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Abiotic stress-induced regulation of antioxidant genes in different *Arabidopsis* ecotypes: microarray data evaluation

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ABSTRACT

Although stresses induce generation of reactive oxygen species (ROS), which are highly reactive and toxic, and cause severe damage to cellular components; plants have very efficient enzymatic ROS-scavenging mechanisms. Despite the substantial knowledge produced about these enzymes, we still have limited knowledge regarding their expression patterns in relation to the stress type, duration and strength. Thus, taking advantage of microarray data, this work evaluated the abiotic stresses (salt, cold, heat and light) induced regulation of six antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), in 10 natural *Arabidopsis* ecotypes. The expression profiles of 36 genes encoding six enzymatic antioxidants including *CSD1-3*, *FSD1-3*, *MSD1-2*, *CAT1-3*, *APX1-6*, *APXT*, *APXS*, *GPX1-8*, *MDAR1-5* and *DHAR1-4* were investigated. In particular, *FSD1*, *FSD2*, *CSD1* and *CSD2* genes coding for SOD; *CAT2* and *CAT3* for CAT; *APX3-6*, *APXT* and *APXS* for APX; *GPX1*, *GPX2*, *GPX5*, *GPX6* and *GPX7* for GPX; *MDAR2-4* for MDHAR; and *DHAR1* and *DHAR3* for DHAR families appeared to be more differentially expressed under given stress conditions. Primarily, high light as well as salt and cold stresses considerably up-regulated the gene expression, whereas cold stress significantly led to the down-regulation of genes. The overall expression pattern of ecotypes suggested that the studied *Arabidopsis* genotypes had different stress tolerance.

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Antioxidant enzymes; ascorbate-glutathione cycle; ROS; oxidative stress; tolerance; expression profile

Introduction

Abiotic stresses induce the overproduction of reactive oxygen species (ROS) which are highly reactive and toxic, and cause severe damage to cellular molecules [1,2]. ROS induce damage (1) via peroxidation of lipids, which increases the membrane leakiness, reduces the membrane fluidity and damages the membrane proteins, ion channels, enzymes and receptors [3–5]; (2) through oxidation of proteins, mostly irreversible, which hinders or changes the protein activities and makes proteins more susceptible to proteolytic attack [6] and (3) by DNA damage with base deletions and modifications, strand breaks, cross-links and pyrimidine dimers [7], resulting in decreased or damaged protein syntheses, cell membrane destruction, replication errors, genomic instability and transcription arrest or induction [8,9]. ROS could be either free radicals such

as OH· (hydroxyl radical), O₂^{·-} (superoxide radical), HO₂· (perhydroxy radical) and RO· (alkoxy radical) or non-radical forms such as H₂O₂ (hydrogen peroxide) and ¹O₂ (singlet oxygen) [1,10–12]. They are mainly generated in mitochondria, chloroplasts and peroxisomes as byproducts of various metabolic pathways resulting from intense electron flow or highly oxidizing activities [13,14]. Besides, cytoplasm, endoplasmic reticulum (ER) and apoplast are also other substantial ROS production sources in plants [15]. ROS generation or accumulation is thus an inevitable outcome of normal metabolic processes in plants [1]. However, in steady state conditions, there is an equilibrium between ROS production and its scavenging; any disturbance of this equilibrium by biotic/abiotic stress factors could increase the intracellular ROS levels [16,17]. Moreover, ROS like H₂O₂ play dual roles in

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plants regulating many crucial metabolic processes, including acclimatory signalling [18], growth and development [19], cell cycle [20], photosynthesis and photorespiration [21], stomatal movement [22] and senescence [2]. Therefore, plants have very efficient ROS-scavenging mechanisms employing enzymatic and non-enzymatic components. For example, ascorbate peroxidase (APX; EC 1.11.1.11), glutathione peroxidase (GPX; EC 1.11.1.9), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2), superoxide dismutase (SOD; EC 1.15.1.1), dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), glutathione-S-transferase (GST; EC 2.5.1.18) and guaiacol peroxidase (GOPX; EC 1.11.1.7) as enzymatic machineries, and phenolic compounds, carotenoids, flavonoids, glutathione (GSH), alkaloids, ascorbic acid (AA), α -tocopherol and some amino acids as non-enzymatic defence molecules [1,23]. SOD is the most effective enzymatic antioxidant catalyzing the removal of $O_2^{\cdot-}$ with its dismutation, one $O_2^{\cdot-}$ being oxidized to O_2 while another reduced to H_2O_2 . There are three known SOD types based on their cofactors: Fe-SOD, Mn-SOD and Cu/Zn-SOD [24]. The *Arabidopsis* genome was reported to contain three *Cu/ZnSOD* and three *FeSOD* genes, and one *MnSOD* gene [25]. CAT has one of the highest turnover rates among all enzymes, removing H_2O_2 by directly converting it into H_2O and O_2 . Its isoforms, including two in barley [26]; three in *Arabidopsis* [27], maize [28], pumpkin [29] and rice [30]; and four in sunflower [31] have been previously reported. APX functions in the scavenging of H_2O_2 in ascorbate-glutathione (ASH-GSH) and water-water cycles using ASH as the electron donor. Studies have reported eight *APX* genes in *Arabidopsis*, eight in rice, and seven in tomato [32–34]. GPX is another important enzyme involved in the reduction of H_2O_2 and some other peroxides using GSH. *Arabidopsis* genome were reported to include eight *GPX* genes [35,36]. MDHAR is a FAD-enzyme catalyzing the reduction of monodehydroascorbate (MDHA) radicals in the presence of NAD(P)H. MDHAR cDNA has been cloned from a number of plant species, including *Arabidopsis*, cucumber, soybean, potato, tomato, pea, rice and leaf mustard [37]. DHAR catalyzes the re-reduction of DHA (dehydroascorbic acid) to ascorbate by glutathione (GSH). The crystal structure of DHARs from *Arabidopsis* and their pivotal role of DHARs under photooxidative stress has been investigated [38,39]. GR is an important enzyme in the ASH-GSH cycle, playing an essential role in ROS defence by maintaining the reduced GSH level via catalyzing the NADPH dependent reaction of glutathione disulphide (GSSG). In a recent genome-

wide study, two and three *GR* genes in *Arabidopsis* and rice (*Oryza sativa*) genomes, respectively, were reported [40]. Considering the presence or abundance of these antioxidant enzymes in almost all plant cells in response to various stress conditions, it is widely acknowledged that ROS-scavenging is significant for plant survivability as well as for crop productivity [24,41,42]. Although a great amount of research has produced substantial knowledge about these enzymes, we still seem to possess just a general idea of their complex roles under various conditions. Thus, taking advantage of microarray data, we investigated the abiotic stress-induced regulation of antioxidant enzyme genes in 10 different *Arabidopsis* ecotypes, with an ultimate goal of understanding the expression profiles of these enzymes under various stress factors. In this study, (1) the regulation of six *Arabidopsis* antioxidant enzyme genes, *SOD*, *CAT*, *APX*, *GPX*, *MDHAR* and *DHAR*, were investigated under cold, heat, high light and salt stress; (2) the type of stress factor that leads to significant up/down regulation of these genes was identified; and (3) the association of the expression pattern in relation to different ecotypes was investigated.

Materials and methods

Microarray data

The microarray data for this study was retrieved from the National Center for Biotechnology Information (NCBI) GEO datasets repository (ncbi.nlm.nih.gov/geo/) with record GSE41935 [43,44]. Three-week-old plants from 10 natural *Arabidopsis* ecotypes, An-1 (Antwerp/Belgium), Cvi (Cape Verdian Islands), Col-0 (Columbia/United States), C24 (Coimbra/Portugal), Eri (Eriksboda/Sweden), Kas-1 (Kashmir/India), Kond (Kondara/Tajikistan), Kyo-2 (Kyoto/Japan), Ler (Landsberg/Poland) and Sha (Shakdara/Tajikistan), were subjected to salinity (100 mmol/L NaCl), cold (10 °C), heat (38 °C) and light (800 μ mol photons $m^{-2}s^{-1}$) stresses for 3 hours. RNA was extracted from leaf samples. GPL16226 [NimbleGen *A. thaliana* 4x72K Array (080306_AT6_expr)] platform was employed for the studies. Three replicates of treatment-vs-control samples were processed using NCBI's GEO2R tool to identify the differentially expressed genes across experimental conditions. Heatmaps were generated using CIMminer tool (discover.nci.nih.gov/cimminer/home.do) [45].

Results and discussion

Utilizing the microarray data with record GSE41935 in NCBI's GEO datasets, the expression profiles of six

Arabidopsis antioxidant enzymes, SOD, CAT, APX, GPX, MDHAR and DHAR, under four different stress conditions, cold, heat, high light and salt, were evaluated in 10 natural *Arabidopsis* ecotypes originating from different geographical locations. Initially, a detailed literature and database search was conducted to compile the *Arabidopsis* antioxidant enzyme genes. A total of 36 genes were identified for six different antioxidant enzymes, of which SODs included eight members (*CSD1-3*, *FSD1-3* and *MSD1-2*), CATs included three ones (*CAT1-3*), APXs eight (*APX1-6*, *APXT* and *APXS*), GPXs eight (*GPX1-8*), MDHAR five (*MDAR1-5*) and DHAR four (*DHAR1-4*) members. Then, the expression profiles of each antioxidant enzyme under given abiotic stresses were individually evaluated in all *Arabidopsis* ecotypes (Table 1).

Superoxide dismutase (EC 1.15.1.1)

It is the most effective intracellular enzyme ubiquitously found in all aerobic organisms and in subcellular compartments exposed to ROS-derived oxidative stress [1]. Many studies have reported significant changes in SOD activities under stress conditions of salt, cold, heat and high light exposure, which were investigated in this study. For example, salt stress treatment increased the SOD activity in tomato (*Solanum lycopersicum*) roots, chickpea (*Cicer arietinum*) and mulberry (*Morus* sp.) plants [46–48]. Salt-treated leaf tissue extracts from chickpea exhibited significant SOD activity bands for MnSOD, FeSOD and Cu/ZnSOD [49]. Salt and drought stresses upregulated the SOD activity in liquorice seedlings (*Glycyrrhiza uralensis*) and a MnSOD isoenzyme was identified in 2% NaCl exposed plants [50]. Tea plants showed high SOD activity following 2 days of cold treatment, but decreased activity after 10 days [51]. SOD activity remained high up to 4 days in cold treated chickpea plants but subsequently slightly declined [52]. Two-day cold acclimation in potato (*Solanum tuberosum*) increased the SOD activity and conferred improved freezing tolerance [53]. SOD activity along with the activities of other H₂O₂-scavenging enzymes were reported to be increased with light intensity, particularly in Mg-deficient leaves [54]. Under high light, drought stress crucially increased the SOD activity in *Picea asperata* seedlings [55]. The expression profiles of 18 cotton (*Gossypium hirsutum*) *GhSOD* genes were analyzed under different abiotic stresses and they showed ectopic expression and important roles in the protection against ROS [56]. The antioxidant enzyme activities in three mulberry cultivars were found high

Table 1. List of major ROS-scavenging enzymes in *Arabidopsis*.

Enzymatic antioxidants	Catalysed reaction	Gene/locus names ^a	NCBI access ^b	Cellular localization ^c
Superoxide dismutase (SOD)	$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow 2H_2O_2 + O_2$	<i>CSD1/At1g08830</i> , <i>CSD2/At2g28190</i> , <i>CSD3/At5g18100</i> , <i>FSD1/At4g25100</i> , <i>FSD2/At5g51100</i> , <i>FSD3/At5g23310</i> , <i>MSD1/At3g10920</i> , <i>MSD2/At3g56350</i>	P24704.2, O78310.2, O9FK60.1, P21276.4, Q9LU64.1, Q9FMX0.1, O81235.2, Q9LYK8.1	Cytoplasm, Chloroplast, Peroxisome, Chloroplast, Chloroplast, Chloroplast, Mitochondria, Mitochondria
Catalase (CAT)	$H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$	<i>CAT1/At1g20630</i> , <i>CAT2/At4g35090</i> , <i>CAT3/At1g20620</i>	O96528.3, P25819.3, Q42547.3	Cytoplasm, Peroxisome, Peroxisome
Ascorbate peroxidase (APX)	$H_2O_2 + AA \rightarrow 2H_2O + DHA$	<i>APX1/At1g07890</i> , <i>APX2/At3g09640</i> , <i>APX3/At4g35000</i> , <i>APX4/At4g09010</i> , <i>APX5/At4g35970</i> , <i>APX6/At4g32320</i> , <i>APXT/At1g77490</i> , <i>APXS/At4g08390</i>	Q05431.2, Q1PER6.3, Q42564.1, P82281.2, Q7XZP5.2, Q8GY91.1, Q42593.2, Q42592.2	Cytoplasm, Cytoplasm, Peroxisome, Chloroplast, Peroxisome, Chloroplast, Chloroplast, Chl./Mitoch.
Glutathione peroxidase (GPX)	$H_2O_2 + GSH \rightarrow H_2O + GSSG$	<i>GPX1/At2g25080</i> , <i>GPX2/At2g31570</i> , <i>GPX3/At2g43350</i> , <i>GPX4/At2g48150</i> , <i>GPX5/At3g63080</i> , <i>GPX6/At4g11600</i> , <i>GPX7/At4g31870</i> , <i>GPX8/At1g63460</i>	P52032.2, O04922.1, O22850.1, Q8L910.1, Q9LYB4.1, O48646.2, Q9S254.2, Q8LBU2.1	Chloroplast, Cytoplasm, Mitochondria, Cytoplasm, Extr./Chl./Nuc., Mitochondria, Chloroplast, Cytoplasm
Monodehydroascorbate reductase (MDHAR)	$MDHA + NAD(P)H \rightarrow AA + NAD(P)^+$	<i>MDAR1/At3g52880</i> , <i>MDAR2/At5g03630</i> , <i>MDAR3/At3g09940</i> , <i>MDAR4/At3g27820</i> , <i>MDAR5/At1g63940</i>	Q9LFA3.1, Q93WJ8.1, Q9SR59.1, Q9LK94.1, P92947.3	Peroxisome, Cytoplasm, Cytoplasm, Peroxisome, Mitoch./Chl.
Dehydroascorbate reductase (DHAR)	$DHA + 2GSH \rightarrow AA + GSSG$	<i>DHAR1/At1g19570</i> , <i>DHAR2/At1g75270</i> , <i>DHAR3/At5g16710</i> , <i>DHAR4/At5g36270</i>	Q9FWR4.1, Q9FRL8.1, Q8LE52.1, Q9FG59.1	Mitochondria, Cytoplasm, Chloroplast, Cytoplasm

^aMajor *Arabidopsis* antioxidant enzyme genes compiled from previous literature and NCBI database search.

^bUniProtKB/Swiss-Prot accession of NCBI.

^cSubcellular localisations were obtained from protein features of enzymes in NCBI as well as predicted by CELLO server if not available there. AA, ascorbic acid; DHA, dehydroascorbic acid; GSH, glutathione; GSSG, glutathione disulphide; MDHA, monodehydroascorbate.

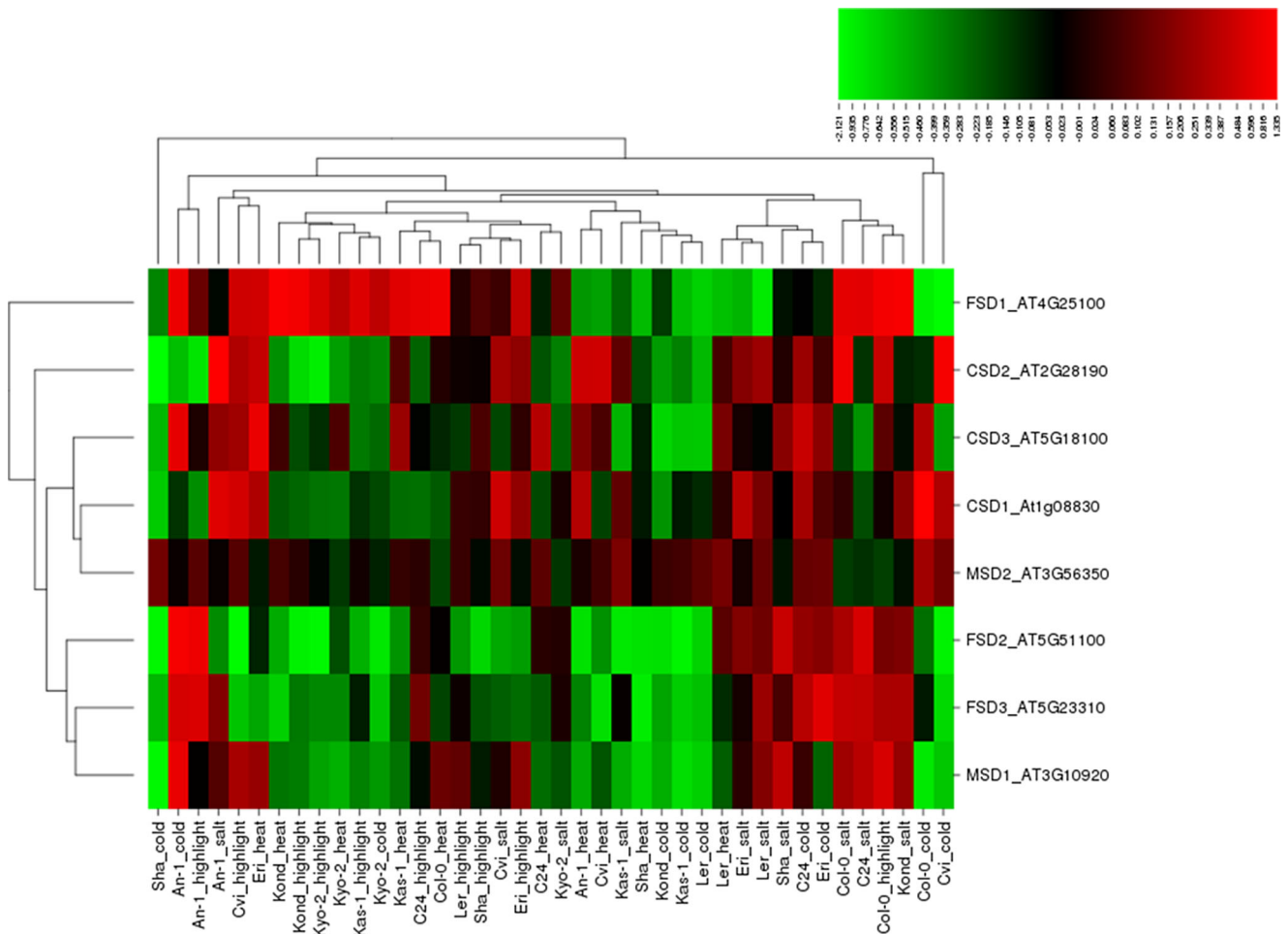


Figure 1. Heatmap showing the expression profiles of eight superoxide dismutase (SOD) genes (*CSD1-3*, *FSD1-3* and *MSD1-2*) in 10 natural *Arabidopsis* ecotypes, An-1, Cvi, Col-0, C24, Eri, Kas-1, Kond, Kyo-2, Ler and Sha, under four different stress conditions, salinity (100 mmol/L NaCl), cold (10 °C), heat (38 °C) and high light (800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Green indicates the down-regulated genes; red shows the up-regulated genes under given stresses. Conditions (up) and genes (left) with similar expression profiles were hierarchically clustered using Pearson correlation.

under high temperature treatment [57]. Moreover, there is an accumulating number of studies reporting increased SOD activities under various biotic and abiotic stress conditions in plants. In this study, eight SOD genes (*FSD1-3*, *CSD1-3* and *MSD1-2*), under four different abiotic stress conditions, salt, cold, heat and high light exposure, in 10 different *Arabidopsis* ecotypes produced a total of 320 expression patterns (Figure 1), of which 142 (~44%) showed up-regulation, whereas 176 (55%) showed down-regulation. Of the up-regulated genes, 45 ones were induced by salt, 37 by high light, 31 by heat and 29 by cold stress. However, of the down-regulated genes, 51 were induced by cold, 48 by heat, 43 by high light and 34 by salt stress. The up-regulated genes demonstrated a distribution varying in the range of 0.001–1.335 fold change (\log_2), whereas the down-regulation varied between -0.001 and -2.121 fold. As a criterion of significant expression, we applied the threshold ≥ 1.0 and

$\leq -1.0 \log_2$ fold change. After applying this threshold, a total of 10 conditions, including four up-regulated and six down-regulated genes were identified. Among them, the most up- and down-regulated gene, with 1.335 and -2.121 folds change, was *CSD2* under salt (An-1) and cold (Sha) stresses, respectively. Other genes that also demonstrated a significant increase in expression were *CSD1* (1.287 fold) under cold stress (Col-0), and *FSD1* with 1.195 and 1.171 fold change under heat (Kond) and salt (Kond) stresses, respectively. However, *FSD2* showed crucial down-regulation with -1.303 (Cvi) and -1.199 (Kyo-2) fold change under high light, and with -1.155 (Sha) and -1.097 (Kas-1) fold change under cold stress. In addition, *MSD1* was also down-regulated with -1.213 folds under cold (Sha) stress. Overall, although all *Arabidopsis* SOD genes exhibited a varying degree of differential expression, some stresses mostly appeared to induce the chloroplastic *FeSOD* genes such as *FSD1*

and 2, and *Cu/ZnSOD* genes, including cytoplasmic *CSD1* and chloroplastic *CSD2*. Moreover, the regulation of *SOD* expression did not demonstrate any particular pattern in relation to ecotypes, giving a hint about the different tolerance and response status of each *Arabidopsis* genotype to given stress factors.

Catalase (EC 1.11.1.6)

CAT has one of the highest turnover rates of all enzymes: it is estimated that one molecule of CAT is able to convert ~ 6 million H_2O_2 molecules into H_2O and O_2 per minute [58]. CAT activity has been extensively studied in many higher plants, including maize, barley, *Arabidopsis*, pumpkin, rice and sunflower [1]. In addition, many studies report the changes in CAT activity under salt, cold, heat and high light stresses, which were evaluated in the present study. For example, CAT activity was reported to increase in chickpea roots in response to salinity [47,49]. In salt-sensitive rice seedlings, CAT activity increased at moderate (7 dS m^{-1} NaCl) salt treatment, whereas its activity declined with higher salinity (14 dS m^{-1} NaCl); however in salt-tolerant seedlings, CAT activity was consistently increased by increased salt levels and duration [59]. Changes in CAT activity under salinity have also been reported in many plants, including common bean, Chinese cabbage, pea and maize [60–63]. The cold stress increased the expression of catalase in rice leaf blades [64]. Cold stress was reported to activate the CAT genes in *Arabidopsis* plants [65]. Transcriptome profiling of cassava plant identified *CAT2* among the early cold-responsive genes [66]. High heat stress significantly increased the CAT activity in five wheat genotypes [67]. High CAT activity was observed under high temperature stress in *Brassica juncea* genotypes but the increase was significantly higher in the tolerant genotype [68]. Under high light, drought stress significantly increased the CAT activity in *Picea asperata* seedlings [55]. CAT-deficiency was reported to significantly affect the gene expression under high light in *Arabidopsis* plants [69]. Moreover, many other studies have also reported changes in the activities of CAT enzymes under diverse panels of biotic and abiotic stresses in plants. In the present study, three CAT genes, *CAT1-3*, under four different abiotic stresses, salt, cold, heat and high light, in 10 *Arabidopsis* ecotypes produced a total of 120 expression conditions (Figure 2), of which 76 conditions ($\sim 64\%$) were up-regulated, while 44 ($\sim 36\%$) were down-regulated. Of the up-regulations, 22 were induced by heat, 21 by high light, 17 by cold and 16

by salt stress. Besides, 14 of the down-regulations were caused by salt, 13 by cold, 9 by high light and 8 by heat. The up-regulated genes showed a fold change of 0.003–1.945, whereas the down-regulation varied between -0.016 and -1.685 . To analyze the significant changes in the expression of the selected CAT genes, we applied a threshold value of ≥ 1.0 and ≤ -1.0 . A total of nine conditions, including six up- and three down-regulated ones, were identified to meet the applied criteria. The most up-regulated gene was *CAT2* with 1.945 fold change (Col-0) under high light, whereas the most down-regulated gene was *CAT3* with -1.685 fold change (Sha) under cold stress. Interestingly, the expression of *CAT2* was also significantly increased in some other cases such as 1.522 fold (Col-0) under heat, 1.274 fold (Col-0) under salt, and 1.385 (Eri), 1.236 (Kas-1) and 1.069 fold (Kond) under high light. However, *CAT2* also showed a significant (-1.322 fold) down-regulation under cold stress (Col-0), implicating a stress-specific regulation pattern. In addition, under heat stress, *CAT3* exhibited decreased (-1.021 fold) expression (Kond). Although all *Arabidopsis* catalase genes (*CAT1-3*) showed some degree of differential expression in response to the studied stress treatments, *CAT2* mainly demonstrated a significant up-regulation, whereas *CAT3* tended to be down-regulated. The clustering pattern of genes also further supported this, showing that *CAT1* and *CAT3* genes are grouped together, while *CAT2* diverged from them. Moreover, a single ecotype, Kyo-2, exhibited very similar expression patterns of the *CAT3* gene under the four stresses, whereas the other genotypes did not show any specific ecotypic distribution, suggesting different stress tolerance of different *Arabidopsis* ecotypes.

Ascorbate peroxidase (EC 1.11.1.11)

APX has higher affinity for H_2O_2 (μmol range) than catalase enzymes (mmol range) [1]. In addition, chloroplastic and mitochondrial APXs are reported to less half-inactivation time (<30 sec) than peroxisomal and cytosolic isoforms (≥ 1 h), therefore making them more sensitive to low level of AsA [70]. Enhanced APX activity in plants has been demonstrated under various abiotic stresses including salt, cold, heat and high light. Studies have reported that salinity increased the transcript levels of *APX1*, 4, 6 and 7 genes in rice leaf tissues, whereas *APX2* expression was not altered but *APX8* was slightly decreased [71]. In sorghum, *APXs/GPXs* genes were mostly up-regulated in leaves, while they were down-regulated in root tissues [72]. Under

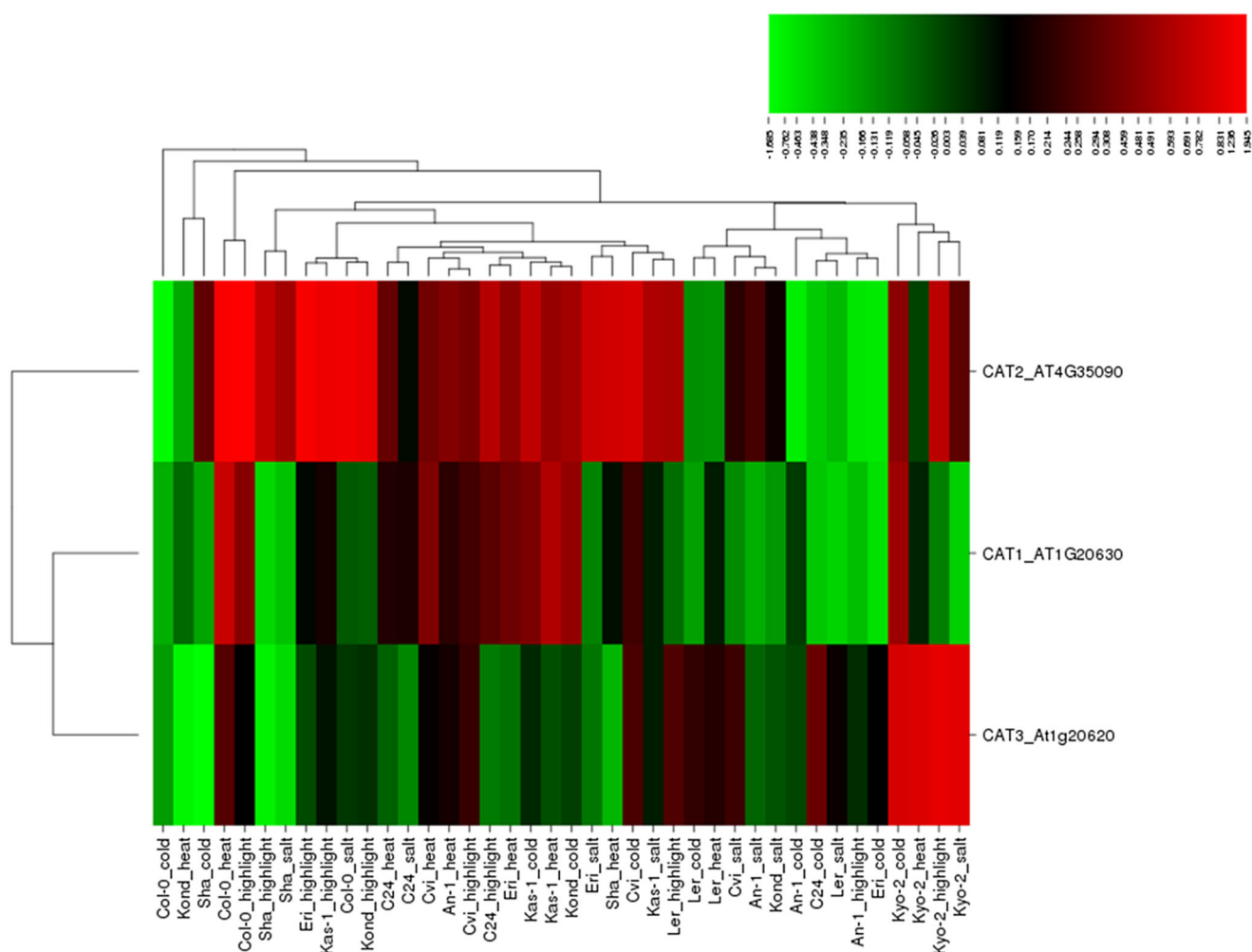


Figure 2. Heatmap showing the expression profiles of three catalase (CAT) genes (*CAT1-3*) in 10 natural *Arabidopsis* ecotypes, An-1, Cvi, Col-0, C24, Eri, Kas-1, Kond, Kyo-2, Ler and Sha, under four different stress conditions of salinity (100 mmol/L NaCl), cold (10 °C), heat (38 °C) and high light (800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Green indicates the down-regulated genes; red shows the up-regulated genes under given stresses. Conditions (up) and genes (left) with similar expression profiles were hierarchically clustered using Pearson correlation.

salinity, salt stress tolerant sweet potato genotypes showed higher APX activity than salt sensitive plants, and APX isoforms demonstrated expression based on tissue type and stress duration [73]. The cytosolic APX2 played a crucial role in rice growth and development by scavenging of ROS under cold, salt and drought stresses [74]. High APX activity improved the recovery of photosynthesis in sugarcane plants under low substrate temperature [75]. APX activity was higher in cold-acclimated barley cultivars than in non-acclimated plants under freezing temperature [76]. The cytosolic APX1 played a crucial role in the acclimation of *Arabidopsis* plants under combined heat and drought stresses [77]. Heat shock increased the expression of APX2 in leaves of rice seedlings [78]. Increased APX activity in broccoli and Chinese cabbage was reported to play a key role in ROS detoxification under heat stress [79]. Thylakoid APX in *Arabidopsis* plants

protects the photosynthetic apparatus under high light stress by employing the water-water cycle [80]. *Arabidopsis* APX1 was reported to be involved in H_2O_2 scavenging under light stress [81]. Excess light in *Arabidopsis* plants caused a reversible photo-inhibition in photosynthesis, resulting in an increase in the mRNA levels of cytosolic APX1 and APX2 genes [82]. Differential regulation of APX activity has been also demonstrated in many other plants under different biotic/abiotic stress conditions. In the present analysis, eight APX genes, APX1-6, APXT and APXS, under four different stress types, salt, cold, heat and high light, in 10 *Arabidopsis* ecotypes formed a total of 320 different expression patterns (Figure 3), of which 149 conditions (~47%) were up-regulated, while 171 (~53%) were down-regulated. Among the up-regulated cases, 46 conditions were induced by high light, 43 by salt, 34 by heat and 26 by cold stress; whereas in the 171

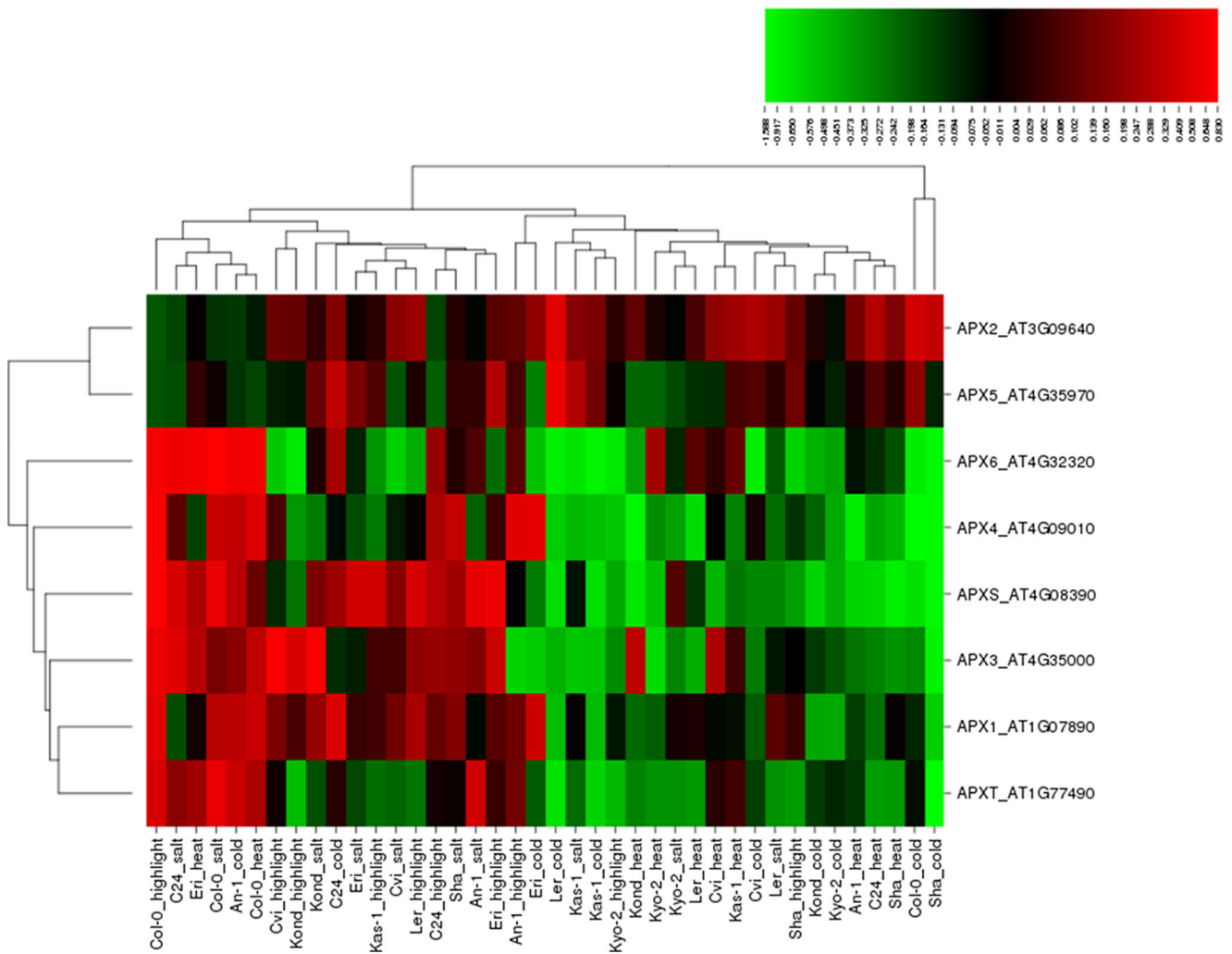


Figure 3. Heatmap showing the expression profiles of eight ascorbate peroxidase (APX) genes (APX1-6, APXT and APXS) in 10 natural *Arabidopsis* ecotypes, An-1, Cvi, Col-0, C24, Eri, Kas-1, Kond, Kyo-2, Ler and Sha, under four different stress conditions of salinity (100 mmol/L NaCl), cold (10°C), heat (38°C) and high light (800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Green indicates the down-regulated genes; red shows the up-regulated genes under given stresses. Conditions (up) and genes (left) with similar expression profiles were hierarchically clustered using Pearson correlation.

down-regulated conditions, 54 were induced by cold, 46 by heat, 37 by salt and 34 by high light. The up-regulation varied in the range of 0.002–0.830 fold change, whereas the down-regulation, between -0.003 and -1.588 . As a significant threshold, in this case we applied the cut-off value of ≥ 0.6 and ≤ -0.6 for differentially expressed genes. A total of 34 conditions, including 13 up- and 21 down-regulated genes were identified by applying this threshold value. APXS was the most up-regulated gene with 0.830 fold change (Col-0) under high light, while APX4 was the most down-regulated gene with -1.588 fold (Col-0) under cold stress. Based on the applied threshold, in some other conditions, the expression of APX3-6 and APXS showed significant up-regulation with 0.603–0.753 fold change. Besides, APX3, APX4, APX6, APXS and APXT demonstrated

significant down-regulation ranging between -1.364 and -0.612 , particularly, chloroplastic APX4, APX6, APXS and APXT genes under cold (Sha) stress with -1.34 , -1.236 , -1.14 and -1.364 fold change, respectively. Although all *Arabidopsis* APX genes were expressed at different levels, chloroplastic APX4, APX6, APXT and APXS and peroxisomal APX3 and APX5 genes appeared to be more responsive under the selected stresses. This is also in agreement with the fact that the chloroplasts and peroxisomes are the main source of ROS production under these stress conditions. Moreover, the gene clustering showed that APX1, APX3, APX4, APX6, APXS and APXT share similar expression profiles. A significant pattern was not observed in relation to the ecotypes, which was indicative of the varying stress tolerance of these *Arabidopsis* genotypes.

Glutathione peroxidase (EC 1.11.1.9)

GPX is another important enzymatic antioxidant involved in the reduction of H_2O_2 as well as organic and lipid hydroperoxides using glutathione (GSH) [1]. Like APX, it helps to protect plants against oxidative stress and its enhanced activity has been reported in many plants under different abiotic stresses, including salt, cold, heat and high light, which the present work aimed to understand in various *Arabidopsis* ecotypes. Previous studies have reported that salinity stress increased the GPX activity in two rice cultivars [83]. Two proteins related to glutathione-based scavenging of ROS were more abundant in the roots of salt-tolerant barley genotypes, showing increased GPX activity [84]. Salt stress highly up-regulated the expression of GPX in pearl millet, and GPX enhanced the salt tolerance in plants [85]. Cold stress and exogenous H_2O_2 treatments induced the GPX gene family in rice plants [86]. Transcriptome profiling of cold-tolerant rice plants revealed up-regulated expression of GPX genes under mild cold stress [87]. Chilling-stressed induced RNA-seq analyses showed significant up-regulation of GPX genes in *Jatropha curcas* [88]. Mung bean seedlings (*Vigna radiata* L. cv. Binamoog-1) treated with exogenous GSH under high temperature increased the GPX activity and showed enhanced high temperature stress tolerance [89]. Heat treatment was reported to increase the GPX activity in *T. aestivum* seedlings [90]. Heat stress-induced transcriptome analysis revealed the increased GPX activity in switchgrass plants [91]. Hairy root culture of wild carrot under light irritation demonstrated the reduced GPX activity [92]. Expression analysis revealed that the cucumber GPX genes showed different expression patterns under abscisic acid treatments and abiotic stress conditions [93]. In addition, most of the *TaGPX* genes indicated higher expression levels in various leaf developmental stages under abiotic stress conditions in wheat [94]. Abiotic stress-induced GPX activity has been also reported in many other plants, showing the importance of GPX members in ROS-scavenging. In this work, eight GPX genes, *GPX1-8*, in 10 *Arabidopsis* ecotypes under the four different stresses that we focussed on, resulted in a total of 320 different expression profiles (Figure 4): 142 (~45%) up-regulated and 177 (~55%) down-regulated conditions. Of the up-regulated conditions, 43 were induced by high light, 42 by salt, 29 by heat and 28 by cold stress, whereas of the down-regulated conditions, 52 were caused by cold, 51 by heat, 38 by salt and 36 by high light. The up-regulated genes showed a variation of 0.005–1.572 fold change, and the down-regulated genes varied between –2.388

and –0.001. Genes with significant differential expression were determined based on a threshold value of ≥ 1.0 and ≤ -1.0 . This cut-off value identified a total of 10 conditions, including four up- and six down-regulated ones. *Arabidopsis GPX6* was identified as the gene with the most pronounced differential expression: it was up- and down-regulated by 1.572 and –2.388 fold (Col-0) under high light and cold stresses, respectively. Thus, the expression of mitochondrial GPX6 could be considered the most responsive gene under this type of stress. It also implies that the most likely source of ROS are mitochondria. Other significantly up-regulated genes were *GPX5* with 1.289-fold (Col-0) under heat, and *GPX6* and 7 with 1.15 fold (Eri) and 1.112 fold (C24) under high light. In contrast, some genes showed down-regulation under cold stress: *GPX1* with –2.152 (Col-0) and –1.248 fold (Eri), *GPX6* with –1.216 (Eri) and –1.062 fold (C24), and *GPX2* with –1.049 fold (C24), indicating that cold stress may appear to significantly down-regulate the expression of some antioxidant genes. Moreover, chloroplastic *GPX1* and mitochondrial *GPX6* demonstrated similar expression patterns; other genes that shared similar expression patterns were *GPX2*, *GPX3*, *GPX4*, *GPX5*, *GPX7* and *GPX8*. Furthermore, the lack of any obvious pattern in the clustering of the ecotypes under these stresses might be attributed to different stress tolerance of these *Arabidopsis* genotypes.

Monodehydroascorbate reductase (EC 1.6.5.4)

MDHAR protects against ROS via involvement in the ascorbate-glutathione (ASH-GSH) cycle, maintaining ascorbate (AsA) in a reduced state in cellular compartments [1,95]. A substantial number of studies have reported the involvement of MDHAR in plant protection against ROS under various abiotic stresses, including salt, cold, heat and high light. For example, Truffault et al. [96] reported that MDHAR activity could be a potential candidate to enhance the yield in crops under drought or light stress. NaCl-salt stress (300 mmol/L) significantly up-regulated the cytosolic MDHAR in *Aeluropus littoralis* shoots and roots but MDHAR accumulation declined at concentrations higher than 300 mmol/L [97]. Reduced MDHAR activity decreased the cold storage tolerance in tomato plants and affected the antioxidant levels in fruits [98]. MDHAR activity was significantly inhibited at either high or low light following cold stress [99]. Prolonged exposure to heat stress increased the transcript levels of MDHAR in rice leaves [100]. MDHAR activity was higher in short-term (3 days) heat stress but its activity

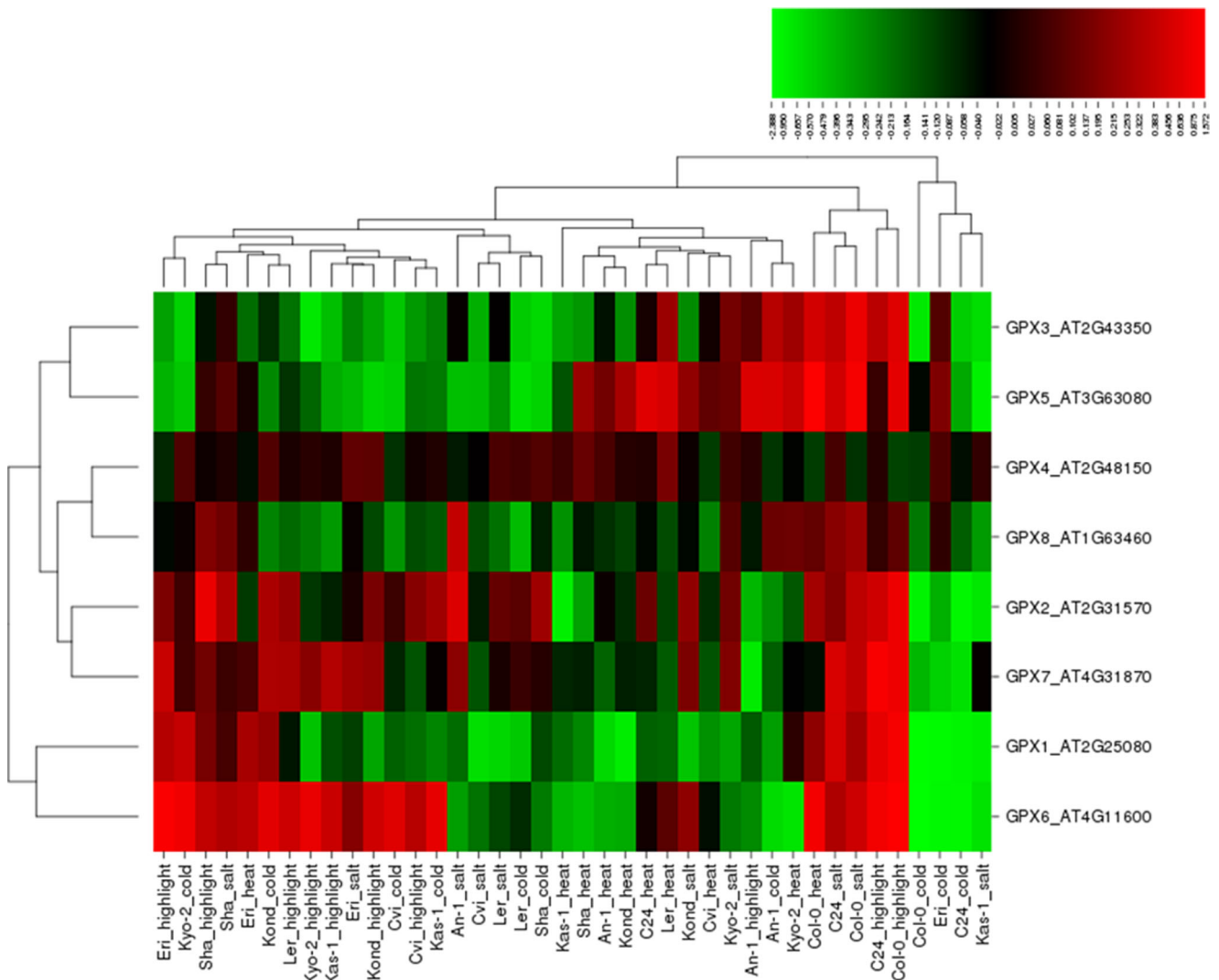


Figure 4. Heatmap showing the expression profiles of eight glutathione peroxidase (GPX) genes (GPX1-8) in 10 natural *Arabidopsis* ecotypes, An-1, Cvi, Col-0, C24, Eri, Kas-1, Kond, Kyo-2, Ler and Sha, under four different stress conditions of salinity (100 mmol/L NaCl), cold (10 °C), heat (38 °C) and high light (800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Green indicates the down-regulated genes; red shows the up-regulated genes under given stresses. Conditions (up) and genes (left) with similar expression profiles were hierarchically clustered using Pearson correlation.

gradually decreased in long-term treatment [101]. Similar results were also reported in a different study, in which heat stress significantly increased the MDHAR activity in a grass species, *Poa pratensis*, but its activity declined under long-term exposure [102]. In *DHAR3 Arabidopsis* mutants, high light stress activated the *MDHAR* gene to compensate the *DHAR3* activity [103]. *MDHAR* is reportedly involved in the regulation of ascorbate in tomato plants in a light-dependent manner [104]. *MDHAR* activity was associated with sugar levels in tomato under high light, and its reduced activity inhibited the plant growth and yield [96]. The involvement of *MDHAR* in ROS-regulation, thereby in plant stress tolerance, has been also demonstrated in many other studies performed under various stress conditions. In this study, five *MDHAR* genes, *MDAR1-5*,

under four different stresses such as salt, cold, heat and high light in 10 *Arabidopsis* ecotypes produced a total of 200 differentially expressed patterns (Figure 5), of which 110 (55%) showed up-regulation, while 89 (~45%) down-regulation. Among the up-regulated conditions, there were 36 ones induced by high light, 29 by cold, 27 by salt and 18 by heat stress. Among the down-regulated conditions, 31 were induced by heat, 23 by salt, 21 by cold and 14 by high light. The extent of up-regulation varied within 0.010–1.576 fold change, while that of down-regulation, between –1.333 and –0.006. To identify the genes whose expression levels were significantly altered, we adopted a threshold range of ≥ 1.0 and ≤ -1.0 . This threshold identified 15 conditions, including eight up- and seven down-regulated ones. *Arabidopsis MDAR3*

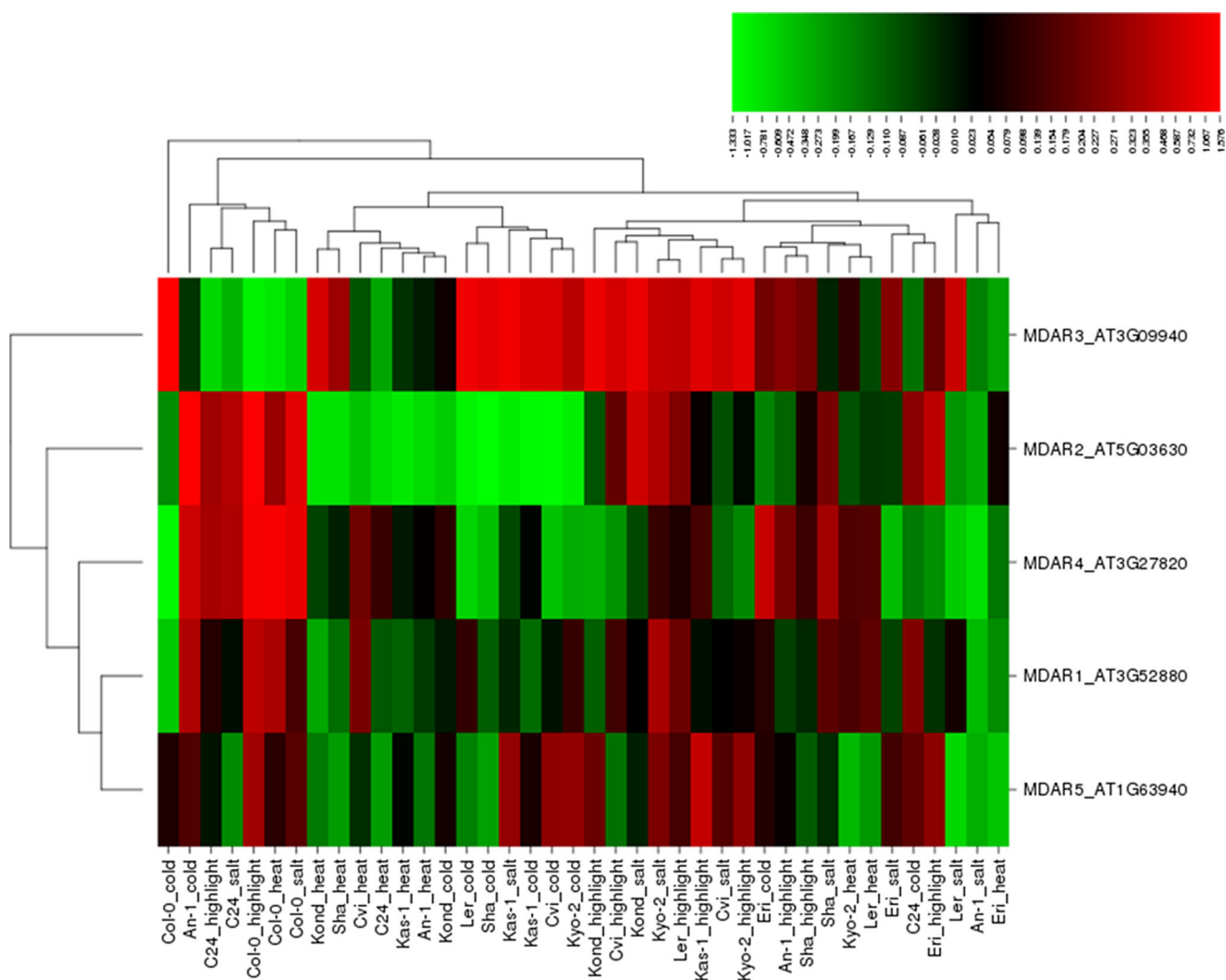


Figure 5. Heatmap showing the expression profiles of five monodehydroascorbate reductase (MDHAR) genes (*MDAR1-5*) in 10 natural *Arabidopsis* ecotypes, An-1, Cvi, Col-0, C24, Eri, Kas-1, Kond, Kyo-2, Ler and Sha, under four different stress conditions of salinity (100 mmol/L NaCl), cold (10 °C), heat (38 °C) and high light (800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Green indicates the down-regulated genes; red shows the up-regulated genes under given stresses. Conditions (up) and genes (left) with similar expression profiles were hierarchically clustered using Pearson correlation.

was the most up-regulated gene with 1.576 fold change (Col-0), while *MDAR2* was the most down-regulated one with -1.333 fold change (Sha) under cold stress. There was also significant up-regulation of *MDAR2* with 1.19 fold (An-1) and 1.17 fold (Col-0) under cold and high light stresses, respectively; *MDAR3* with 1.14 (Kas-1), 1.067 (Ler) and 1.041 fold (Kond) under salt, cold and high light, respectively; and *MDAR4* with 1.146 and 1.144 fold (Col-0) under heat and high light, respectively. However, significant down-regulation was observed for *MDAR2* by -1.274 fold (Cvi), -1.224 (Kas-1), -1.098 (Ler) and -1.017 fold (Kyo-2) under cold stress; *MDAR3* with -1.023 fold (Col-0) under high light; and *MDAR4* with -1.205 (Col-0) under cold stress. As such, the cytosolic *MDAR2* and *MDAR3* and the peroxisomal *MDAR4* were the most

responsive genes under these stresses, particularly under cold stress. Moreover, *MDAR2-4* demonstrated different expression patterns in clustering, while *MDAR1* and *MDAR5* had similar expression. Furthermore, the clustering pattern of ecotypes under these stresses also suggested different stress tolerance of the *Arabidopsis* genotypes included in this study.

Dehydroascorbate reductase (EC 1.8.5.1)

DHAR maintains the redox pools of AsA through recycling of oxidized AsA to reduced AsA. Thus, it is of crucial significance in the regulation of ROS-scavenging in plants, especially under stress conditions [1,39]. The important role of DHAR in ROS detoxification has been widely reported under various abiotic stresses.

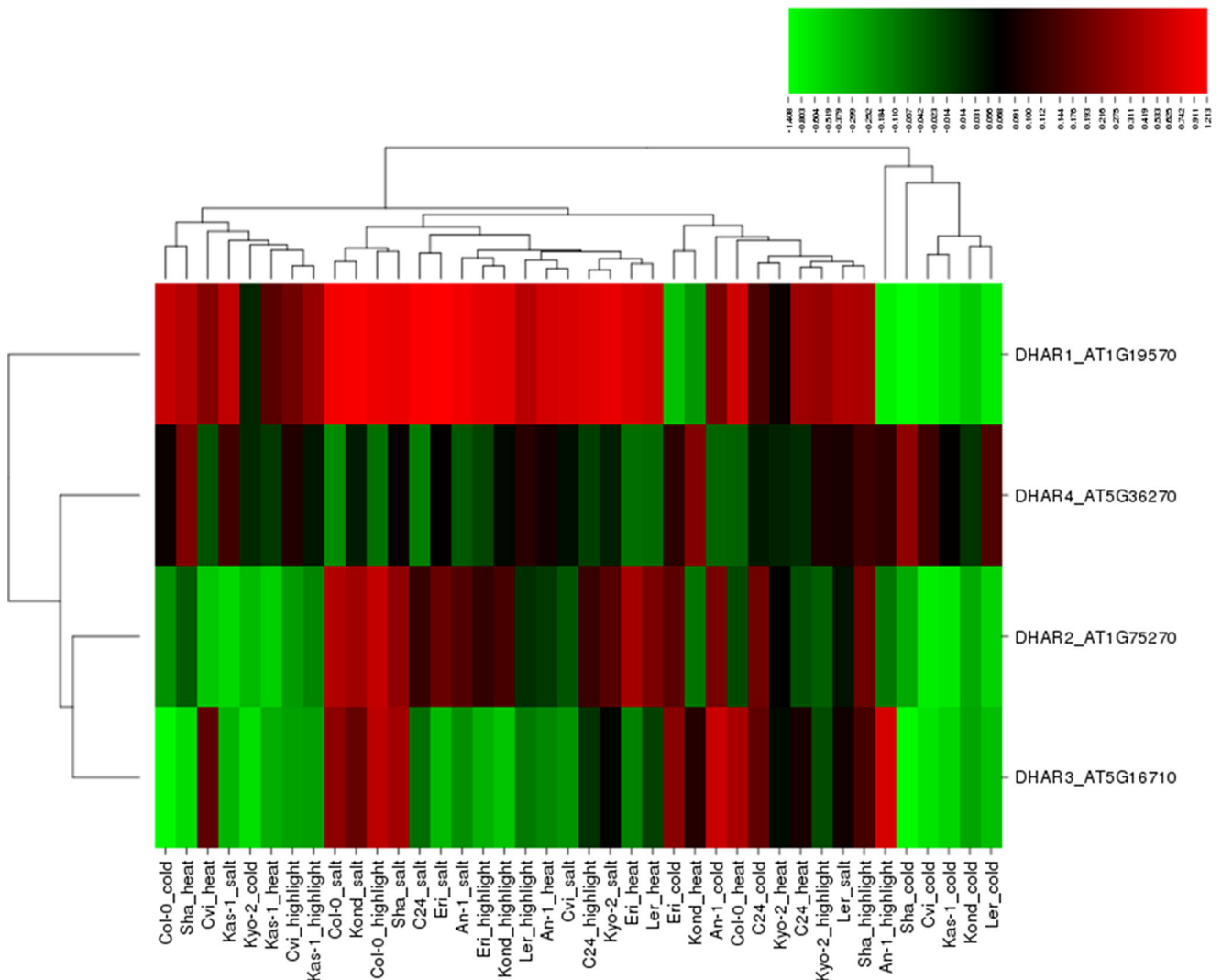


Figure 6. Heatmap showing the expression profiles of four dehydroascorbate reductase (DHAR) genes (*DHAR1-4*) in 10 natural *Arabidopsis* ecotypes, An-1, Cvi, Col-0, C24, Eri, Kas-1, Kond, Kyo-2, Ler and Sha, under four different stress conditions of salinity (100 mmol/L NaCl), cold (10 °C), heat (38 °C) and high light (800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Green indicates the down-regulated genes; red shows the up-regulated genes under given stresses. Conditions (up) and genes (left) with similar expression profiles were hierarchically clustered using Pearson correlation.

For example, salt stress treatment decreased the DHAR activity in cucumber [105]. In salt-sensitive rice seedlings, the DHAR activity increased at moderate salinity (7 dS m^{-1} NaCl) but declined with higher salinity (14 dS m^{-1}) [55]. High or low light following cold stress significantly inhibited the DHAR activity in cucumber (*Cucumis sativus*) [99]. RNA-Seq analysis revealed up-regulation of the *DHAR* gene in cold-induced *Jatropha curcas* [88]. DHAR exhibited higher activity in short-term (3 days) heat stress but declined activity in further treatments [101]. Upon heat stress, the *DHAR* gene demonstrated an increased transcript level in rice leaves [100]. The DHAR activity significantly increased in heat-tolerant grass species, *Poa pratensis* under heat stress but its activity declined under long-term exposure [102]. High light stress

induced the *MDHAR* gene in *DHAR3 Arabidopsis* mutants, compensating *DHAR3* activity [103]. Proteomic analysis showed increased DHAR activity in *Arabidopsis* chloroplasts under high light stress [106]. Under high light, the transcript level of *DHAR* markedly increased in salt-stressed tomato fruits [107]. Many other studies also report on the significant role of DHAR in plant stress tolerance under various stresses. In the present study, four *DHAR* genes, *DAR1-4*, under four different abiotic stress conditions, salt, cold, heat and high light, in 10 *Arabidopsis* ecotypes gave a total of 160 different expression patterns (Figure 6), of which 95 (59%) displayed up-regulation, while 65 (~41%) down-regulation. Among the up-regulated conditions, 30 were induced by salt, 25 by high light, 22 by heat and 18 by cold stress. Among

the down-regulated conditions, 22 were induced by cold, 18 by heat, 15 by high light and 10 by salt stress. The up-regulated genes showed a distribution with 0.007–1.213 fold change, whereas the down-regulated ones, between –1.408 and –0.004 fold. Genes with significantly differential expression were determined based on a cut-off value of ≥ 1.0 and ≤ -1.0 . According to this threshold, six conditions, including four up- and two down-regulated ones showed the significant expression. *Arabidopsis DHAR1* was identified to be the most up- and down-regulated gene with 1.213 (Eri) and –1.408 fold change (Sha) under salt and cold stress, respectively. *DHAR1* was significantly up-regulated with 1.199 (C24), 1.155 (Kond) and 1.148 fold change (Col-0) under salt stress, whereas *DHAR3* with -1.364 fold change (Sha) under cold stress was considerably down-regulated. Thus, the mitochondrial *DHAR1* and the chloroplastic *DHAR3* appeared to be the most responsive genes under the selected abiotic stresses, especially in response to cold and salt stress. Moreover, the hierarchical clustering of *DHAR2-4* genes under these stresses showed a more similar expression pattern than *DHAR1*. Furthermore, although some ecotypes shared similar expression patterns for particular stress types, the overall clustering pattern under the selected stresses mainly indicated different stress tolerance of the *Arabidopsis* genotypes.

Overall, the expression profiles of the major *Arabidopsis* antioxidant genes varied based on stress and enzyme type and plant ecotype. However, among them, the ones that showed the most pronounced up- and down-regulation respectively included *CSD2* (An-1; salt; 1.335 fold) and *CSD2* (Sha; cold; -2.121 fold) in *SODs*; *CAT2* (Col-0; high light; 1.945 fold) and *CAT3* (Sha; cold; -1.685 fold) in *CATs*; *APXS* (Col-0; high light; 0.830 folds) and *APX4* (Col-0; cold; -1.588 fold) in *APXs*; *GPX6* (Col-0; high light; 1.572 fold) and *GPX6* (Col-0; cold; -2.388 fold) in *GPXs*; *MDAR3* (Col-0; cold; 1.576 fold) and *MDAR2* (Sha; cold; -1.333 fold) in *MDHARs*; and *DHAR1* (Eri; salt; 1.213 fold) and *DHAR1* (Sha; cold; -1.408 fold) in *DHARs*. In terms of stress type, mainly high light as well as salt and cold treatment significantly up-regulated the gene expression, whereas cold stress caused mainly down-regulation. The *Arabidopsis* ecotypes showed different levels of stress tolerance. The pronounced expression of the genes from the *SOD*, *CAT*, *APX*, *GPX*, *MDHAR* and *DHAR* families under given conditions highlighted their crucial roles in ROS-scavenging under abiotic stress.

Conclusions

Taking advantage of microarray data, the expression profiles of six *Arabidopsis* antioxidant enzyme genes encoding *SOD*, *CAT*, *APX*, *GPX*, *MDHAR* and *DHAR*, under four different abiotic stress conditions, cold, heat, high light and salt, were evaluated in 10 natural *Arabidopsis* ecotypes originating from different geographical locations. A total of 36 gene isoforms, including *CSD1-3*, *FSD1-3*, *MSD1-2*, *CAT1-3*, *APX1-6*, *APXT*, *APXS*, *GPX1-8*, *MDAR1-5* and *DHAR1-4*, were investigated. Under these stresses, *CAT* genes had the most up-regulated expression with about 64% of the conditions, whereas *SOD* and *GPX* demonstrated the most down-regulated expression with about 55% of the conditions. The expression levels of the up-regulated antioxidant genes ranged from 0.001 to 1.945 fold change, whereas the expression of the down-regulated genes varied between –0.001 and –2.388 fold. Among the enzyme isoforms, *CAT2* showed the highest increase by 1.945 fold under high light in Col-0 ecotype, followed by *MDAR3* with 1.576 fold (Col-0) under cold stress, *GPX6* with 1.572 fold (Col-0) under high light, *CSD2* and *DHAR1* with 1.335 (An-1) and 1.213 (Eri) fold under salt stress, respectively, and *APXS* with 0.830 fold (Col-0) under high light exposure. As such, the types of abiotic stresses that considerably up-regulated the gene expression were high light as well as salt and cold stress, implicating that *Arabidopsis* ecotypes could be more vulnerable to photo-oxidative damage. However, *GPX6* was the most down-regulated antioxidant gene, with –2.388 fold change under cold stress in Col-0 ecotype, followed by *CSD2* with –2.121 (Sha), *CAT3* with –1.685 (Sha), *APX4* with –1.588 (Col-0), *DHAR1* with –1.408 (Sha) and *MDAR2* with –1.333 fold change (Sha) under cold stress. Thus, cold stress seemed to exert down-regulation of some of the analyzed genes. In addition, differential expression of Sha and Col-0 genotypes suggested that these varieties could be more sensitive to given stresses. However, overall the expression pattern of the ecotypes suggested different stress tolerance of these *Arabidopsis* genotypes. Moreover, *Arabidopsis FSD1*, *FSD2*, *CSD1* and *CSD2* genes from the *SOD* family; *CAT2* and *CAT3* from *CAT*; *APX3-6*, *APXT* and *APXS* from *APX*; *GPX1*, *GPX2*, *GPX5*, *GPX6* and *GPX7* from *GPX*; *MDAR2-4* from *MDHAR*; and *DHAR1* and *DHAR3* from the *DHAR* family appeared to be more differentially expressed under given stress conditions, emphasizing their crucial roles in ROS-scavenging. Thus, the results from this study provide insight into better understanding the enzymatic antioxidant

mechanisms in plants, with an ultimate goal of engineering abiotic stress tolerant crops.

Disclosure statement

No potential conflict of interest was reported by the authors.

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