the hypothesis that epigenetic changes might play roles beyond immediate adaptive responses, potentially contributing to evolutionary processes at a longer timescale.

Analysis of DMCs showed that F25UV plants had more differentially methylated cytosines than F2C group at all contexts; it also had more DMCs in comparison to F25C groups at CHG and CHH contexts, while F25C in comparison to F2C group had the lowest number of DMCs. The accumulation of C-to-T transitions, especially in the F25UV group, likely results from deamination of methylated cytosines, consistent with findings from Kusmartsev et al. (2020). Methylated cytosines deaminate at higher rates than unmethylated cytosines, and the lesions they produce are repaired less efficiently. As a result, methylated cytosines are mutational hotspots.

The increased mutability of 5-methylcytosine (5mC) has influenced patterns of genetic diversity and genomic evolution. In mammalian populations, CpG dinucleotides are more likely than other dinucleotides to exhibit polymorphism (Xia et al., 2012). Additionally, changes from CpG to TpG are disproportionately prevalent among variants linked to infection (Zemojtel et al., 2009, 2011; Cooper et al., 2010) and adaptation to new environments (Stoltzfus & McCandlish, 2017; Storz et al., 2019). Transitions from CpG to TpG often dominate substitution patterns observed between different species (Ebersberger et al., 2002; Hwang & Green, 2004). As discussed by Pértille et al. (2019) CpG mutation rates in natural populations could be influenced by environmental exposures affecting their methylation status. Interestingly, epigenetic alterations can be maintained for several generations (at least eight in plants) (Johannes et al., 2009; Cortijo et al., 2014). In addition to influencing point mutations, DNA methylation at CpG sites also contributes to the formation of larger genomic rearrangements. It plays a key role in regulating transposable element activity, which can lead to the creation of new genomic structures, including insertions, deletions, and duplications (Zhou et al., 2020).

Our methylation analysis also revealed significant differences in DMCs across cytosine contexts, with the F25UV group showing a dramatic increase in DMCs at CHH positions. These findings echo those of Wibowo et al. (2016), who noted stress-induced CHH methylation changes in plants, although they, too, did not observe direct resilience traits arising from these epimutations.

In a recent study, Laanen et al. (2021) utilized a multigenerational setup in which three generations (Parent, Generation 1, and Generation 2) of 7-day-old *Arabidopsis thaliana* plants were exposed to different gamma radiation levels (30, 60, 110, or 430 mGy/h) or to natural background radiation as a control for a duration of 14 days. They also employed whole-genome bisulfite sequencing to identify DMRs across three generations of plants under three radiation conditions: control, 30 mGy/h, and 110 mGy/h. When considering methylation context, it was found that the greatest number of changes occurred in cytosines followed by guanine (CG methylation), with fewer changes observed in CHG methylation and none in CHH methylation. Over the three generations exposed to the lowest radiation dose, there was a clear increase in IR-induced DMRs, with Generation 2 showing a substantially higher number than the Parent and Generation 1. Surprisingly, plants exposed to the highest dose rate showed no significant differences. They hypothesized that this could indicate that a certain threshold was crossed at which the plants switch to a different method of coping with the IR exposure. Probably a similar response may occur in UV-C radiation at different intensity levels.

The clustering and heatmap analyses of DMCs and DMRs across F25UV, F2C, and F25C groups reveal key insights into the methylation patterns associated with UV exposure and its potential epigenetic impacts on plant adaptation and stress response (Figure 3.12). The distinct separation of F25UV plants from F2C in both symmetrical (CG and CHG) and non-symmetrical (CHH) contexts suggests that UV exposure triggers specific and consistent changes in DNA methylation profiles, particularly in regions that are typically more conserved (CG and CHG). Our results are comparable to what was found by Yadav et al. (2022), where hierarchical clustering of epimutations and the heat map analysis of changes in both hypo- and hypermethylated DMPs revealed that the F25 heat-stressed (F25H) progeny of stressed plants showed a clear separation from plants of the parental and parallel control progenies, indicating directional epimutations in the F25H stressed progeny due to multigenerational exposure to heat stress. Similar results were found by Rahman et al. (2024), with clustering of DMCs and DMRs in F25 cold-stressed (F25Cd) vs. F2C comparison group showed a clear separation of F25Cd samples and F2C samples in all three methylation contexts. Similarly, the analysis of the relatedness of the samples using heatmaps and hierarchical clustering done for DMCs in the CG context demonstrated clear separation between F25Cd and F2C and F25C in most of the samples.

In contrast, F25C and F2C samples showed much less distinction, especially in CG and CHG contexts, where clustering was more random, suggesting that plants without UV exposure may retain a baseline or homeostatic methylation profile in the absence of environmental stress. The less pronounced separation between F25C and F2C could imply that, under controlled conditions, these groups maintain a similar epigenetic landscape, supporting the notion that methylation profiles remain stable in a non-stressful environment.

The heatmap analysis further supports these observations, with clear differentiation of F25UV from F2C and F25C, reinforcing that UV exposure induces a distinguishable epigenetic signature in the F25UV group, indicating directional epimutations in the F25UV stressed progeny due to multigenerational exposure to UV-C stress. This result could imply that CHH methylation, which is often transient and environmentally responsive, may serve as an adaptive mechanism under UV stress to cope with environmental challenges.

The location of hypo- or hyper- methylated DMCs was compared to the annotated Arabidopsis genes using *genomation* Bioconductor package (Akalin et al., 2014). Both hyper- and hypo-methylated DMCs at the CG context were predominantly located in the genic regions, with higher fraction found in the gene body, and specifically in the exons (Figure 3.13A). However, no substantial differences between comparison groups were found, although we noticed that F25UV group in comparison to F2C or F25C groups had lower percentage of hypermethylated DMCs in the exon region and higher percentage in the promoter region as compared to F25C vs F2C group comparison (Figure 3.13A). Both CHG and CHH genic methylation types are associated with reduced expression levels, as is CG methylation in promoter regions (Niederhuth et al. 2016). However, the exons of some genes (∼20% of *A. thaliana* genes) are methylated only in the CG context, a phenomenon called gene body methylation (gbM) (Takuno & Gaut, 2011). This type of methylation that occurs specifically in the CG context, positively correlates with gene expression, and is enriched over constitutively expressed genes, mostly moderately and constitutively expressed housekeeping genes (Horvath et al., 2019). However, as reviewed by Muyle et al. (2022), evolutionary and comparative studies based on genetic diversity or species comparisons have frequently, though not universally, shown a link between gbM and gene expression, particularly in relation to expression stability. Determining whether these associations are causal is challenging due to various complexities, such as functional overlap among epigenetic marks and the difficulty in distinguishing genetic from epigenetic influences. Nevertheless, this observed association between gbM and expression is noteworthy as it may represent a phenotype that natural selection could act upon. While further research is required to confirm if gbM is shaped by selection, both phylogenetic and population genetic analyses indicate that selection may preserve gbM in certain genes. Additionally, variations in gbM within populations have been linked to fitness under water stress and selection for flowering time (Shahzad et al., 2021). Furthermore, it has been found that transgenerational inheritance of acquired gbM helps environmental canalization of gene expression, facilitating long-term stress adaptation of mangroves facing of a severe reduction in genetic diversity (Wang et al., 2021).

When we analyzed the location of hypo- or hyper- methylated DMCs in CHG and CHH contexts, we found large fraction of DMCs in promoter or intergenic regions. For hypomethylated CHG, drastic difference was found for DMCs in the intergenic regions. Opposite was observed for promoter regions – F25UV comparison groups had lower percentage of hypomethylated DMCs as compared to F25C vs F2C group (Figure 3.13D). The transgenerational inheritance of the CHG methylation context remains unclear (Muyle et al., 2022). Although CHG methylation is retained during gametogenesis, epimutation accumulation lines in *A. thaliana* do not diverge for CHG methylation over generations (Van Der Graaf et al., 2015). Yadav et al. (2022) found that in the CHG context, plants exposed to 25 generations to heat stress showed a significantly lower global methylation level compared to controls. In accordance with these results, Rahman et al. (2024) data revealed that the average percentage of global genome methylation was significantly higher in F25 generation under cold stress in the case of the CpG, while statistically similar in CHG and CHH contexts, as compared to the controls. Also, even when methylation at CHG in F25Cd group was higher than in the other two control groups, the difference was not significant. These results suggest the lack of transgenerational inheritance relevance of this methylation context, supporting the idea that in our plants these changes may not play an adaptative role.

In the CHH context, we also found the decrease in hypermethylated and hypomethylated cytosines in the promoter region in F25UV as compared to F25C and F2C groups, and the increase in the intergenic region. CHH methylation is largely removed by demethylation in the male germline of *A. thaliana* and subsequently re-established during embryonic development. As a result, CHH methylation is only partially inherited, typically persisting for, at most, one or a few generations—though interestingly, it can be maintained in certain asexual plants that lack meiosis (Boquete et al., 2021). In terms of biological significance, the shift observed in methylation from promoter regions to intergenic regions may imply that UV stress causes distinct changes in gene regulation mechanisms, potentially impacting gene expression stability. As changes in CHG and CHH in the gene body, likely contributing to changes in phenotypes (Bewick & Schmitz, 2017), it has been also reported that the promoters of stress-responsive genes may be hypomethylated under stress conditions (Yao & Kovalchuk, 2011; Bilichak et al., 2012).

Biological Enrichment Analysis. The GO analysis of DMRs in the hyper- and hypomethylated CG contexts for UV stress did not reveal significant differences in the enrichment of biological processes (Figure 3.14). Notably, the presence of “unknown biological processes” in the F25UV comparison groups, which were absent in the F25C vs. F2C comparison, raises questions about the functional implications of these methylation changes. Although differences in “DNA and RNA metabolism” were observed, they did not reach statistical significance. However, the analysis of the CHG context revealed enrichment in “DNA or RNA metabolism” for the F25UV hypomethylated group, alongside an underrepresentation of crucial processes related to “response to stress,” “response to biotic and abiotic stimulus,” and “signal transduction” in the F25UV hypermethylated group. This suggests that UV-induced hypermethylation may disrupt essential stress response mechanisms.

Rahman et al. (2024) reported that the GO analysis in their study showed that while all groups had similar enrichment in CG contexts, significant differences were found in the CHG and CHH contexts. In the F25 cold-stressed progeny, they observed substantial enrichment in processes related to “response to abiotic and biotic stimuli,” “stress response,” and “other biological and metabolic processes” compared to control progenies. This indicates that methylation changes in the F25Cd group significantly impact CHG and CHH contexts, influencing various biological processes crucial for plant development and stress adaptation. In accordance with them, Yadav et al. (2022) found that GO analysis identified that epimutations in the F25H stressed progeny showed mainly enrichment in the processes such as response to abiotic and biotic stimulus, cell organizations and biogenesis, and DNA or RNA metabolism. It is important to highlight that the nature of the stress may have an impact on the mechanisms responsible for transgenerational adaptation. Considering that both studies already mentioned were focused on antithetical conditions (cold and heat), but still in the same category of stress (temperature), their results might have a strong correlation, even though the same model plant was used. In a different study, Laanen et al. (2021) reported that *Arabidopsis thaliana* plants exposed over multiple generations to ionizing radiation (gamma radiation), performed a functional analysis of the genes with DMRs (either in their promoter regions or their gene bodies) that showed an enrichment for genes related to development as well as various stress responses, including DNA repair, RNA splicing, and (a)biotic stress. On the other hand, our enrichment analysis of InDels revealed that cellular components “cell wall” and “extracellular” were significantly enriched in F25UV group, but not in the other groups (Figure 3.9A). Molecular functions “kinase activity” and “nucleotide binding” and biological processes “cell organization and biogenesis”, “response to stress”, “response to abiotic and biotic stimulus” and “other biological processes” were also enriched in F25UV as compared to other groups. In SNPs, molecular function “other binding” was enriched in F25UV group (Figure 3.9B).

Recently, Zhang et al. (2024) showed the complex biochemical and genetic foundations that govern the metabolite dynamics underlying the response, tolerance, and adaptive strategies of rice to UV-B stress. In their research, a comprehensive metabolic profiling was performed using leaves from 160 diverse rice accessions under UV-B and normal light conditions using a widely targeted metabolomics approach. Overall, it was found through GO enrichment and KEGG pathway enrichment that majority of associated with UV-B stress were involved in biological processes, cellular components, and molecular functions by GO analysis. These findings reinforce our results suggesting that UV radiation may have a role in modeling the organism in, for example, the cellular wall in order to adapt to UV. As reported in the same study, KEGG pathway analysis also revealed that the genes that were induced under normal conditions were primarily involved in amino acid metabolism, fatty acid metabolism, and glycolysis, whereas those induced under the UV-B conditions were predominantly involved in phenylpropanoid biosynthesis (which is an important way to produce lignin, a cell wall component), phenylalanine metabolism, and starch and sucrose metabolism (probably related to the cell wall composition).

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Our genomic study revealed that UV-stressed progeny showed higher number of genetic variations, where deletions were more prevalent than insertions. Also, UV-stressed progeny had a significantly higher percentage of nonsense mutations than either control groups. Analysis of DMCs showed that F25UV plants had more differentially methylated cytosines than F2C group at all contexts; it also had more DMCs in comparison to F25C groups at CHG and CHH contexts, while F25C in comparison to F2C group had the lowest number of DMCs. The most striking difference was observed for CHH context, where F25UV comparison to F2C revealed over 10-fold more DMCs at CHH position as compared to the control group comparison F25C to F2C. Multigenerational UV-C stress results in the increased number of hypermethylated cytosines at CG sites, equally increased the number of hyper- and hypomethylated cytosines at CHG sites and drastically increased the number of differentially methylated cytosines at CHH sites, with hypomethylated cytosines prevailing. The accumulation of genomic and epigenomic alterations related to stress responsiveness over multiple generations due to extended UV exposure may be adaptively guided and maintained by differential methylation and the expression of non-coding RNAs. This phenomenon has been observed in previous studies by our research group focusing on heat and cold stress, and in present study, likely as a result in conjunction with the increased mutation rate caused by UV radiation.

Future research should focus on identifying specifically both genetic and epigenetic components that contribute to UV resistance in plants, with the goal of advancing breeding programs for improved resilience. More specific genetic studies, including genome-wide association studies and quantitative trait loci mapping, can reveal specific genes involved in UV-C response pathways, such as those related to DNA repair, antioxidant production, and stress signaling.

Further experiments would be to examine whether the genetic and epigenetic changes observed in F25UV plants persist after the removal of stress. By allowing the F25UV progeny to recover under normal conditions for several generations, we could determine if these modifications are reversible or stable across non-stressed generations. This would provide insights into the long-term evolutionary impact of transgenerational stress, particularly whether epigenetic changes serve as a transient adaptive mechanism or a more permanent feature.

A comprehensive analysis of the generational transition from F1 to F25, including intermediate generations, could provide valuable information on the rate and nature of mutation accumulation. Such an approach would clarify the timeline of genetic and epigenetic changes and identify critical points where stress may exert the greatest influence. Comparing both control and UV-C-stressed lineages at each stage will help isolate the direct impacts of stress from natural generational variability.

A crucial aspect in our research was our limitation to find a phenotypic resilience in our UV-stressed progeny. Therefore, given the well-established role of flavonoids as UV protectants and their involvement in plant stress responses, measuring flavonoid content across generations under UV stress could elucidate whether these compounds play a role in the lack of UV resilience observed in F25UV plants. Additionally, analyzing chlorophyll a fluorescence could provide insights into the efficiency of the photosynthetic apparatus under UV exposure, offering potential explanations for the observed growth patterns.

To better understand the molecular impact of UV stress, future work should quantify pyrimidine (6-4) pyrimidine photoproducts and cyclobutane pyrimidine dimers, which are common forms of UV-induced DNA damage. Measuring photolyase, the enzyme responsible for repairing these lesions, activity of gene expression would further clarify the UV repair capacity in all the studied plant lines. A comparison of DNA damage levels and repair efficiency between F25UV and F25C groups could highlight any transgenerational modifications in DNA repair mechanisms.

However, since UV-C is not naturally present on Earth’s surface, no specific receptor to this type of radiation or responsive mechanism have been found on plants so far. Consequently, the stress induced by UV-C may lack significant biological relevance for plants in the short term from an evolutionary perspective. That said, an intriguing experiment, beyond our interest and probably reasonable possibilities, involving the exposure of plants to UV-C over many generations could lead to the evolution of a more refined response mechanism in plants. This might parallel Syuiti Mori’s experiments (Izutsu et al., 2016), where a captive colony of fruit flies were kept in a dark environment for sixty years and approximately 1500 generations, resulting in numerous genetic variations that could assist the flies' descendants in adapting to a life devoid of light.

In addition, while our study focused on UV-C stress, it would be valuable to explore whether the observed genetic and epigenetic changes in F25UV plants enhance resilience to other types of abiotic stresses, such as drought, salinity, or high temperatures. Testing F25UV plants under multifactorial stress combinations could reveal whether these modifications confer a broader adaptive advantage or are specific to UV stress.

Finally, chloroplasts play a vital role in plant stress responses, and their genome could potentially contribute to transgenerational inheritance mechanisms. Investigating the role of chloroplast DNA (cpDNA) in transgenerational epigenetic inheritance would add another layer of complexity to our understanding of how stress-induced changes are passed down. Exploring whether cpDNA undergoes stress-induced modifications related to nuclear DNA could help clarify the interplay between these organelles in long-term adaptation.

In conclusion, our study complements the various investigations conducted by our research group, enhancing the understanding of how different abiotic factors can adaptively shape plants. Direct applications in crop improvement, using the specific molecular changes identified in our research as a reference, could represent the main goal of our work.

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SUPPLEMENTARY MATERIAL

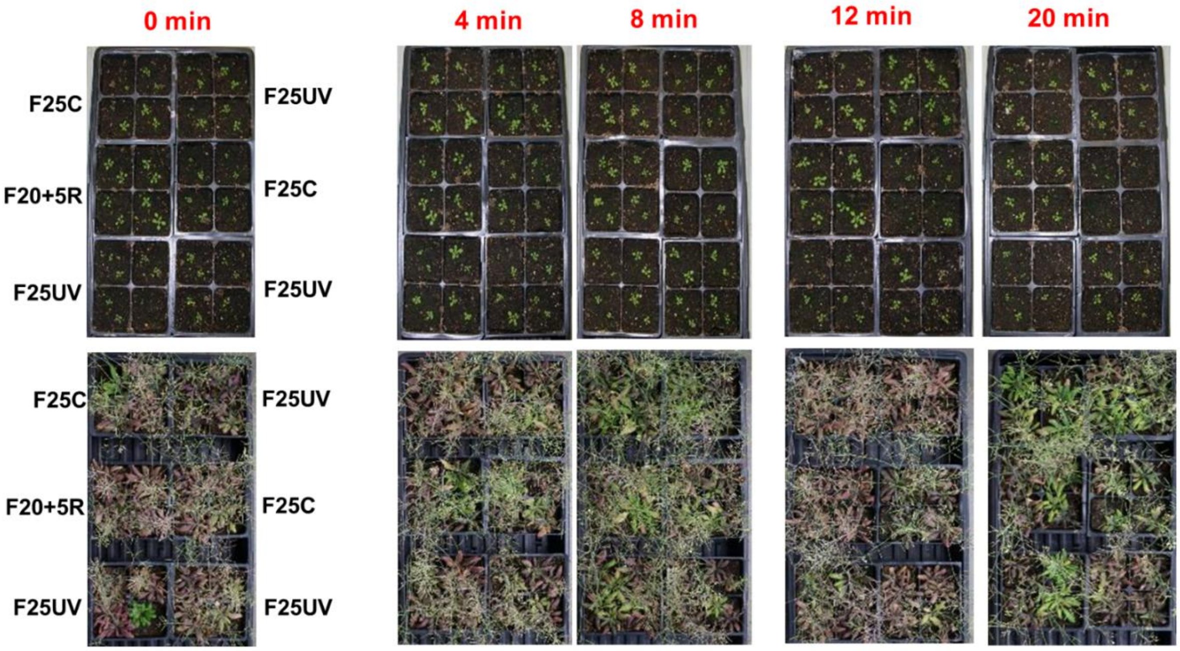

Figure S1. UV Stress phenotype at mature stage. UV stress progeny (F25UV) did not show any difference in growth as compared to parallel control progeny (F25C) under UV stress. An additional F20UV after 5 more generations without stress were included. UV stress was given at 10-days old seedlings. Growth conditions were the same as in the other experiments. Recovery photograph after 11-days of UV treatment.

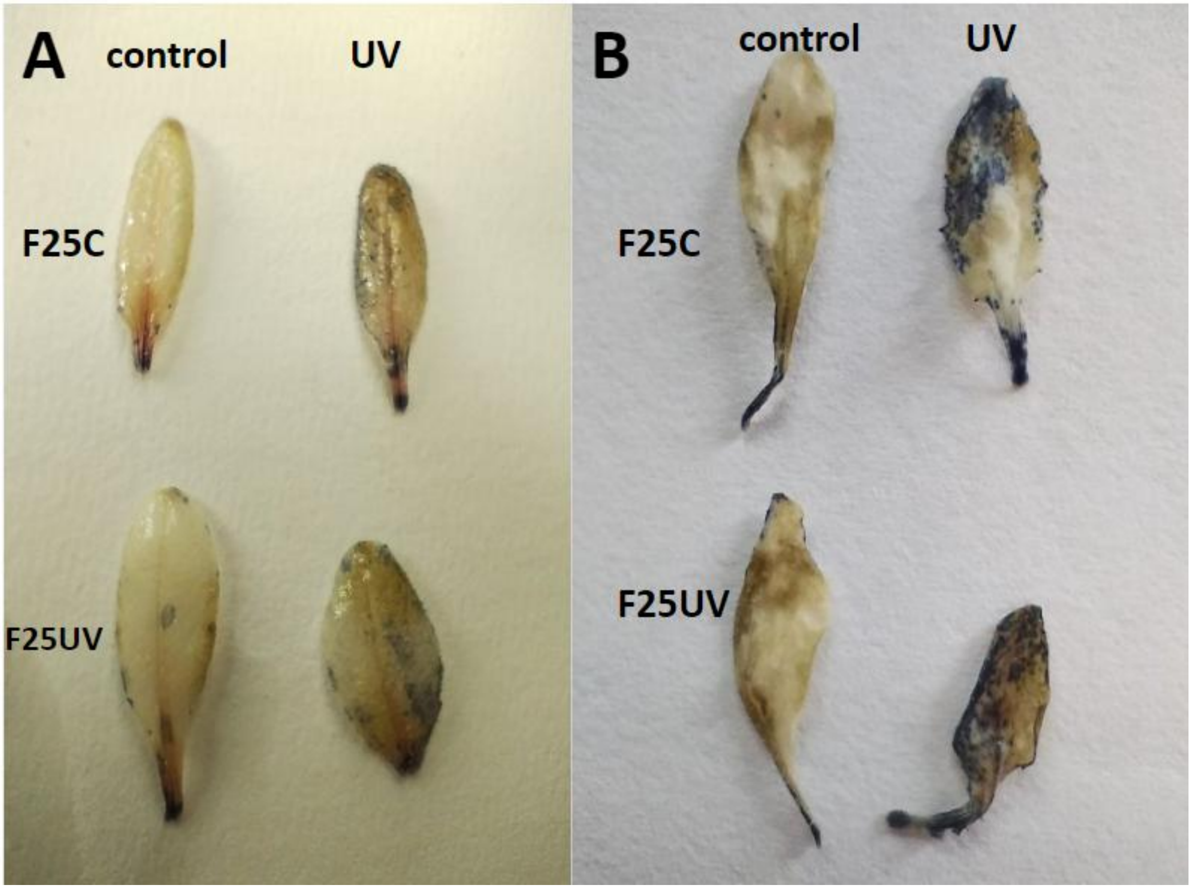

Figure S2. Figure S2. Oxidative stress assessment in leaves using DAB and NBT staining protocols. Images show leaves assessed using the DAB (**A**) and NBT (**B**) protocols to evaluate oxidative stress in control plants and plants exposed to UV-C radiation. Leaves were exposed to UV-C for 30 minutes using a germicidal lamp positioned 30 cm above the pots. Assessment of oxidative stress was performed visually. The NBT protocol detects superoxide anions, producing formazan that forms a blue coloration, while the DAB protocol detects hydrogen peroxide through oxidation, resulting in a brownish coloration. The intensity of the coloration correlates with the concentration of reactive oxygen species (ROS) in the sample. The figure presents the most representative leaves for each treatment, selected from a total of at least three leaves per protocol, sampled from five different plants per group.

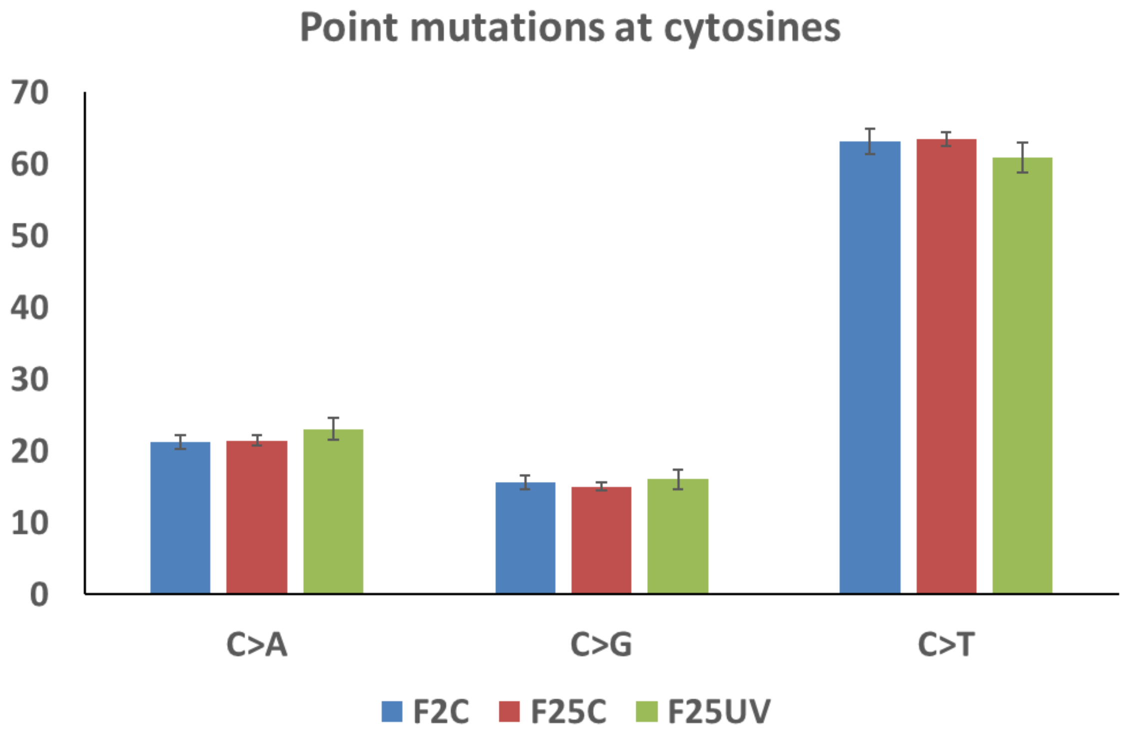

Figure S3. Analysis of point mutations at cytosines. Y axis shows the percentage of total. Bars show average with standard error calculated from 5 samples.

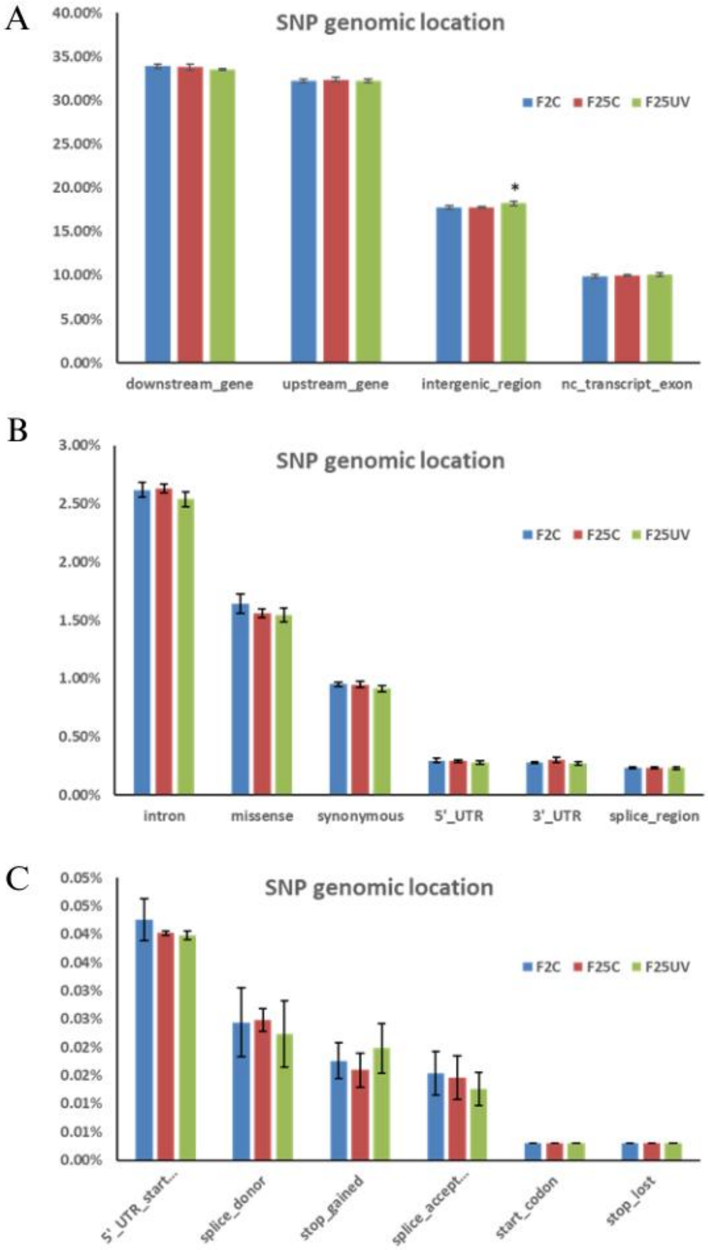

Figure S4. Genomic location of single nucleotide substitutions in F2, F25C and F25UV groups. A, B and C show different genomic locations according to the frequency of occurrence. Y axis demonstrates the percentage of mutations among all mutations, while X axis shows the genomic location groups. Data are shown as an average from five samples, with SE. Asterisk shows significant differences (t-test, two-sample assuming unequal variances; $p<0.05$).

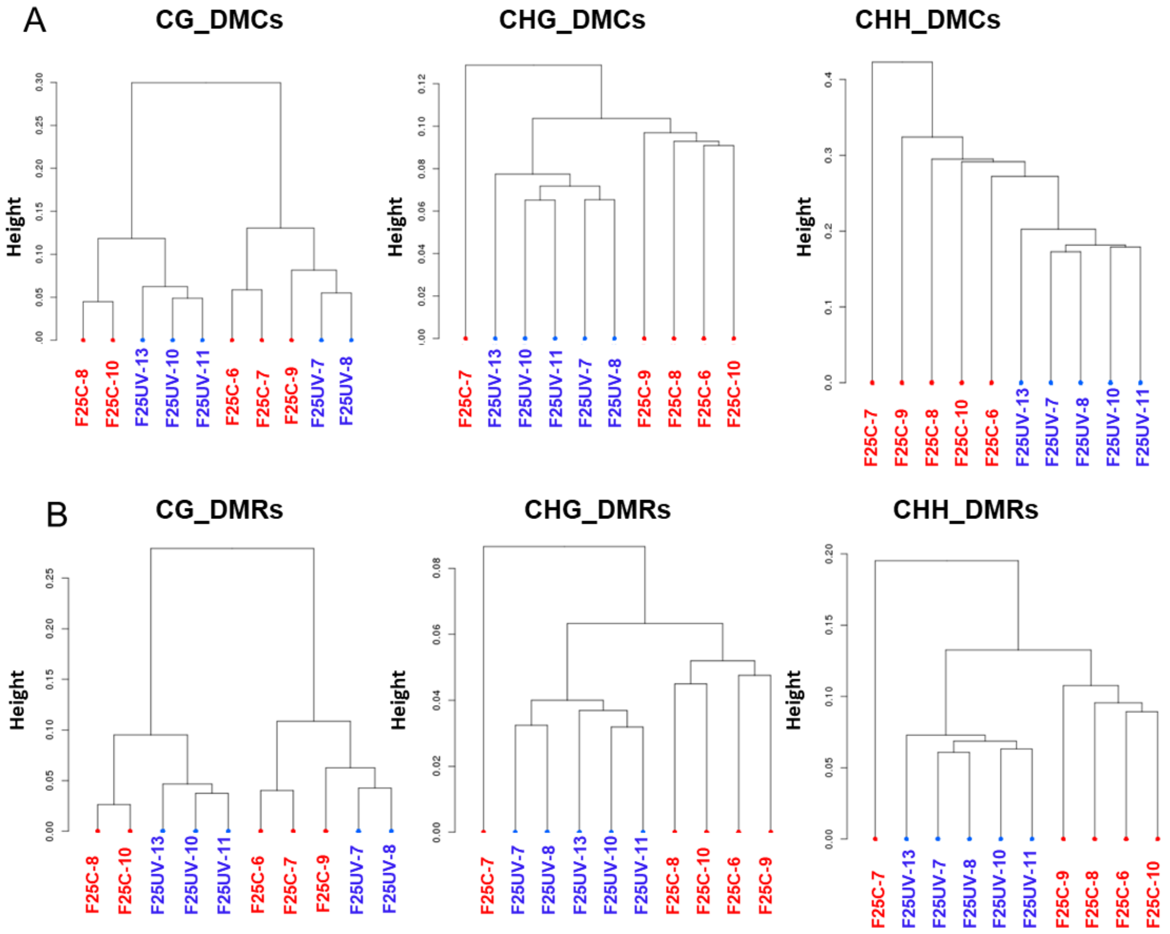

Figure S5. Hierarchical clustering of DMCs (A) and DMRs (B) at CG, CHG and CHH context in F25UV vs F25C comparison using 1-Pearson's correlation distance. "Height" indicates the distance of split.

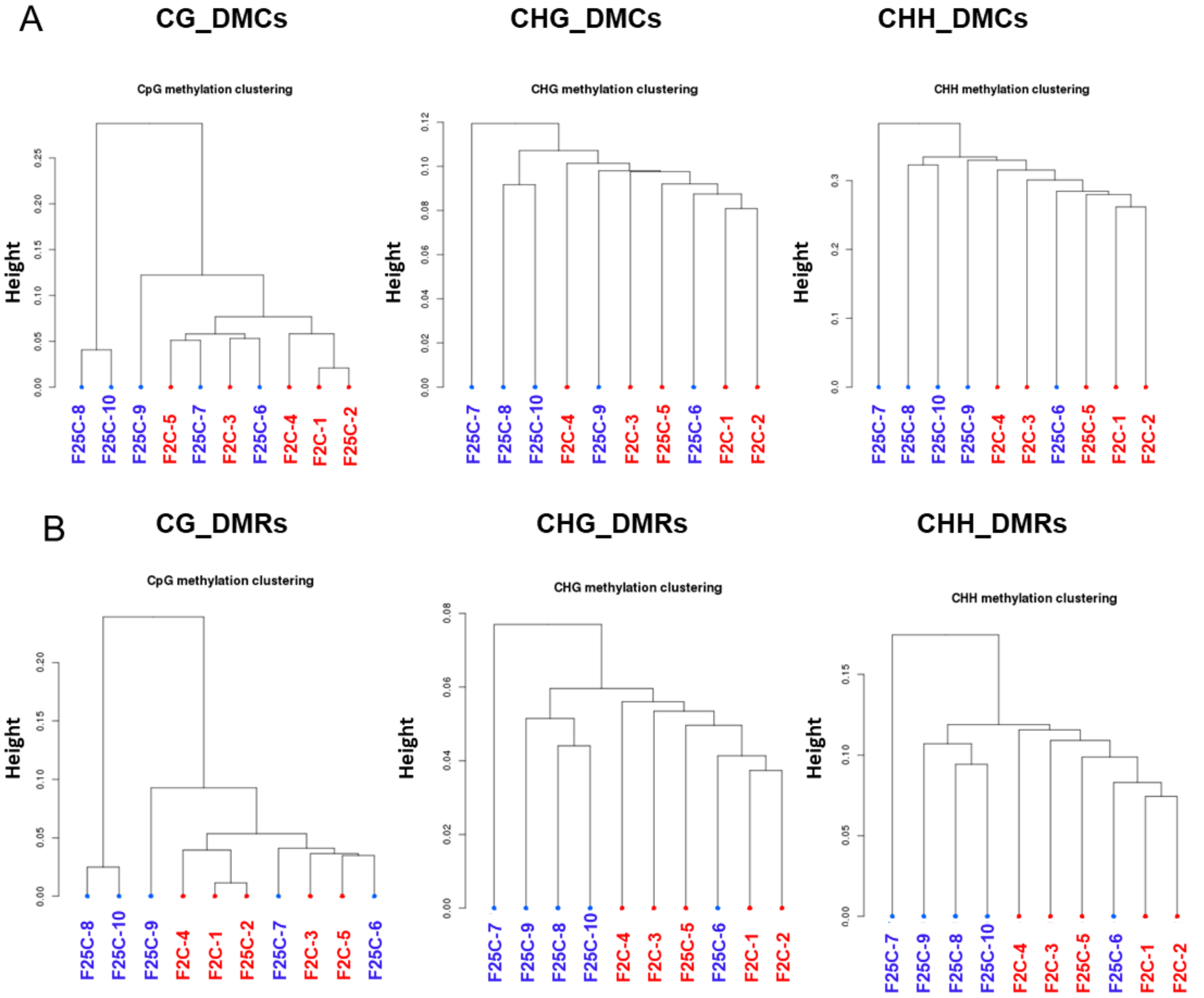

Figure S6. Hierarchical clustering of DMCs (A) and DMRs (B) at CG, CHG and CHH context in F25UV vs F2 comparison using 1-Pearson's correlation distance. "Height" indicates the distance of split.

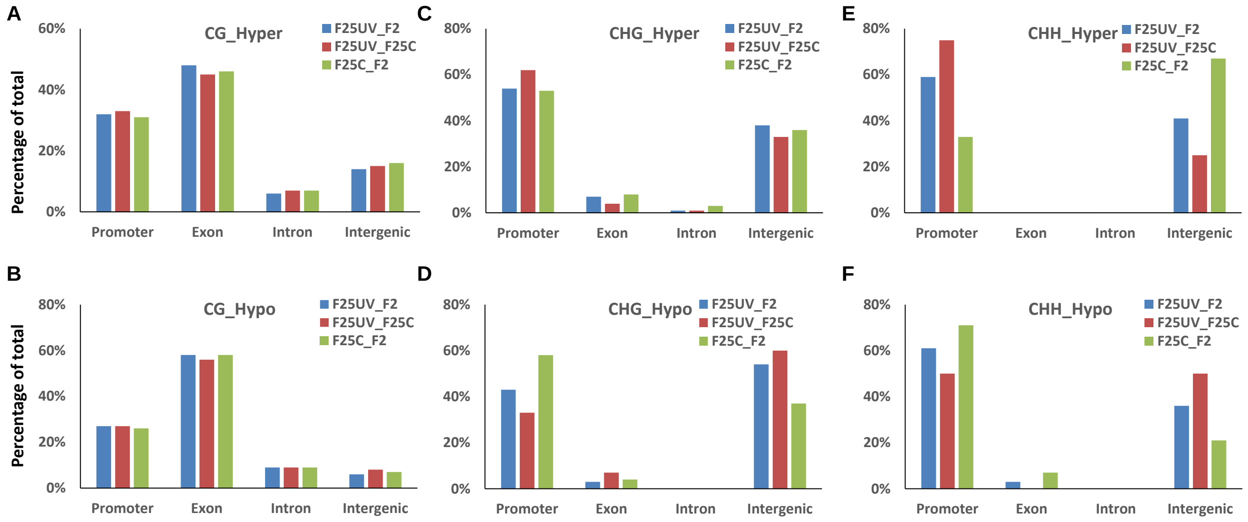

Figure S7. The distribution of hyper-and hypo-methylated DMCs in the genic and intergenic regions in F25UV vs. F2C, F25UV vs F25C and F25C vs. F2C comparison groups. (A) Distribution of CG DMCs and DMRs. (B) Distribution of CHG DMCs and DMRs. (C) Distribution of CHH DMCs and DMRs.