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Contact

Sari Agricultural Sciences and Natural Resources University, Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari, P.O. Box: 578, Iran.
Tel: +98-11-33687577
Email: jpmbjournal@sanru.ac.ir
www.jpmb-gabit.ir

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The efficiency of *Agrobacterium*-mediated gene transfer in *Arabidopsis thaliana* mutants

Mohammad Sadegh Taghizadeh *[1], Mohammad Mehdi Sohani [2], Reza Shirzadian-Khorramabad [2]

[1] Institute of Biotechnology, Faculty of Agriculture, Shiraz University, Shiraz, Iran.
[2] Department of Agricultural Biotechnology, Faculty of Agriculture, University of Guilan, Rasht, Iran.

Original paper

Abstract: A few small molecular weight signals, including jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), regulate the expression of defense-related genes in plants. These signals serve to inhibit the activation of plant defense genes against aggressors and can manipulate the plant's defense signaling pathways. In this study, the impact of acetosyringone on the induction of virulence genes was examined in *Agrobacterium tumefaciens* A348 (MX311) and A348 (MX243) at three different levels: 0, 100, and 200 µM. The concentration that demonstrated the highest induction of virulence genes was then used for transforming *Arabidopsis* mutants using *A. tumefaciens* EHA105, with the aim of inducing virulence genes. Results revealed that *virD2* expression reached its peak at 200 µM acetosyringone, while *virB2* expression was highest at 0 µM. Additionally, transformation experiments indicated that the SA mutants (nahG) exhibited the highest transformation efficiency, while the control plants (Col-0) displayed the lowest efficiency. Therefore, the efficiency of gene transfer in SA-suppression mutants suggests a more significant role for SA in plant defense against pathogens compared to the other hormones. Enhancing gene transfer efficiency in these mutants could unlock the potential for increased expression and production of recombinant proteins compared to the wild type.

Keywords: acetosyringone, in Planta, salicylic acid mutant, jasmonic acid mutant, ethylene mutant, PCR.

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Correspondence
Dr. Mohammad Sadegh Taghizadeh
ms.taghizadeh@shirazu.ac.ir

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Introduction

Plants display a range of defense mechanisms in response to biotic and abiotic stresses. Some compounds such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play an important role in regulating the defense responses of plants against pathogens, pests and abiotic stresses such as wounds (Loake and Grant, 2007; Balbi and Devoto, 2008) and thus, the expression of genes involved in the defense responses increase with these compounds. SA generally plays an important role in activating plant defense responses against biotrophic and hemibiotrophic pathogens as well as systemic acquired resistance (Grant and Lamb, 2006) while JA and ET are usually associated with plant defense responses to necrotrophic pathogens and herbivorous insects. SA, ET, JA, and camalexin phytoalexin, alone or in together combination, are involved in *Arabidopsis thaliana* defense against various pathogens. SA is also known to protect plants against many pathogens, including fungi, bacteria, and viruses (Kuć, 1982; Tripathi et al., 2019). In several studies, JA concentration has been increased in the pathogen-infected area or damaged tissue and also its external application has been stimulated the expression of genes dependent on plant defense responses (Wasternack, 2007; Siddiqi and Husen, 2019). In addition, plant treatment with ET or its derivatives, as well as ethylene inhibitors, have demonstrated the clear relevance of this plant hormone to plant defense responses(Beckman, 2000; Xu et al., 2018). Although SA and JA/ET defense pathways are antagonistic, evidence of synergistic interactions between these pathways has also been reported (Beckers and Spoel, 2005; Nie et al., 2012). This suggests that the relevance among plant signaling pathways is very complex. On the other hand, in response to *Agrobacterium* infection, the high levels of SA and ET reduce the *Agrobacterium* virulence by inhibiting vir gene expression and T-DNA transfer into plant cells (Yuan et al., 2007; Anand et al., 2008) Therefore, the cross talk of phytohormones plays an important role in the interaction between the host plant and *Agrobacterium*.

The floral dip transformation method, a modified vacuum-infiltration method, was introduced by Clough and Bent (Clough and Bent, 1998) for the transformation of *Arabidopsis*. Immersing the plants containing many unopened flower buds, in the suspension of *Agrobacterium* along with sucrose and Silwet L-77 surfactant, the rate of gene transfer reaches 3-5%. To have a successful gene transfer, it is necessary to pay attention to the growth stage of the plant such as many unopened flower buds, the presence of sugar sources, and the use of surfactants or suction to help penetrate bacteria.

The study of the effect of defense hormones in the presence of *Agrobacterium* leads to understanding the role of those hormones in the efficiency of transformation. Whole plant regeneration from transformed somatic cells occasionally results to generate somatic mutations, so that the presence of phytohormones increases the chances of these mutations occurring (Bent, 2006; Hwang et al., 2017). One solution is to use mutants that lack the ability to produce those hormones. Hence, in this study, we used some *Arabidopsis* mutants to better understand the role of phytohormones, including ethylene, jasmonate, and salicylate, in increasing gene transfer efficiency by *Agrobacterium*. Understanding this will improve our knowledge to increase gene transfer efficiency followed by increase the expression and production of recombinant proteins in plants.

Materials and Methods

Plant materials

jar1 (JA suppression), *etr1-8* (ET-suppression), and *nahG* (SA-suppression) plant mutants and Col-0 (Wild type) were used for transformation. The plants were grown in a growth chamber at 21 °C and a photoperiod of 16/8 darkness/light with a relative humidity of 70%. Jasmonate resistance 1 (JAR1) is a jasmonate-amino synthase that catalyzes the formation of a biologically active jasmonyl-isoleucine (JA-Ile) conjugate (an active form of JA) so that this gene has knocked out in *jar1* mutants (Staswick and Tiryaki, 2004). In *etr1-8* mutants, there is a G-to-A transition in the *ETR1* gene, which results in a stop codon at Trp563 (Hua and Meyerowitz, 1998). On the other hand, nahG plants are containing a gene from the bacterium *Pseudomonas putida* that encodes SA hydroxylase to metabolize SA to catechol, which results in a

dramatic decrease in SA content (Rosas-Díaz et al., 2017).

A. tumefaciens strains and gene construct

Two strains of *A. tumefaciens*, called A348 (MX311) and A348 (MX243), were used. In these strains, pCM110 binary vector carrier Tn3 transposons (containing a gene without lacZ promoter) have been mixed with the promoter of vir genes. Accordingly, A348 (MX243) and A348 (MX311) stains are carrier virB2::lacZ and virD2::lacZ, respectively (Figure 1). These strains were used to determine a concentration of acetosyringone, which induces the expression of vir genes highly, using the measure of β-galactosidase activity assay. Subsequently, those concentrations were used to transform the plant mutants by the *Agrobacterium* EHA105 containing pCAMBIA1105.1.

vir genes induction

A348 (MX243) and A348 (MX311) strains were grown in 5 mL YEP culture medium (10 g/l peptone, 10 g/l yeast extract, 5 g/l NaCl) containing rifampicin (100 µg/ml) and carbenicillin (100 µg/ml) antibiotics at 28 °C overnight with shaking. 0.5 ml of the culture was diluted into 50 ml AB-sucrose minimal medium (50 ml 20X AB-buffer (60 g/l K2HPO4, 20 g/l Na2HPO4, pH=7), 50 ml 20X AB-salts (20 g/l NH4Cl, 6 g/l MgSO4.7H2O, 3 g/l KCl, 0.2 g/l CaCl2, 50 mg/l FeSO4.7H2O), 900 ml 0.05% sucrose solution) containing rifampicin and carbenicillin antibiotics and grown overnight at 28 °C until the bacteria were in late log phase (OD600 = 0.8). The bacteria were centrifuged at 9000 xg for 5 min and the pellet was re-suspended in two volumes of induction medium (1X AB-salts, 2 mM phosphate buffer (pH=5.6), 50 mM 2-(4-morpholino)-ethane sulfonic acid, 0.5% glucose) containing the different concentrations of acetosyringone (0, 100 and 200 µM) and shaken very gently (approx. 50 rpm) for 14-24 h at 25 °C (Gelvin, 2006).

β-galactosidase activity assay

After induction of vir genes, an aliquot of them was centrifuged for 1 min and then, re-suspended in a final volume of 4 mL Z-buffer (16.1 g/l Na2HPO4.H2O, 5.5 g/l NaH2PO4.H2O, 0.74 g/l KCl, 0.246 g/l MgSO4.7H2O, 2.7 ml β-mercaptoethanol, pH=7), and OD600 was adjusted to 0.1-0.25. Two drops of 0.1% SDS and four drops of chloroform were added to the 2 ml cell culture, vortexed and incubated in a 30 °C water bath for 10 min. In the next step, 400 µl of O-Nitrophenyl-β-D-galactoside (ONPG, 4 mg/ml in Z-buffer) solution was added, vortexed, and started timing until 60 min to detect β-galactosidase activity. Finally, the reaction was terminated by the addition of 1 ml 1 M Na2CO3 and read the absorption at both 420 and 550 nm. The β-galactosidase activity was calculated as follows (Miller, 1972):

$$Miller\ unit = \frac{1000\ (A420\ nm - 1.75 \times A550\ nm)}{time\ (min.) \times A600}$$

Plant transformation

The floral dip method was used to plant transformation. First, the vir genes of the *Agrobacterium* EHA105 containing the pCAMBIA1105.1 vector were induced as above in a 200 µM concentration of acetosyringone (as treatment) and 0 µM concentration of acetosyringone (as control). The bacteria were centrifuged at 9000 xg for 5 min, resuspended in 5% sucrose solution containing 0.02% (v/v) silwet L-77.

Figure 1. Structure and organization of Tn*3*-HoHol. The coding region within the element and the transcriptional orientation of each gene are indicated by an arrow. *tnpR* and *bla* are wild-types, while *tnpA* is non-functional owing to the *lac* sequences inserted into its 3' end. The *lac* operon sequences are intact but lack a functional promoter. Translation can potentially initiate at *virB2 / virD2* that occurs upstream of the *lacZ* structural gene. Symbols: IRL, left-inverted repeat; IRR, right-inverted repeat; *lac*ZYA, *E. coli lac* operon; *tnpA*, transposase; *tnpR*, resolvase; *bla*, β-lactamase.

Afterward, the plant mutants were inoculated with bacteria suspension. To maintain 100% moisture, the plants were kept in dark boxes covered with plastic for 24 hours and finally, the plants returned to normal growth conditions. After a week, this work was repeated to increase the efficiency of the transformation.

Screening of putative transgenic plants

Hygromycin resistance analysis

Surface sterilization of harvested seeds was done with 70% ethanol for 1 minute and afterward with 5% sodium hypochlorite for 10 minutes, then washed several times with sterilized water. The sterile seeds were cultured in an MS plant tissue culture medium containing hygromycin antibiotic (50 µg/mL) at 4 °C for two days and then, were transferred to a growth chamber at 20-22 °C with photoperiod 8/16 darkness/light. After 10 days, grown seedlings and green were transferred to the pot.

PCR analysis

The DNA extraction from the putative transgenic plant mutants was carried out using the Dena Zist kit (S-1030-1) according to the manufacturer's instructions. To confirm the presence of hyg in the plant mutants, a PCR reaction was performed using specific primers. To confirm the absence of bacterial contamination of the plants, spect specific primers were used (Table 1). Finally, the PCR products were run on the 1% agarose gel.

Statistical analysis

All experiments were carried out in a completely randomized design with two biological replications and three technical replications. Analysis of variance of the data obtained from the experiment and comparison of the mean of treatments with Tukey test at P-value ≤ 0.01 were performed using SAS 9.0 software and the charts were drawn using GraphPad prism software.

Results

The induction of vir promoter activity in the MX311 and MX243 strains

In the MX311 strain, the results showed that the highest and lowest of β-galactosidase activity was at 200 and 0 µM concentrations of acetosyringone respectively, while in the MX243 strain, the highest and lowest of β-galactosidase activity was at 0 and 100 µM concentrations of acetosyringone respectively (Figure 2).

Also, the results of the analysis of variance showed that β-galactosidase activity in the MX311 strain has a significant difference in the different concentrations of acetosyringone at P-value ≤ 0.01 (Table 2). However, β-galactosidase activity in MX243 strain had a significant difference among 0 and 100 µM concentrations of acetosyringone at P-value ≤ 0.01, while no significant difference was observed between each of concentrations of 0 and 100 µM with the concentration of 200 µM at P-value ≤ 0.01 (Figure 2 and Table 3).

Table 1. The sequences of specific primers used in PCR.

Gene	Sequence	Tm (°C)	PCR product (bp)
hyg	F: 5′-GATGTTGGCGACCTCGTATT-3′ R: 5′-GTGCTTGACATTGGGGAGTT-3′	63.7 63.9	450
spect	F: 5′-ATTTGCCGACTACCTTGGTG-3′ R: 5′-GAACATAGCGTTGCCTTGGT-3′	63.7 63.9	450

Table 2. Analysis of the variance of β-galactosidase activity in different concentrations of acetosyringone in MX311 strain.

Source of changes	DF	Sum of Squares	Mean Square	*P-value*
Treatment	2	2230874.918	1115437.459	< 0.0001
Error	6	38.987	6.498	

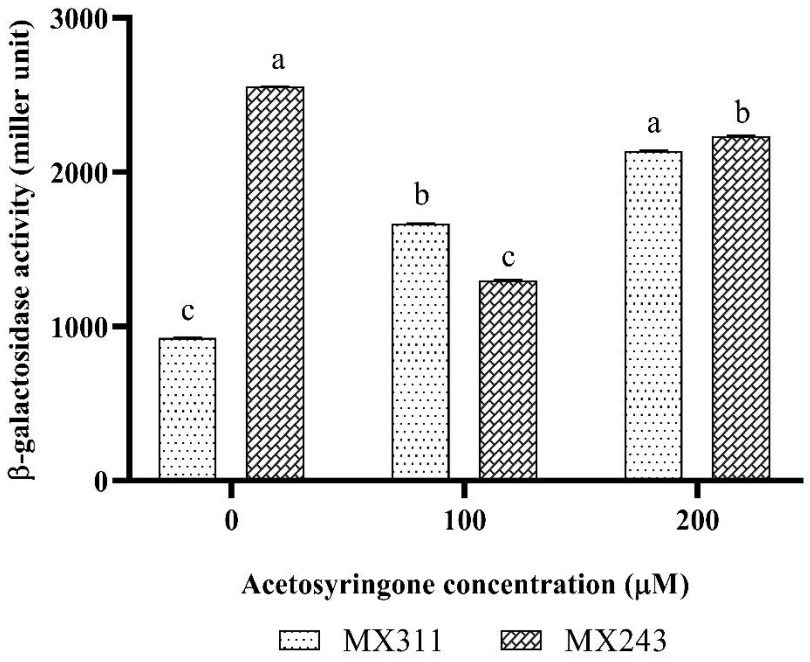

Figure 2. β-galactosidase activity in the presence of different concentrations of acetosyringone in MX311 and MX243 strains. Letters show a significant level at *P-value* of ≤ 0.01.

Table 3. Analysis of the variance of β-galactosidase activity in different concentrations of acetosyringone in MX243 strain.

Source of changes	DF	Sum of Squares	Mean Square	*P-value*
Treatment	2	2550991.162	1275495.581	< 0.0001
Error	6	38.599	6.433	

Molecular analysis and resistance to hygromycin antibiotic

Resistance test to hygromycin antibiotic

By counting the number of putative transgenic plants on the culture medium containing hygromycin, the transformation efficiency was calculated according to Lin et al. (Lin et al., 2009) method. The results showed that the plant mutants, which were inhibited in SA biosynthesis (nahG), had the highest transformation efficiency among other mutants, and the lowest transformation efficiency was related to the control ecotype (Col-0) in the absence of acetosyringone (Figure 3). The results of mean comparisons and variance analysis showed that there is a significant difference among plant mutants in the presence of acetosyringone at P-value ≤ 0.01, while there isn't a significant difference among the plant mutants in the absence of acetosyringone at P-value ≤ 0.01.

Molecular analysis by PCR

To confirmation of putative transgenic plants, the extracted DNA from each plant mutant was used as the template of PCR reaction using the specific primers of hyg. Transgenic plants were confirmed with the presence of a 450 bp fragment (Figure 4). The non-transgenic plant was a negative control for hyg. Also, the absence of *Agrobacterium* contamination was confirmed using the extracted DNA from transgenic plants as the template of PCR and the specific primers of spect. The absence of 450 bp fragment was confirmed the absence of *Agrobacterium* contamination (data not shown).

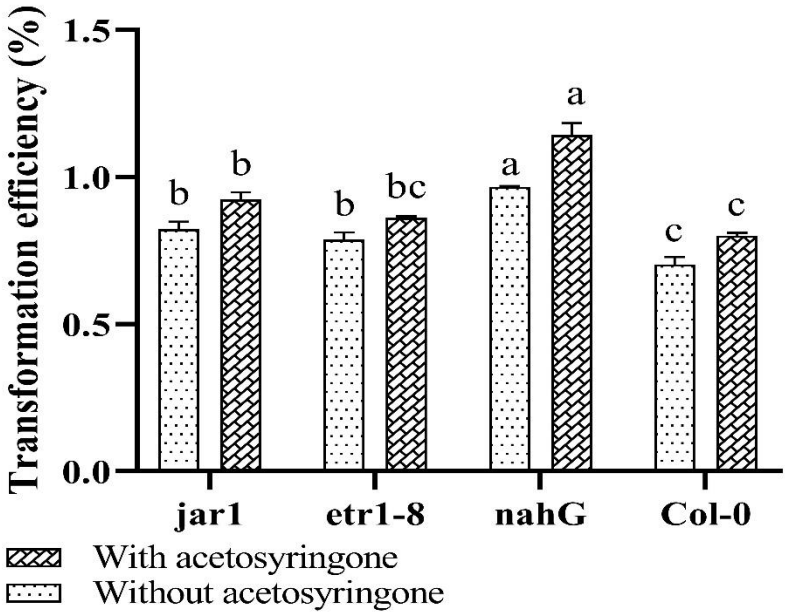

Figure 3. Comparison of transformation efficiency in mutant plants using hygromycin antibiotic resistance test. Letters show a significant level at *P-value* of ≤ 0.01.

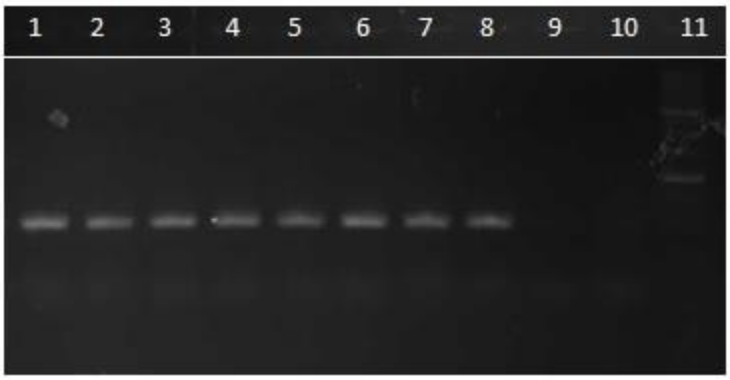

Figure 4. Confirmation of putative transgenic plants using specific primers of *hyg*. Lane 1-4: DNA from transgenic plants of Col-0, *etr1-8*, *nahG*, and *jar1* in the present of acetosyringone, respectively. Lane 5-8: DNA from transgenic plants of Col-0, *etr1-8*, *nahG*, and *jar1* in the absence of acetosyringone, respectively. Lane 9: DNA from the non-transgenic plant. Lane 10: negative control in the absence of DNA. Lane 11: 1 kb molecular ladder.

4. Discussion

The vir expression induction with phenolic compounds, such as acetosyringone, naturally is a prerequisite for transformation, and in normal conditions, a minimal medium is used for the induction of vir gene (Gelvin, 2006). Generally, the optimum temperature for the induction of vir (25 °C) is lower than the optimum temperature for *Agrobacterium* growth (28-30 °C), in which this principle was followed in this experiment. Also, increasing the expression of the *virD2* results in the transfer of the T-DNA more efficiently to the host cell and thus increases the efficiency of the transformation, which according to the results of this study, increases the concentration of acetosyringone enhanced the induction of *virD2*. On the other hand, *Vir B1-11* genes are involved in forming a communication channel between the bacterial cell and the plant cell to transmit T-DNA, resulting in the minimal expression of Vir B2 would

be sufficient for this operation. Also, the sugars like glucose in the presence of limited concentrations of acetosyringone increase the induction of vir, which mainly is used instead of sucrose in a plant growth medium (Wang, 2006).

Identification of the main hormones involved in plant defense provides an ideal model for dealing with interactions between pathogenic bacteria and plants. Plant hormones of SA, JA, and ET are essential for contributing to regulating plants' defense(Glazebrook, 2001; Thaler et al., 2004). After infection, the amount of SA increases in response to acquired systemic resistance, which results in prolonged resistance to the pathogen (Durrant and Dong, 2004). Thus, in this study, disrupting the pathway of SA biosynthesis in the mutants of *nahG*, increased their sensitivity against Agrobacterium and the transformation efficiency. In *nahG* plants, the expression of PR-genes has greatly reduced and they thus exhibit enhanced susceptibility to different pathogens (Heck et al., 2003; Dobon et al., 2013). In addition, it has been reported that the accumulation of ET decreases after infection with *Xanthomonas* compestris at *nahG* plants (O'Donnell et al., 2003). Therefore, it shows that *nahG* plants display hormonal disorders related to SA and ET, two important hormones in plant defense, and it may be a reason to increase the transformation efficiency of these plants compared to others in this study. In a study, *Agrobacterium*-mediated transformation efficiency is increased in *sid2* and *nahG* plants, which both are deficient in salicylic acid production (Rosas-Díaz et al., 2017). Interestingly, lack of salicylic acid production is thought to not affect bacterial growth, bacterial attachment to plant cells, inhibiting the expression of vir genes, and virulence (Hwang et al., 2017; Rosas-Díaz et al., 2017).

The role of JA response in resistance to some pathogens in several plants such as *Arabidopsis* (Thomma et al., 1998), tomatoes (Diaz et al., 2002) Norway spruce (Kozlowski et al., 1999) and barley (Mitchell and Walters, 1995) is reported. Mechanisms that affect the response of JA can induce pathogenesis-related genes in *Arabidopsis* (Thomma et al., 1998; Hamamouch et al., 2011). Therefore, JA has a lower role in plant defense against pathogens than SA, as they are mutually antagonistic(Li et al., 2019). Therefore, our results showed the transformation efficiency of JA-suppression mutants is lower than SA-suppression mutants. In addition, although SA and JA show a negative regulation of each other, they also sometimes have synergistic effects (Dobon et al., 2013).

ET controls the extent and development of plant disease symptoms after inoculation with pathogenic bacteria or fungi (Bent et al., 1992; Zhou et al., 2019). Therefore, it can be assumed that the *etr1-8* mutants should have high efficiency in transformation with *Agrobacterium* compared to the control. Further, studies indicate that the growth of *Agrobacterium* was not affected by ET, but the presence of ET at the start of the infection with *Agrobacterium* showed significant inhibitory activity in the vir expression (Nonaka et al., 2008). Such inhibitory effects can be eliminated through supplementation with acetosyringone, as a vir inducer (Nonaka et al., 2008). These observations indicate that ET affects the interaction between *Agrobacterium* and plants due to its inhibitory effects on bacterial pathogenicity. Therefore, in plant defense against pathogens, SA plays a more important role than JA and ET (Anand et al., 2008). As expected, in the SA mutants, the efficiency of transformation is higher than other mutants due to the lack of SA production.

Up to today, the low production yield of recombinant protein in plants has become a challenge and this encouraged us to look up other aspects of increasing gene transfer efficiency to increase the expression and yield of recombinant proteins. In this study, one of these aspects was found by determining the role of defense hormones in the efficiency of *Agrobacterium*-mediated gene transfer. According to the results, when in the absence of salicylate, the efficiency of gene transfer increases, these results can be very promising for the production of a recombinant protein. Hence, by increasing the efficiency of gene transfer, the amount of recombinant protein produced may increase (Zhao et al., 2017).

Conclusion

In this study, the effect of acetosyringone on increasing the transformation efficiency, three old-day culture was investigated using YEP, AB-sucrose, and induction media based as described by

Gelvin (Gelvin, 2006).The results showed that the presence of acetosyringone increased the efficiency of the transformation. Also, given the role of plant hormones of SA, JA and ET in plant defense against pathogens and disrupting their pathway of biosynthesis causes plant sensitivity to the pathogen. According to the findings of this study, in mutants with impaired regulation of biosynthesis of each of the above hormones exhibited higher transformation efficiency than the control plants. The use of mutants offers an effective approach to enhance the efficiency of *Agrobacterium*-mediated gene transfer because direct application of the hormone may activate some other defense mechanisms potentially impede gene transformation s by *Agrobacterium*. On the other hand, the presence of inducer compounds like acetosyringone has been shown to significantly increase the efficiency of the transformation when compared conditions lacking these inducers. Finally, since SA-suppression mutants have the highest transformation efficiency compared to others, it can be concluded that SA may play a greater role in plant defense against *Agrobacterium* than two other hormones. Therefore, the use of mutants lacking the ability to biosynthesize SA is a promising strategyto increase the production of recombinant proteins in the plant.

Supplementary Materials:

No supplementary material is available for this article.

Author Contributions:

Conceptualization, M.S.T. and M.M.S.; methodology, M.S.T.; software, M.S.T. and M.M.S.; validation, M.S.T. and R.S.; formal analysis, M.S.T. and R.S.; investigation, M.M.S and R.S.; resources, M.M.S.; data curation, M.S.T., M.M.S. and R.S.; writing—original draft preparation, M.S.T.; writing—review and editing, M.S.T., M.M.S. and R.S.; visualization, M.S.T., M.M.S. and R.S.; supervision, M.M.S. and R.S..; project administration, M.M.S.; funding acquisition, M.M.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest:

There is no conflicts of interest.

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مقاله پژوهشی

کارایی انتقال ژن به‌واسطه آگروباکتریوم در موتانت‌های آرابیدوپسیس تالیانا

محمد صادق تقی‌زاده*[۱]، محمد مهدی سوهانی[۲]، رضا شیرزادیان‌خرم‌آباد[۲]

[۱] پژوهشکده زیست فناوری، دانشکده کشاورزی، دانشگاه شیراز، شیراز، ایران
[۲] گروه بیوتکنولوژی کشاورزی، دانشکده کشاورزی، دانشگاه گیلان، رشت، ایران

چکیده: تعداد کمی از سیگنال‌های با وزن مولکولی پایین، از جمله جاسمونیک اسید (JA)، سالیسیلیک اسید (SA) و اتیلن (ET)، بیان ژن‌های مرتبط با دفاع را در گیاهان تنظیم می‌کنند. این سیگنال‌ها جهت مهار فعالسازی ژن‌های دفاعی گیاه در برابر مهاجم‌ها فعالیت می‌کنند و می‌توانند مسیرهای سیگنالینگ دفاعی گیاه را دستکاری کنند. در این مطالعه، تاثیر استوسیرینگون بر روی القا ژن‌های بیماری‌زا در آگروباکتریوم تومه‌فاشینس سویه‌های A348 (MX311) و A348 (MX243) در سه سطح مختلف ۰، ۱۰۰ و ۲۰۰ میکرومولار مورد ارزیابی قرار گرفت. غلظتی که بالاترین القا ژن‌های بیماری‌زا را داشت متعاقبا برای القا ژن‌های بیماری‌زا آگروباکتریوم تومه‌فاشینس EHA105 جهت ترانسفورماسیون موتانت‌های آرابیدوپسیس استفاده شد. نتایج نشان داد که بیان virD2 در غلظت ۲۰۰ میکرومولار استوسیرینگون به حداکثر مقدار خود رسید در حالی که بیشترین بیان virB2 در غلظت ۰ میکرومولار بود. علاوه بر این، آزمایشات ترانسفورماسیون نشان داد که موتانت‌های سالیسیلیک اسید (nahG) بالاترین کارایی ترانسفورماسیون را نشان دادند در حالی که گیاهان کنترل (Col-0) پایین‌ترین کارایی را داشتند. بنابراین، کارایی انتقال ژن در موتانت‌های سرکوب کننده SA در مقایسه با سایر هورمون‌ها نقش قابل توجه‌تر SA در دفاع گیاه در برابر پاتوژن‌ها را پیشنهاد می‌دهد. افزایش کارایی انتقال ژن در این موتانت‌ها می‌تواند راهی برای افزایش بیان و تولید پروتئین‌های نوترکیب در مقایسه با نوع وحشی باز نماید.

کلمات کلیدی: استوسیرینگون، In planta، جهش‌یافته اسید سالیسیلیک، جهش‌یافته اسید جاسمونیک، جهش‌یافته اتیلن، PCR.

ویراستار علمی
دکتر غلامرضا احمدیان،
پژوهشگاه ملی مهندسی ژنتیک و زیست‌فناوری

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دکتر محمدصادق تقی‌زاده
ms.taghizadeh@shirazu.ac.ir

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Genetic evaluation of some safflower genotypes under salinity stress

Ayman Ebrahim Badran*, Rasha M. Khalil, Ehab Mostafa

Genetic Resources Department, Desert Research Center, Cairo, Egypt.

Original paper

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Isfahan University of Technology, Iran

Abstract: In the context of the increasing demand for cultivation of marginal lands affected by salinity, it became necessary to assess the most important crop traits that can be relied upon as well as to identify genotypes that have the potential to withstand adverse environmental conditions. In this study, ten genotypes of safflower were evaluated under two salinity levels during two successive agricultural seasons. According to correlation and path coefficient analysis and positive direct effect of studied traits, 100 seed weight followed by number of capitulum per plant strongly related to seed yield. Also, the results confirmed based on tolerance indices to salt stress, that line 6 was followed by local cultivar recorded the highest values as an indicator of tolerance. Protein analyses were conducted to identify protein markers associated with salinity tolerance for selection of promising lines tolerant to salt stress. This investigation revealed substantial polymorphism in protein markers that could be useful tools to assist breeders in the selecting and breeding of safflower lines tolerant to salinity stress.

Keywords: safflower, salinity, path coefficient, tolerance indices, protein marker.

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Correspondence
Dr. Ayman Ebrahim Badran
dr.ayman_badran@yahoo.com

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Introduction

The cultivated area of arable land is decreasing as a result of unsustainable agriculture, climate change, and soil degradation (Lobell et al., 2008). Both salinity and drought are the most dangerous abiotic stresses that threaten the productivity of diverse crops around the world (Guo et al., 2014). Based on these environmental changes, plant breeders work to develop and identify the genotypes that are more capable of tolerating salinity and water stress (Janmohammadi et al., 2008). In general, it is possible to benefit from the use of the lands affected by the high salt content by cultivating crops that tolerate salinity. However, it is necessary to assess the variation within and between genotypes available for tolerance to salinity. For example, this has been done in different types of crops such as cotton, *Brassica napus* (Akram and Jamil, 2007) and Safflower (Siddiqi et al., 2007).

Safflower (*Carthamus tinctorius* L.) is a diploid (2n = 24) and belonging to oilseed crops because its seeds contain an oil percentage, ranging from 32 to 40% (Weiss, 1983). Due to the tolerance of safflower to salt stress compared to commonly cultivated oilseed crops, it is usually grown in dry and semi-arid regions where soil salinity is one of the most important areas a threat to agriculture (Kaya, 2009). Simple correlation analysis is not sufficient to give an accurate indication of the relative importance of the crop, so path analysis is an important tool to assist plant breeders in dividing the correlation coefficient into direct and indirect contributions, which gives a better insight about character and its relationship with the yield. Biochemical markers like proteins are considered a powerful tool for assessment of crop germplasm, characterization of genetic diversity and determination of differentiation among crop plants (Jan et al., 2017; Ibrar et al., 2018). Assessments of genetic diversity based on biochemical markers of different crops have been studied by different researchers. Akbar et al. (2012) used SDSPAGE technique to study genetic diversity of *Sesamum indicum* for total seed protein and got satisfactory results. Likewise, Zada et al. (2013) utilized this method to study protein based genetic diversity in *Brassica carinata* (Ethiopian mustard) germplasm.

This study aims to select the best traits in the selection process for the yield as well as the selection of the best genotypes to tolerate salt stress through some indications represented by path coefficient analysis, tolerance indices, and genetic expression of the tested genotypes of safflower.

Materials and Methods

Experimental conditions

The field experiments were carried out during the two growing seasons 2017/2018 and 2018/2019 in the experimental farm of Ras-Sudr Research Station Desert Research Center, South Saini Governorate. Nine lines of safflower (*C. tinctorius* L.) were obtained from International Center for Bio-saLine agriculture (ICBA), and a local cultivar obtained from the Agriculture Research Center (ARC), Egypt (Table 1). The genotypes were grown under two levels of salinity (75 and 150 mM NaCl). The experiments were carried out in a split-plot design where salinity levels in main plots and safflower genotypes in sub-plots with three replicates. The t-test and least significant difference (LSD) test were used to compare salinity levels and genotype means at 0.05% level respectively. The soil sample and irrigation water were collected from the experiment wells and analyzed as shown in Table 2. With regard to meteorology, the lowest temperature (9.46 C°) was recorded during January, while the highest temperature was recorded during November and April (29.23 and 30.80 C°, respectively), and the relative humidity percentage ranged from 66.87 in December to 88.01% in April. On the other hand, the lowest wind speed was in January (19.58), while the highest wind speed was recorded in March (35.52 km/h). The average rainfall was monitored during the two growing seasons and ranged from 0 to 5 mm.

Measurements

At harvesting, a number of measurements were recorded as follow: plant height (cm), capitulum length (cm), capitulum diameter (cm), No. of seeds/capitulum, No. of capitulum / plant, 100 seed / plant and seed yield/plant (g).

Parameters

Correlation and path coefficient analysis using SPSS/AMOS program. Tolerance indices were used as follow:

Salinity tolerance index (STI); STI = (Yn) × (Ys)/(Ýn)² (Fernandez 1992).

Table 1. The describe of tested genotype based on pedigree, institution and origin.

Name	Pedigree	Institution offered	Origin
Line 1	(PI 167390)	ICBA	UAE
Line 2	(PI 173885)	ICBA	UAE
Line 3	(PI 181866)	ICBA	UAE
Line 4	(PI 199892)	ICBA	UAE
Line 5	(PI 239707)	ICBA	UAE
Line 6	(PI 248836)	ICBA	UAE
Line 7	(PI 250714)	ICBA	UAE
Line 8	(PI 250840)	ICBA	UAE
Line 9	(PI 251267)	ICBA	UAE
Giza 1	(local cultivar)	Agricultural Research Center	Egypt

UAE: United Arab Emirates, International Center for BiosaLine Agriculture: ICBA

Table 2. Chemical analysis of soil and irrigation water in two wells.

Well	Irrigation water analysis									
	pH	Salt mM	Cations (meq/L)				Anions (meq/L)			
			Ca^{++}	Mg^{++}	Na^{+}	K^{+}	CO_3^{--}	HCO_3^{-}	Cl^{-}	SO_4^{--}
First well	7.81	75	10.8	7.15	53.6	0.35	-	5.30	39.1	26.8
Second well	7.66	150	19.3	13.8	105.1	0.90	-	7.50	93.1	38.7
Soil	Soil analysis									
	7.77	106	4.6	3.2	88.3	0.67	-	4.95	65.7	26.1

Yield injury % (YI); YI = (Yn-Ys)/Yn × 100 (Blum et al., 1983). Superiority measure (SM); SM = Ys/Yn (Lin & Binns 1988). Relative performance (RP); RP = (Ys/Yn)/ (Ýs/Ýn) (Abo-Elwafa and Bakheit, 1999). Where, Yn = yield of genotype under normal conditions; Ys = yield of genotype under salinity stress conditions; Ýn = mean yield of all genotypes under normal conditions; Ýs = mean yield of all genotypes under salinity stress.

Protein pattern was studied using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). It was washed till such time till the color of background disappeared and bands could be observed. The protein bands were scored as 0 for absence or 1 for presence for polymorphism. The similarity matrix was calculated using NTSYS-pc version 2.02e and this similarity matrix was used in cluster analysis using an unweighted pair group method with arithmetic averages (UPGMA) to obtain a dendrogram.

Results

t test in Table 3 showed that there were significant differences between the two salinity levels under this study, as well as clear differences between the genotypes tested under the conditions of the experiment for all the traits that were monitored depending on the values of least significant difference (L.S.D) at 0.05% level.

Correlation and path coefficient analysis

The estimation of correlation coefficient between the studied traits and the yield is one of the important implications in plant breeding for direct or indirect selection. Therefore, it is of great value in guiding the procedures of the breeding program. The results of the correlation between the traits in Table 4 showed that both the weight of 100 seeds and the number of capitulum / plant had the highest correlation with the seed yield / plant (0.667 and 0.521, respectively).

Path coefficient analysis can be used to divide the correlation into direct and indirect effects of the traits used in contributing to the seed yield as a

useful criterion in selection through the different traits of the safflower plant. Path coefficient analysis was performed by looking at the traits associated with the seed yield as shown in Table 5. The results indicated that the highest positive direct effect for the seed yield was recorded by the weight of 100 seeds (0.537). While, both number of seeds/capitulum and plant height were negative direct effects however these traits can be benefited from it using indirect positive selection, passing through of the weight of 100 seed followed by number of capitulum / plant and as showed in Table 5.

Tolerance indices

The evaluation of salinity tolerance index in Table 6 confirms that the genotypes can be divided into two groups based on the general mean (0.631), the first group is tolerant to salinity, and genotype 10 ranked the first place in as tolerant to stress followed by genotype 9 while, the second sensitive group, genotype 4 occupies the highest place of sensitivity to salinity (0.351).

Table 3. Means of some yield-related traits for two salinity levels and ten genotypes across two seasons.

	Plant height (cm)	Capitulum length (cm)	Capitulum Diameter (cm)	No. of seeds/ capitulum	No. of capitulum / plant	100 seed weight (g)	Seed yield/ plant (g)
Salinity levels							
Low salinity	73.017	2.266	2.436	31.858	12.602	5.406	46.490
High salinity	63.683	1.907	2.070	21.850	5.686	4.584	34.836
t-test (0.5)	*	*	*	*	*	*	*
Genotypes							
Line 1	64.17	2.158	2.200	16.63	6.822	4.861	38.51
Line 2	75.08	2.417	2.283	24.08	8.220	4.812	40.48
Line 3	67.92	2.158	2.117	27.75	8.211	4.269	38.55
Line 4	67.08	1.858	2.217	29.67	9.872	5.185	47.02
Line 5	61.08	2.425	2.092	25.50	8.687	5.264	42.51
Line 6	74.08	2.000	2.100	19.75	8.669	5.418	37.66
Line 7	70.08	2.183	2.483	36.67	9.142	4.666	33.08
Line 8	67.17	2.117	2.258	20.67	7.807	4.683	44.29
Line 9	67.17	2.117	2.483	41.00	7.955	5.114	31.68
Giza 1	69.67	1.908	2.300	26.83	10.87	5.679	52.86

Table 4. Correlation coefficient among studied traits under overall treatments and seasons.

Trait	Plant height	Capitulum length	Capitulum Diameter	No. of seeds/ capitulum	No. of capitulum / plant	100 seed weight
Capitulum length	0.360*					
Capitulum diameter	0.400*	0.476*				
No. of seeds/capitulum	0.185$^{n.s}$	0.288*	0.632*			
No. of capitulum/ plant	0.466*	0.488*	0.603*	0.493*		
100 seed weight	0.243*	0.335*	0.390*	0.311*	0.532*	
Seed yield/ plant	0.241*	0.424*	0.323*	0.136$^{n.s}$	0.521*	0.667*

^{ns}: not significant; * significant at the 0.05 level of probability, respectively.

Table 5. Partition of correlation coefficients into direct and indirect effect for mean seed yield under.

Trait	Direct effect	Indirect effect						Correlation with
		Plant height	Capitulum length	Capitulum diameter	No. of seeds/ capitulum	No. of capitulum / plant	100 seed weight	
Plant height	-0.047		0.067	0.011	-0.041	0.121	0.130	0.241
Capitulum length	0.186	-0.017		0.013	-0.063	0.126	0.180	0.424
Capitulum diameter	0.027	-0.019	0.088		-0.139	0.156	0.209	0.323
No. of seeds/capitulum	-0.220	-0.009	0.054	0.017		0.128	0.167	0.136
No. of capitulum / plant	0.259	-0.022	0.091	0.016	-0.108		0.285	0.521
100 seed weight	0.537	-0.011	0.062	0.010	-0.068	0.138		0.667

With regard to the percentage of yield injury, we note that there is a large variation between the tested genotypes and the least percentage of yield injury was in the genotype 6 followed by genotype 10 while both genotypes 1 and 7 recorded the highest of yield injury (73.92, and 51.62 %, respectively) under the high salinity level compared to low salinity level. It is evident from the data in Table 6 for the tolerance indices that the results of superiority measure (SM) and relative performance (RP) are completely in harmony with the results of the yield injury (%) for all genotypes tested under conditions of salt stress.

SDS-PAGE for Protein electrophoresis

A total of 45 protein bands were detected as a result of SDS-PAGE technique. On the basis of these proteins ten safflower Lines (Table 7) Figure 1 were evaluated under salinity conditions. Out of these, 5 protein bands were consistently present throughout the genotypes and considered as monomorphic while 40 (89%) protein subunits reflected variations and were polymorphic. Size of the protein bands ranged from 14.787 to 450.871kDa. Lines with minimum proteins bands were Giza 1, Line8, Line5 and Line1. They have 6, 9, 10, and 10 protein bands, respectively. Some lines showed maximum protein bands, such as line9 and line2 showed 14 and 18 bands, respectively. Genotypes of line10 and line9, regarded as salt tolerant, exhibited a high number of specific bands with different molecular weights, which appeared in the salt treatment. These bands could be considered as a positive biochemical marker for salt tolerance.

Table 6. Salinity tolerance indices of tested genotypes grown under low salinity and high salinity levels for seed yield during two seasons.

Genotype	Salinity tolerance index (STI)			Yield injury (YI) (%)			Superiority measure (SM)			Relative performance (RP)		
	2107	2018	Mean	2107	2018	Mean	2107	2018	Mean	2107	2018	Mean
Line 1	0.638	0.610	0.624	72.77	75.06	73.92	0.272	0.249	0.261	0.421	0.398	0.410
Line 2	0.628	0.579	0.604	30.18	29.65	29.92	0.698	0.704	0.701	1.078	1.122	1.100
Line 3	0.603	0.557	0.580	37.01	44.29	40.65	0.630	0.557	0.594	0.973	0.888	0.931
Line 4	0.880	0.909	0.895	42.70	43.14	42.92	0.573	0.569	0.571	0.885	0.907	0.896
Line 5	0.676	0.607	0.642	27.41	21.21	24.31	0.726	0.788	0.757	1.121	1.256	1.189
Line 6	0.480	0.463	0.472	04.76	9.20	06.98	0.952	0.908	0.930	1.471	1.448	1.460
Line 7	0.456	0.458	0.457	50.39	52.85	51.62	0.496	0.471	0.484	0.766	0.752	0.759
Line 8	0.695	0.704	0.700	16.34	19.87	18.11	0.837	0.801	0.819	1.292	1.278	1.285
Line 9	0.369	0.332	0.351	19.62	21.36	20.49	0.804	0.786	0.795	1.241	1.254	1.248
Giza 1	0.985	0.992	0.989	15.72	18.12	16.92	0.843	0.819	0.831	1.302	1.305	1.304
Mean	0.641	0.621	0.631	31.69	33.47	32.58	0.683	0.665	0.674	1.055	1.061	1.058

Table 7. SDS-PAGE pattern of total proteins of ten safflower Lines.

Parameters	Number
Monomorphic bands	5
Polymorphic (without Unique)	8
Unique bands	32
Polymorphic (with Unique)	40
Total number of bands	45
Polymorphism (%)	89%

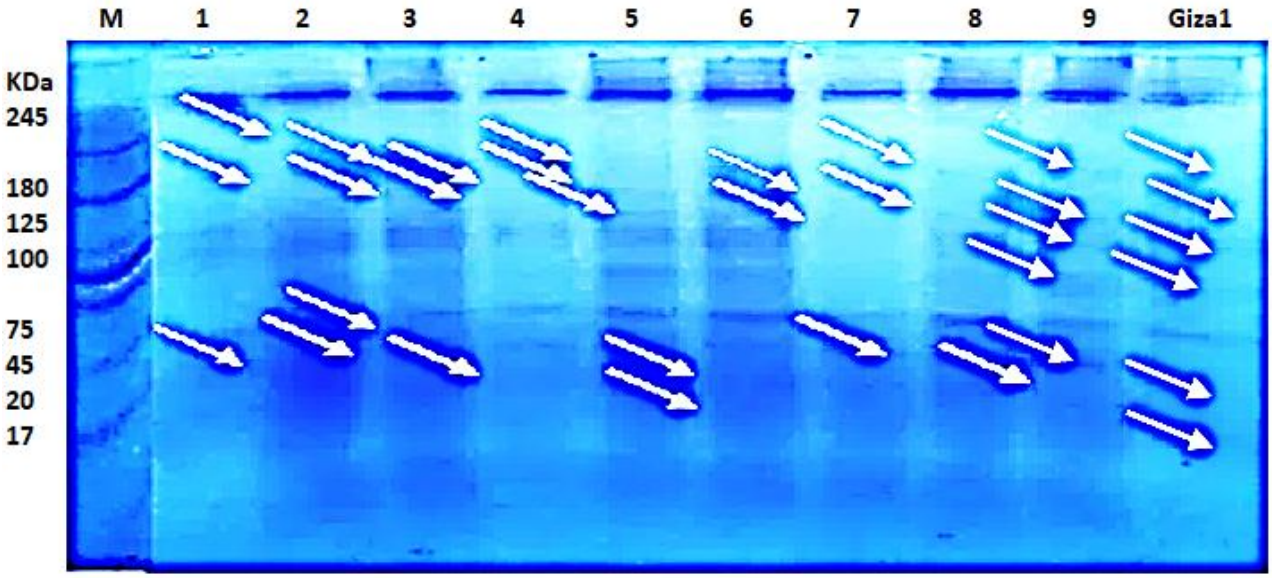

Figure 1. Protein profile of ten safflower lines using SDS-PAGE technique.

A dendrogram, representing the relationships among the genotypes of ten lines of safflower, indicated that lines 10 and line 9 were the most diverse among the studied lines with tolerance to salinity (Figure 2). The dendrogram separated the ten lines of safflower into two main clusters, first cluster included line1 by itself and the second cluster included the remain lines.

Discussion

The context of increasing the demand for the cultivation of salt-affected lands, it has become necessary to focus on the most important plant traits that can be relied upon for selection for high yield, directly or indirectly, depending on the correlation and path coefficient analysis. It is also necessary to determine the genotypes of crop that have the ability to resist harsh environmental changes, depending on tolerance indices and gene expression.

Correlation and path coefficient analysis

Dewey and Lu (1959) asserted that simple correlation coefficient analysis to elucidate the relationship between a single variable and grain yield may not provide a complete understanding of the selection process. Also, Badran et al., 2015 stated that reliance on the direct selection of the grain yield is very difficult because it depends on a large number of genetic factors, so it is necessary to rely on the components of the yield because these variables are controlled through limit genetic factors and this facilitates direct or indirect selection of grain yield through it.

By discussing the results in Table 5, it is evident that the direct effect of 100 seed weight followed by the number of capitulum / plant contributes to the seed yield / plant is greater than the direct effect of other traits indicating that direct selection through them may be good traits for improving the seed yield / plant. It is also possible to depend

on the other traits through indirect selection using 100 seed weight / plant to improve the seed yield/ plant. The previous discussion is in harmony with those by Singh and Chaudhary (1977), who reported that if the correlation between a causal trait and the effect is almost equal to its direct effect, then correlation explains that a direct selection through this trait well be effective.

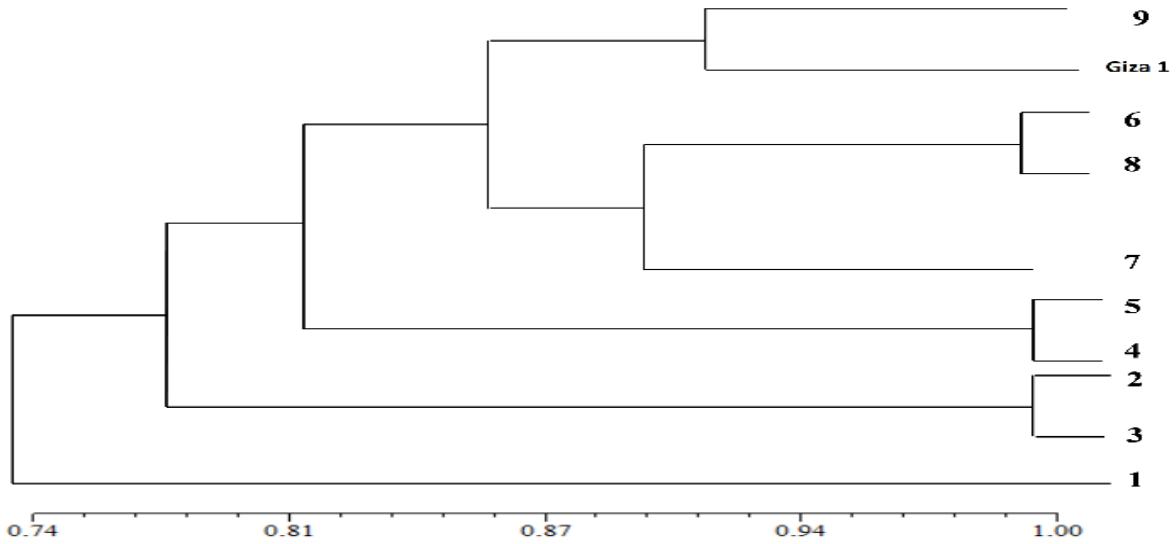

Figure 2. Dendrogram representing the relationships among ten Lines of safflower based on similarity matrix derived from protein profile.

Tolerance and sensitive indices

This investigation separated the tested safflower genotypes to tolerant and sensitive under low salinity and high salinity levels based on seed yield. Assessment based on the salinity tolerance index (STI and means productivity (MP) are a reliable predictor for determining high-yielding genotype of crops under high stress conditions compared to low stress or normal conditions according to previous report (Pourdad, 2008) on safflower. While, Ramirez-Vallejo and Kelly (1998) on common bean and Guttieri et al. (2001) on wheat relied on the stress sensitivity index (SSI) to identify high yield genotypes to improve resistance to abiotic stresses in crops. Therefore, I believe that it is better to rely on more than one of the environmental stress tolerance indices for classifying genotypes tested under stress conditions compared to optimal conditions or less stress. These previous findings are in harmony with Fernandez (1992) and Badran (2015) who reported this, genotypes are classified into four groups according to their performance under non-stress and water stress conditions.

Protein analysis under salinity stress

Plants have different types of proteins on the basis of which they are diverse from each other and this could be a source of identifying diversity at protein level using SDS-PAGE (Shah, 1999). SDS-PAGE technique is mostly thought as a reliable mean and has proved to be an important way of revealing the differences and relations between and within taxa (Iqbal et al., 2005). Few investigations showed diversity in the germplasm of safflower (Ramirez-Vallejo and Kelly, 1998; Mukhlesur and Hirata, 2004; Ali et al., 2007). Žilić et al. (2010) also reported a very similar proteins profiling pattern in sunflower protein bands. One possible explanation for appearance of some proteins under salt stress is that the gene(s) responsible for certain proteins had been completely enhanced as a result of stress (Harb et al., 2010). Our results are parallel with those of several authors, who used some total protein pattern electrophoresis variations to evaluate some cultivars under different salt stress to be used as biochemical genetic markers for early evaluation (Sayed and Gabr, 2013; Mahgoub et al.,

2016). The present results indicates that SDS-PAGE technique succeed in presenting a means for studying genotypes discrimination based on genetic variation in proteins as useful methods to detect specific markers for salinity tolerance, in the genotypes of safflower under salinity conditions.

Conclusion

Our research work will provide valuable information on safflower breeding and genetic variation to researchers and genetic resources managers working with this crop. Considering these facts, correlation and path coefficient analysis as well as tolerance indices of ten lines of safflower, demonstrate a strong correlation with biochemical proteins markers indicating significantt polymorphism. This offers a promising avenue for identifying specific markers related to salinity tolerance and constructing adaptive strategies for salt stress. Identification of tolerance indices and specific positive markers could greatly benefit breeding programs by enabling the prediction of the most tolerant cultivars.

Supplementary Materials:

No supplementary material is available for this article.

Author Contributions:

AEB wrote the initial draft of this manuscript and conceived the original ideas. AEB and RMK designed the research and AEB and EM conducted the experiment and analysis. AEB, RMK and EM contributed to idea refinement, writing, and revising of the manuscript. AEB supervised the work and AEB and RMK revised and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest:

The authors have no conflict of interest.

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مقاله پژوهشی

ارزیابی ژنتیکی برخی از ژنوتیپ‌های گلرنگ تحت تنش شوری

ایمن ابراهیم بدران *، راشا م. خلیل، ایهاب مصطفی

گروه ذخایر ژنتیکی، مرکز تحقیقات بیابان، قاهره، مصر.

چکیده: با توجه به افزایش تقاضا برای کشت در اراضی حاشیه‌ای تحت تأثیر شوری، ضروری است تا مهمترین صفات زراعی موثر و همچنین ژنوتیپ‌هایی با پتانسیل مقاومت بالا در برابر شرایط نامساعد محیطی شناسایی شوند. در این مطالعه ۱۰ ژنوتیپ گلرنگ در دو سطح شوری در دو فصل زراعی مورد ارزیابی قرار گرفتند. با توجه به تجزیه همبستگی و ضریب مسیر و اثرات مستقیم مثبت صفات مورد مطالعه، وزن صد دانه و بعد از آن تعداد طبق در بوته، با عملکرد دانه در ارتباط بودند. همچنین نتایج شاخص‌های تحمل به تنش شوری نشان داد که لاین شماره ۶ و بعد از آن رقم محلی بالاترین شاخص‌های تحمل را به خود اختصاص داده بودند. ارزیابی پروتئین‌ها برای شناسایی نشانگرهای پروتئینی مرتبط با تحمل به شوری جهت انتخاب لاین‌های امید بخش مقاوم به تنش شوری نیز انجام شد. چند شکلی بالایی در نشانگرهای پروتئینی مشاهده شد که می‌توان از آن به عنوان ابزار مولکولی کارا جهت انتخاب لاین‌های متحمل به تنش شوری در گلرنگ استفاده نمود. از نتایج این تحقیق می‌توان در برنامه‌های اصلاحی شناسایی و انتخاب ژنوتیپ‌های متحمل به تنش شوری بهره‌مند شد.

کلمات کلیدی: گلرنگ، شوری، ضریب مسیر، شاخص‌های تحمل، نشانگر پروتئینی.

ویراستار علمی
دکتر احمد ارزانی،
دانشگاه صنعتی اصفهان، ایران

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نویسنده مسئول
دکتر ایمن ابراهیم بدران
dr.ayman_badran@yahoo.com

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Expression of miR9863a in responding to drought stress in some wheat and *Aegilops* species

Morteza Barati[1], Mohamad Reza Azimi*[1], Mohamad Reza Naghavi[2], Ehsan Mohseni Fard[1]

[1.] Department of Production Engineering & Plant Genetics, University of Zanjan, Iran.
[2.] Agricultral & Natural Resourses Collage, University of Tehran, Karaj, Iran.

Original paper

Abstract: MicroRNAs are small RNAs known for their essential roles in regulating both biotic and abiotic stress responses. Drought stress poses a significant challenge to wheat productivity in Iran. The present study evaluated the expression of miR9863a and its target genes in wheat, as well as three *Aegilops* species under drought stress. The results revealed differential expression of miR9863a in the shoots of the studied plants under drought stress conditions. Specifically its expression was increased in *Ae. tauschii* and *Ae. crassa*, while decreasing in *Ae. cylindrica*. The observed differential expression could be explained by the inherent nature of miRNA as a mediator molecule in various biological processes. Analyzing the expression pattern of miR9863a and its target genes in *Ae. tauschii* suggests that the effect of miR9863a in response to drought stress may be attributed to *PLGG1*, impacting glycerate/glycolate transfers and *SAR1A*, influencing trafficking of transcription factors from the endoplasmic reticulum to the nucleus. In addition to complementing previous studies on the role of miR9863 in countering plant diseases, the results presented here illustrate how this miRNA assists the abiotic stress-response mechanism in plants, particularly in the context of drought stress.

Keywords: crassa, cylindrica, *PLGG1*, qRT-PCR, *SAR1A*, tauschii.

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Correspondence

Dr. Mohamad Reza Azimi
azimi@znu.ac.ir

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Introduction

Drought stands as the most limiting factor for crop productivity worldwide. Approximately 88% of the total arable area of Iran is located in arid and semi-arid regions (Vaghefi et al., 2019). Therefore, conducting research on the mechanisms of drought-stress tolerance is crucial. Plants employ various mechanisms to tolerate drought, often utilizing osmotic adjustment, desiccation tolerance, and antioxidant capacity (Zhang, 2007). Numerous genes contribute to drought tolerance, initially involving protein kinase-encoding genes and transcription factors (TFs) (Hadiarto and Tran, 2011). Beyond their defense function, drought-related genes can be categorized into three groups; transcriptional regulation, post-transcriptional RNA and osmoprotectant metabolism or molecular chaperons (Yang et al., 2010).

The plastidial glycolate/glycerate translocator 1 (*PLGG1*) gene is responsible for encoding a chloroplast protein that facilitates transports glycolate and glycerate- two of compounds that are required for the photorespiratory cycle to function appropriately (Pick et al., 2013). The *PLGG1* is involved in other major ABA responses, including ABA-inhibited seed germination, ABA-mediated stomatal movement, and drought tolerance (Dong et al., 2018). Secretion Associated Ras Related GTPase 1A (*SAR1A*) is a member of a large family of small GTP-binding proteins. These proteins exist in many organisms and regulate various processes such as signal transduction, cell duplication, cytoskeletal formation, and intracellular membrane exchange. Also, they are involved in the hydrolysis of GTP and transfers from the endoplasmic reticulum to the Golgi apparatus (Vernoud et al., 2003). Endomembrane trafficking is a fundamental cellular process in all eukaryotic cells, the regulatory mechanisms of which have been studied extensively (Wang et al., 2020). A higher amount of GTP-binding proteins can mediate plasma membrane trafficking, affect the plasma membrane proteome, and increase drought tolerance (Ambastha et al., 2021).

miRNAs are a class of short and non-coding RNAs. They are about 20 to 25 nucleotides in length and regulate gene expression in plants and animals. Generally, miRNAs are transcribed from DNA (pri-miRNAs) and processed into stem-loop regions (pr-miRNAs) and mature miRNAs (Ha and Kim, 2014). In plants, miRNAs are extremely complementary to target mRNAs and usually direct cleavage by silencing specific complexes (Bartel, 2004). miRNAs have important roles in gene regulation at different points of the plant life cycle and are involved in several biological processes such as plant development (Curaba et al., 2012), stress response (Budak et al., 2015) and pathogen resistance. The miR9863 family has been identified in *Aegilops tauschii* (Jia et al., 2013). Recently, miR9863a-3p (MI0031654) was reported in *Triticum aestivum* (Naghavi and Fard, 2021). The function of miR9863a-3p is not well-known but is reportedly associated with disease resistance (Liu et al., 2014; Tang and Chu, 2017) and abiotic stress tolerance (Ferdous et al., 2017).

Bread wheat (*T. aestivum*) is one of the most widely grown crops (Mochida and Shinozaki, 2013) and an important food source in the world (Kurtoglu et al., 2014). *T. aestivum* has a complex genome of 6x ploidy (AABBDD) (Marcussen et al., 2014). *Ae. tauschii* is the diploid (2n = DD) progenitor of the D genome of hexaploid wheat and is an important genetic resource in the wheat family (Luo et al., 2017).

Ae. cylindrica is a tetraploid species (CCDD) that shows meiotic pairing with the D genome chromosomes of hexaploid wheat (Dubcovsky and Dvorak, 1994). *Ae. crassa* is tetra- (DDMM) and hexaploid (DDDDMM) and is mainly distributed in Afghanistan, Iraq, Iran, Palestine, Syria, and Turkestan (Eig, 1929). Previous studies have demonstrated the most crucial pathways associated with drought-stress response in *Aegilops* species (Noori et al., 2015; Falaknaz et al., 2019; Zhao et al., 2020). As a wild relative of wheat, *Aegilops* species are regarded as an important source of drought-tolerance genes that can be transferred to wheat cultivars.

To date, only a limited number of studies have investigated the association between miR9863a and abiotic stress response, particularly in the context of drought. The present study aimed to identify and compare the expression of miR9863a-3p and its target genes (*PLGG1* and *SAR1A*) in response to drought stress across wheat and three species of *Aegilops*. The results may help illustrate the roles of miR9863a-3p in

drought-stress response in the *Triticeae* tribe.

Materials and Methods

Plant samples and drought stress treatments

An Iranian bread wheat cultivar (Qods) and three species of *Aegilops*, i.e. *Ae. tauschii* (Acc. No.: TN012189), *Ae. cylindrica* (Acc. No.: KC50180) and *Ae. crassa* (Acc. No.: TN01300), were obtained from the Seed and Plant Improvement Institute of Iran and the National Plant Gene Bank of Iran, respectively. The seed surface was sterilized by rinsing under tap water and then soaking them in 3% NaOCl for 10 minutes. After washing with distilled water, the seeds were transferred to petri dishes and incubated at 4°C for 72 h. Germinated seeds were planted in pots (12 cm in diameter) containing a mixture of loess and sand (5:1 v:v) for 1 month. After two weeks of normal irrigation, drought stress was applied by limiting the irrigation, maintaining 20% of field capacity for 14 days, followed by 3 days without water supply, (Supplementary Figure 1) (Zivcak et al., 2013). The plants were visually assessed for the effects of drought. After 30 days, shoot samples of drought-stressed and control plants were immediately frozen in liquid N_2 and stored at -80ºC.

RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from the shoots of plants at the 4-leaf stage by the CTAB method, with three repetitions (Barman et al., 2017). RNA quantity and quality were measured by NanoDrop 2000 (Thermo Co.). After the treatment of total extracted RNA with *DNase* I, the cDNA was synthesized using the YT4500 kit (YektaTajhizAzma Co.). Oligo dT primers were used for the first strand cDNA synthesis according to the manufacturer's instructions. According to the confidence sequence of *miR9863a* (Acc. No. MIMAT0037105) (tgagaaggtagatcataatagc), stem-loop RT, forward and universal primers were designed according to previously published protocols (Chen et al., 2005; Varkonyi-Gasic et al., 2007). 18s rRNA (Acc. No. AY059462.1) was used as an internal wheat control gene in each reaction (Paolacci et al., 2009; Safarzadeh et al., 2014). qRT-PCR was performed on cDNA samples of wheat and the three species of *Aegilops*, with three biological and two technical repeats using QIAGEN's Rotor-Gene Q and Ampliqon's RealQ Plus 2X Master Mix Green (Table 1).

Prediction of miRNA drought-related target genes

The psRNATarget software was used for predicting the target genes. The miR9863 sequence was used as a query against the *T. aestivum* cDNA library (Ensemble Plants, release 43) with default parameters, including a maximum expectation value of 3, a target accessibility of 25, a mismatch for translational inhibition between 9 and 11 nucleotides, and a maximum mismatch at the complementary site which was equal to or less than 4 without any gaps (Dai and Zhao, 2011). Relevant target genes for drought stress were selected from the results. Primers for qRT-PCR reactions were designed from the deduced sequence, corresponding to the two drought-related target genes (*PLGG1*and *SAR1A*) using the OLIGO 7 primer analysis software (Table 2).

Statistical analysis

Shoot samples from the control and drought-stress-treated plants were collected randomly. The relative level of gene expression for each gene of interest was calculated using the $2^{-\Delta\Delta}$ method described by the Relative Expression Software Tool (REST) (Pfaffl et al., 2002).

Table 1. List of the primers used in cDNA synthesis and qRT-PCR.

Primers	Sequences (5′–> 3′)
miR-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTATT
miR-F	ATGCCGTGAGAAGGTAGATCA
Universal	GTGCAGGGTCCGAGGT
18s rRNA F	GATGAGCCAAGTGCATATCTCG
18s rRNA R	CTTGTCCGCTAAGTAGGTTGC

RT: stem-loop Reverse Transcription primer, F: Forward primer, R: reverse primer

Table 2. List of primer pairs used in the qRT-PCR target genes.

Gene Accession number	Gene name	Primers	Sequences (5′–> 3′)	Product length
XM_020343245.3	*PLGG1*	Forward Reverse	TCTCCGTGCTCCTCGTC AACCAGCCACCGAATGTG	202
XM_020327846.3	*SAR1A*	Forward Reverse	CTCCTCATCCTTGATGCTCTG TCACCTTACACACGAACACTC	138

Table 3. List of identified function for miR9863 reported in *Triticeae* and monocots.

Plant	Function	Reference
Aegilops	relative to MPV (mid-parent value)	Li et al. (2014)
wheat	predicted to target R gene analogs	Wei et al. (2009)
barley	confirmed to regulate distinct Mla alleles	Liu et al. (2014)
monocots	regulated the NB-LRR genes with CC domains (Disease resistance-associated)	Zhang et al. (2019)
barley	associated with fungal pathogen	Tang and Chu (2017)
barley	regulation under drought stress and their role in mediating expression of target genes for abiotic stress response	Ferdous et al. (2017)

Results and Discussion

Identification, confirmation and expression analysis of miR9863a

According to previous indications, the miR9863 family is mainly expressed in wheat and barley and might be *Triticeae*-specific (Table 3).

The miR9863 family was previously known in *Ae. tauschii*. The family has two members, ata-MIR9863a (Acc. No.: MI0031654) and ata-MIR9863b (Acc. No.: MI0031721). They are located on chromosome 1. (Jia et al., 2013). In particular, *miR9863a* was recently identified and confirmed to exist in *T. aestivum* (Naghavi and Fard, 2021). Despite the difference in the precursor sequence, the sequence of mature *miR9863a* was identical in both plants (Figure 1).

Identification of miR9863a target genes

The target genes were identified for miR9863a using the psRNATarget. Among the 208 genes, *PLGG1* and *SAR1A* were selected and regarded as effective in inducing the drought-stress response based on Gene Ontology (GO) analysis via the UniProt database. The alignment of target gene sequences with miR9863a is shown in Figure 2.

The expectation score to search targets having a poor complementary matching with miRNA or long gaps in alignment were 3 and 4 in the case of *PLGG1* and *SAR1A*, respectively. Unpaired energy (UPE) is defined as the energy required to open secondary structures around the target site on mRNA. The UPE was -1 in both target genes. A lower amount of energy represented a higher likelihood of being an effective target site because the secondary structures may prevent small RNAs and target sites from coming into contact with each other. From a total of 22 co-aligned sequences between *PLGG1* and miR9863a, there were 4 mismatches and two wobble base pairs (G–U). Also, there were 2 mismatches and 3 wobble base pairs between the *SAR1A* and miR9863a alignment. The protein coding region of *PLGG1* gene is located between 117 to 1712 nucleotides, therefore the target site in *PLGG1* gene is located in the coding region, while in *SAR1A* gene, the protein coding region is located from 209 to 790 nucleotide, and as a result, the target site is located in the 3'UTR region.

Quantitative analysis of miR9863a expression

The expression patterns of the *miR9863a* in the drought-stress treatment demonstrated an up-

regulation in *Ae. tauschii* and *Ae. Crassa*, whereas a down-regulation was observed in T. aestivum and *Ae. cylindrica* (Figure 3).

Different expressions of miR9863a were observed in plants under the drought stress treatment. For example, miR9863a was 6-fold up-regulated in Ae. tauschii during drought stress, whereas the miRNA in wheat was 2-fold down-regulated. There are many indications in the available literature that miRNAs have different expression patterns in response to abiotic stresses such as drought (Mehri et al., 1970; Anderberg and Walker-Simmons, 1992; Bakhshi et al., 2013; Hua et al., 2019). In response to drought stress, plants activate their drought-response mechanisms, involving morphological and structural changes, expressions of drought-resistant genes, synthesis of hormones, and osmotic regulatory substances that assist in alleviating drought stress (Yang et al., 2021).

It seems that miR9863a is an intermediate molecule which modulates other protein-coding genes and takes different functions among plants when drought stress is applied. Also, differential expression may due to the fact that overexpression in one tissue may be masked by down regulation in another one (Mone et al., 2018).

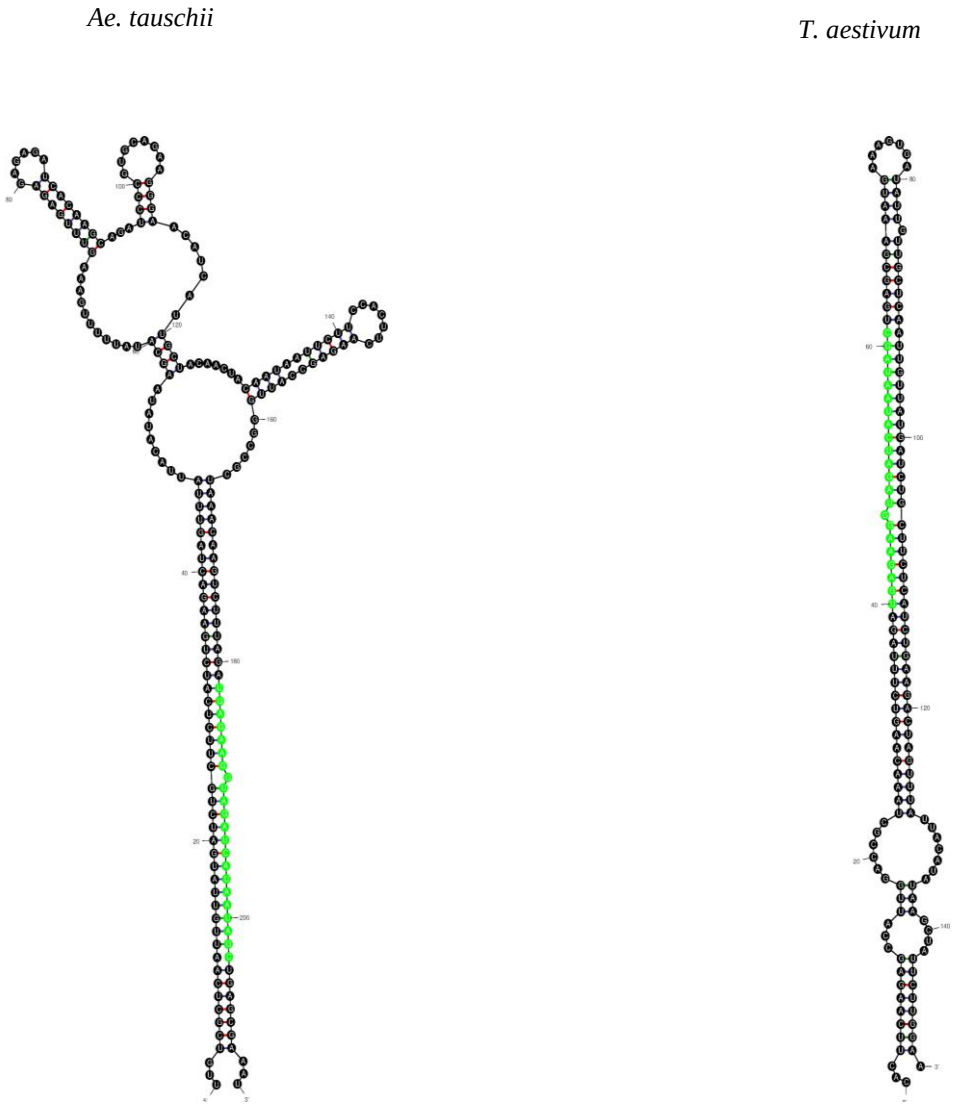

Figure 1. miR9863a structure in *Ae. tauschii* and *T. aestivum.* Identical mature sequences with a length of 22 nucleotides are marked with green spots.

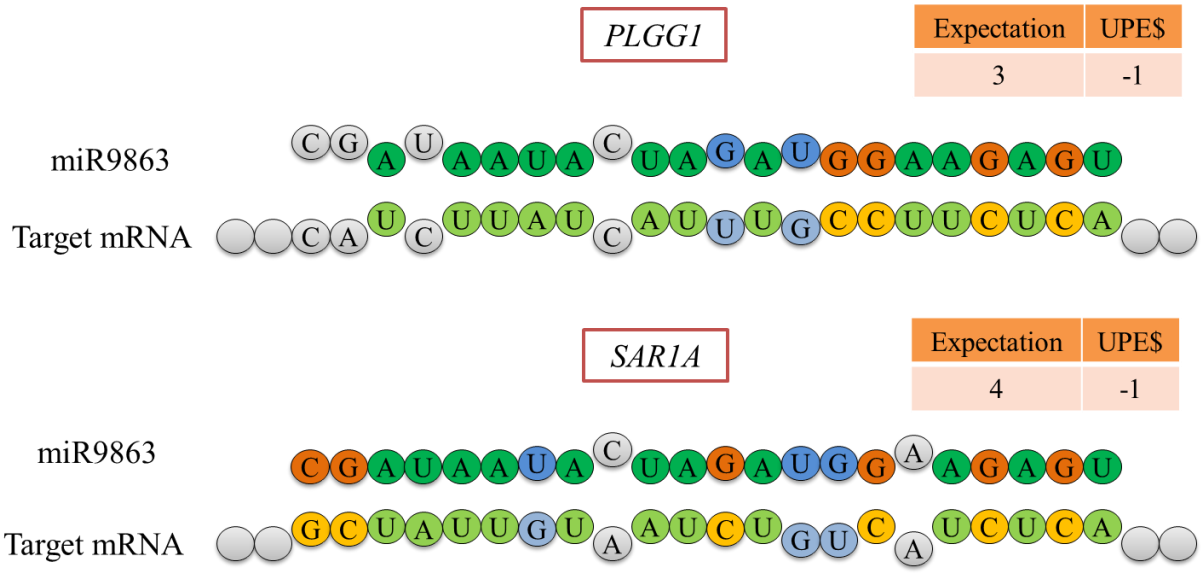

Figure 2. Alignment of *PLGG1* nd *SAR1A* with miR9863a sequences by psRNATarget software.

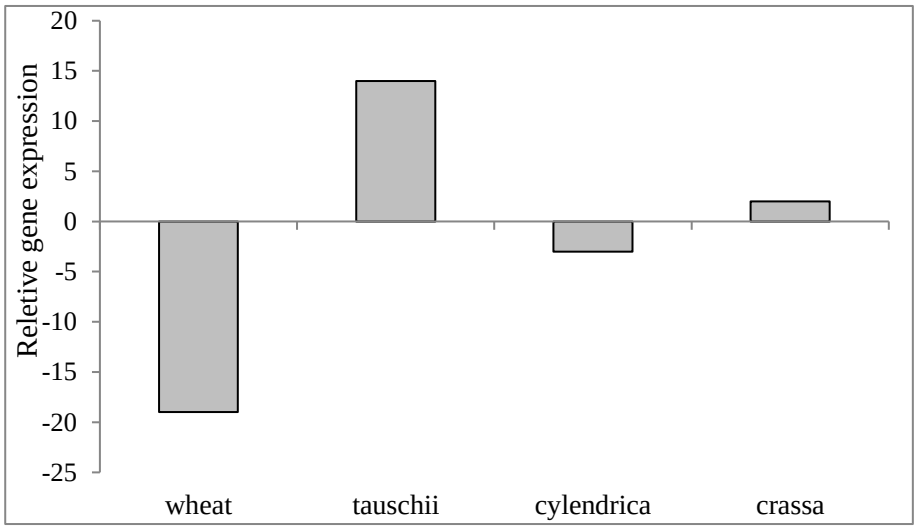

Figure 3. The relative expression rate of miR9863a in drought-stress treatments on *T. aestivum*, *Ae. tauschii*, *Ae. cylendrica* and *Ae. crassa*.

miR9863a expression and regulation of the glycolate/glycerate translocator in photorespiration

To evaluate miR9863 target genes, the relative expression of drought-related genes in wheat and the three *Aegilops* species was evaluated using qRT-PCR. The *PLGG1* was not amplified or had a small level of expression in wheat, *Ae. cylindrica*, and *Ae. crassa* under normal and drought stress conditions. In the *Ae. tauschii*, despite a high level of *PLGG1* expression in the normal condition, its expression was significantly suppressed under drought stress. Referring to the increase in miR9863a expression and the decrease in *PLGG1* expression in *Ae. tauschii* during drought stress, it seems that drought stress may cause an increase in miR9863a expression which suppressed its target gene.

The *PLGG1* gene encodes a chloroplastic protein named plastidial glycolate/glycerate translocator 1 which is required for photorespiration. During plant growth and development, drought stress is one of the most severe environmental restrictions that lead to the accumulation of ABA. Under drought stress, ABA regulates the transpiration

rate via guard cells by triggering stomatal closure and inhibiting stomatal opening. When the plant closes its stomata, carbon dioxide cannot enter the plant. As a result of the stomatal closure, while oxygen is produced, it is entrapped inside the plant. Thus, the amount of oxygen increases and photorespiration takes place. Photorespiration is a metabolic repair pathway that is activated in oxygenic photosynthetic organisms to degrade a toxic product of oxygen fixation. Within the metabolic pathway, energy is consumed and carbon dioxide is released (Dong et al., 2018). PR is frequently regarded as a wasteful process because the loss of CO2 and the occurrence of energy consumption can lower the photosynthetic efficiency and the plant yield. To mitigate the yield penalty, scientists have suggested different strategies, one of which is the synthetic bypass of PR to circumvent mitochondrial glycine decarboxylation, thereby avoiding the release of CO_2 (Figure 4). In this way, a large portion of the carbon in glycolate is released as CO_2 in close proximity to Rubisco within the chloroplast, thereby enhancing CO_2 fixation (Kuhnert et al., 2021).

In the present study, considering the miR9863a and *PLGG1* expression pattern in *Ae. tauschii*, it seemed that the drought stress induced the expression of miR9863a which then down-regulated the *PLGG1* expression. A decrease in *PLGG1* expression causes synthetic bypass. Also, *PLGG1* is involved in the drought-response via ABA (Pfaffl et al., 2002). Possibly, miR9863a is indirectly implicated in the drought-response by inhibiting the *PLGG1* gene.

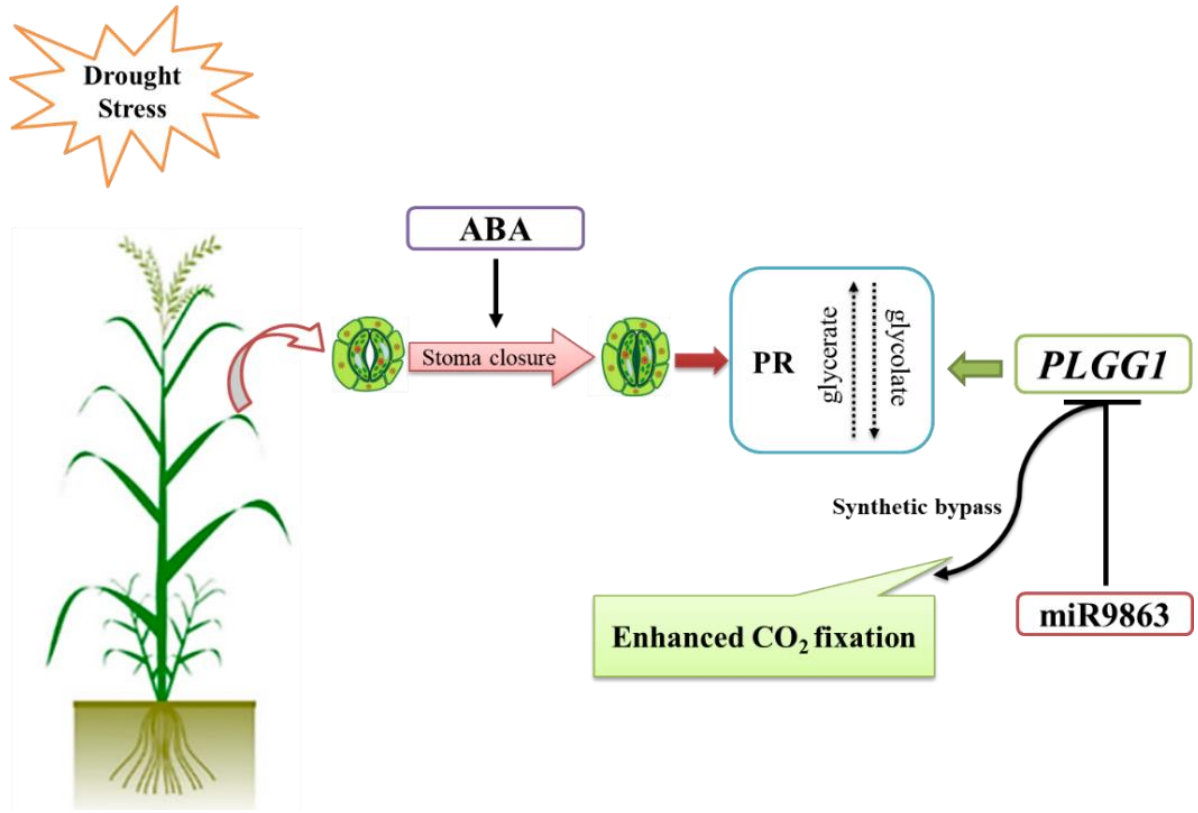

Figure 4. Significance of the *PLGG1* in anticipated flux distribution between native and synthetic route in PR metabolism. Silencing the plastidial glycolate/glycerate transporter allows synthetic bypass. Image adapted from (Nawkar et al., 2018; Wang et al., 2020).

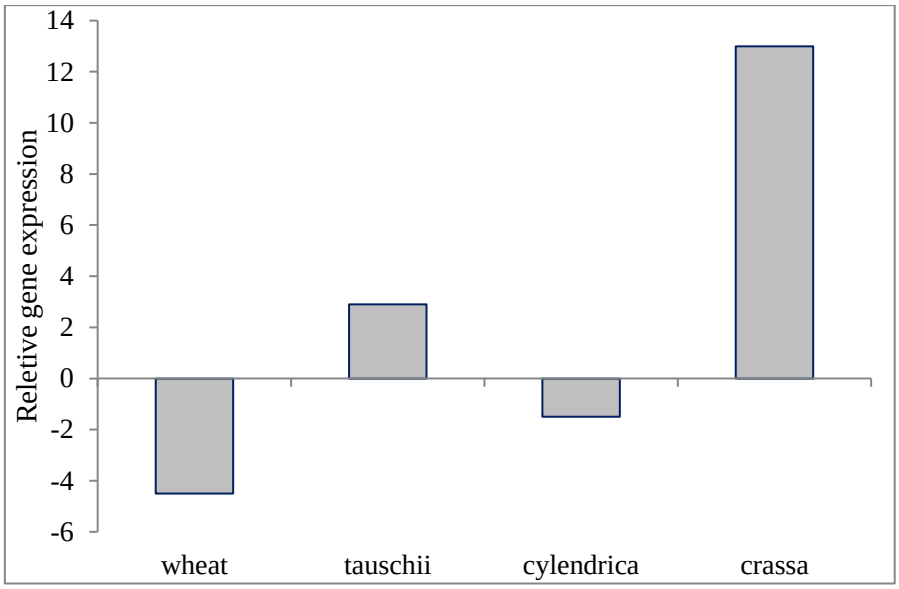

Figure 5. The relative expression rate of *SAR1A* target gene in response to the drought-stress treatment on *T. aestivum*, *Ae. tauschii*, *Ae. cylendrica* and *Ae. crassa*.

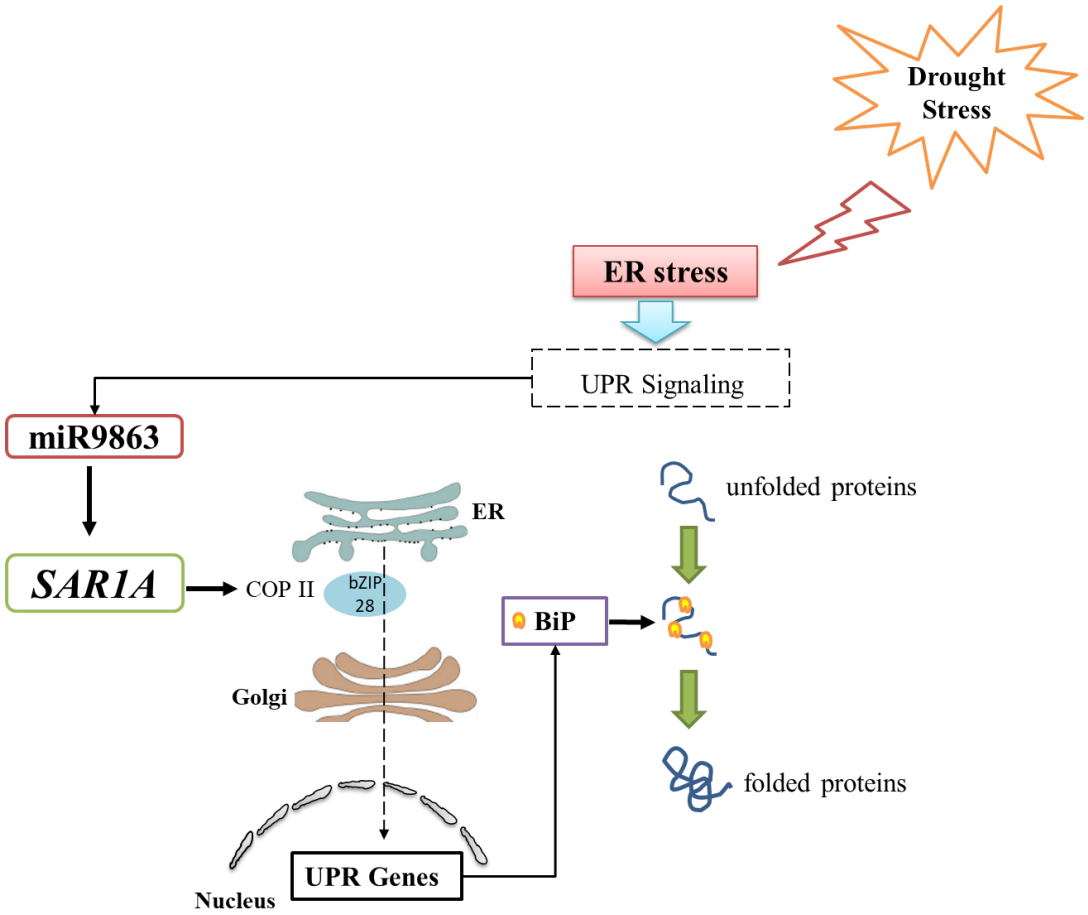

Figure 6. The role of miR9863a and its target gene in response to drought stress. Endoplasmic reticulum stress due to drought stress activates the Unfolded Protein Response (UPR) signaling and the *SAR1A* gene. The translocation of bZIP28 by COP II vesicles from ER to the nucleus induced responsive genes and the encoded BiP protein assisted in folding the proteins despite drought stress.Image adapted from (Nawkar et al., 2018; Wang et al., 2020).

miR9863a and regulation of intracellular protein trafficking

The results of qRT-PCR analysis for the relative expression of the *SAR1A* gene in the plants are shown in Figure 5. As it's depicted in Figure 5, *SAR1A* was up-regulated in *Ae. tauschii* and *Ae. crassa*, but was down-regulated in *T. aestivum* and *Ae. cylindrica* during drought stress. The expression pattern of *SAR1A* was similar to the expression pattern of miR9863a in all of the plants under drought stress. In other words, there was a direct relationship between miR9863a and the *SAR1A* target gene expression. The *SAR1A* gene encodes GTP-binding proteins that are involved in transport from the endoplasmic reticulum (ER) to the Golgi apparatus. Many small GTPases are involved in the regulation of intracellular protein trafficking (Wang et al., 2020). The SAR family of GTPases are essential for the formation of transport vesicles while having functions in the endoplasmic reticulum and assist in the ER-to-Golgi transport in plant cells. Endomembrane trafficking is a fundamental cellular process in all eukaryotic cells and signaling pathways. It mainly transducts signals during stress (Nawkar et al., 2018). *SAR1A* defines a specific colony of COPII vesicles which moderate the export of ER proteins to the Golgi apparatus. Drought stress leads to ER stress which disturbs protein folding and causes a set of signaling pathways, collectively called the Unfolded Protein Response (UPR). In the UPR mechanism, COPII vesicles by SAR1Aassist the export of bZIP28 as a transcription factor to the Golgi apparatus. The cytosolic part of bZIP28 can be translocated into the nucleus to activate the expression of ER stress-responsive genes (Wang et al., 2020). Binding proteins (BiP) encoded relevant responsive genes that assisted in the proper folding of the unfolded proteins (Nawkar et al., 2018) (Figure 6).

Therefore, considering the miR9863a and *SAR1A* expression pattern in *Ae. tauschii*, it seems that an increase in miRNA expression under drought stress signaled the increase in *SAR1A* expression and consequently produced small proteins that became bound to GTP, thereby becoming involved in endomembrane trafficking and the drought-stress response pathway.

Conclusion

While previous publications primarily focused on describing the involvement of miR9863 in biotic-stress response (Wei et al., 2009; Li et al., 2014; Liu et al., 2014; Zhang et al., 2019), the current research offers an analysis of miR9863a under drought stress conditions. In this research, we report that miR9863a exhibits distinct expression patterns in response to drought stress. Differential expression may result from the fact that overexpression in one tissue may be counteracted by down regulation in another (Mone et al., 2018). It will be the focus of future attempt to investigate expression variations tissue by tissue. In addition, when considering miR9863a and its target genes in *Ae. tauschii*, it is likely that miR9863a probably exerts its effects through a relationship between this miRNA and its target genes, regulating photorespiration and endomembrane trafficking in the drought-stress response pathway. As miR9863a is considered a newly explored miRNA, these results can illuminate the regulatory role of miR9863a in abiotic-stress response, particularly in the context of drought stress.

Supplementary Materials

The Supplementary Material for this article can be found online at: https://www.jpmb-gabit.ir/article_704821.html.

Supplementary Figure 1. Stages of cultivation and the application of drought stress on the plants.

Author Contributions

Investigation and writing, M.B.; supervision, M.R.A and M.R.N; project administration, E.M.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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مقاله پژوهشی

بیان miR9863a در پاسخ به تنش خشکی در برخی از گونه‌های گندم و *Aegilops*

مرتضی براتی[۱]، محمدرضا عظیمی*[۱]، محمدرضا نقوی[۲]، احسان محسنی فرد[۱]

[۱].گروه مهندسی تولید و ژنتیک گیاهی، دانشگاه زنجان، ایران.
[۲].دانشکده کشاورزی و منابع طبیعی، دانشگاه تهران، کرج، ایران.

چکیده: MicroRNAها، RNAهای کوچک حدود ۲۲ نوکلئوتیدی هستند که نقش مهمی در اثرات تنظیمی پاسخ به تنش‌های زیستی و غیرزیستی دارند. تنش خشکی یکی از مهمترین چالش‌های تولید گندم در ایران است. در مطالعه حاضر، بیان *miR9863a* و ژن‌های هدف در گندم و سه گونه اجیلوپس تحت تنش خشکی مورد بررسی قرار گرفت. نتایج نشان داد که *بیان miR9863a* در اندام هوایی گیاهان مورد مطالعه در شرایط تنش خشکی متفاوت بود به طوری که بیان آن در اجیلوپس تائوشی و کراسا افزایشی و در اجیلوپس سیلندریکا کاهشی بود. این اختلاف بیان در گیاهان تحت تنش می‌تواند به دلیل ماهیت miRNA به عنوان مولکولی واسطه‌ای در فرآیندهای مختلف زیستی باشد. با در نظر گرفتن الگوی بیان *miR9863a* و ژن‌های هدف در اجیلوپس تائوشی، می‌توان تا حدودی به نقش *miR9863a* در پاسخ به تنش خشکی از طریق ژن هدف *PLGG1* به واسطه نقش در نقل و انتقالات گلیسرات/گلیکولات و ژن هدف *SAR1A* به دلیل نقش داشتن در نقل و انتقالات عناصر تنظیمی از شبکه اندوپلاسمی به هسته متصور شد. همچنین با توجه به اینکه مطالعات گذشته بر ارتباط *miR9863a* با بیماری‌های گیاهی متمرکز بوده، نتایج مطالعه حاضر می‌تواند بر نقش این miRNA در مکانیسم‌های پاسخ به تنش‌های غیر زیستی با تأکید بر تنش خشکی کمک نماید.

کلمات کلیدی: تائوشی، کراسا، سیلندریکا، *PLGG1*، qRT-PCR.

ویراستار علمی
دکتر سیدحمیدرضا هاشمی‌پطرودی، پژوهشکده ژنتیک و زیست‌فناوری کشاورزی طبرستان، دانشگاه علوم کشاورزی و منابع طبیعی ساری

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نویسنده مسئول
دکتر محمدرضا عظیمی
azimi@znu.ac.ir

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Comparison of comet assay parameter patterns between self-pollinated and cross-pollinated diploid *Medicago* species, their resulting tetraploids and cultivated cultivars

Elham Ansari[1], Mahmood Khosrowshahli *[1], Alireza Etminan[2], Ali Ashraf Jafari [3]

[1.] Department of Biotechnology and Plant breeding, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2.] Department of Plant breeding and Biotechnology, Kermanshah Branch, Islamic Azad University, Kermanshah, Iran.
[3.] Research Institute of Forests and Rangelands, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.

Original paper

Abstract: Three parameters of comet assay (tail length, tail intensity, and tail moment) were used to compare the autotetraploids produced from three populations of cross pollinated *Medicago sativa* spp. *caerulea* and five diploid self-pollinated species from *Medicago* genus. These specimens were subjected to three concentrations of colchicine (0.1, 0.5 and 1%) alongside five alfalfa cultivated cultivars. In the concentrations of 0.1% and 0.5%, a similar level of increase and pattern was observed in the two autotetraploids medic categories. Increasing of concentration from 0.1% to 0.5% resulted in a more pronounced augmentation of comet parameters. Autotetraploids induced by the two mentioned colchicine concentrations exhibited increases in the value and pattern of the three comet parameters compared to the cultivated cultivars and the two categories medics in diploid level. At the concentration of 1% colchicine, only two annual medic species produced tetraploids showing very pronounced augmentation of comet parameters in comparison with 0.1% and 0.5% of colchicine. Changes in patterns and values of the three parameters in induced tetraploids compared to cultivated alfalfa and the two categories medics in diploid level, demonstrate differential effects of damages of colchicines from one concentration to another. A new variability in each concentration change will be expected.

Keywords: comet analysis, autopolyploidy, alfalfa, colchicine, medic species.

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Correspondence
Dr. Mahmood Khosrowshahli
mkhosrowchahli@yahoo.com

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Introduction

The pattern of DNA migration from a single cell on the electrophoresis gel, resembling a comet, has been designated as the "comet assay" by Ostling O. and Johansson K. J. (Ostling and Johanson, 1984). Currently, the comet assay (i.e., the single-cell gel electrophoresis assay) is being used as a quick, popular, sensitive, and relatively cost-effective technique to detect transient genetic damages and their repair at the DNA level in eukaryotic cells. This technique has been used to detect the genotoxic effects of toxic agents (Forchhammer et al., 2012; Amaeze et al., 2015). The procedure was initially developed by Ostling and Johansson (Ostling and Johanson, 1984) at the level of the individual mammalian cells after irradiation, and it was later modified by Singh and coworkers (Singh et al., 1988). The use of higher plants for the detection of cytotoxicity and genotoxicity and monitoring its mutagenesis was recommended by the Royal Swedish Academy of Sciences (1973), the Council of the Environmental Mutagen Society (1975), and the World Health Organization (1985). In 1989, the assay was approved by the Swedish Board of the Protection of the Environment (Fiskesjó, 1993; Lanier et al., 2015). Before using plants in comet assay, some cytotoxic and genotoxic agents were used in plant breeding programs as physical or chemical mutagens. For many years, cytogenetic techniques have been used as a practical tool for detecting genetic and chromosomal damages and changes caused by genotoxic agents in crops, independent of the change being useful or useless. Such studies have focused on different species, including *Allium cepa* (onion) (Levan, 1938; Firbas and Amon, 2014) tobacco and eggplants (Kostoff and Kendall, 1931), or the gene mutation test in *Hordeum vulgare* (barley) (Gustafsson, 1940; 1947). Nowadays, the comet assay plays an important role in detecting the cytotoxic and genotoxic effects of mutagen agents. Duplication of the plant genomes, or polyploidy induction, which leads to changes in some horticultural, pharmaceutical, and agronomic traits in the plant species, is based on the application of different drugs, such as colchicine, oryzalin, trifluralin, and so on. Colchicine is the main chromosome doubling agent, which is extracted from the bulbs and seeds of the autumn crocus (*Colchicum autumnal*). It prevents the polymerization of tubulin, preventing spindle formation; hence, chromosomal segregation cannot occur in the dividing cells (Ade and RAI, 2010; Kumar and Rani, 2013; Manzoor et al., 2019). Taking into account the importance of polyploidy in plant improvement and the existence of two main categories of crops (i.e., self-pollinated and cross-pollinated crops), and endeavor to obtain more and healthier food and the importance of *Medicago* species in livestock feeding, it was important to study the behavior of two different categories of medic species versus using colchicine as a chromosome doubling agent.

Therefore, on the one hand, considering the importance of polyploidy in improving plants and on the other hand, as a factor in increasing the number of chromosomes, it is important to investigate the effects of colchicine as a genotoxic agent in these two categories of products. Due to the simplicity and sensitivity of the comet assay, it will be possible to demonstrate the genotoxic effects of colchicine in self-pollinating and cross-pollinating species of *Medicago*, where these two categories of products have differences in plant breeding methods.

Materials and Methods

The design of the study

The current study was carried out in the Genetic Laboratory of the Ahvaz Branch of the Islamic Azad University, Ahvaz, Iran. Three populations of *Medicago sativa* ssp. *caerulea* (i.e., Karaj1, Karaj2, and Tehran /IRAN as perennial diploid *Medicago*); five species of annual diploid *Medicago* (i.e., *M. radiata, M. lupulina, M. rigidula, M. truncatula, and M. turbinate*); four native alfalfa cultivars (i.e., Bamy, Hamadany, Bagdady, and ghareyonjeh); and one exotic cultivar (i.e., Ranger) were used in this study (Table 1). The polyploidy induction was already performed on the aforementioned diploid *Medicago* species by application of three concentrations of colchicine (0.1, 0.5, and 1%) in the auxiliary buds of the one node cuttings in our previous experiments (Ansari et al., 2021; Ansari et al., 2022).

The Comet assay

The seventh to twelfth leaves of the 12 to 18 cm cuttings of five annual diploid species, three populations of one perennial diploid species, eight tetraploid populations obtained from the induction of the diploid species, and five cutivated alfalfa cultivars were used for the comet

assay. The plant cell nuclei were isolated from the leaf tissues, and the alkaline single cell gel electrophoresis (SCGE) assay, developed for leaf tissues by Gichner and Plewa (1998) was used. The different steps of the comet assay for identifying the genotoxic and cytotoxic effects of three concentrations of colchicine in the diploid species, the tetraploids obtained from the diploids, and the cultivated tetraploid cultivars were followed. These steps are discussed in the following.

Step 1: Nuclei isolation from plants tissue

All operations were carried out under yellow or faint light. Leaves were removed from each species in three plant categories (annual diploid and perennial diploid species, induced tetraploids and cultivated tetraploids), and they were then placed in 60-mm petri dishes. Then, 500 ml of cold modified Sorensen buffer (50 mM of sodium phosphate at pH 6.8, 0.1 mM of ethylene diamine tetra acetic acid (EDTA), and 0.5% dimethyl sulfoxide (DMSO) was spread on the leaves that were kept on ice. Using a sharp razor blade, leaves were sliced to form fringes over most of the leaves' surface. The petri dishes were inclined in a way that the buffer was collected on the side, and the leaf fringes were immersed in the buffer and gently stirred five times. The fringes were spread over the bottom of the plate and rinsed with Sorensen buffer for several times using a cut plastic pipette tip. The plate was kept tilted in the ice so that the nuclei would be collected in the buffer.

Step 2: Slide preparation

The objective of the slide preparation step was to obtain a uniform gel to ensure easily viewable comets, a better attachment of the gel to the slides, and non-shedding. Regular microscopic slides were dipped into a solution of 1% normal melting point agarose (NMA) prepared with water at 50°C. The bottom of the slides was wiped to remove the agarose, then they were placed horizontally on a level surface and dried overnight at room temperature. Prepared slides were kept dry in slide boxes until use.

Step 3: Single Cell Gel Electrophoresis

Slides that were previously coated and dried with NMA were marked (labeling, numbering, and scoring).

The surface of each slide was supplemented with 30 µl of the nuclear suspension, then 60 µl of 0.75% low melting agarose (LMA), prepared with PBS, was added to each slide at 37 °C to bring the final concentration of LMA in the mixture to 0.5%. Using gentle and repeated pipetting with a disposable and cut micropipette tip, the cell nuclei and LMA were mixed, and then cover slips were placed on the mixtures.

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Table 1. Name and characteristics of plant material.

Medic characteristics	Species	Cultivars	Population
Annual, Diploid medics species/ Self-pollinated	*M. radiata*,	-	1
	M.rigidula,	-	1
	M. truncatula,	-	1
	M. turbinata,	-	1
	M. lupulina	-	1
Perennial, Diploid Medic species/ Cross-ollinated	*M. sativa* ssp. *caerulea*		3
Cultivated cultivars of alfalfa (Tetraploid)/ Cross pollinated	*M. sativa* L.	Bamy,	1
		Hamadany,	1
		Bagdady,	1
		Ghareyonjeh	1
		Ranger	1

The slides were placed on an ice surface for at least 5 minutes, after which the cover slips were removed, and a final layer of 90 µl of 0.5% LMA was placed on each slide. Cover slips were placed on the LMA, and the slides were maintained at 48°C for 5 min. Then, the cover slips were removed, and all the SCGE slides were immersed in a lysis solution composed of 2.5 M of NaCl, 1% sodium sarcosinate, 100 mM of Na_2 EDTA, and 10 mM of Tris (pH = 10) with 1% Triton X- 100 and 10% DMSO at 48°C. After a minimum of 1 h in the lysis solution, the slides were placed in a horizontal gel electrophoresis tank, containing a freshly prepared and cold electrophoresis buffer (1 mM of Na2 EDTA and 300 mM of NaOH, pH > 13). The nuclei were incubated for 20 min to allow the DNA to unwind, and then electrophoresis at 0.74 V/cm (25 V, 300 mA) at 48° C was carried out for 20 min. After electrophoresis, the slides were rinsed three times with 400 mM of tris at pH=7.5, and then they were stained with 60 µl of ethidium bromide (20 µ gram/ ml) for 5 min. The SCGE slides were immersed in ice water to remove excess ethidium bromide, and then they were covered with cover slips. For each slide, 15 cells were randomly selected to be analyzed under a fluorescence microscope with an excitation and a barrier filter. A computerized image analysis system (Open Comet version 1.3.1, 2016) was used to measure various parameters of the comets. SAS version 9.1 was used for statistical analysis and to draw the graphs. The tail length was measured from the leading edge of the head image. The most important parameters of the comet assay, i.e., the criteria for DNA damages, include the percentage of DNA in the tail (tail intensity), the length of the tail, and tail moment (tail intensity x the length of the tail), which were used in the statistical analysis.

Results

Comet assay parameters pattern and value in the diploid level of annual and perennial medic species

After performing the comet assay at the diploid level of annual and perennial *Medicago* species, three parameters of the comet tail (i.e., length, intensity, and moment) were measured in the annual and perennial diploid species of *Medicago*. The results showed no significant differences in comet parameters patterns and values between the genetic structures of the annual self-pollinated and perennial cross-pollinated species at the diploid level in terms of genome structural stability. For instance, the average of tail lengths for both cases were 10.81±0.26 and 10.78±0.26 microns, respectively (Table 2 and Fig. 1a).

Slides that were previously coated and dried with Eight distinct *CsCBL* were found in *C. sinensis* (Table 1). All *CsCBL* have 7 introns and 8 exons, except *SsCBL7*, which has 8 introns and 9 exons. They all code for between 213. Therefore, the two genetic structures have almost similar stability in gel electrophoresis field (Table 2).

Effects of 0.1% colchicine on comet parameters patterns and values

The pattern and values of the three comet parameters did not show any significant difference between the induced tetraploids resulted from annual diploid species and the tetraploids resulted from the perennial diploid populations treated with 0.1% colchicine concentration (Table 3, Fig. 1b, 2b, and 3b).

Table 2. Comet parameters in 3 populations of perennial and 5 diploid annual medic species.

Growth type	Diploids	Tail Length (µ)	Tail Intensity (%)	Tail Moment (µ)
Perennials (2n=2x=16)	Karaj 1	10.38	7.21	0.84
	Karaj 2	11.29	7.49	0.94
	Tehran	10.76	7.88	0.81
Mean values		10.81±0.26	7.52±0.19	0.86±0.04
Annuals (2n=2x=16)	*M. truncatula*	10.60	7.75	0.88
	M. lupulina	10.51	7.27	0.82
	M. rigidula	10.92	7.55	0.87
	M. radiata	10.67	7.19	0.86
	M. turbinata	11.22	7.59	0.84
Mean values		10.78±0.29	7.47±0.23	0.85±0.024

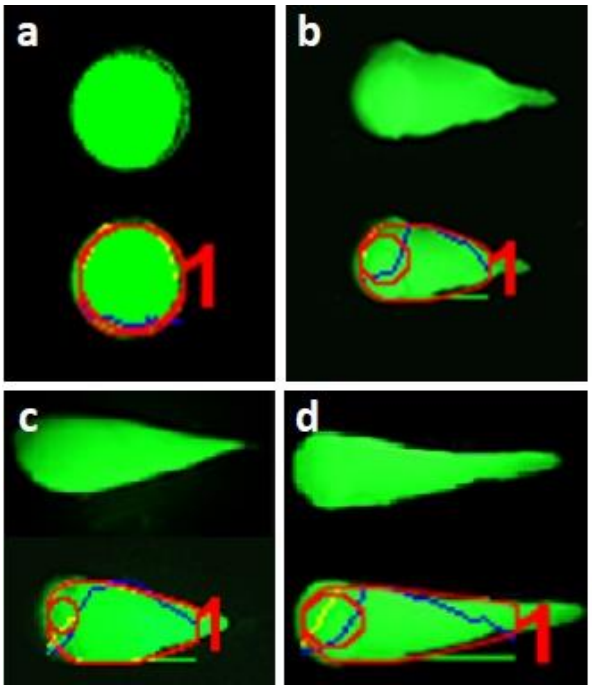

Figure 1. Comet assay in *M. truncatula* at diploidy level (a) and induced tetraploids at different concentrations of colchicine, 0.1% (b), 0.5% (c) and 1.0% (d).

Table 3. Comet parameters in the induced tetraploids from perennial and annual diploid species at 0.1% colchicine concentration.

Growth habit	Induced tetraploids	0.1% colchicine		
		Tail length (μ)	Tail intensity (%)	Tail moment (μ)
Perennials *M. sativa ssp. caerulea*	Karaj 1	15.17	12.94	1.96
	Karaj 2	15.26	13.03	1.98
	Tehran	15.28	13.39	2.08
Mean values		15.24 ± 0.03	13.12 ± 0.14	2.01 ± 0.04
Annuals	*M. truncatula*	15.37	13.56	2.17
	M. lupulina	15.74	12.86	2.02
	M. rigidula	15.51	13.27	2.07
	M. radiata	15.47	13.65	2.16
	M. turbinata	16.10	13.61	2.17
Mean values		15.64 ± 0.13	13.39 ± 0.15	2.12 ± 0.03

This means that the self-pollinated genome structure of the tetraploid from the annual *Medicago* species has shown a behavior similar to the cross-pollinated genome structure of the tetraploid from the perennial *Medicago* species against colchicine damages at the concentration of 0.1%. (Table 3).

Effects of 0.5% Colchicine on Comet parameters pattern

At the 0.5% colchicine concentration, tetraploids were obtained only in three species of annuals, i.e., *M. radiata*, and *M. truncatula*. The study of these tetraploids resulting from the aforementioned self-pollinated diploid species showed a similar comet parameters pattern (Table 4, Fig. 1c and 3c). Given the absence of the tetraploids originating from *M. rigidula* and *M. turbinata* self-pollinated diploid species, one could have imagined that they behaved differently compared to the three other annual species examined in this study at this colchicine concentration. Parameters pattern of tetraploids from three populations of perennial diploid

species, treated with 0.5% colchicine, was similar to each other (Table 3, Fig. 2c) and to the three annual species.

Effects of 1% Colchicine on Comet parameters pattern

Only the genomes of *M. truncatula* and *M. lupulina* have tolerated the damages of colchicine at 1% concentration (Fig. 1d and 3d). Then, genotype-dependent (species-dependent) genotoxic effects were shown. Genotype-depending effects of genotoxic agents were already demonstrated in *V. faba*, which appears to be more sensitive than *A. cepa* to Cd-induced genotoxicity (Arya and Mukherjee, 2014). The tetraploid plants resulting from the two aforementioned diploid species with tail lengths of 32.14 (*M. lupulina*) and 31.03(*M. truncatula*) microns showed greater but tolerable damages (Table 5).

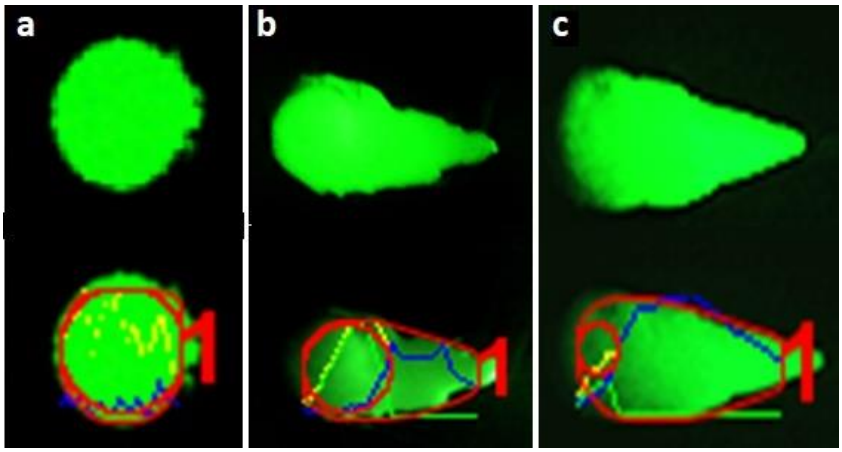

Figure 2. Comet assay in interphasique nucleus of: *M. sativa* cultivar Hamadany (a), and induced tetraploids at different concentrations of colchicine from *M sativa* ssp. caerulea Karaj 1 population at 0.1% (b) and 0.5% (c) colchicine.

Table 4. Comet parameters in the induced tetraploids in 3 annual and perennial species at 0.5% colchicine.

Growth habit	Induced tetraploids	Colchicine 0.5%		
		Tail length (µ)	Tail intensity (%)	Tail moment (µ)
Perennials	Karaj 1	25.03	23.55	5.80
	Karaj 2	25.18	23.84	6.04
	Tehran	25.06	24.37	6.14
Mean value		25.09 ±0.05	23.92 ±0.24	5.99 ±0.10
Annuals	*M. truncatula*	25.21	24.81	6.26
	M. lupulina	26.13	23.66	6.28
	M. radiata	25.22	24.84	6.34
Mean value		25.52 ±0.31	24.44 ±0.39	6.29 ±0.02

Table 5. Comet parameters in the induced tetraploids from two annual species at 1% colchicine concentration.

Annual species	1% colchicine		
	Tail length (µ)	Tail intensity (%)	Tail moment (µ)
M. truncatula	31.03	36.22	11.34
M. lupulina	32.14	34.54	11.18

None of the three populations of the perennial *M. sativa* ssp. *caerulea* could tolerate the damages caused by this concentration of colchicine, and no survivors were observed. According to these results, it might be possible to think about the more stable genomic structure of some self-pollinated species compared to the cross-pollinated species in this genus.

Comet parameters pattern in cultivated alfalfa cultivars

Examination of the comet parameters pattern in alfalfa cultivars without treatment with colchicine (Table 6) showed that the average tail length in these 5 cultivars was 10.64 microns (Fig. 2a and 3a); however, the percentage of DNA in the tail was lower in Hamedany and Qarehyonjeh cultivars (9.19 and 9.31, respectively) versus three others (Table 6). These two Iranian cultivars are generally grown in the cold regions of the country and two others which are also Iranian cultivars are grown in the warm regions of the country, therefore, adaptation of the genome according to the region can be concluded. the cultivar Ranger as a foreign cultivar cultivated in the different regions of the country behaved like the cultivars cultivated in the warm regions and it has shown the percentage of DNA in the tail, like Bamy and baghdady cultivars.

The tail length among the tetraploids derived from the population of the diploid *M. sativa* spp. *Caerulea* at the concentration of 0.1% of colchicine (Fig. 2b) was about 15.24 microns (Table 3), while it was about 25.09 microns at the concentration of 0.5% (Table 4 and Fig. 2c). The tail length of these tetraploids was longer than that of the cultivated cultivars with 10.64 microns (Table 6, Fig. 2a and 3a). Alfalfa cultivars generally have the genotype A1A2A3A4 (tetragenic form), then the full heterozygote form Demarly (1977), while the tetraploids derived from the diploid subspecies of *M. sativa* ssp. *Caerulea* had the genotype A1A1A2A2 or A3A3A4A4, etc (digenic form) because they were originated from Demarly (1977), while the tetraploids are derived from the diploid subspecies of *M.sativa* ssp. *Caerulea* had the genotype A1A1A2A2 or A3A3A4A4, etc (digenic form) because they were originated from A1A2 or A3A4, etc (diploid genotypes), and it has been demonstrated that these induced tetraploids have much lower cultural performance compared to the cultivated cultivar (Khosrowchahli, 1974).

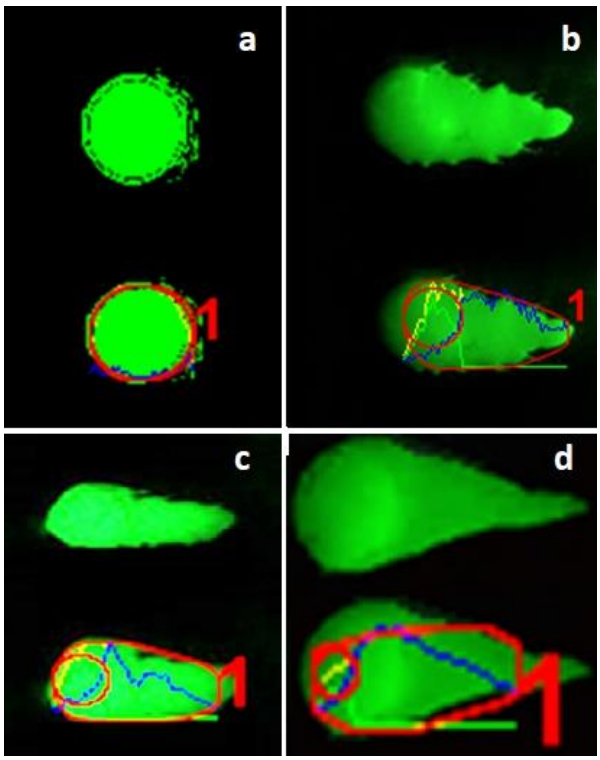

Figure 3. Comet assay in interphasique nucleus of *M. sativa* cultivar ghareyonjeh (a) and tetraploids from *M. lupulina* at 0.1% (b), 0.5% (c) and 1% (d) colchicine.

The high rates of comet parameters in these induced tetraploids (Fig. 2b and 3c) are probably due to the lack of sufficient opportunity and time to repair and rearrange their genome after the doubling process to arrive at the full heterozygote form (or to occur diploidization).

The tetraploids of two annual diploid self-pollinated species, i.e., *M. truncatula* and *M. lupulina* (at 1% colchicine concentration), showed a tail length of 31.01 and 32.14 microns, and a percentage of DNA in the tail (tail intensity) of 36.22 and 34.54%, respectively. Despite serious damage to their genomes (Fig. 1d and 3d), they were able to show a percentage of survival, and tetraploid plants were produced.

According to Table 7, DNA damages increased with increasing colchicine concentrations (changes in the size of comet parameters), which may lead to new and different rearrangements for DNA fragments damaged by each colchicine concentration in the chromosomes. This may also bring about a disruption of the organization of chromatin in the interphase nucleus of newly-produced tetraploids. Dose-dependence of the genotoxic effects of some chemical compounds, such as lead (Pb) in *A. cepa* (Jiang et al., 2014a; Jiang et al., 2014b), cadmium as a soil polluter in *A. cepa*, *V. faba* (Gichner and Plewa, 1998; Arya and Mukherjee, 2014) and *P. sativum* (Hattab et al., 2009) and ethyl methane sulfonate (EMS) as a mutagenic agent in tobacco (Gichner and Plewa, 1998) was already confirmed through the comet assay.

According to Table 7, the comparison of the three parameters of the comet assay between cultivated alfalfa cultivars and annual and perennial diploid species did not show significant differences. However, a significant difference was observed between the aforementioned species and all the tetraploids induced from the diploid species. Therefore, genome instability after treatment with colchicine, as well as the chromosomal doubling process, changes the genome so much that a performance similar to that of natural tetraploids cannot be expected in the short run.

Table 6. Comet parameters in cultivated alfalfa cultivars.

Cultivar	Tail length (μ)	Tail intensity (%)	Tail moment (μ)
Hamadani	10.84	9.19	1.04
Bamy	10.62	10.47	1.21
Ghareyongeh	10.77	9.31	1.07
Baghdady	10.45	10.36	1.53
Ranger	10.53	10.48	1.22
Mean value	10.64± 0.07	9.96 ± 0.29	1.21 ± 0.09

Table 7. Comet assay parameters in cultivated cultivars alfalfa, annual and perennial diploids and tetraploids from perennial and annual diploid species under different concentrations of colchicine.

Growth type of medic species	Colchicine %	Tail length (μ)	Tail intensity (%)	Tail moment (μ)
Cultivated cultivars *M. sativa* L.	Control	10.64± 0.07 d	9.96 ± 0.29 d	1.21± 0.09 d
Perennials diploids(2x)	Control	10.81±0.26 d	7.52±0.19 d	0.86±0.04 d
Annuals diploids(2x)	Control	10.94±0.15 d	7.50±0.08 d	0.87±0.01 d
Perennial diploids(2x)	0.1%	15.24 ± 0.03 c	13.12 ± 0.14 c	2.01 ± 0.04 c
Annual diploids(2x)	0.1%	15.64 ± 0.13 c	13.39 ± 0.15 c	2.12 ± 0.03 c
Tetraploids(4x) from perennial diploids(2x)	0.5%	25.09 ± 0.05 b	23.92 ± 0.24 b	5.99 ± 0.10 b
Tetraploids(4x) from annual diploids(2x)	0.5%	25.52 ± 0.31 b	24.44 ± 0.39 b	6.29 ± 0.02 b
Tetraploids(4x) from annual diploids(2x)	1.0%	31.59 ± 0.56 a	35.38 ± 0.84 a	11.26 ± 0.08 a

Means of columns followed by same letters have no significant difference based on Tukey mean comparison.

4. Discussion

While polyploidy induction has been an important technique in plant breeding for several decades (Dhooghe et al., 2011), it's significance particularly pronounced in horticulture, vegetative-propagated crops, and some forages, such as alfalfa, where it plays a pivotal role in refining the quality and enhancing the biomass. Polyploidy was first discovered in 1907, resulting in an increase in the number of chromosomes (Alam and Razaq, 2015). Polyploidy in nature can develop through cytological mechanisms, such as the crossing of unreduced gametes (Pereira et al., 2014), or by the doubling of the number of chromosome at the zygotic or somatic levels due to undesirable environmental conditions (Alam and Razaq, 2015). However, the natural processes to create an adequate and profitable species are slow. Colchicine is an antimitotic agent which has speed up this process. Since the discovery of colchicine in the 1930s as a mitoclasic agent, the induction of polyploidy has experienced a great boom (Marzougui et al., 2011). In addition, the polyploidization effects of colchicine and its mutagenic effects on plants have also been shown (El-Nashar and Ammar, 2016). Moreover, its genotoxic effects have been confirmed using the comet assay, where it is shown to induce DNA damages (Kiffe et al., 2003).

The current study has compared the pattern of comet parameters in non-treated and treated self and cross pollinated diploid medic species with colchicine using the comet assay. In the polyploidization of two types of diploid species, two types of induced tetraploids were obtained (from annual self-pollinated and perennial cross-pollinated species). At a concentration of 0.1% to 0.5% of colchicine, a significant increase was observed in the three comet parameters in the tetraploids from those two types of *Medicago* species compared to each other and to the cultivated cultivars and diploid species which the tetraploids were obtained. However, the comet parameters pattern (evaluating the damages caused by colchicine) at the tetraploids treated with 0.1% and 0.5% concentration of colchicine, did not show a significant difference between the genetic structures of self-pollinated and cross-pollinated *Medicago*. In the concentration of 1%, no survivors were observed in perennial cross-pollinated species. However, in the two annual self-pollinated *Medicago* species (i.e.,*M. lupulina* and *M. truncatula*), tetraploid survivors were observed, showing that these two annual diploid *Medicago* species were more tolerant against the genotoxic effects of colchicine.

Due to the heterozygote genetic structure of the diploid perennial *Medicago* populations like A1A2, A2A3, A1A4 (*M. sativa* spp. *Caerulea*), the tetraploids induced from this perennial *Medicago* diploid species will be heterozygous as well like A1A1A2A2, A2A2A3A3 and so on, therefore the members of the resulting tetraploid population will be different from each other and in the digenetic or duplex state. Consequently, in each colchicine concentration different structural damages and different repair systems may have occurred resulting in a different genome rearrangement in each member of the resulting tetraploid population because the diploid parents had been genetically different from each other from the start (Khosrowchahli, 1974). Tetraploids obtained at the concentrations of 0.1% and 0.5% will also differ from each other due to different damages and restructurings inflicted on the genome at each concentration, as shown in the values of the comet parameters. This can be true for tetraploids obtained from the cross-pollinated perennial species of *M. sativa* spp. *caerulea* and the self-pollinated annual *Medicago* species. Of course, due to the limited number of polyploid plants obtained during the polyploidy induction, these issues are not usually taken into account. The comparison of comet assay parameters between the cultivated alfalfa cultivars which are fully heterozygous, that means in the tetragenetic or quadruplex state (Demarly, 1977) and the tetraploids obtained from the diploid species has shown that the pattern of the three comet parameters in the induced tetraploids were different and their values were much higher than those of the cultivated tetraploids. Therefore, the induced tetraploids may have an unstable and unfixed genetic structure caused by the colchicine treatment. Obtaining, stability in the genome and performance similar to cultivated alfalfa in the induced tetraploids from cross-pollinated species will probably be possible by the establishment of the new rearrangement of chromosomal structures and territory reorganization in the interphase nucleus after the damages inflicted by colchicine and arriving at the state of complete heterozygosity. This is probably not the case of self-pollinated species because these treated

genetic structures are homozygous and the tetraploids derived, can acquire appropriate performance after the damages is repaired. Investigating on the pattern of the three parameters of the comet assay in the two diploid categories of medic species and the auto-tetraploids obtained from them showed that the pattern and their values were significantly different and this difference increased by colchicine concentrations, it was demonstrated that the induced auto-tetraploids despite the damages inflicted by colchicine have revealed increases in the performance of the many characters studied (Ansari et al., 2021).

Conclusion

Owing to the variations in genetic structure of the diploid cross-pollinated species populations, such as *M. sativa* spp. *caerulea* populations, where plants may exhibit the heterozygous nature (A1A2, A1A3 or A2A3, A1A4, etc,) of genetic structures, the disparities observed in morphological and other characteristics of the induced autotetraploids can be attributed partly to these inherent structural genetic differences and partly to the pattern of genotoxic effects of colchicine at each level of concentration. At each concentration level of colchicine, the genetic structure of treated plants will give rise to distinct plant variations. Another source of variability in the resulting autotetraploids in this cross-pollinated species must be acknowledged. This variability arises from the varying concentration of colchicine, each exerting a distinct genotoxic effect. Consequently, different genotypes will respond differently to different colchicine concentrations, leading to additional variability. While this variability is valuable for breeding programs, its significance may be overlooked due to the limited number of autotetraploid plants obtained. This holds particular significance for the alfalfa breeding program, given the current narrowness of the alfalfa gene pool. Due to the homozygous structure inherent in self-pollinated diploid species, such as the various annual diploid species of *Medicago*, a diverse range of tetraploids will only emerge when colchicine concentrations change. Interestingly, comparable patterns and comet parameter values were observed for the cross-pollinated and self-pollinated Medicago species at 0.1% and 0.5% colchicine concentration. This suggests a parallel behavior of these distinct genetic structures in response to these two levels of colchicine concentrations.

Supplementary Materials:

No supplementary material is available for this article.

Author Contributions:

The authors confirm contribution to the paper as follows: conceptualization, M.Kh.; methodology, E.A.; formal analysis: A.A.J.; writing—review: M. Kh., A.E. All authors have read and agreed to the published version of the manuscript.

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The authors declare no conflict of interest.

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مقاله پژوهشی

مقایسه الگوهای پارامترهای سنجش دنباله‌دار بین گونه‌های *Medicago* دیپلوئید خودگرده‌افشان، دگرگرده‌افشان و تتراپلوئیدهای حاصل از آنها و ارقام زراعی

الهام انصاری[۱]، محمود خسروشاهلی*[۱]، علیرضا اطمینان[۲]، علی‌اشرف جعفری[۳]

[۱].گروه بیوتکنولوژی و اصلاح نباتات، واحد علوم و تحقیقات، دانشگاه آزاد اسلامی، تهران، ایران.
[۲].گروه اصلاح نباتات و بیوتکنولوژی، واحد کرمانشاه، دانشگاه آزاد اسلامی، کرمانشاه، ایران.گروه
[۳].پژوهشکده جنگل‌ها و مراتع، سازمان تحقیقات، آموزش و ترویج کشاورزی (AREEO)، تهران، ایران.

چکیده: سه پارامتر آزمون دنباله دار (طول دم، شدت دم و گشتاور دم) بین اتوتتراپلوئیدهای تولید شده از سه جمعیت گونه *Medicago sativa* زیر گونه *caerulea* دگر گرده افشان، پنج گونه دیپلوئید خود گرده افشان از جنس *Medicago* تیمار شده با سه غلظت کلشیسین (۰/۱ ، ۰/۵ و ۱٪) و پنج رقم یونجه زراعی مقایسه شد. در غلظت‌های ۰/۱ و ۰/۵ درصد، افزایش و الگوی مشابهی در دو گروه اتوتتراپلویدالقا شده مشاهده شد. همچنین افزایش غلظت از ۰/۱٪ و ۰/۵٪ منجر به افزایش بارزتر پارامترهای دنباله‌دار شد. اتوتتراپلوئیدهای القا شده از دو غلظت کلشی سین مذکور در مقایسه با ارقام کشت شده و دو رده یونجه در سطح دیپلوئید، افزایش در ارزش و الگوی پارامترهای آزمون دنباله دار نشان دادند. در غلظت ۱ درصد کلشیسین، تنها دو گونه یونجه یکساله اتوتتراپلوئید در مقایسه با غلظت های ۰/۱ و ۰/۵ درصد کلشیسین و افزایش واضح تر پارامترهای آزمون دنباله دار را نشان دادند. تغییرات در الگوها و مقادیر هر سه پارامتر در تتراپلوئیدهای القایی در مقایسه با یونجه کشت شده و گونه‌های دیپلوئید یونجه، اثرات متمایز آسیب‌های کلشیسین را از غلظتی به غلظت دیگر نشان داد، در نتیجه تغییرات جدیدی در هر تغییر غلظت مورد انتظار است.

کلمات کلیدی: آزمون دنباله‌دار، اتوتتراپلوییدی، یونجه، کلشیسین، گونه‌های یونجه.

ویراستار علمی
دکتر سیدکمال کاظمی‌تبار،
دانشگاه علوم کشاورزی و منابع طبیعی ساری، ایران

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نویسنده مسئول
دکتر محمود خسروشاهلی
mkhosrowchahli@yahoo.com

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Investigating members of *Arabidopsis* WRKYs transcription factors with differential expression under various stresses using bioinformatics approaches

Elham Younesi-Melerdi

Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University (SANRU), Sari, Iran.

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Correspondence

Dr. Elham Younesi-Melerdi
eyonesi@gmail.com

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Abstract: In the current study, a comprehensive analysis was performed on Arabidopsis WRKY transcription factor (TF) family members that their differential expression (DE) was reported under various stresses in GEO profile database. First, WRKY members with DE were extracted from GEO profile and information of the data set, sequence and their gene structure were obtained. Then, the concept of the intersection of sets was used to select some of WRKY TFs for downstream analysis. DE of candidate members was compared by t-test. The protein– protein interaction network were predicted by STRING web server. A total of 16 *WRKY* genes were identified in the 11 dataset of GEO profile. Analysis of the gene structure showed that 56% of the studied *WRKY* genes have 3 exons and all 16 members are distributed across all five chromosomes of *Arabidopsis*. Also, the results showed that *WRKY40*, *WRKY46*, *WRKY18*, and *WRKY33* were most frequently responsive to various stresses. The protein-protein interaction network showed that WRKY40, WRKY46, WRKY18, and WRKY70 have high interactions with four genes: *MPK4*, *ACS6*, *MKS1*, and *STZ*. Therefore, WRKY40, WRKY46, WRKY18, and WRKY33 can be considered the most important WRKY TFs of *Arabidopsis* at response to various stresses and may find applications in genetic and metabolic engineering projects.

Keywords: GEO profile, exon number, high light, chitooctaose.

Introduction

The concept of stress in plants has been a subject of discussion in scientific communities for more than 8 decades and refers to any adverse conditions and substances that impact or impede the growth, development, and metabolism of plants (Mochizuki et al., 2001; Liu et al., 2016). In plants, due to the nature of their immobility, the definition of stress is completely different from humans and animals. Consequently, plants employ various tolerance and response mechanisms to cope with adverse conditions. Different factors of natural and anthropogenic can be known as stressors and these factors can lead to damage or death of plants depending on the duration and intensity of the stressor. Drought, salt, high and low temperatures, nutrient deficiency, long rainy periods, various insects, and pathogens are some of the known natural stressors. Also, acid rain, various herbicides, and poisons, ozone, increasing UV radiation, and changing the pH of soil and water can be mentioned as stresses with anthropogenic origin (Jäger et al., 2022). In the last three decades, with the development of the concept of plant stress, research technologies have also improved in this field. So that, the number of articles on subject of investigating the response of plants to different types of stress has increased. The response of plants to stress is modulated through deep changes in physiological, biochemical and genomic levels.

In the genomic level, the regulation of gene expression or change of transcriptome is a vital step of the plant's response to stimuli. Recently, high-throughput technologies such as microarray and RNAseq have made it possible to comprehensively investigate the response of plant to stress at the transcriptome level. Transcription factors (TFs) are one of the key factors regulating gene expression. TFs are proteins possessing DNA-binding domains and can change the genes expression and perform the first phase of the genome decoding under different conditions (Lambert et al., 2018; Deng et al., 2022). A significant portion of the plants genome is assigned to TFs, for example in *Arabidopsis* 1771 loci assigned to 2296 TFs, which are divided into 58 families (Jin et al., 2017). Some families of TFs are specifically found in plants and algae, including AP2, NAC, ERF, and WRKY families.

The family of WRKY TFs has a different number of members in various plant species. For example, In *Arabidopsis*, the WRKY TF family with 90 members is one of the most numerous families of TFs (Li et al., 2020). All WRKY proteins have one or two WRKY domains with a length of 60 amino acids, contain a highly conserved amino acid sequence of WRKYGQK, and a zing finger like motif. Based on the type of zing finger-like motif and the number of domains, they are divided into three groups (Jiang et al., 2021). The members of this family regulate the expression of their target genes by binding to W-box, (T)TGAC(C/T), cis-elements in the genes promoter. The members of WRKY family are involved in various processes including growth and development and response to various biotic and abiotic stresses (Jiang et al., 2021; Yu et al., 2023). Overexpression of the *MxWRKY55* gene in transgenic *Arabidopsis* has led to increased resistance to salt stress. Also, the study of transgenic *Arabidopsis* with overexpression of the *GhWRKY33* gene showed this WRKY as a negative regulator mediated the response of plants to drought stress (Wang et al., 2019). Li et al. (2020) reported that the high expression of the *TaWRKY46* gene in *Arabidopsis* led to increased resistance to osmotic stress (Li et al., 2020).

Considering that TFs are one of the important factors in the regulation and coordination of biochemical pathways that are widely used in metabolic engineering (Deng et al., 2022). So that, identification of the most important TFs involved in response to most stress can provide valuable information for genetic and metabolic engineers. Accordingly, in this research, efforts were made to identify and introduce the most important members of the *Arabidopsis* WRKY family of TFs in response to various stresses using information available in databases and through bioinformatics analyses.

Materials and Methods

Dataset extracting

The GEO profile database was searched on the NCBI website (https://www.ncbi.nlm.nih.gov) to identify members of the WRKY TF family with differential expressions. Then, the explanation of the experiment of each of the resulting records was studied through the GEO dataset, the records

related to mutant samples of *Arabidopsis* were removed, and only the samples related to the wild type were included in the analysis. Types of stress were assayed in the present study, which included salinity stress, dehydration stress, highlight, auxin, paraquat, selenate, phosphate deficiency, chitoactase and NEP1. Summary of Information about the dataset studied in this experiment is reported in Table 1.

Table 1. Information of the GEO datasets (expression profiling by array) of assayed in present study.

Dataset name	Dataset ID	No. sample	Stress type	Stress level	Replicates	Time course	PMID	Publication year
Salt stress effect on multiple genotypes: leaf	GSE16765	12	salinity stress	2	2	_	21821598	2011
Gene expression from *Arabidopsis* under high light conditions	GSE22671	9	high light	3	3	_	21531897	2011
Leaf response to paraquat: time course	GSE10464	6	paraquat	2	3	3	14508004	2008
Selenate effect on roots	GSE9311	4	Selenate	2	2	_	18251864	2007
Auxin signaling inhibitors effect on seedlings	GSE1491	21	Auxin	6	3 or 4	_	15466695	2004
Abiotic stress-inducing agents effect on suspension cell cultures	GSE3709	37	a range of abiotic treatments.	17	1 or 3 or 4	_	14730085, 16027974	2005
Whole seedling roots response to salinity stress	GSE7642	12	salinity stress	2	2	5	18436742	2008
Phosphate deficiency effect on roots: time course	GSE25171	24	phosphate deficiency	2	2	4	21248074	2011
Chitin oligomer chitooctaose effect on seedlings	GSE4746	6	chitooctaose (Chitin oligomer)	2	3	_	17722694	2006
Necrosis- and ethylene-inducing peptide effect on dicots	GSE4638	4	Nep1	2	2	_	16698904	2006
mRNA translation and dehydration stress	GSE2268	4	dehydration	2	2	_	15716313	2005

Results and Discussion

In this experiment, eleven datasets were examined, encompassing 139 samples originated from various conditions and organs of the *Arabidopsis* plant. The evaluation of GEO profile database demonstrated that 16 genes of *WRKY* family members showed differential expression under different stimuli. In order to provide better information from each of these members, some nucleotide and protein characteristics are shown in Table 2. The lengths of sequences of *WRKY* members ranged between 810 (*WRKY75*) to 2028 (*WRKY33*) nucleotides. Also, *WRKY75* and *WRKY33* have the minimum and maximum length of the amino acid sequence, respectively.

Table 2. Information of sequence of *Arabidopsis* WRKY members studied in present study.

Gene symbol	Gene ID	Transcript ID	Protein ID	Gene name	Length (nt)	Length (aa)	Chr
WRKY33	818429	NM_129404.4	NP_181381.2	WRKY DNA-binding protein 33	2028	519	2
WRKY40	844423	NM_106732.4	NP_178199.1	WRKY DNA-binding protein 40	1325	302	1
WRKY46	19248	NM_130204.3	NP_182163.1	WRKY DNA-binding protein 46	1354	295	2
WRKY18	829308	NM_119329.4*	NP_567882.1	WRKY DNA-binding protein 18	1740	310	4
WRKY53	828481	NM_118512.3	NP_194112.1	WRKY family transcription factor	1514	324	4
WRKY30	832476	NM_122316.3	NP_568439.1	WRKY DNA-binding protein 30	1290	303	5
WRKY75	831147	NM_121311.5	NP_196812.1	WRKY DNA-binding protein 75	810	145	5
WRKY70	824807	NM_115498.4	NP_191199.1	WRKY DNA-binding protein 70	1470	294	3
WRKY22	827896	NM_116355.3	NP_192034.1	WRKY family transcription factor	1326	298	4
WRKY25	817575	NM_128578.4	NP_180584.1	WRKY DNA-binding protein 25	1776	393	2
WRKY28	827542	NM_117927.3	NP_193551.1	WRKY DNA-binding protein 28	1453	318	4
WRKY48	835012	NM_124329.3	NP_199763.1	WRKY DNA-binding protein 48	1921	399	5
WRKY38	832320	NM_122163.3	NP_197649.2	WRKY DNA-binding protein 38	1306	289	5
WRKY54	818670	NM_129637.3	NP_181607.1	WRKY DNA-binding protein 54	1432	346	2
WRKY26	830601	NM_203017.2*	NP_974746.1	WRKY DNA-binding protein 26	1576	216	5
WRKY45	821270	NM_111063.4	NP_186846.1	WRKY DNA-binding protein 45	1359	147	3

The symbol * indicates that more than one transcript was detected for the desired gene, and here the features of the longest transcript are reported as the canonical transcript

The distribution of the number of exons in the studied members of the WRKY is shown in figure 1. The results showed that 56% (9 members) of the studied members of this family have the exon number of 3. Considering that the amino acid sequence of the members of WRKY family is not very long, the number of exons is predictable. The *WRKY33*, *WRKY18* and *WRKY25* with five exons have the largest number of exons. Also, three genes, *WRKY53*, *WRKY45*, and *WRKY75* have only two exons. Also, the evaluation of the number of transcripts of the studied *WRKY* genes showed that only two genes, *WRKY18* and *WRKY26*, were affected by alternative splicing and produced 3 and 5 transcripts, respectively. The map and chromosomal distribution of the WRKY members revealed that the studied genes are scattered in all chromosomes and chromosome 5, with 5 genes, showed the highest density, and chromosome 1 showed the lowest density (Figure. 2).

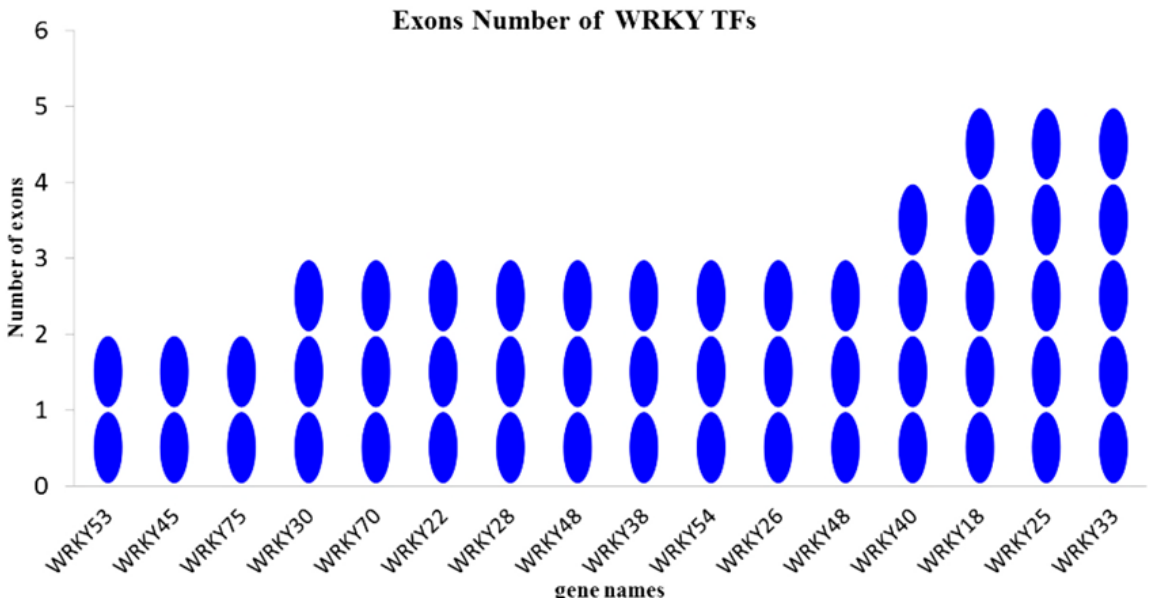

Figure 1. The exons number of the *Arabidopsis WRKY* members studied in present research.

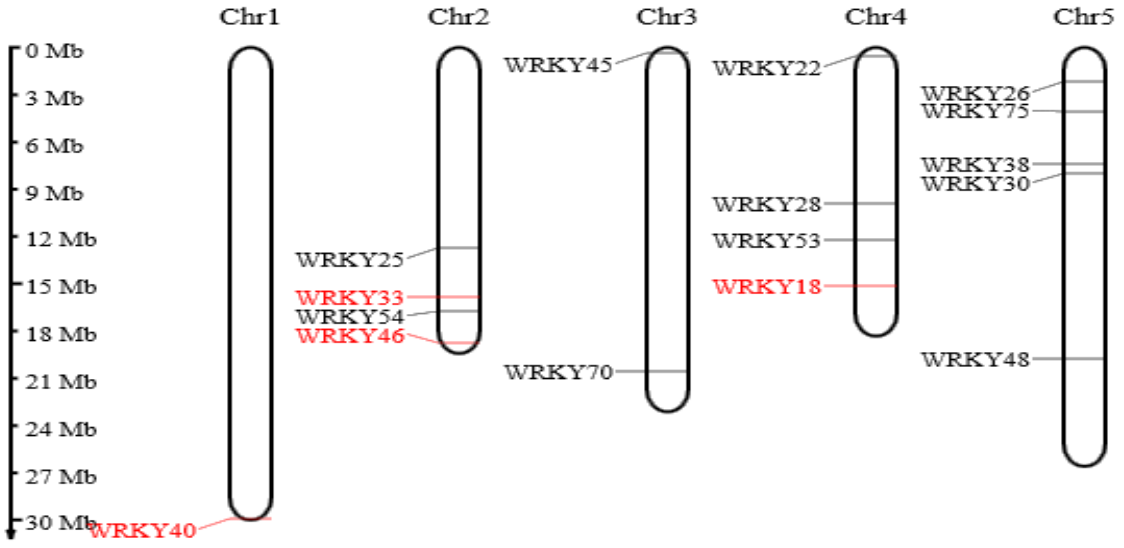

Figure 2. Distribution the *Arabidopsis WRKY* members studied in present research on 5 chromosomes of *Arabidopsis* . Four candidate members for further investigation are marked in red font.

The evaluation of the frequency of WRKY members in the dataset revealed that four genes of *WRKY40*, *WRKY46*, *WRKY18* and *WRKY33* showed differential expression in more than 50% of the studied datasets, such that *WRKY40*, *WRKY33*, and *WRKY46* have been observed in 8, 7 and 6 studies, respectively (Figure. 3). On the other hand, more detailed investigations revealed that all four mentioned members are present in three datasets of GSE4746, GSE9311 and GSE22671. Therefore, in the

following we focused on the investigation of *WRKY40*, *WRKY46*, *WRKY18* and *WRKY33* genes in three datasets of GSE4746, GSE9311 and GSE22671. The expression values of these four genes in the three datasets were reported in the Figure 4.

In the GSE9311 dataset, the effect of selenate (Se) on the expression of genes in the root was evaluated. Se and sulfate are chemical analogs and can be taken up by plants through the same transporters and enzymes. Unlike many other organisms, Se is not essential for higher plants. In plants, its excessive amount is toxic and limits growth. Both Se deficiency and toxicity doses are dangers worldwide (Van Hoewyk et al., 2008). The results of the expression of four *WRKY* in this dataset showed that the expression of all four studied genes increased significantly, so that *WRKY40* showed the highest expression value equal to 5627 and 95 FPKM in control and treatment samples, respectively. Also, the *WRKY46* gene was showed the lowest expression value with means 75 and 1975 in control and treatment samples, respectively (Figure. 4a).

In the GSE4746 dataset, the changes of *Arabidopsis* transcriptome were evaluated by chitectase treatment. Chitectase is an oligomer of chitin that is found in the cell wall of fungi, insects and nematodes and is used as a fungal elicitor in researches (Libault et al., 2007).

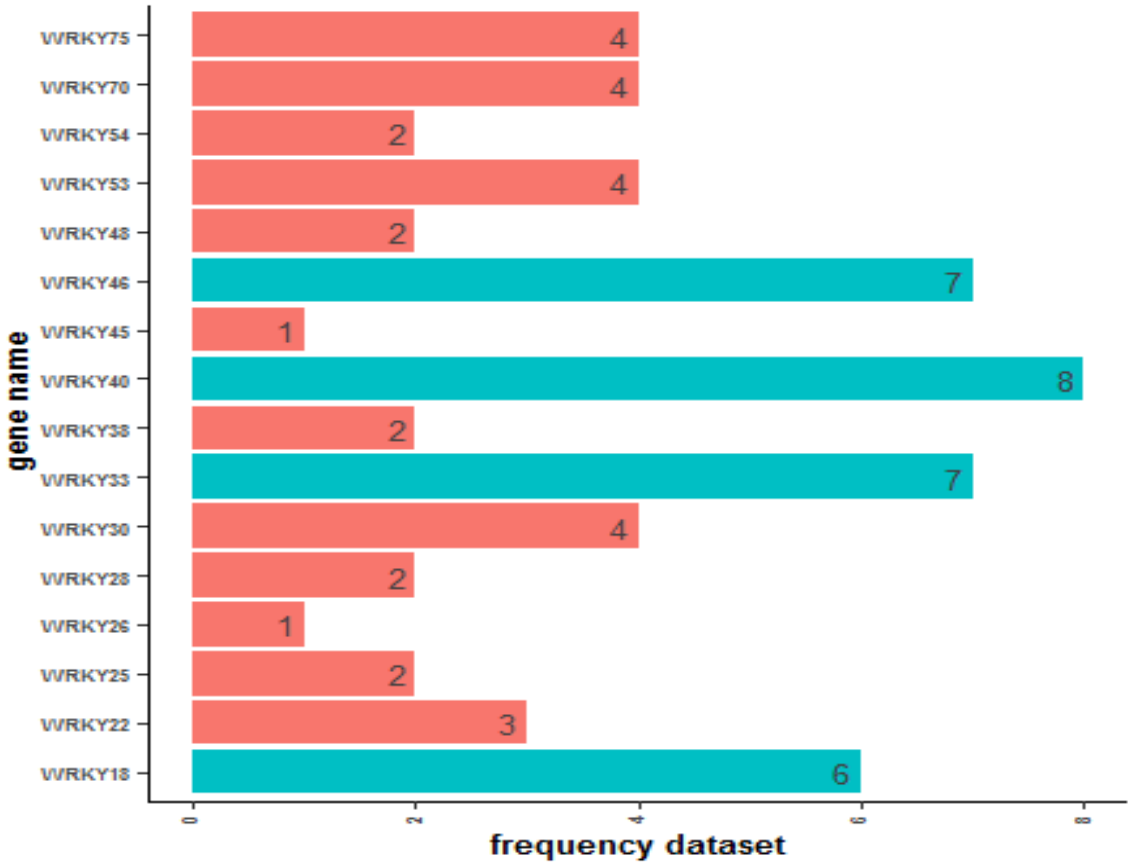

Figure 3. The number of datasets that reported the differential expression of each *Arabidopsis WRKY* member in present study. The genes that present in more than 50% of the data sets were marked with blue columns.

The results of investigations of this dataset showed that the expression of all four candidate genes increased significantly, so that *WRKY33* showed the highest expression value (mean FPKM = 3588) and *WRKY46* showed the lowest value (mean FPKM =1727). Also, in the control (water) samples, the mean expression of these two genes was estimated 359 and 118, respectively (Figure 4b).

The evaluation of differential expression in the GSE22671 data set is shown in the Figure 4c. In this dataset, cell culture suspension, that has active chloroplast cells, was exposed to high light and its defense response was investigated against high light stress. The results showed that the expression of *WRKY33*, *WRKY40* and *WRKY43* genes increased significantly in response to high light, but our statistical analysis did not confirm the significant increase in expression of *WRKY18* gene.

When plants sense stress, the signals of stress perception are activated and transformed into cell. Inside the cells, plants recruit a complex signaling transduction network for trigger chemical and molecular responses.

The change of balance of reactive oxygen species (ROS) and the concentration of calcium play the role of an intercellular second messenger, as well as the activation of kinases cascade pathway such as MAPK (mitogen activated protein kinase). Subsequently, the MAPK cascade pathway activates transcription factors as cis-regulatory elements of responsive genes. Among the TFs, the expression of *WRKY* TFs changes rapidly in response to various stresses (Gonzalez-Perez et al., 2011). Recently, the WRKY TFs were introduced as negative and positive regulators in response to various stresses in plant species. Zhang et al. (2006) reported that WRKY33 regulates the antagonistic interaction between defense pathways mediating responses to necrotrophic pathogens and *P. syringae* (Zheng et al., 2006). In addition, the role of WRKY33 as a negative regulator in oxidative and abscisic acid (ABA) stresses has been confirmed in *Arabidopsis*. Also, the results showed that AtWRKY18, AtWRKY40 and AtWRKY60, control resistance to pathogens by forming homologous or heterologous dimers (34). It has also been reported that WRKY40 is involved as a negative regulator of ABA during the stages of seed germination and post-germination growth (Geilen and Bohmer, 2015).

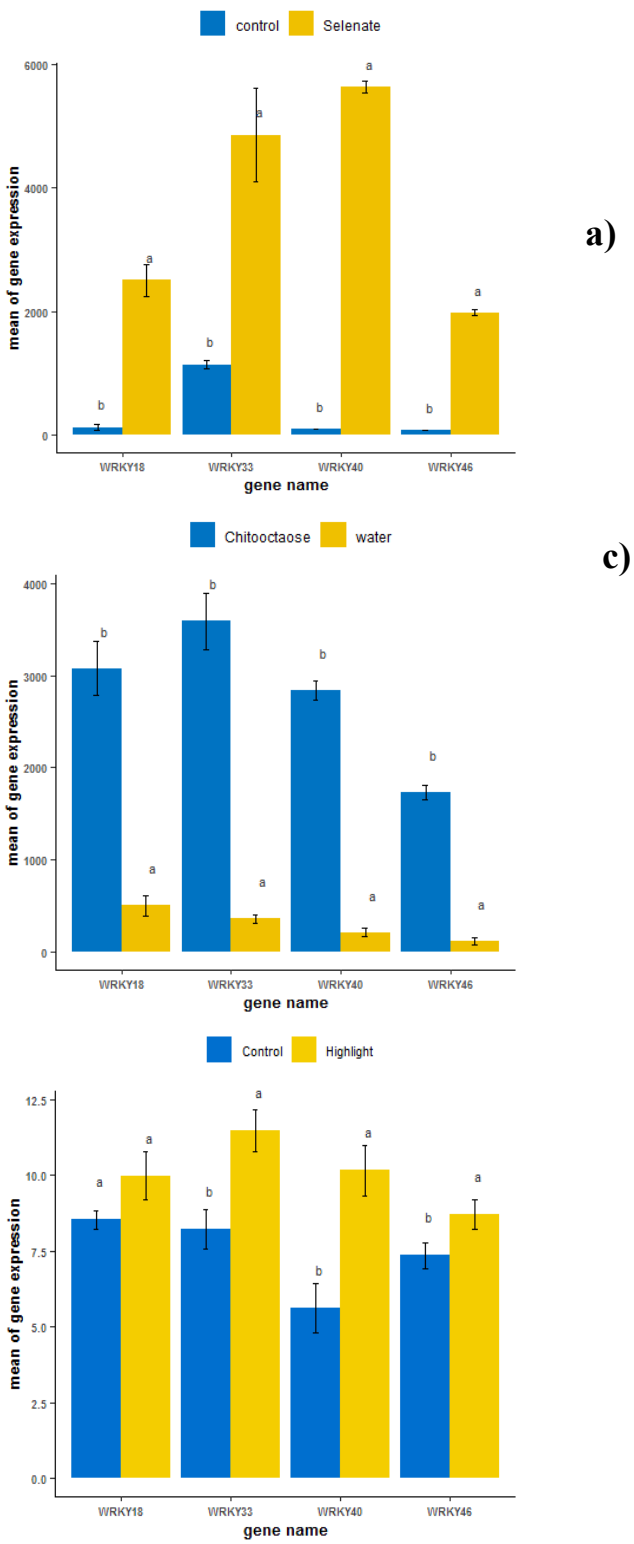

Figure 4. The differential expression of the *Arabidopsis* WRKY members selected in present study. a) GSE9311 dataset (selenate treatment) b) GSE4746 dataset (chitectase treatment) c) GSE22671 dataset (high light treatment).

More studies also showed that other members of the WRKY family of transcription factors in *Arabidopsis* are affected by various stresses. For example, WRKY26, WRKY25, and WRKY39 have been introduced as transcription factors involved in heat stress and WRKY34 as a negative regulator of cold resistance in *Arabidopsis* (Zheng et al., 2006; Li et al., 2011).

The number of the WRKY family members is different in various species, so the concept of orthology and orthologous genes in this family is very broad. Until now, the effective role of the WRKY members in the response to various stresses has been reported in different species. It has been reported that WRKY25, WRKY63 and WRKY21 are involved in the tolerance of salt and drought stresses (Jia et al., 2015; Liu et al., 2016; Wang et al., 2021). In rice, the WRKY74 and WRKY76 have been introduced as key factors in response to cold stress, and WRKY89 is a TF involved in UV light resistance (Wang et al., 2007; Yokotani et al., 2013; Dai et al., 2016).

The interactions between WRKYs members and between WRKYs with other proteins regulate the various pathways involved in response to stress. Analysis of protein-protein interaction network showed that among the four candidate proteins, WRKY40, WRKY18, and WRKY33, have a very strong interaction with each other and with another group of proteins. Another member of WRKY family, WRKY70, was also present in the predicted protein network (Figure 5). The WRKY70 showed differential expression in only two of the studied datasets in current study (Figure 3).

Also, the protein network formed two distinct group of protein-protein network. The GUN5 protein plays role of connected node for formed two groups (Figure 5). The GUN5 protein is a multifunctional protein that plays important role in the production of chlorophyll, a photosynthetic pigment, by catalyzing the metabolism of porphyrin-containing components. Also, due to the function of magnesium clathase, this enzyme plays an important role in the plastid-to-nucleus signaling pathway, by coordinating the genes involved in photosynthesis with the two origins of the chloroplast and the nucleus. In addition, the study of ABA sensitive phenotypes showed this gene is a positive regulator of the signaling pathway of ABA hormone in stomatal guard cells during seed germination (Mochizuki et al., 2001).

The protein- protein interaction network also showed that the four genes of MPK4, ACS6, MKS1 and STZ interact with all four WRKY members that are present in the predicted protein network. The thickness of the edges between these nods confirms interactions with very strong data support (Figure 5). Studies have reported the role of each of these proteins in response to various types of stress. The MAPK signal transduction cascade is utilized by eukaryotic cells to transduce a wide variety of extracellular signals such as growth factors, hormones, and stress stimuli (Li et al., 2015).

The results showed that mapk4 is one of the key members of the signaling pathway in *Arabidopsis* and regulating the expression of genes responding to various biotic and abiotic stresses and also plays an important role as a negative regulator in pathogen defense (Li et al., 2015).

Also, Liu and Zhang (2004) showed that MAPK6 enzyme leads to the stimulation of ethylene production in response to various stresses by phosphorylation of the ACS6 enzyme. The enzyme ACS6 which converts S-adenosyl-L-methionine (SAM) to 1-aminocyclopropene 1-carboxylase (ACC) and is involved in the production of ethylene is stimulated by bacterial flagellin. The ACC is considered as a central precursor of ethylene, and the increase in ethylene production in response to a variety of biological stresses have been confirmed in plants (Chen et al., 2020). It has also been reported that the activity of ACS6 enzyme leads to an increase in stomatal density of the epidermis of *Arabidopsis* leaves in response to drought stress (Jia et al., 2021).

Another protein present in protein- protein interaction network is MKS1, which is a defense response regulator in plants, and acts as a downstream substrate of the MAPK4 enzyme in the salicylic acid-dependent pathway. Its interaction with the transcription factors of WRKY33 and WRKY25 has been proven, and may act as a mediator between MAPK and downstream transcription factors (Andreasson et al., 2005).

#node	Gene name
ACS6	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6
ALB1	Magnesium-chelatase subunit ChlD, chloroplastic
AT1G74470	Geranylgeranyl diphosphate/geranylgeranyl-bacteriochlorophyllide a reductase
BHLH92	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
CHLI1	P-loop containing nucleoside triphosphate hydrolases superfamily protein
CHLI2	Magnesium-chelatase subunit ChlI-2, chloroplastic
CHLM	Magnesium protoporphyrin ix methyltransferase, chloroplastic
CML38	Calcium-binding protein CML38
ERF11	Ethylene-responsive transcription factor 11
GUN4	Tetrapyrrole-binding protein, chloroplastic
GUN5	Magnesium-chelatase subunit chlh, chloroplast
HEMG2	Protoporphyrinogen oxidase 2, chloroplastic/mitochondrial
MKS1	MAP kinase substrate 1
MPK4	Mitogen-activated protein kinase 4
PPOP1	Protoporphyrinogen/coproporphyrinogen iii oxidase
RHL41	C2H2-type zinc finger family protein
STZ	Related to Cys2/His2-type zinc-finger proteins found in higher plants.
WRKY18	Arabidopsis thaliana wrky dna-binding protein 18
WRKY33	Probable WRKY transcription factor 33
WRKY40	Probable WRKY transcription factor 40
WRKY70	Arabidopsis thaliana wrky dna-binding protein 70

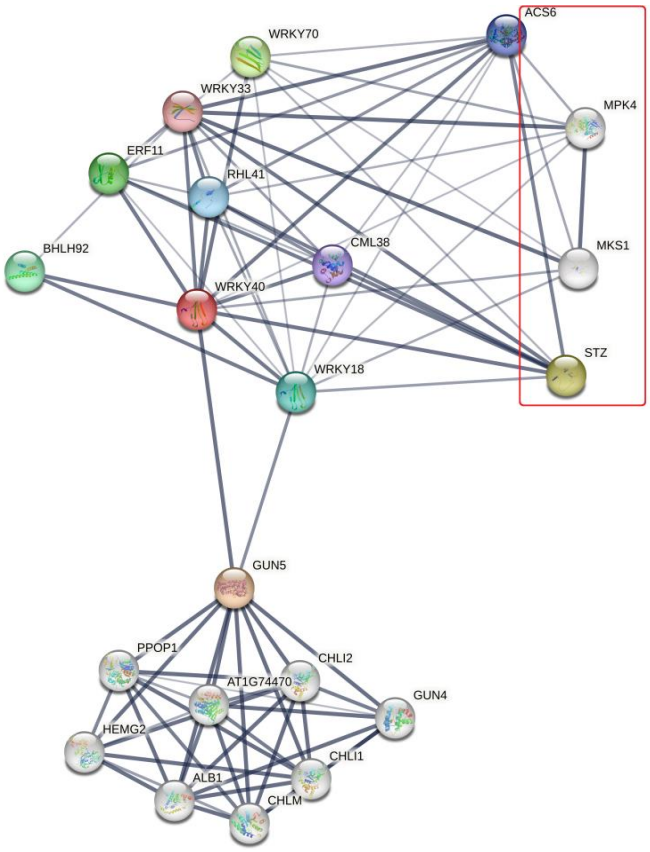

Figure 5. The protein-protein interaction network with present WRKY40, WRKY18, WRKY33 and WRKY70. The confidence level of interactions was considered more than 0.7 (high level). Full names of nodes defined in table.

Its interaction with the transcription factors of WRKY33 and WRKY25 has been proven, and may act as a mediator between MAPK and downstream transcription factors (1). STZ protein is known as a transcription repressor in abiotic stresses. It controls the expression of LOX3 (jasmonic acid biocenter gene) and *JAZ1* genes (key repressor in jasmonic acid cascade signaling pathway) (Mittler et al., 2006; Xie et al., 2012). In transgenic *Arabidopsis* plants, overexpressing STZ1 growth retardation has been observed to increase tolerance to drought, salt, heat and osmotic stresses (Sakamoto et al., 2004).

Conclusion

The findings of this study reveal 16 *Arabidopsis WRKY* genes exhibiting differential expression in response to diverse stress conditions. Subsequent bioinformatics-based analyses such as expression analysis and the examination protein- protein interaction network of WRKY proteins revealed that four members of WRKY40, WRKY46, WRKY18 and WRKY33 are the most crucial within this family. These members can be used in genetic and metabolic engineering projects aimed at developing stress-tolerant plants.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

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Conflicts of Interest

The author declares no conflict of interest.

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مقاله پژوهشی

بررسی اعضای فاکتورهای رونویسی WRKYs عربیدوپسیس با بیان افتراقی تحت تنش‌های مختلف با استفاده از رویکردهای بیوانفورماتیک

الهام یونسی ملردی

پژوهشکده ژنتیک و زیست‌فناوری کشاورزی طبرستان، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، ایران

چکیده: در مطالعه حاضر، تجزیه و تحلیل بر روی اعضای خانواده فاکتورهای رونویسی WRKY در گیاه عربیدوپسیس که بیان افتراقی آنها (DE) تحت تنش‌های مختلف در پایگاه داده GEO profile گزارش شده بود، انجام شد. ابتدا اعضای *WRKY* همراه با DE از GEOprofile اخذ و اطلاعات مجموعه داده‌ها، توالی و ساختار ژنی آنها به دست آمد. سپس از مفهوم اشتراک مجموعه‌ها برای انتخاب برخی از WRKYها برای تحلیل پایین دستی استفاده شد. بیان افتراقی اعضای انتخاب شده با آزمون t مقایسه و شبکه برهمکنش پروتئین – پروتئین توسط وب سرور STRING پیش‌بینی شد. در مجموع ۱۶ ژن *WRKY* در ۱۱ مجموعه داده GEO profile شناسایی شد. تجزیه و تحلیل ساختار ژن نشان داد که ۵۶ درصد از ژن‌های *WRKY* مورد مطالعه دارای ۳اگزون هستند و هر ۱۶ عضو در هر پنج کروموزوم عربیدوپسیس توزیع شده‌اند. همچنین نتایج نشان داد که *WRKY40*، *WRKY46*، *WRKY18* و *WRKY33* بیشترین فراوانی را در پاسخ به تنش‌های مختلف داشتند. شبکه برهمکنش پروتئین–پروتئین نشان داد که WRKY40، WRKY46، WRKY18 و WRKY70 برهمکنش بالایی با چهار ژن *MPK4*، *ACS6*، *MKS1* و *STZ* دارند. بنابراین، WRKY40، WRKY46، WRKY18 و WRKY33 می‌توانند مهم‌ترین TFهای WRKY عربیدوپسیس در پاسخ به تنش‌های مختلف باشند و در پروژه‌های مهندسی ژنتیک و متابولیک مورد استفاده قرار گیرند.

کلمات کلیدی: GEO profile، تعداد اگزون، نور زیاد، کیتوکتاز.

ویراستار علمی
دکتر قربانعلی نعمت‌زاده،
گروه بیوتکنولوژی و اصلاح نباتات، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ایران

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نویسنده مسئول
دکتر الهام یونسی ملردی
eyonesi@gmail.com

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Exogenous hydrogen peroxide enhances the response of corn (*Zea mays* L.) plants to drought stress

Hamid Valizade[1], Saeid Navabpour*[1], Ali Dehestani[2], Pooyan Mehrabanjoubani[3]

[1.] Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.
[2.] Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.
[3.] Department of Basic Sciences, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

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Correspondence
Dr. Saeid Navabpour
s.navabpour@gau.ac.ir

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Abstract: Drought stress is a significant factor limiting crop growth and production. In this experiment, the effect of hydrogen peroxide (H_2O_2) application on water-stressed corn plants was investigated using various biochemical and molecular methods. Corn seedlings grown in hydroponic culture were treated with 2 mM H_2O_2 and subsequently exposed to water stress using polyethylene glycol 6000 at three levels: 0, -2 bar, and -4 bar. The results showed that drought stress significantly altered all of the studied traits. With an increase in stress levels, the activity of the catalase enzyme was decreased, and the highest drop, 50%, occurred eight days after stress. It was revealed that catalase activity increased by up to 18% on the second day after the stress, but it decreased significantly over time. The indigenous accumulation of H_2O_2 increased significantly in the -4 bar treatment four days after stress, while it was reduced by 50% on the eighth day post-stress. It was revealed that H_2O_2 application increased *PAO* gene expression 1.7 fold compared to the control plants. Its expression was decreased by 35% at -4 bar in control plants, while H_2O_2 treatment increased its expression by 2.8 times. These results indicate that H_2O_2 application enhanced tolerance to drought stress in corn plants.

Keywords: antioxidant enzymes, drought stress, hydrogen peroxide, maize.

Introduction

Corn is a vital crop used for both human consumption and livestock feed. Similar to other plants, corn is affected by many abiotic stresses throughout its growth period. Among these stresses, water scarcity stands out as one of the most important environmental factors strongly impacting corn production. Insufficient water during the initial growth phase diminishes the survival rate of seedlings, elevates abortion rates, and ultimately leads to a decrease in yield (Farooq et al., 2009). To overcome the problems caused by drought stress, plants deploy several strategies, including escape, tolerance, and resistance mechanisms. Many of these responses result from the expression of inducible genes during physiological, biochemical, and molecular defense mechanisms. For instance, the physiological processes involved in biotic (Ramezani et al., 2017) and abiotic (Mahdavian et al., 2021) defense responses. Different genetic engineering approaches have been employed for more than four decades to improve plant tolerance to different biotic and abiotic stresses (Dehestani et al., 2010; Dolatabadi et al., 2014). To achieve this goal, studying physiological responses and the diverse expressions of genes is crucial for comprehending the intricate physiological mechanisms involved in responding to drought stress.

Corn seedlings subjected to drought stress conditions exhibit various physiological responses, including reduced cell expansion, leaf curling, reduced CO_2 exchange, diminished chlorophyll content and photosynthetic efficiency (Mittler, 2006). Gonçalves et al. (2019) observed a decrease in net photosynthesis rate, relative growth rate, leaf area index, grain yield, and harvest index due to drought stress. Chen et al. (2015) demonstrated in their study that drought stress damages both the recipient and receptor components of photosystem II, including the photosystem reaction center II, and the photosystem receptor part I which eventually reduces the efficiency of electron transfer and photosynthesis.

Numerous studies have been conducted to identify genes associated with metabolic and regulatory enzymes, as well as photosynthesis in response to drought stress. In an experiment, Wang et al. (2018) investigated the effects of drought stress on the physiological responses and gene expression of corn seedlings. They observed that drought stress strongly affected water content, leaf size, and photosynthetic parameters, resulting in a substantial decrease in seedling growth. They also observed that several genes with different expression patterns under stress conditions, influencing both photosynthetic and hormone biosynthesis systems. These genes with distinct expressions have the potential to be utilized in enhancing and refining drought-tolerant corn lines. When plants are subjected to stress, the plant's antioxidant system is activated. This activation involves an increase in the activity of the catalase enzyme, which serves as the primary defense barrier against the assault of oxygen radicals. In this way, the system resists the damage caused by drought stress (Maiti and Satya, 2014). As long as the plant can control the amount of superoxide produced in the plant, this process continues. To achieve this, non-enzymatic antioxidants collaborate with scavenging enzymes like catalases and peroxidases to eliminate hydrogen peroxide (H_2O_2). Therefore, knowledge of antioxidant genes and enzyme activities at a specific level of drought tolerance can be beneficial. Studies indicate a correlation between drought tolerance and antioxidant defense capacity (Farooq et al., 2009).

Tolerance inducers are biological or chemical factors that trigger various defense responses during stress, leading to notable changes in physiologic (Barzegargolchini et al., 2017), antioxidative (Moradi et al., 2016), and metabolic (Ramezani et al., 2017) functions. The foliar application of H_2O_2 results in an increase in photosynthetic pigments, water content, and lipid oxidation rate, coupled with a reduction in cellular H_2O_2. Simultaneously, it enhances the activity of antioxidant enzymes such as catalase, superoxide dismutase and ascorbate peroxidase, glutathione reductase, and malondialdehyde (MDA). This increased tolerance to drought stress is attributed to the modulation of the antioxidant system (He and Gao, 2009; Guler and Pehlivan, 2016). In a study investigating the foliar application of H_2O_2 to alleviate the effects of drought stress in soybean, Ishibashi et al. (2011) demonstrated that the application of this substance reduced the wilting of

leaves and increased the rate of net photosynthesis and stomatal conductance in the leaves. This research aims to investigate the effect of the externally applied H_2O_2 on drought stress, and the biochemical and molecular responses of corn plants.

Materials and Methods

Plant growth and treatment

The seeds of a single cross hybrid "704" corn plant were grown under hydroponic conditions using Hoagland's solution in a greenhouse with 16 hours of light at a temperature of 25 ˚C and 8 hours of darkness. After germination and establishment of seedlings, foliar application of H_2O_2 (2 mM) was administered at 21 days of age. Subsequently, at 28 days of age, drought stress was applied using polyethylene glycol 6000 at three different levels (0, -2 bar, - 4 bar). After the application of drought treatment, leaf samples were collected on day 28 at the 4-leaf stage, with stress duration (period) of 1, 2, 4, and 8 days. This experiment was carried out with three replications in a factorial experiment designed with a completely randomized (CRD) layout. Leaf samples were placed in liquid nitrogen immediately and transferred at -80 ˚C for further molecular and enzyme analysis. The data were subjected to the analysis of variance using SPSS-19 software. Means were statistically compared by Duncan test at $P < 0.01$ level.

Measurement of metabolites

Hydrogen peroxide

Leaf H_2O_2 content was measured using 0.1% trichloroacetic acid solution. Thus, 0.2 grams of powdered leaves were mixed with 1800 microliters of TCA solution in liquid nitrogen. After 12000g centrifugation, the liquid phase was used to measure H_2O_2. To 0.5 ml of liquid phase prepared above, we add one ml of 1M KI and 0.5 ml of 10 mM phosphate buffer (pH=7). The sample was placed in the dark at room temperature for 15 minutes and then the absorbance was read at 390 nm. H_2O_2 standard in concentrations of 100 to 250 nanomoles was used to draw the standard curve (R2=0.98, Y=0.4349X+ 0.0771), (Alexieva et al., 2001).

Measurement of total phenolic contents

About 0.2 gr of powdered leaves were combined with 1800 microliters of pure methanol. Then it was placed in an ultrasonic bath for 30 minutes. After centrifugation (10 minutes – 1000 g), the liquid phase was used to measure the amount of phenolic compounds. 100 microliters of methanol extract were combined with 200 microliters of 10% folin reagent, and then 800 microliters of sodium carbonate were added to it and left at room temperature for 3 hours. The absorbance was read at 765 nm, and the amount of total phenolic compounds was calculated based on gallic acid standard (concentrations 0 to 10 µg/ml, Y=0.0197X + 0.0019, R2=0.98) (Singleton and Rossi, 1965).

Antioxidant enzyme activity assay

Preparation of enzyme extract and estimation of protein content

Powdered leaves (0.1 gr) were homogenized in liquid nitrogen with 1800 microliters of cold potassium phosphate buffer (pH=7.5) and subsequently centrifuged for 20 minutes at 12,000 g at 4 °C. Then the liquid phase was removed and kept in a freezer at -20 °C for further studies. The amount of extracted protein was estimated using the method of Bradford. For this purpose, 40 microliters of enzyme extract were combined with 960 microliters of Bradford's reagent and after 20 minutes, the absorption of the samples was recorded at a wavelength of 530 nm. A standard curve was drawn using Bovine Serum Albumin (BSA) (Y=0.019 X + 0.027, R2=0.98). This parameter was used to correct the amount of enzyme activity.

Catalase enzyme activity

Catalase enzyme activity was assessed by recording the decrease in absorbance at 240 nm (Aebi, 1984). The reaction components included 2.450 ml of 50 mM phosphate buffer (pH=7), 0.5 ml of 7.5 mM H_2O_2, and 50 microliters of enzyme extract. After adding H_2O_2, the reaction started and the 240 nm decrease in absorbance was estimated in 3 minutes (every 30 seconds). Catalase enzyme activity was calculated by the method of Bergmayer (1983).

RNA extraction, cDNA, and RT-PCR

Total RNA was extracted from leaf samples by Threezol (Riragene, Iran), and RNA quality was determined by spectrophotometry and agarose gel electrophoresis. cDNA was made using the SinnaClone kit. For this purpose, first, 5 microliters of RNA treated with Dnase1 was mixed with 1

microliter of primer oligo(dt), 1 microliter of10 mM dNTP mix and 3 microliters of DEPC water, and placed at 70 °C for 5 minutes. Then it was placed on ice for 2 minutes. Four microliters of 5X buffer, one microliter of MMuLV enzyme, one microliter of RNase inhibitor, and four microliters of DEPC treated water were added to the above solution and placed at 42 °C for 50 minutes. Then it was placed at a temperature of 85 ˚C for 5 minutes. Finally, the reaction product was kept in a freezer at -20 °C.

ABI-step one plus real-time PCR machine was used to check gene expression. Each 12-microliter reaction contained 6 microliters of SYBR green master mixs (Amplicon), 15 micromoles of each primer, and 2 microliters of diluted cDNA (five-fold), and nuclease-free water. The *PAO* gene was designed using prime software and NCBI database data (Table 1). The *GAPDH* gene was used as a reference gene.

Results

The overall results demonstrated significant effects of the treatments, namely drought stress and exogenous application of H_2O_2, on the studied traits (Table 2).

Catalase activity

The amount of catalase enzyme activity did not show significant changes on the first day after drought stress. However, on the second day, it significantly decreased under -4 bar stress compared to the control. In addition, it was decreased at -2 and -4 bar levels. Upon eighth-day stress period, it showed a decrease of 18% at -4 bar stress level. The most substantial decrease in the amount of catalase enzyme activity, compared to the initial control, occurred at -4 bar stress on the eighth day following the stress, reaching less than half of the initial level (Figure 1-a). The use of H_2O_2 on the first day resulted in a decrease in the amount of catalase enzyme, but on the second day, we saw a significant increase in the enzyme level, and on the eighth day, a 35% decrease in the amount of catalase activity was observed (Figure 1-b). With the increase of drought stress, the amount of catalase enzyme decreases, and in the stress of -4 bar, we saw a decrease in the activity of catalase enzyme by about 16%, and the use of peroxide inducer increased the amount of enzyme at the beginning, and there was a significant decrease in the activity of catalase enzyme on the eighth day stress period (Figure 1-c).

Hydrogen peroxide assay

On the first and second days after drought stress, the amount of H_2O_2 decreases at the level of -4 bar. However, over the four-day drought stress period, we observed an increase in the amount of H_2O_2 at the -4 bar level and a decrease at the -2 bar level. On the eighth day after the stress, we again observed a significant decrease in the amount of H_2O_2 at the -4 bar level (Figure 2-a). By foliar application with H_2O_2 inducer on the second and fourth days of the stress, the amount of internal H_2O_2 was increased. But on the eighth day period, compared to the first day, its quantity was reduced by 50% (Figure 2-b). The foliar application of H_2O_2 at the stress of -2 bar level resulted in a 20%increase in the H_2O_2 concentration (Figure 2-c).

Table 1. Primer sequences used for the real-time PCR.

Gene name	Forward Sequence	Reverse Sequence
GAPDH	CCCTTCATCACCACGGACTA	CTCACCCCACGGGATTTC
Polyamine oxidase (*PAO*)	GCAAGTACCATGTCCAGGG	CGAGGGAACATGGCTGTCA

Table2. Analysis of variance for the effect of treatments on studied traits.

S.O.V	df	CAT	Phenol	H_2O_2	Protein
Treat(T)	1	0.001**	0.009*	0.529**	0.009^{ns}
Stress(S)	2	0.001**	0.002^{ns}	2.018**	0.001^{ns}
Period (P)	3	0.009**	0.047**	6.797**	0.002^{ns}
T×S	2	0.001^{ns}	0.018**	1.453**	0.145**
T×P	3	0.003**	0.015**	0.479**	0.012**
S×P	6	0.001**	0.01**	0.846**	0.012*6
T×S×P	6	0.002**	0.012**	1.101**	0.005^{ns}
Error	48	0.001	0.001	0.07	0.002
% CV		8.65	13.81	11.32	10.23

*, ** Significant at the 5% and 1% levels of probability respectively, and ns (non-significant)

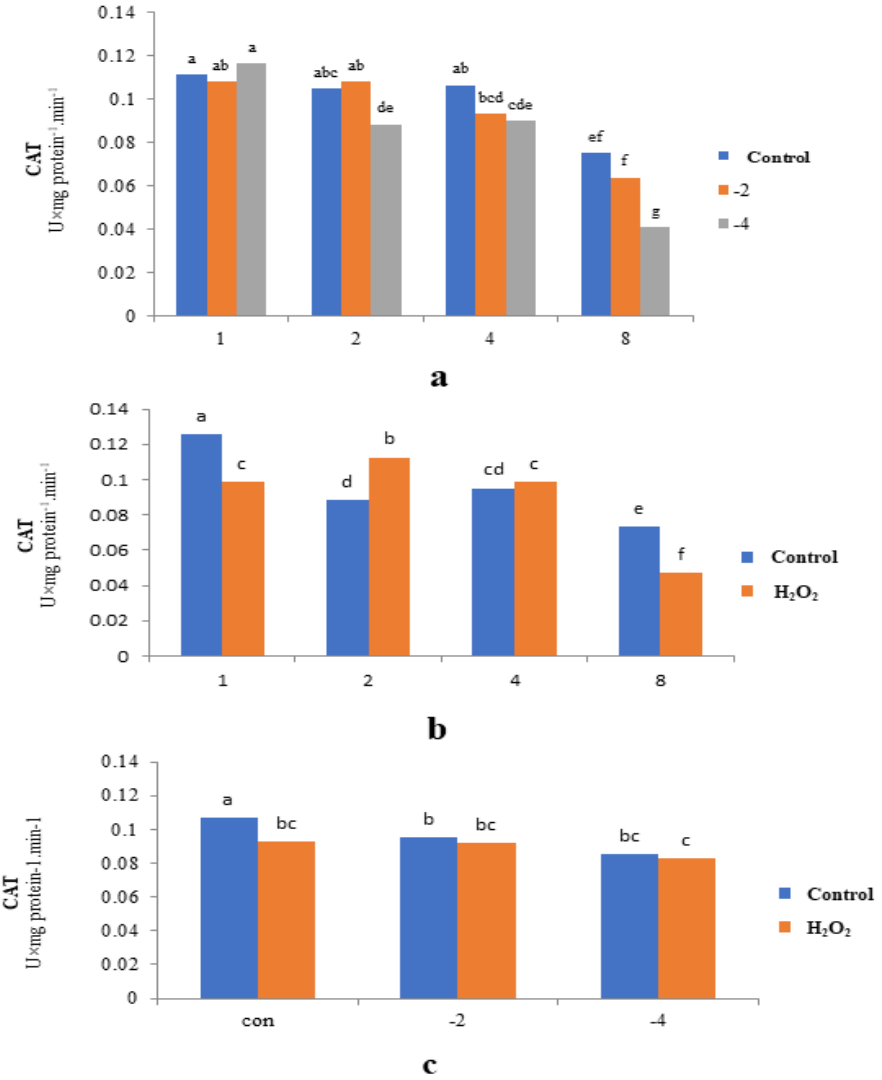

Figure 1. Effects of drought stress (3 levels) at four periods (a), inducer and periods (b), and inducer and drought stress on catalase activity (c).

Total phenolics content

On the first day, there was no change in the phenol content. However, on the second day, a significant increase in the amount of phenol was observed at the stress of -2 bar level. From the fourth day, the phenol content was increased by 14% and 26%, at the -2 and -4 bar levels, respectively. On the eighth day after stress, the amount of phenol was reduced again at the level of -4 bar (Figure 3-a). Using H_2O_2 on the first days after stress increased the amount of phenol. But from the 8th day after stress, the amount of phenol decreased (Figure 3-b). Application of H_2O_2 at -2 bar stress level caused a 20% increase in the amount of phenol. In the first days after the stress, the application of H_2O_2 at the level of -2 bar caused a 20% increase in the amount of phenol, and with the continuation of the drought stress and on the eighth day after the stress, we saw a 15% decrease in the amount of phenol (Figure 3-c).

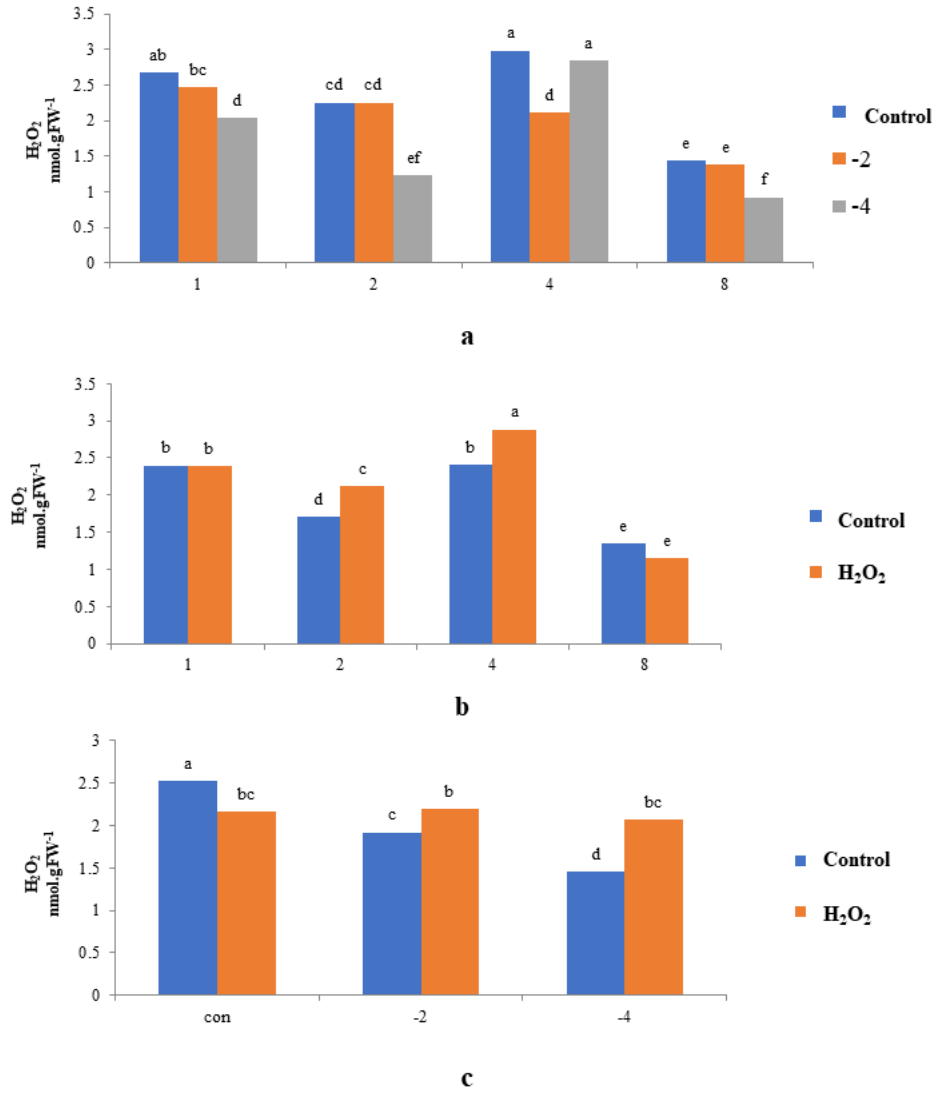

Figure 2. Effects of drought stress (3 levels) at four periods (a), inducer and period (b,) and inducer and drought stress on H_2O_2 content (c).

Total protein content

The protein content did not change significantly on the first days of stress. However, on the eighth day following drought stress at -2 and -4 bar, an increase of 18.5% and 21.5% was observed, respectively (Figure 4-a). The application of H_2O_2 on the first days after drought stress did not have any significant changes in the amount of protein. But on the eighth day period of stress, the protein concentration increased by 17% (Figure 4-b). In non-stressed conditions, the use of H_2O_2 led to a decrease in the amount of protein, but in drought stress of -4 bar, it increased by 31% (Figure 4-c).

PAO gene expression:

The expression of the *PAO* gene was increased 1.7 fold compared to the control under normal conditions with the use of a H_2O_2 inducer. Applying -4 bar stress during four-day stress period, decreased the expression level in the control sample by 35%, and the treatment with H_2O_2 stimulus at this stress level increased the expression level of this gene by 2.8 fold. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control gene (Figure 4-d).

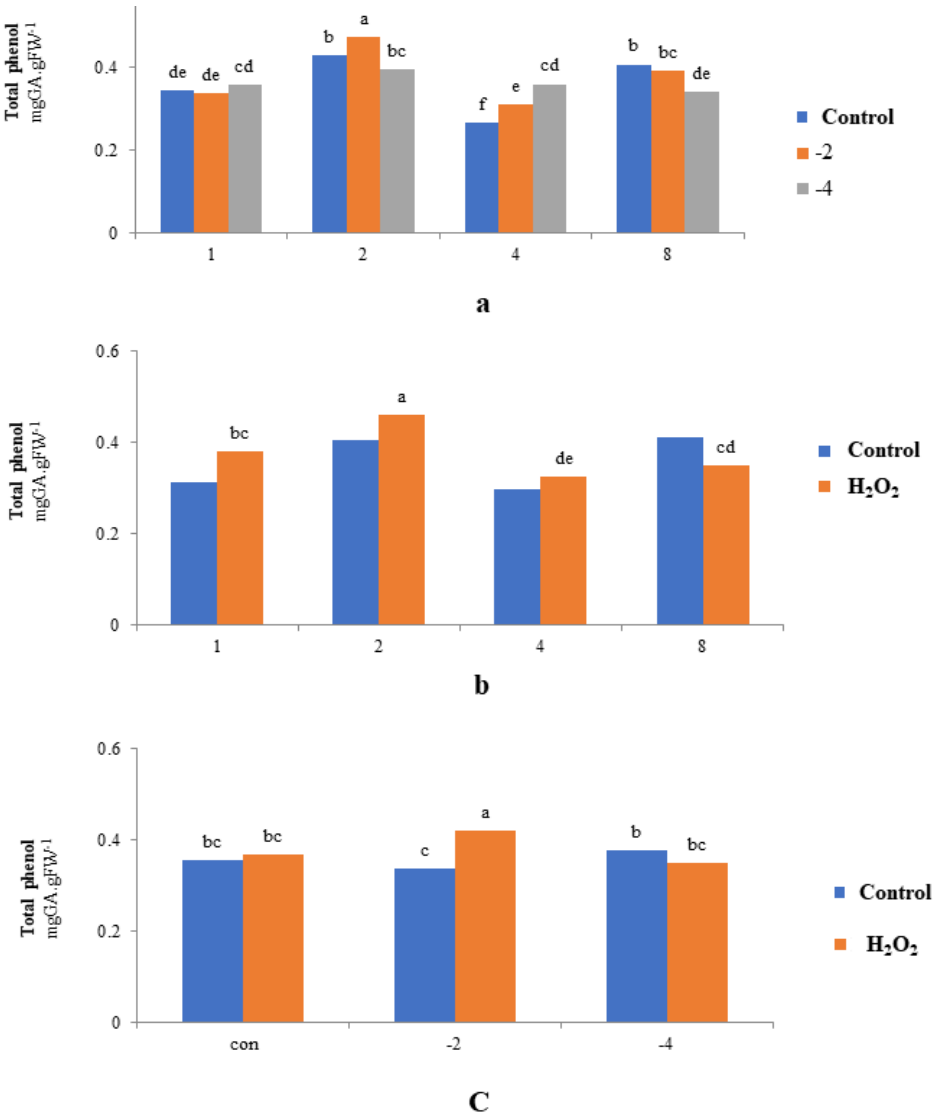

Figure 3. Effects of drought stress (3 levels) at four periods (a), inducer and period (b) and inducer and drought stress on total phenolic content (c).

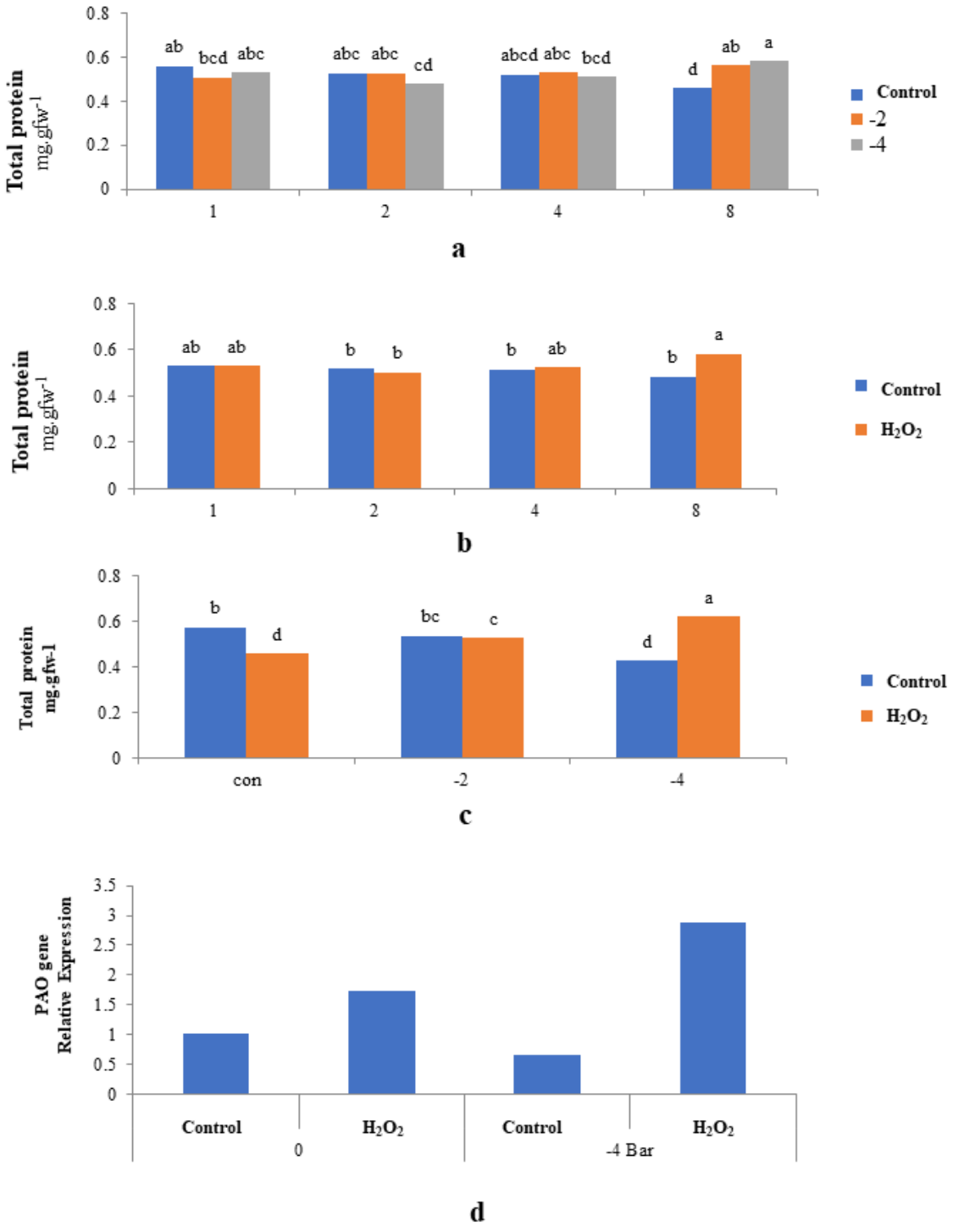

Figure 4. Effects of drought stress (3 levels) at four periods (a), inducer and period (b) and inducer and drought stress on total phenolic content (c), and effects of drought stress and inducer on *PAO* gene expression (d).

4. Discussion

Drought is critical environmental stress, especially prevalent in arid and semi-arid regions worldwide, significantly impacting plant yield. Drought stress disrupts physiological and biochemical processes, posing challenges to crop production. The response to drought stress is a highly intricate process. Indeed, drought stress influences plants from various aspects and manifests at different morphological, biochemical, and molecular levels. This includes impeding growth, the accumulation of compatible organic substances, and alterations in the expression of stress-responsive genes. Drought stress results in the generation of large amounts of reactive oxygen species (ROS), leading to oxidative stress (Hussain et al., 2018). Drought stress also leads to alteration in metabolic processes, steering them towards biosynthesis of the secondary metabolites. Consequently, there will be an upsurge in the biosynthesis of important bioactive compounds such as polyphenols, terpenoids, and alkaloids. To alleviate the damage caused by ROS, plants deploy antioxidant mechanisms that encompass non-enzymatic components, polyphenols, ascorbate, and glutathione, carotenoids, as well as enzymes such as catalase, superoxide dismutase, ascorbate peroxidase, peroxidase, polyphenol oxidase, and glutathione reductase. The activity of both enzymatic and non-enzymatic antioxidants is crucial in scavenging ROS. This will enhance the plant's ability to tolerate stress (Agarwal and Pandey, 2004).

Antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD)play a protective role in safeguarding the photosynthetic systems of plants exposed to environmental stress (Cavalcanti et al., 2004). Catalase enzyme destroys H_2O_2 produced in photorespiration pathways inside peroxisomes (Mittler, 2002). Catalase is an antioxidant enzyme that halts the chain reactions of free radicals and protects plants against oxidative stress by eliminating H_2O_2 (Rukmini et al., 2004). In the present study, a significant decrease in catalase enzyme activity was observed concomitant with the increase in drought levels. The most substantial decrease occurred at the -4 bar level, specifically on 8 days post- stress, where the activity was halved. The elevation in the amount of H_2O_2-type ROS under -4 bar stress conditions, coupled with the persistence of these conditions, resulted in the reduced activity of antioxidant enzymes including catalase.

The H_2O_2 foliar application led to an increase in the amount of catalase enzyme by up to 18% on the second day following stress. However, its concentration was decreased significantly with ongoing drought stress particularly by the eighth day. Similar results were reported in drought stress (Ahmed et al., 2022) and salinity stress (Abdel Latef et al., 2019). Catalase is one of the iron-containing proteins and it is activated in plant cells when the amount of h2o2 in the environment is high. Catalase plays an important role in removing H_2O_2 in peroxisome. Antioxidant enzymes such as catalase play an effective role in tolerance to drought stress (Hameed et al., 2013). Catalase enzyme, as a compatible osmolyte and anti-ROS enzyme, while protecting macromolecules and cell membranes, neutralizes the damage caused by ROS caused by drought stress (Hameed et al., 2013).

Reactive oxygen species (ROS) are produced in various plant cellular organelles including chloroplast, mitochondria, and peroxisomes under normal conditions as part of the processes involved in the electron transport chain of respiration and photosynthesis. Disturbance in oxidant-antioxidant balance and growth conditions in plants increases the cellular concentration of ROS (O_2, H_2O_2, and OH). Among these, H_2O_2 acts as a stress signal transmitter due to its relative stability compared to other ROS and its diffusion capacity through the membrane and intercellular space and is involved in various physiological functions in plants (Anjum et al., 2022). During evolution, plants develop an effective ROS removal system that includes a set of enzymatic and non-enzymatic antioxidants to control excess ROS generated in cells (Singh et al., 2020). Treatment with H_2O_2 helps in stress tolerance by starting the ROS removal mechanism in different plants (Dikilitas et al., 2020). Ahmed et al. (2022) stated during an experiment that drought stress increases the concentration of internal H_2O_2 and spraying with H_2O_2 reduces its concentration, which effectively reduces the effects of oxidative stress. In this experiment, we saw a 22% increase in the amount of internal H_2O_2 at -4 bar stress and on

the fourth day after the stress, and with the continuation of the stress on the eighth day, we again had a decrease in the amount of internal H_2O_2 at the level of -4 bar. The external application of H_2O_2 caused a 32% decrease in the amount of internal h2o2 on the eighth day after stress. H_2O_2 foliar application at the level of -2 bar increased the concentration of internal H_2O_2 by 20%, which is due to the low intensity of stress and the activation of the internal messenger system. Similar results were reported by Guler and Pehlivan (2016) in soybean, in mustard under stress (Alam et al., 2013), and in wheat under stress (Abdel Latef et al., 2019). H_2O_2 serves as a messenger molecule that plays multifaceted functions at different levels in plants. This molecule, in collaboration with hormones and other messenger molecules, regulates the metabolism of plants, thereby contributing in stress tolerance (Smirnoff and Arnaud, 2019).

In an experiment, the effect of different stresses including drought, salinity, cold, and heat was investigated on four wheat cultivars by Kamal et al. In this experiment, on the 8th day after drought stress at -2 and -4 bar levels, the amount of total protein increased by 18.5% and 21.5%, respectively. The use of H_2O_2 under stress conditions of -4 increased the amount of protein by 31%. Drought stress leads to the alteration of metabolic processes towards biosynthesis activities. Therefore, the biosynthesis of proteins increases (Agarwal and Pandey, 2004).

Phenolic compounds, encompassing flavonoids and tannins, are the most abundant secondary metabolites that assume diverse molecular and biochemical functions within plants. They contribute to essential processes, including the scavenging of free radicals, mediation of auxin transport, participation in signaling pathways, and bolstering plant defense mechanisms (Soleimani et al., 2022). These multifaceted roles underscore the significance of phenolic compounds in orchestrating various physiological and protective aspects vital for the overall well-being and resilience of plants (Kiani et al., 2021; Soleimani et al., 2022). In the current study, an elevation in phenol levels was observed on the second day following exposure to stress level of -2 bar. By the fourth day, under stresses of -2 and -4 bar, the phenol content demonstrated an increase of 14% and 16%, respectively. Additionally, the foliar application of H_2O_2 foliar application on the first and second days after stress contributed to the augmentation of phenol levels. On the eighth day under a stress level of -4 bar, the amount of phenol decreased by 15%. Desoky et al. (2021) stated that increasing the levels of drought stress led to an increase in proline, phenol, and flavonoid content; thereby the highest amount was observed in the drought stress of -10 bar. Increasing the concentration of phenol is effective in eliminating free radicals and inhibiting lipid peroxidation (Blasco et al., 2013). In many studies, a positive correlation between phenolic content and antioxidant activity has been reported, and some of the mechanisms of antioxidant activity of these compounds are inactivating lipid free radicals and preventing the decomposition of hydroperoxides into free radicals, as well as their ability to chelate metal ions (Banerjee and Roychoudhury, 2019). Researchers stated that the tolerance of some plants against environmental stresses including drought, can be related to the accumulation of phenolic compounds in these plants. One of the important features of phenolic compounds is the antioxidant property that is related to the hydroxyl groups in their molecular structure (Halliwell and Gutteridge, 2015; Sehar et al., 2021).

Polyamines (spermidine and spermine) are involved in a complex messenger network and play an essential role in stress tolerance (Pal et al., 2015). Polyamine oxidase (PAO) oxidizes spermidine (Spd) and spermine (Spm) (Flores and Filner, 1985). Most of the polyamine biosynthetic genes are up-regulated by abiotic stresses despite a difference in timing and the degree of induction (Liu et al., 2011; Wang et al., 2011). The application of polyamines can modulate drought responses (Li et al., 2014; Ebeed et al., 2017). In this experiment, the expression level of the *PAO* gene in the control sample decreased by 35% on the fourth day of -4 bar stress, whereas the exogenous application of H_2O_2 increased the expression by 2.8-fold. As a result, the expression of *PAO* gene increases with H_2O_2 spraying.

Conclusion

The results of the analysis indicate that drought stress significantly altered the amount of internal

H_2O_2, catalase enzyme, phenolic compounds, and protein content. Additionally, this stress condition induced oxidative damage and an increase in ROS. The expression level of the *PAO* gene experienced a 35% decrease under -4 bar drought conditions. Treatment with H_2O_2 stimulus at this stress level increased the expression level of this gene by 2.8 times, thereby mitigating the negative effects of drought stress. As a result, foliar application of a low dose of H_2O_2, taking into account its role in signaling and the regulation of gene expression, yielded significant effects on the levels of investigated compounds. Ultimately, this approach is anticipated to reduce the damage caused by drought stress.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

AD designed research; HV performed research and analyzed the data; SN, PM, and AD interpreted the data and were major contributors in writing the manuscript and revising it critically; All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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کاربرد پراکسید هیدروژن موجب افزایش واکنش گیاه ذرت (*Zea mays* L.) به تنش خشکی

حمید ولی زاده[۱]، سعید نواب پور[۲]*، علی دهستانی[۲]، پویان مهربان[۳]

[۱].گروه اصلاح نباتات و بیوتکنولوژی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران
[۲].مؤسسه ژنتیک و بیوتکنولوژی کشاورزی طبرستان، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، ایران
[۳].گروه علوم پایه، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، ایران

چکیده: تنش خشکی عامل مهمی است که رشد و تولید محصول زراعی را محدود می کند. در این مطالعه، اثر کاربرد پراکسید هیدروژن (H_2O_2) بر روی گیاهان ذرت تحت تنش آبی با استفاده از روش‌های مختلف بیوشیمیایی و مولکولی مورد بررسی قرار گرفت. بوته‌های ذرت رشد یافته در کشت هیدروپونیک با ۲ میلی‌مولار H_2O_2 تیمار شدند و متعاقباً با استفاده از پلی اتیلن گلیکول ۶۰۰۰ در سه سطح ۰، ۲- و ۴- بار در معرض تنش آبی قرار گرفتند. نتایج نشان داد که تنش خشکی به طور معنی‌داری تمامی صفات مورد مطالعه را تغییر داد. با افزایش سطح استرس، فعالیت آنزیم کاتالاز کاهش یافت و بیشترین افت به میزان ۵۰ درصد هشت روز پس از استرس مشاهده گردید. فعالیت آنزیم کاتالاز در روز دوم پس از آغاز تنش تا ۱۸ درصد افزایش یافت، اما با گذشت زمان به طور قابل توجهی کاهش یافت. تجمع داخلی H_2O_2 در بافت‌های گیاهی بطور قابل توجهی در تیمار ۴- بار چهار روز پس از استرس افزایش یافت، در حالی که در روز هشتم پس از استرس ۵۰ درصد کاهش یافت. نتایج نشان داد که کاربرد پراکسید هیدروژن بیان ژن *PAO* را ۱/۷ برابر نسبت به گیاهان شاهد افزایش داد. بطوری که بیان آن در سطح تنش بار ۴- به میزان ۳۵ درصد در گیاهان شاهد کاهش یافت، در حالی که تیمار H_2O_2 بیان آن را ۲/۸ برابر افزایش داد. این نتایج نشان دهنده افزایش تحمل به تنش خشکی در گیاهان ذرت تحت تاثیر تیمار H_2O_2 می‌باشد.

کلمات کلیدی: آنزیم‌های آنتی اکسیدان، تنش خشکی، پراکسید هیدروژن، ذرت.

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نویسنده مسئول
دکتر سعید نواب پور
s.navabpour@gau.ac.ir

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Contents:

Address:

Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT)
Sari Agricultural Sciences & Natural Resources University (SANRU)
Khazar Abad road, Sari, Mazandaran, Iran P.O.Box: 578

www.jpmb-gabit.ir Tel : +981133687744