THE EFFECT OF MELATONIN ON SOME PHYSIOLOGICAL PARAMETERS, SALT SENSOR GENES AND DNA METHYLATION IN SALT STRESSED LETTUCE (Lactuca Sativa L.)

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Abstract

Salinity, an important abiotic stress, affects arable land worldwide, reducing crop growth, yield and quality. Lettuce (*Lactuca sativa L.*), which is very sensitive to salt stress, is one of the crops affected by soil salinity and salt (NaCl) stress also affects lettuce cultivation. Different plant growth regulators (salicylic acid (SA), polyamines, glycine betaine (GB) and melatonin (MEL)) are used to reduce the harmful effects of soil salinity. In this study, the effects of NaCl and MEL treatments on physiological parameters (cell membrane damage (%), leaf relative water content (RWC%), chlorophyll amount), DNA methylation and some NaCl tolerance genes (*SOS1*, *SOS2*, *AKT1*, *NHX1*, *HKT1*) have examined in two different lettuce genotypes (Yedikule and Ivanka). When the effect of NaCl stress on physiological parameters was examined, it was detected that this stress increased cell membrane damage and decreased chlorophyll content and RWC %. The effects of MEL applications together with NaCl stress on these parameters were positive. In the gene expression analysis results, it was determined that *SOS1*, *SOS2*, *AKT1*, *NHX1* and *HKT1* gene expressions increased in both lettuce genotypes and decreased in MEL applications. On the flip side, according to methylation sensitive amplification polymorphism (MSAP) findings, while a decrease in methylation level was determined in NaCl stress compared to the control in both genotypes. Methylation levels increased compared to NaCl stress in MEL applications with NaCl stress, but this rate was still lower than the control. According to the results obtained, it was concluded that exogenous MEL application in lettuce genotypes is important in response to NaCl stress.

INTRODUCTION

Salinity is an abiotic stress factor that limits crop yields. Salinization is increasing rapidly in total irrigated areas in the world. About 50% of arable land is expected to be lost by 2050 due to salinization (Ahanger et al., 2017). High salt concentration in the growth medium causes strong detrimental effects on plant biomass, physiology, formation of reactive oxygen species (ROS), accumulation of mineral ions and PSII reactions (Ali et al., 2021; Parvin et al., 2020).

While excess NaCl intake in the plant decreases the water potential, it disrupts the ion balance in the cell and negatively affects plant growth. While Na^+ and Cl^- levels increase in the cell, Ca^{+2}, K^+ and Mg^{+2} concentrations decrease. Plants maintain ion balance by removing excess Na^+ ions through vacuolar Na^+/H^+ antiporters such as SOS1 and NHX1 in the plasma membrane, and by keeping K^+ ions inside the cell through different functions of K^+ channels (Zhang et al., 2010).

Biochemical and molecular tolerance mechanisms have been developed by plants against the negative effects of NaCl stress. Genes encoding salt tolerance proteins, genes regulating the activity of enzymes in the pathways that lead to the protection and repair of cells and transcription factors that activate these genes are expressed (Flowers 2004; Munns 2005). Genetic changes that increase the expression of NaCl stress

sensor genes (*SOS1* , *SOS2* , *AKT1* , *HKT1* ,*NHX1*) or endogenous antioxidant activity are important in crop tolerance to stress.

One of the molecular level responses of plants to soil fertility changes and abiotic stresses is DNA methylation mechanism and this mechanism is also used to understand some physiological, biochemical or adaptation processes (Erturk et al., 2014; Taspinar et al., 2017; Zenda et al., 2018). It is known that salinity causes changes in both hypermethylation and hypomethylation stages of DNA methylation in plants (Demirkiran et al., 2013).

MEL, an indolic compound derived from tryptophan, has an important role in the general processes of plants such as stress relief, germination, circadian rhythms, flowering, photosynthesis and senescence (Rajora et al., 2020). MEL increases tolerance to NaCl stress in two ways, directly (by providing electrons to ROSs) and indirectly (by increasing the activity of antioxidant enzymes, photosynthetic efficiency, metabolite content and regulating stress-related transcription factors) (Ayyaz et al., 2022; Yan et al., 2022). Moreover; Exogenous MEL application can regulate the expression of ion channel genes (NHX1 and AKT1) involved in ion homeostasis in leaf tissue to improve salinity resistance in plants (Li et al., 2019; Rajora et al., 2020). Other MEL-related precursors such as tryptophan and 5-hydroxylase and metabolites can also contribute to tolerance against NaCl stress (Fan et al., 2018). In addition, It has been reported that MEL increase NaCl tolerance in cucumber (*Cucumis sativus*) by regulating the expressions of genes related to gibberellic acid and abscisic acid metabolism (Zhang et al., 2014).

The study aimed to investigate the effect of MEL on DNA methylation, expression of salt stress sensor genes and physiological parameters in lettuce genotypes subjected to NaCl stress.

MATERIAL and METHOD

Plant Material

Ivanka and Yedikule lettuce genotypes used in the study have been commercially provided.

Sterilization, germination, growth of plants, MEL and NaCl applications

Seed sterilization was performed by using sodium hypochlorite (NaOCl, 6.15%) and hydrochloric acid (HCl, 12M) solutions. The research was established according to a randomized trial plan with 3 replications and 25 plants in each replication. Sterilized seeds were sown in petri dishes containing 1/2 MS medium and 1% (w/v) phytagel. Stratification was carried out in a growth chamber at 5°C for 16 hours. Then the seeds germinated at 20°C for 7 days in a 16/8 hour photoperiod. After this period, the plant seedlings were grown in a hydroponic system (pH=5.5-5.7) containing 1/2 Hoogland solution (Hoagland and Arnon, 1938) for 3 weeks. Different doses (0, 50, 100 and 200 µm) of MEL were applied in the form of a foliar spray (Yu et al., 2018). It was grown in a hydroponic system for 15 days by applying different doses of NaCl (0 and 150 mM) 24 hours after MEL application (Zapata et al., 2003). Physiological parameter measurements were made 15 days after the stress application. Plant samples were stored at -80 °C for gene expression and MSAP analyses.

Measurement of physiological parameters

Cell membrane damage (%)

Cell membrane damage was done according to Lutts et al., (1996). Cell membrane damage of the treatment groups was calculated based on the following formula.

Cell membrane damage (%) = (EC1/EC2) x 100

Relative water content (RWC %)

In order to determine RWC (%) in leaf tissue, 3 leaves from each repeat group were randomly selected and their fresh weight (FW) was measured. The leaves of the plant were soaked in pure water for 4 hours and the turgor weights (TW) were determined. Then, to determine the dry weights (DW), the leaf samples were

kept in the oven at 80°C for 48 hours and DW was measured. The leaf's relative water content was calculated based on the following formula (Barrs and Weatherley 1962).

RWC (%): [(FW – DW) / (TW – DW)] x 100

Amount of chlorophyll (SPAD)

Five randomly selected leaves from each recurrence were used to determine the amount of chlorophyll. The amount of chlorophyll was measured with SPAD-502 Chlorophyll Meter (Konica Minolta, Tokyo, Japan).

Analysis of data on physiological parameters

The data of physiological parameters were subjected to variance analysis with SAS 9.3 program (SAS Institute 1999) with PROC GLM in a factorial order of 2 (genotype) x 2 (NaCl dose) x 4 (MEL dose) according to the trial plan depending on the exact chance. The differences between the averages were made by Fisher's LSD multiple benchmark test at a 5% significance level.

Gene expression analysis

RNA isolation, cDNA synthesis, qRT-PCR analysis

For RNA isolation of 100 mg plant sample, Trizol reagent (Invitrogene, USA) chemical and product protocol were optimized and applied. The amount of RNA was determined by using the Qiagen Nanodrop (Qiaxpert 200061) Instrument (A260/280 O.D.). The sample amount for cDNA synthesis was calculated as µl corresponding to 0.5 g RNA concentration. cDNA synthesis was performed by using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit according to the kit protocol. SYBRGreen/Hi-ROX qPCR Kit was used in the qRT-PCR process and was carried out in accordance with the manufacturer's instructions. The primers for the *SOS1* , *SOS2* , *AKT1* , *NHX1* and *HKT1* genes were designed using the Primer3 program (http://frodo.wi.mit.edu/) by using databases related to bioinformatics studies on the *Lactuca sativa* genome. *Tubulin* (*TUB*) and *Ubiquitin* (*UBQ*) genes were used as reference genes (Borowski et al., 2014). The primer sequences of target genes and reference genes are shown in Table 1. Expression of genes with RT-PCR analysis was determined according to reference gene expression with $2^{-\Delta\Delta^{^}\tau}$ proportional calculation algorithm according to Livak and Schmittgen (2001).

MSAP analysis

DNA isolation, MSAP technique and data analysis

Genomic DNA isolation was performed using the cetyl trimethyl ammonium bromide (CTAB) method according to Taspinar et al., (2009). 200-400 ng of genomic DNA was cut with EcoRI and MspI/HpaII. DNA fragments were ligated to MspI-HpaII (50 pmol) and EcoRI (5 pmol) adapters by incubation at 37°C for 3 hours by using ATP and T4 DNA ligase. In the pre-amplification step, 4 µl of DNA fragments, 2.5 pmol of primers (E01 and HM0), 200 µM dNTPs, 1X PCR buffer and 0.5 U of Taq DNA Polymerase were used. Pre-treatment was carried out for 2 minutes at 65 °C and 95 °C, respectively. Then, 20 cycles of amplification (20 seconds at 94 °C, 30 seconds at 56 °C, 2 minutes at 72 °C, 2 minutes at 72 °C and 30 minutes at 60 °C) were applied. Selective amplifications were made using the specified primer combinations. These reactions included 4 µl of pre-amplification mix with a total volume of 20 µl, 1.5 pmol EcoRI primer labeled with both 6-FAM and JOE fluorescent dyes, 5 pmol MspI-HpaII primer, 200 µM dNTPs, 1X PCR buffer, and 0.5 pmol of MspI-HpaII primer. It was carried out with a mixture containing U Taq DNA polymerase. Touch Down PCR technique was used in the application. The samples were run and visualized on a Metaphor agarose gel at 120 Volts for 120 min. The primers used by Ding et al., (2019) were used in the MSAP technique. The base sequences of the primers used in the study have given in Table 2. As a result of the MSAP analysis, the total, complete and hemi-methylation classes and ratios of the primers were calculated with the help of Microsoft Excel program. DNA methylation rates and classes of lettuce genotypes were determined according to Tang et al., (2014).

RESULTS

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Physiological parameter results

It has been determined that the effect of genotype on cell membrane damage (%) is significant (p<0.05), and the effect of application and genotype application (GxU) interaction is very important (p<0.01) (Table 3). Cell membrane damage (%) has decreased in both genotypes due to the increase in MEL dose compared to the control treatment (Table 3). In both genotypes, % cell membrane damage has increased under NaCl stress compared to the control. In MEL applications with NaCl stress, cell membrane damage has reduced compared to stress applications alone (Table 3).

It has been determined that the effect of genotype and applications on the relative water content value is very important (p<0.01), while the effect of genotype application (GxU) interaction is significant (p<0.05) (Table 3). RWC% in only MEL applications have differed according to genotype. In Ivanka genotype, RWC% has decreased MEL treatments compared to the control. In Yedikule genotype, RWC% has increased in 50 μM and 100 μM MEL applications. In both genotypes, RWC% has decreased in NaCl treatment without MEL compared to the control (Table 3). On the other hand, in both genotypes, it has increased in MEL applications with NaCl parallel with the MEL dose increase compared to only NaCl application (Table 3).

When Table 3 is examined, it has determined that the effect of genotype on the amount of chlorophyll is significant (p<0.05), and the effect of the interaction of application and genotypexapplication (GxU) is very important (p<0.01). In both genotypes, amount of chlorophyll has increased MEL dose dependent in MEL applications without NaCl compared to the control. In both lettuce genotypes, NaCl application has been decreased the amount of chlorophyll compared to the control. In addition, the amount of chlorophyll has increased in a dose-dependent manner in MEL applications along with NaCl in both genotype (Table 3).

Gene expression analysis results

When gene expression analyzes is examined in leaves of lettuce genotypes, *SOS1* gene expression has decreased in both lettuce genotypes in all only MEL treatments compared to control (+1)(Figure 1).*SOS1* gene expression has increased in both lettuce genotypes along with NaCl treatment. When MEL applications along with NaCl treatments are examined, this gene expression has increased only in 150 mM NaCl + 50 μM MEL application in the Ivanka genotype. The same gene expression has decreased in Yedikule genotype in all MEL applications along with NaCl stress (Figure 1). *SOS2* gene expression for both genotypes has decreased in all MEL treatments without NaCl compared to the control (Fig. 2). *SOS2* gene expression has decreased in all genotypes in only NaCl treatment. In all MEL+NaCl treatments,*SOS2* gene expression has decreased in both genotypes except for 50 μM MEL + 150 mM NaCl treatment in Yedikule genotype (Figure 2).*AKT1* and *NHX1* gene expressions have increased for both genotypes in 150 mM NaCl and 50 μM MEL + 150 mM NaCl treatments compared to control but decreased in all other treatments (Figure 3-4).*HKT1* gene expression has increased in both genotypes in NaCl treatment alone. In both lettuce genotypes, the same gene expression has decreased in all MEL and MEL+NaCl treatments compared to the control (Figure 5).

MSAP analyzes and polymorphism results

Bands identified in MSAP analysis results are divided into four classes according to Wang et al. (2011). The overall DNA methylation level in the Ivanka genotype was 62% in the control and 53.7% in the NaCl application. Compared to the control, the rate of DNA methylation decreased in MEL applications alone and in MEL applications with NaCl, depending on the dose. It has been determined that the mimethylated thylated DNA band (type II) regions increased about 0.9% in NaCl (19.4%) application compared to the control (18.5%). In control and NaCl applications, 43.5% and 34.3% fully methylated DNA bands (type III + IV) were observed respectively (Table 4). In Yedikule genotype, the DNA methylation level was 54.2% in the control and 48.6% in NaCl stress. Compared to the control, it was observed that the rate of DNA methylation decreased depending on the dose increase in MEL applications alone. Depending on the MEL dose, DNA methylation levels decreased 3.7%, 2.8% and 0.9% respectively in NaCl applications with MEL. Hemi-methylated DNA band ratio was determined as 14% in control and 16.8% in NaCl stress. Fully methylated DNA bands (type III + IV) were detected as 40.2% and 31.8% in control and NaCl stress

applications respectively (Table 5).

DISCUSSION

It is known that the salt concentration in the soil, which is one of the most important stress factors, negatively affects the growth and development of plants, the quality and quantity of the product. MEL, a pleiotropic molecule with various functions in animals and plants, has proven to be an abiotic stress inhibitor in plants (Li et al., 2016).

MEL also controls the regulation of a number of stress-sensitive genes and their signaling cascades in plant cells (Ayyaz et al., 2022). The first sign of abiotic stress factors occurs when cell membranes are damaged (Holmberg and Bülow 1998). In our findings, it has determined that cell membrane damage increase under salt stress condition in both genotypes. It is thought that this damage caused by NaCl stress on the membranes is related to the high concentration of Na$^+$ions accumulated in the apoplast by affecting the ionic bonds of structural elements such as pectin in the structure of the cell membrane or the apoplastic enzymes and disrupting the basic functions of the membrane (Çulha and Çakırlar 2011). In addition, the deterioration of cell membrane structures due to membrane peroxidation by ROS whose accumulation increases in stress can lead to increased relative electrical conductivity (RC, Relative Electrical Conductivity) (Wang et al., 2021). Similar to our research results, the deterioration of membrane structures due to salt stress have reported in studies conducted on lettuce (Campos et al., 2019), barley (Zhang et al., 2022), pork loin (Dutta Gupta 2007), and tomato (Ali et al., 2021).

Decreased membrane stability under NaCl stress and increased membrane stability in MEL+NaCl application have been reported in two different tomato genotypes (Ali et al., 2021). In addition, It has been reported that MEL completely inhibits cell membrane damage under stress (Zhang et al., 2014).

The osmotic stress induced by the increase in the amount of NaCl in the roots causes a decrease the amount of usable water and the RWC% decreases (Tuteja 2007).

In the study, it has determined that NaCl stress decreases RWC% in both genotypes compared to the control. This situation is thought to be related to the decrease in the amount of usable water with the effect of osmotic stress caused by salt stress. Decreased RWC% has been reported in lettuce (Kalleli et al., 2021), sunflower (Naz et al. 2015), sugar beet (Jamil et al. 2012), tomato (Ali et al., 2021) and quinoa (Parvez et al., 2020) in the condition of NaCl. In both genotypes, when compared to the control, it was determined that alone MEL applications and MEL applications along with stress against NaCl stress caused an increase in RWC%. Similar results were recorded in paddy (Yan et al., 2021), cotton (Shen et al., 2021) and broad bean (Abd El-Ghany and Attia 2020). It is known that genotypes with low cell membrane damage have high relative water content (Jamil et al. 2012). High MEL concentrations can severely reduce ROS in cells, thereby inhibiting damage by affecting ROS-dependent signal transduction (Afreen et al. 2006).

In our study, chlorophyll content decreased with NaCl stress in both genotypes. It has been determined that there are a gradual increase in SPAD values in MEL applications along with NaCl. It has been reported that the chlorophyll content of lettuce decreased against NaCl application (Garrido et al., 2014; Kalleli et al., 2021). In general, it has been reported that chlorophyll content decreases with increasing NaCl stress, possibly due to the increase in chlorophylase activity and adversely affecting membrane stability (Kaleli et al., 2021). The effect of MEL against NaCl stress similar to our findings has been recorded in alfalfa (Niu et al., 2022), melon (Castañares and Bouzo, 2019), broad bean (Abd El-Ghany and Attia 2020), rice (Yan et al., 2021). It has been noted that exogenously applied MEL increases photosynthetic efficiency by regulating chlorophyllase (CHLASE) gene activity in drought stress (Sharma et al., 2020).

Only overly sensitivity (SOS) is effective in controlling the uptake of Na+ ions and the entry of Na+ ions into the cytosol in salinity tolerance, and the SOS1 protein is known as a very important component in the protection of plants against Na+ ions entering the cytoplasm. The findings have determined that *SOS1* gene expression increases under NaCl stress in both genotypes. It has been noted that the*SOS1* gene expression increased at different time intervals in tolerant and sensitive genotypes in rice genotypes with different salt

tolerance (Hossain et al., 2017). Increased *SOS1* gene expression has been reported in bread wheat (Karadayı 2017), transgenic Arabidopsis (Yang et al. 2009), cotton genotypes (Wang et al., 2017), sorghum (Youssef et al., 2021) and sugarcane (Brindha et al., 2021) under salinity stress conditions. The research results exposed that*SOS1* gene expression only increased at the lowest MEL dose+NaCl treatment in Ivanka genotype. The same gene expression decreased in other applications and genotype. Similarly, it has been noted that*SOS1* gene expression increased in bitter melon under low-dose NaCl stress+MEL application (Sheikhalipour et al., 2022).

The other protein of the SOS pathway, SOS2 (a CBL-Interacting Protein Kinase [CIPK]), is thought to increase both SOS1 protein activity and *SOS1* gene expression through direct protein-protein interaction in salt tolerance (Brindha et al., 2021). In addition, SOS2 modulates vacuolar V-ATPase and regulates Na^+/H^+ and Ca^{+2}/H^+ exchange in the vacuolar membrane (Batelli et al., 2007). In our study, it was determined that*SOS2* gene expression increased in both lettuce genotypes under NaCl stress. Increased *SOS2* gene expression was noted in Arabidopsis roots (Liu et al. 2000), sorghum (Assem et al., 2017), transgenic rice (Kumar et al., 2022) and sugarcane (Brindha et al., 2021) under salt stress. In another study conducted in Arabidopsis, it was reported that overexpression of the S*OS2* gene contributes to salt tolerance by causing the activation of the *GRIK1* gene (Barajas Lopez et al., 2018). In the study, in NaCl+MEL applications, it was determined that *SOS2* gene expression increased only in Yedikule genotype at the lowest MEL dose compared to the control, but the gene expression rate decreased compared to only NaCl application. Unlike our results, it was reported that MEL applications with stress increased the *SOS2* gene expression level more than NaCl stress in cucumber. This is thought to be due to the interaction between MEL and other signal transduction pathways in response to environmental stress (Zhang et al., 2020).

AKT1 , *NHX1* and *HKT1* genes are genes involved in ion homeostasis under NaCl stress and contribute to salt tolerance. The*AKT1* gene encodes a carrier protein that has a high affinity for K+ and a low affinity for Na^+, allowing K^+ to be taken into the cell (Li et al. 2012). The ion balance is controlled by storing excess Na^+ ions in the vacuole via Na^+/H^+ carriers (NHX) (Botella et al., 2005). In Arabidopsis, it has been reported that the transport of Na^+ from the shoot to the root tissue is mediated by *HKT1* gene product carriers (Berthomieu et al. 2003). In our study, it was determined that *AKT1* , *NHX1* and*HKT1* gene expressions increased in leaf tissue in both lettuce genotypes with only NaCl application. Similar to our findings, these gene expressions have been reported to increase in cotton genotypes (*NHX1* and *AKT1*)(Wang et al. 2017), *Malus hupehensis*(*AKT1*)(Li et al., 2012), salt-resistant wheat and barley genotypes (*AKT1* , *NHX1* and *HKT1*)(Zeeshan et al., 2020), soybean (*AKT1*)(Wang et al., 2021), salt-tolerant and sensitive rice genotypes (*NHX1*)(Hossain et al., 2017), maize (*HKT1*)(Zhang et al., 2018), tomato (*NHX1*)(Abdelaziz et al. 2019), transgenic tobacco (*HKT1*)(Ali et al., 2019), and Arabidopsis (*NHX1*)(Krishnamurthy et al. 2019), sorghum (*NHX1*)(Youssef et al., 2021) under salt stress. When our findings are examined for both genotypes in NaCl+MEL applications, it has been determined that *AKT1* and *NHX1* gene expressions increased in at the lowest MEL dose + NaCl aplication, *HKT1* gene expression decreased in all of the applications. Similar to our findings, it has been reported that MEL application against salt stress increases*NHX1* and *AKT1* gene expressions in apples (Li et al., 2010). In another study, it has noted that exogenously applied MEL decrease the accumulation of Na^+ in leaves by affecting the expression of *NHX1* and *AKT1* , which increase salt tolerance in plants (Li et al., 2016). It has also been reported that the synergistic effects of the *SOS1* gene, rather than the expression of the NHX1 gene alone, alleviate salt tolerance (Gedik et al., 2020). Also, the same synergistic effect with salt sensor genes (*NHX1* and *SOS1*) has been reported in the *HKT1* gene (Wang et al., 2020).

DNA methylation/demethylation, which is one of the adaptation and regulation mechanisms of plants against abiotic stresses, is one of the oldest and most studied epigenetic mechanisms that can regulate genome functioning and induce plant resistance (Sun et al., 2022). The methylation level greatly contributes to the plant's ability to respond to stress and is used to understand physiological, biochemical or adaptation processes (Zenda et al., 2018). In this study, it has observed that total DNA methylation rate and fully methylated DNA rate decrease in genotypes under NaCl stress compared to the controls. It has been reported that methylated cytosines are generally more than 20% in plants and the methylation status differs between

various plants and tissues (Gruenbaum et al. 1981; Yaish 2013). The methylation level in Arabidopsis is about 20% (Zhang et al., 2006). Karan et al., (2012) observed that the methylation level in the salinity sensitive IR29 and tolerant Geumgangbyeo rice genotypes decreased under NaCl stress. Wang et al., (2011) stated that the methylation level of rice decreased under drought stress. All MSAP studies suggest that NaCl stress may have an important role in regulating gene expression specifically in each genotype (Zhang et al., 2006; Wang et al., 2010; Wang et al., 2011). In our only MEL application results, decreased total DNA methylation and fully methylated DNA rates were observed in both genotypes. The results to similiar with our findings have been reported in grapes in MEL applications alone (Sun et al., 2020).

CONCLUSION AND RECOMMENDATIONS

As a result, when all the properties examined in the study are evaluated, it can be suggested that the negative effects of stress can be mitigated by the application of MEL and MEL contributes to NaCl stress tolerance.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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Table 1: Primer sequences used in the study (F:Forward (5'-3'/R:Reverse 3'-5')

Primer-Gene	Base Sequence	Tm
SOS1	F: TGATAATAGCGGCGGAGGAG	54°C
	R: TGGAGGTCGTGTGAAAGAGA	52°C
SOS2	F: TGCTAGCTATCAAATCAGTGGAG	53°C
	R: CTCCAGCAGCTTTTCGAACA	52°C
AKT1	F: GGGTTTCAATGTGTGGCGAT	52°C
	R: TCACTCATCCGGTTTGCATC	52°C
NHX1	F: TCGCGTTATTGCTTGGGAAA	50°C
	R: TACTCTGTTCGGTTGGTGGT	52°C
HKT1	F: GGAGCCACGGAATCCTATGA	54°C
	R: TGCTGGAGAGATGAGGGAAA	52°C
TUB	F: TAGGCGTGTGAGTGAGCAGT	54°C
	R: AACCCTCGTACTCTGCCTCTT	54°C
UBQ	F: AAGACCTACACCAAGCCCAA	52°C
	R: AAGTGAGCCCACACTTAC	48°C

Table 2: Base sequencing of primers and adapters used in the MSAP technique

	Primer	**5'-3'**
	Eco RI primer +1 (50 ng/µL)	GACTGCGTACCAATTCG
	Hpa II– Msp I primer +1 (50 ng/µL)	GATGAGTCTCGATCGGT
A	EcoRI primer +3 (12 ng/µL)	GACTGCGTACCAATTCATG
B		GACTGCGTACCAATTCACC
C		GACTGCGTACCAATTCACG
D		GACTGCGTACCAATTCAGA
E		GACTGCGTACCAATTCATC
A	HpaII- MspI primer +3 (50 ng/µL):	ATCATGAGTCCTGCTCGGTCG
B		ATCATGAGTCCTGCTCGGTTC
C		ATCATGAGTCCTGCTCGGTTG
D		ATCATGAGTCCTGCTCGGTGA
E		GATGAGTCTCGATCGGTTAC
	HpaII–MspI adapter	GACGATGAGTCTCGAT
		CGATCGAGACTCAT
	EcoRI-adapter	CTCGTAGACTGCGTACC
		AATTGGTACGCAGTC

Table 3. Cell Membrane Damage (%), RWC (%) and Chlorophyll Content of genotypes according to applications (SPAD-502)

Uygulama	Cell Membran Damage (%)	Cell Membran Damage (%)	Cell Membran Damage (%)	RWC (%)	RWC (%)	RWC (%)	Chlorophyll Content (SPAD-502)	Chlorophyll Content (SPAD-502)	Chlorophyll Co (S 50
	Yedikule	İvanka	Average	Yedikule	İvanka	Average	Yedikule	İvanka	Av
Control	38,08^c	32,03^e	**35,06^e**	72,84^{ab}	74,08^a	**73,46^a**	39,10^c	42,40^{bc}	**40**
50 μM Mel	31,49^{de}	29,00^f	**30,25^f**	75,33^a	72,60^a	**73,97^a**	47,97^b	45,37^{ab}	**46**
100 μM Mel	31,24^{de}	30,02^{ef}	**30,63^f**	74,93^a	68,40^b	**71,66^{ab}**	52,10^{ab}	46,53^a	**49**
200 μM Mel	27,72^e	30,61^{ef}	**29,17^f**	72,43^{ab}	68,43^b	**70,43^{bc}**	54,87^a	48,37^a	**51**
150 mM NaCl + 0 μM Mel	72,64^a	66,84^a	**69,74^a**	57,56^d	52,48^d	**55,02^f**	32,87^d	36,93^d	**34**
150 mM NaCl + 50 μM Mel	53,94^b	60,31^b	**57,12^b**	64,84^c	56,43^c	**60,63^e**	38,63^c	41,20^c	**39**
150 mM NaCl + 100 μM Mel	36,25^{cd}	56,35^c	**46,30^c**	68,09^{bc}	65,49^b	**66,79^d**	41,23^c	42,63^{bc}	**41**
150 mM NaCl + 200 μM Mel	41,88^c	42,97^d	**42,43^d**	69,30^{bc}	67,84^b	**68,57^{dc}**	42,27^c	43,37^{bc}	**42**
Average (Avg)	**41,66^B**	**43,52^A**	42,59	**69,41^A**	**65,72^B**	67,57	**43,63^A**	**43,35^A**	43,
F Value (Genotype) (G)	-	-	6.67*	-	-	28.84**	-	-	0.1
F Value (Application) (A)	66.34**	301.94**	205.63**	15.72**	37.25**	46.37**	19.13**	13.43**	31.

Uygulama	Cell Membran Damage (%)	Cell Membran Damage (%)	Cell Membran Damage (%)	RWC (%)	RWC (%)	RWC (%)	Chlorophyll Content (SPAD-502)	Chlorophyll Content (SPAD-502)	Chlorophyll Co... (S... 50...
F Value (GxA)	-	-	17.35**	-	-	2.40*	-	-	4.3...
Variation Coefficient (%)	7.61	3.55	5.86	3.75	3.25	3.53	6.73	3.83	5.4...

Table 4. DNA methylation levels in Ivanka genotype

Primers	Classes	0 µM Mελ 0 mM NaCl	0 µM Mελ 150 mM NaCl	50 µM Mελ 0 mM NaCl	50 µM Mελ 150 mM NaCl	100 µM Mελ 0 mM NaCl	100 µM Mελ 150 mM NaCl	200 µM Mελ 0 mM NaCl	200 µM Mελ 15... mM Na...
A	Class I	9	7	8	8	7	7	4	6
	Class II	2	5	2	2	4	4	2	4
	Class III	2	3	2	2	4	3	7	2
	Class IV	5	3	6	6	3	4	5	6
B	Class I	9	9	8	9	9	8	9	10
	Class II	5	5	7	6	5	7	8	6
	Class III	5	5	5	4	5	5	3	4
	Class IV	6	6	5	6	6	5	5	5
C	Class I	8	15	12	12	12	10	14	12
	Class II	4	3	4	4	4	3	4	4
	Class III	5	4	4	3	2	3	2	4
	Class IV	6	1	3	4	5	7	3	3
D	Class I	9	12	10	11	9	12	10	6
	Class II	4	6	4	5	8	5	6	6
	Class III	7	2	4	4	4	3	3	7
	Class IV	5	5	7	5	4	5	6	6

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Primers	Classes	0 μM Meλ	0 μM Meλ	50 μM Meλ	50 μM Meλ	100 μM Meλ	100 μM Meλ	200 μM Meλ	20... μM M
E	Class I	6	7	4	5	6	7	7	8
	Class II	5	2	4	3	4	4	3	3
	Class III	2	4	5	5	2	3	4	2
	Class IV	4	4	4	4	5	3	3	4
Total band count	**Total band count**	**108**	**108**	**108**	**108**	**108**	**108**	**108**	**10**
Hemi methylation rate (%)	*Hemi methylation rate (%)*	*18,5*	*19,4*	*19,4*	*18,5*	*23,1*	*21,3*	*21,3*	*21,*
Fully mehylation rate (%)	*Fully mehylation rate (%)*	*43,5*	*34,3*	*41,7*	*39,8*	*37,0*	*38,0*	*38,0*	*39,*
Total methylation rate (%)	*Total methylation rate (%)*	*62,0*	*53,7*	*61,1*	*58,3*	*60,2*	*59,3*	*59,3*	*61,*

A score of 1 or 0 indicates the presence or absence of groups, respectively.

Total methylated band ratio = [(II + III + IV) / (I + II + III + IV)] x100

Ratio of fully methylated band = [(III + IV) / (I + II + III + IV)] × 100

Hemi-methylated band ratio = [(II) / (I + II + III + IV)] x100

Type I are unmethylated bands; Type II are hemi-methylated bands and types III + IV are fully methylated bands.

Table 5. DNA methylation levels in Yedikule genotype

Primers	Classes	0 μM Meλ	0 μM Meλ	50 μM Meλ	50 μM Meλ	100 μM Meλ	100 μM Meλ	200 μM Meλ	20... μM M
		0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl	0 mM NaCl	15... Na...
A	Class I	5	7	4	6	5	5	6	2
	Class II	2	3	2	3	5	5	2	2
	Class III	6	5	5	4	4	4	4	7

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Primers	Classes	0 μM Mελ	0 μM Mελ	50 μM Mελ	50 μM Mελ	100 μM Mελ	100 μM Mελ	200 μM Mελ	20 μM M
	Class IV	4	2	6	4	3	3	5	6
B	Class I	13	14	18	17	17	17	18	19
	Class II	2	5	4	4	4	6	4	5
	Class III	4	4	3	4	4	3	4	4
	Class IV	11	7	5	5	5	4	4	2
C	Class I	12	11	10	9	11	13	9	10
	Class II	3	4	3	6	2	2	6	4
	Class III	2	2	2	2	4	2	2	3
	Class IV	2	2	4	2	2	2	2	2
D	Class I	10	10	9	10	9	10	9	10
	Class II	5	4	5	5	5	4	5	4
	Class III	2	3	4	3	2	4	4	4
	Class IV	4	4	3	3	5	3	3	3
E	Class I	9	13	9	11	9	7	10	9
	Class II	3	2	4	2	4	4	5	3
	Class III	5	2	4	3	4	5	3	4
	Class IV	3	3	3	4	3	4	2	4
Total band count	**Total band count**	**107**	**107**	**107**	**107**	**107**	**107**	**107**	**10**
Hemi methylation rate (%)	*Hemi methylation rate (%)*	*14,0*	*16,8*	*16,8*	*18,7*	*18,7*	*19,6*	*20,6*	*16,*
Fully mehylation rate (%)	*Fully mehylation rate (%)*	*40,2*	*31,8*	*36,4*	*31,8*	*33,6*	*31,8*	*30,8*	*36,*

16

Primers	Classes	0 μM Mελ	0 μM Mελ	50 μM Mελ	50 μM Mελ	100 μM Mελ	100 μM Mελ	200 μM Mελ	20... μM M
Total methylation rate (%)	Total methylation rate (%)	54,2	48,6	53,3	50,5	52,3	51,4	51,4	53,

A score of 1 or 0 indicates the presence or absence of groups, respectively.

Total methylated band ratio = [(II + III + IV) / (I + II + III + IV)] x100

Ratio of fully methylated band = [(III + IV) / (I + II + III + IV)] × 100

Hemi-methylated band ratio = [(II) / (I + II + III + IV)] x100

Type I are unmethylated bands; Type II are hemi-methylated bands and types III + IV are fully methylated bands.

Φιγυρε 1. $\Sigma O\Sigma 1$ γενε εξπρεσσιον ςηανγες ($2^{-\Delta\Delta^{\cdot}\tau}$)

Figure 2. *SOS2* gene expression changes ($2^{-\Delta\Delta^{\cdot}\tau}$)

Figure 3. *AKT1* gene expression changes ($2^{-\Delta\Delta^{\cdot}\tau}$)

Figure 4. *NHX1* gene expression changes ($2^{-\Delta\Delta^{\cdot}\tau}$)

Figure 5. *HKT1* gene expression changes ($2^{-\Delta\Delta^{\cdot}\tau}$)