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A mutation in *ELA1*, an age-dependent negative regulator of *PAP1/MYB75*, causes UV- and cold stress-tolerance in *Arabidopsis thaliana* seedlings

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ABSTRACT

UV light is one of several severe abiotic stresses encountered by terrestrial plants. It is impairing cellular metabolism and causing cell death by damaging DNA, proteins, and lipids. Phenolics including various flavonoids serve as "sunscreens" to UV exposure; in particular, flavonoids play pivotal roles in absorbing free radicals, quenching singlet oxygen, and decomposing peroxides. Here, we screened *Arabidopsis thaliana* for a UV-tolerant mutant with elevated flavonoid levels, and identified the recessive *ela1* mutant. We demonstrate that the enhanced UV stress-tolerance shown in the *ela1* mutant seems to be conferred by high levels of UV-absorbing pigments such as anthocyanins. The *ela1* mutant exhibited novel characteristics as a negative regulator of *PAP1* and cold stress-tolerance, and its role is specific to early stages of *A. thaliana* development. Our results clearly suggest that flavonoid biosynthesis is regulated by a developmental program where *ELA1* participates as an important regulatory component.

1. Introduction

UV light is one of the more severe abiotic stresses encountered by an organism as induces the generation of reactive oxygen species, resulting in deleterious effects by damaging biologically essential macromolecules such as DNA, proteins, and lipids ([1]; Schulte-Frohlinde et al., 1991; [2,3,42]). Plants exhibit an increased tolerance to UV stress compared to microbes or mammalian cells due to the aid of various secondary metabolites, such as polyphenolics, that absorb UV light and prevent its penetration into the cell [4]. Based on these findings, myriad studies have been performed to elucidate the molecular events surrounding UV stress in plants.

Several studies have shown that the accumulation of flavonoid, including red and purple pigments, is strongly associated with UV stress [4–7]. Flavonoids appear to accumulate in epidermal cells following UV exposure, due to enhanced expression of their biosynthetic genes [8]. In addition to UV stress, abiotic and biotic stresses can also trigger flavonoid accumulation [9,10]. The role for flavonoids in these various processes likely arises from their ability to remove dangerous stress-response elements from the cell, including free radicals, singlet oxygen molecules, and peroxides [11,12]. Some flavonoids also play various roles in plant growth and development, including phyto-hormone signaling, pollen tube growth, and allelopathy [13–15]. This functional diversity manifested by flavonoids is due to the structural diversity of these compounds.

Genetic and molecular approaches in last few decades have uncovered many important genetic loci that control expression of UV stress associated flavonoid biosynthetic genes [4,16–18]. For example, UV-hypersensitive *transparent testa* mutants have defects in flavonoid biosynthesis (see review by Grotewold [19]), whereas UV-tolerant mutants reportedly exhibit accumulation of UV-absorbing compounds [20,21]. For example, UV-B-absorbing compounds were more abundant in *rcd1-2* mutants than in wild-type *Arabidopsis* following UV-B exposure for 24 h [22]. This mutation in *RCD1* also caused reduced growth. Additionally, there are several lines of evidence that suggest that DNA repair enhanced active oxygen species-scavenging activities, or higher ploidy levels can also account for UV-tolerance in plants [23,24].

The pivotal roles played by flavonoids explain why their biosyntheses are tightly regulated by various transcription factors. Of these transcription factors, the maize *myb* genes *C1* and *pl* are known to increase anthocyanin production by inducing *chalcone synthase* (*CHS*) expression. It was reported that a hybrid transgenic *Arabidopsis* expressing both *C1* and *R* produced anthocyanins in

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tissues that normally do not express these flavonoids [14,25]. Certain types of *myb* transcription factors require other transcription factors to turn on their downstream genes. For example, the WD40 proteins TTG1 and AN11 are required for MYB control of flavonoid biosynthesis in both *Arabidopsis* and *Petunia* [26,27]. However, when constitutively over-expressed the *myb* gene *PAP1* can independently activate *phenylalanine ammonia lyase* (*PAL*), *CHS*, and the genes encoding *dihydroflavonol reductase* (*DFR*) and *glutathione S-transferase* [28].

Despite the large body of accumulating knowledge concerning flavonoids, the mechanisms underlying regulation of their biosynthesis have not yet been fully elucidated. In particular, very few components involved in the negative control of flavonoid biosynthesis have been isolated. Therefore, the objective of this study was to isolate an *Arabidopsis* mutant with elevated flavonoid levels in order to identify a novel negative regulator of flavonoid biosynthesis. We performed a large-scale screen for UV-B-tolerant mutants using commercially available ethylmethanesulfonate (EMS) M_2 seeds of *Arabidopsis thaliana*, and recovered the e*nhanced level of anthocyanin* 1 (*ela1*) mutant. We used this mutant to investigate the possible role of the ELA1 genetic locus on the regulation of flavonoid biosynthesis.

2. Materials and methods

2.1. Plant materials and mutant screening

A. thaliana (Colombia ecotype) plants were grown in MS (Murashige and Skoog salt)-agar medium containing 2% sucrose or in potting soil at a constant temperature of 23 °C with of 16 h light /8 h dark photocycles. *A. thaliana* seeds were sterilized and germinated on various agar MS media for stress treatment and growth measurement after vernalization for 3 days at 4 °C. Ethylmethanesulfonate (EMS)-exposed Col-0 seeds were employed for mutant screening. Approximately 70,000 seeds were sown and grown on normal MS media for 1 week, then irradiated with treated 7 W m^{-2} s^{-2} UV-B for 2.5 h. Mutants were selected based on their healthier phenotype compared with wild-type after a 5-day recovery under normal conditions.

2.2. Flavonoid staining

One-week-old wild-type and mutant seedlings were grown under normal conditions and stained for 15 min using saturated (0.25%, w/v) diphenylboric acid 2-aminoethyl ester (DPBA) with 0.005% Triton X-100. Flavonoid localization was visualized by confocal laser scanning microscopy. Fluorescence was imaged using an LSM 5 Exciter (Carl-Zeiss) with an EC Plan-NEOFLUAR 10x/0.30an objective lens, two HeNe and Ar ion lasers, and emission filters (LP 560 nm for rhodamine, BP 505-530 nm for GFP, and BP 530-600 nm for YFP).

2.3. Anthocyanin level measurement

Determination of anthocyanin levels was performed according to the method of Mancinelli et al. (1988). Four-day-old seedlings were transferred to medium and were treated with various stresses or hormones, including cold (4 °C), sucrose, auxin, ethylene, and kinetin for 3 d. The samples (40 mg) were ground in liquid nitrogen and extracted overnight in 200 μl of 1% (v/v) HCl in methanol at room temperature. After extraction, anthocyanins were separated from the chlorophyll by addition of 250 μl distilled water and 500 μl chloroform. The samples were then vortexed and centrifuged for 2 min at 3000 × *g* for the removal of chlorophyll. Relative anthocyanin levels in the aqueous phase were determined spectrophotometrically by measuring A_{530}.

2.4. RNA analysis

Wild-type and mutant seedlings at various developmental stages (4-, 7-, and 11-day-old) were grown on a standard MS media. The seedlings were then exposed to UV-B irradiation (0.3 W m^{-2} s^{-2}) for serial time periods. Total RNA was purified using the RNeasy Plant Mini kit (QIAGEN). Northern analysis was performed as described previously [29]. Total RNA (20 μg) was subjected to electrophoresis on 1.5% (w/v) agarose gels containing formaldehyde, and then transferred to a nylon membrane. Probes for *CHS*, *PAP1*, *PAP2*, *CHI*, *DFR*, 4*CL* and *CAD* were obtained from RIKEN *Arabidopsis* full-length cDNA clones. Specifically, *PAP1* and *PAP2* probes were obtained from PCR to obtain *PAP1* or *PAP2* specific probes (PAP1-F: 5′-GAGAGACATTACGCCCATTC; PAP1-R: 5′-AGGTGTCCCCCTTTTCTGTT & PAP2-F:5′-ATTATTTCCCCTCCTACAAC;PAP2-R:5′-GGAACAATCGCATCAGCTTC). Radiolabeled probes were prepared using a random primer DNA Labeling System (Invitrogen). After pre-hybridization for 1 h, the hybridization of ^{32}P-random-primed probes onto an RNA blot was carried out at 65 °C for over 16 h. The RNA blot was then washed twice in 1 × SSC, 0.1% SDS and once more in 0.1 × SSC, 0.1% SDS at 42 °C (for high stringency, we used 48 °C). The blot was then exposed to a film and developed.

2.5. Microarray analysis (commercial service)

Total RNA (5 μg) from fibroblast cultures was used for labeling. Probe synthesis from total RNA samples, hybridization, detection, and scanning were performed according to standard protocols from Affymetrix, Inc. The following trade-named reagents were obtained from Affymetrix unless otherwise specified. In brief, cDNA was synthesized from total RNA using the One-Cycle cDNA Synthesis Kit. Single stranded cDNA was synthesized using Superscript II reverse transcriptase and T_7-oligo(dT) primers at 42 °C for 1 h. Double stranded cDNA was obtained using DNA ligase, DNA polymerase I and RNase H at 16 °C for 2 h followed by T_4 DNA polymerase at 16 °C for 5 min for gap filling. After clean up with a Sample Cleanup Module, ds-cDNA was used for *in vitro* transcription (IVT). cDNA was transcribed using the GeneChip IVT Labeling Kit in the presence of biotin-labeled CTP and UTP. After clean up, 10–15 μg of labeled cRNA was fragmented from 35 to 200 bp by a fragmentation buffer. Fragmented cRNA was hybridized to Arabidopsis ATH1 gene chips (Affymetrix) at 45 °C for 16 h according to the manufacturer's protocol. After hybridization, the arrays were washed in a GeneChip Fluidics Station 450 with a non-stringent wash buffer at 25 °C followed by a stringent wash buffer at 50 °C. After washing, the arrays were stained with a streptavidin–phycoerythrin complex and intensities were determined with a GeneChip scanner 3000, controlled by GCOS Affymetrix software.

3. Results

3.1. Identification of ela1 mutant

In an effort to isolate UV tolerance-associated mutants with a high level of flavonoids, we screened *A. thaliana* Col-0 M_2 seeds treated with EMS (obtained from *The Arabidopsis Information Resources*). These seeds were germinated in normal MS medium containing 2% sucrose and 4-day-old seedlings were exposed to strong irradiation by UV-B (7 W/m^2) for 2 h, then allowed to recover 5 d under white light. A total of 75,000 M_2 seeds were screened and 5 candidate mutants were recovered. To ensure that the mutation causing the phenotype was transmitted through the germ line, mutants from next generation were re-tested for their UV-B tolerance. Finally, two promising mutants