

# Two splice variants of the DsMEK1 mitogen-activated protein kinase kinase (MAPKK) is involved different way in salt stress regulation in *Dunaliella salina*

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## Research

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# Abstract

**Background** *Dunaliella salina* can produce a large amount of glycerol under salt stress, which can quickly adapt to the change of external salt concentration, and glycerol is one of the ideal energy sources. In recent years, it has been reported that Mitogen-activated protein kinase cascade pathway plays an important role in regulating salt stress, and in *Dunaliella tertiolecta* DtMAPK can regulate glycerol synthesis under salt stress. Therefore, it is urgent to study the relationship between MAPK cascade pathway and salt stress in *D. salina*, and help it to increase the content of glycerol.

**Results** In our study, we identified and analyzed the alternative splicing of DsMEK1 (DsMEK1-X1, DsMEK1-X2) from the unicellular green alga *D. salina*. DsMEK1-X1, DsMEK1-X2 both localized in the cytoplasm. The qRT-PCR assays showed that DsMEK1-X2 induced by salt stress. Overexpression of DsMEK1-X2 revealed a higher increase rate of glycerol compared to the control and DsMEK1-X1-oe under salt stress. The expression of DsGPDH2/3/5/6 increased in DsMEK1-X2-oe strains compared to the control under salt stress. It means that DsMEK1-X2 is involved in the regulation of DsGPDHs expression and glycerol overexpression under salt stress. Overexpression of DsMEK1-X1 increasing the proline content and reducing the MDA content under salt stress, and DsMEK1-X1 can regulate oxidative stress, thus we speculate that DsMEK1-X1 can reduce the damage of oxidative under salt stress. Yeast two-hybrid analysis showed that DsMEK1-X2 can interact with DsMAPKKK1/2/3/9/10/17 and DsMAPK1, however, DsMEK1-X1 interacted with neither upstream MAPKKK nor downstream MAPK. DsMEK1-X2-oe transgenic lines increased the expression of DsMAPKKK1/3/10/17 and DsMAPK1, and DsMEK1-X2-RNAi lines decreased the expression of DsMAPKKK2/10/17. DsMEK1-X1-oe transgenic lines do not increased genes expression, except for DsMAPKKK9.

**Conclusion** Our findings demonstrate that DsMEK1-X1 and DsMEK1-X2 can response to salt stress in two different pathways, DsMEK1-X1 response to salt stress by reducing oxidative damage, however, DsMAPKKK1/2/3/9/10/17- DsMEK1-X2-DsMAPK1 cascade is involved in the regulated of DsGPDH expression and thus glycerol synthesis under salt stress.

## Background

Saline soil is a severely adverse environmental factor, and more than 800 million hectares of land is affected by excess salt concentrations[1]. Adaptive responses to salt stress can be grouped into three processes: osmotic stress, ionic stress, and detoxification response[2]. Among the various reported regulation mechanisms of salt stress[3–5], there are many studies on the regulation of salt stress by the MAPK cascade pathway[6]. MAPK cascade pathway is minimally composed of three kinase modules, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK)[7]. PLD $\alpha$ 1-derived PA binds to AtMPK6 and the activated AtMPK6 phosphorylates the Na<sup>+</sup>/H<sup>+</sup> antiporter, AtSOS1, reduced Na<sup>+</sup> accumulation in Arabidopsis leaves under salt stress[8]. DtMAPK in *Dunaliella tertiolecta* accumulation of intracellular glycerol under hyperosmotic shock[9]. In Arabidopsis, MKK4-MPK3 and MKKK20-MPK6 cascades mediate osmotic stress responses [10, 11]. MEKK1-MKK5/6

mediates the salt-induced expression of iron superoxide dismutases in Arabidopsis[12]. It is precise that MAPK cascade pathway plays an important role in the three processes caused by salt, so it is necessary to further study of MAPK cascade pathway.

The activation of these MAPK cascade pathway is a continuous process. In the MAPK cascade pathway, MAPKKK is phosphorylated by external stimuli as the first step of signal transduction, while MAPK regulates the downstream target to respond to external stimuli. So the current research focus is mainly on MAPKKK and MAPK. For example, AtMAPKKK20 mediates osmotic stress induced by salt[10]. OsDMS1, a Raf-like MAPKKK gene, responds to oxidative stress caused by salt by regulating the scavenging of ROS[13]. Furthermore, AtMAPK3/6-HSFA4A is involved in the modulation of ROS metabolism and responses to salt stress[14]. OsWRKY30 could interact with and be phosphorylated by OsMPK3/7/14. Phosphorylation of OsWRKY30 by MAPKs plays a crucial role in the process in which OsWRKY30 performing its biological function under salt stress[15]. However, there are few reports that MAPKK is involved in the regulation of salt stress.

MAPKK plays a pivotal role in MAPK cascade pathway, and perform different functions in signal distribution. It has been reported that OsMEK1 from *Oryza sativa*, is involved in different signal transduction processes. OsMEK1–OsMPK1 interaction might be involved in defense against pathogens as well as salt and drought stresses[16]. Meanwhile, the OsMEK1–OsMPK5 pair plays a role in the response to cold stress[17]. This phenomena occur in other species. In Arabidopsis, the AtMKK1–AtMPK6 pair was reportedly a critical mediator common to both sugar and ABA signaling during seed germination[18], while the AtMKK1–AtMPK4 pair was shown to be involved in the innate immunity[19]. On the contrary, this pattern also found in MAPKKK-MAPKK pairs. The ABA-activated MAPKKK17/18-MKK3 module in the crosstalk between ABA and auxin signaling[20]. According to the existing results, it is common for the same MAPKK to participate in the transduction of various signals in the different modules. In recent analysis, 19 MAPKs, 9 MAPKKs and 71 MAPKKK were identified in the maize genome[21]; 16 MAPKs, 6 MAPKKs and 89 MAPKKKs in tomato[22]; 15 MAPKs, 7 MAPKKs and 21 MAPKKKs in *Homo sapiens* (<http://genome.ucsc.edu/>); 5 MAPKs, 4 MAPKKs and 4 MAPKKKs in *Saccharomyces cerevisiae*[23]. In MAPK cascade pathway, the number of MAPKK gene families is the least. It is a puzzle that the number of MAPKK is limited, how does it deal with the complexity of the MAPK cascade pathway. The inspiration for solving this problem comes from the related researches of human MEK1 and its variant MEK1b. In human MEK1-ERK1/2 was directly involved in activating CDC25 during the G<sub>2</sub>/M transition to regulate mitosis[24]. Then Shaul et al. found out that MEK1b interacted with ERK1c that specifically regulates Golgi fragmentation[25]. After that, we found MEK1b, which lacks 26 amino acids within its kinase domain, was the alternative spliced isoform of MEK1[26]. This study suggests that the switch of the signaling route may be caused by alternative splicing of MEK1. These results imply that the alternative splicing of MAPKK is responsible for distributing a variety of signal transduction, which allows the MAPK cascade pathway to participate in a wider range of processes. However, the AS regulation mechanism of MAPKK was relatively unexplored, and the AS regulation mechanism of MAPKK has not been reported in salt stress.

*Dunaliella salina* is a kind of unicellular green algae without a rigid cell wall, which can survive in media containing a wide range of NaCl concentrations, ranging from about 0.05 M to saturation (around 5.5 M) [27]. The response of *D. salina* to the change of external salt concentration is mainly manifested in two ways: one is to rapidly change its cell size to maintain the balance of intracellular and extracellular osmotic pressure, and the other is to alleviate the change of osmotic pressure through the synthesis and transformation of intracellular glycerol [28]. However, there are no reports on how changes in external salt concentration trigger salt tolerance in *D. salina* cells. In plants, the MAPK cascade pathway mediates the regulation of abiotic stress as a signal transduction pathway, and DsMAPK1 participates in abiotic stress, so it is speculated that there are MAPK cascade pathways in *D. salina* involved in the regulation of salt stress.

Here, we report that DsMEK1 gene undergoes alternative splicing, producing two protein splice variants, the full-size DsMEK1-X2 form and the truncated DsMEK1-X1 form that contains the disrupted protein kinase domain. DsMEK1-X2 can interact with DsMAPK1 and DsMAPKKK1/2/3/10/17. However, so far, no interacting protein of DsMEK1-X1 has been found. DsMEK1 alternative splicing is induced under salt stress, producing more DsMEK1-X2 protein. DsMEK1 alternative splicing is also induced under oxidative stress, producing more DsMEK1-X1 protein. The expression of DsGPDHs was induced and the Glycerol content accumulated in the DsMEK1-X2-oe transgenic lines under salt stress. DsMEK1-X1-oe transgenic lines increase the content of proline and decrease the content of MDA under salt stress. These results suggest that DsMEK1 produces two different salt stress response pathways through alternative splicing: DsMEK1-X1 responds to salt stress by reducing oxidative damage, and DsMEK1-X2 responds to salt stress by regulating glycerol synthesis to maintain osmotic pressure balance.

## Results

### cDNA cloning and sequence analysis of the DsMEK1 gene

One specific band of the expected length was detected with 2% agarose gel electrophoresis, but DsMEK1 had an additional lower band (Fig. 1A). Considering the potential for AS, these bands were purified and transformed into *Escherichia coli* DH5 $\alpha$ , screened by colony PCR, and clones containing fragments of different lengths were then sequenced and a total of two transcript variants of DsMEK1 were obtained.

Two splicing variants, one in which thirteen introns were complete spliced (DsMEK1-X2), the other transcript variant DsMEK1-X1 lacked four exons (Fig. 1B). The DsMEK1-X1 gene's CDS is 1020 bp that encodes a 340 amino acid protein with an expected molecular weight of 37.07 kDa. The DsMEK1-X2 gene's CDS is 1464 bp which encodes a 488 amino acid protein with an expected molecular weight of 53.66 kDa. DsMEK1-X2 code for the full-length protein, whereas DsMEK1-X1 codes for a truncated protein, lacking a partial protein kinase domain and NTF2 domain (Fig. 1C).

A dendrogram was built based on the translated amino acid sequences of DsMEK1-X1, DsMEK1-X2, 10 *Arabidopsis* members (AtMAPKK1-10), 8 *O. sativa* members (OsMAPKK1-8), and 1 *C. reinhardtii* member

CrMAPKK1 (Fig. 2). The MAPKK proteins used in the analysis were divided into four groups, A–D[29]. DsMEK1-X1 and DsMEK1-X2 were categorized into subfamily B, which contains AtMKK3 and OsMKK3 suggesting that DsMEK1-X1 and DsMEK1-X2 possessed the close evolutionary relationship with AtMKK3 and OsMKK3. Previous studies revealed that AtMKK3 was induced by ABA or salt stress treatments[30], and treatment with methyl jasmonate (MeJA) or salicylic acid (SA) could induced the expression of OsMKK3[31]. Thus, the phylogenetic tree analysis suggested that DsMEK1-X1 and DsMEK1-X2 may involve in abiotic stress.

Database search (WoLF PSORT) with the DsMEK1 splice variant sequences pointed to the same targeting (cytoplasm). For validating the prediction, DsMEK1-X1::GFP, and DsMEK1-X2::GFP were expressed in Arabidopsis protoplasts (Fig. 3). As expected the DsMEK1-X1::GFP and DsMEK1-X2::GFP localized to cytoplasm. This suggested that the splicing does not alter the localization of DsMEK1s variants in the *D. salina*.

## DsMEK1 is regulated by alternative splicing

For many genes, AS leads to the production of functionally different protein isoforms, which may exhibit alterations in activity, interactive partners[32], localization[33], and patterns of expression[34]. To solve the last issue, we firstly analyzed the transcription levels of DsMEK1 isoforms under salt stress. DsMEK1-X2 was significantly up-regulated along with the salt treatment, while DsMEK1-X1 was nearly unaffected under salt stress (Fig. 4).

Given that DsMEK1-X2 was found to be involved in the regulate of salt stress, we constructed DsMEK1-X1 and DsMEK1-X2 overexpression lines named DsMEK1-X1-oe and DsMEK1-X2-oe, respectively, furthermore, we constructed a DsMEK1-X2 knock down mutant, DsMEK1-X2-RNAi (Fig. 5A). In all the transformants, the *Cmr* gene (573 bp) was found, confirming the correct insertion of DsMEK1s in the genome of the *D. salina* (Fig. 5B)[9]. Furthermore, qRT-PCR assays of DsMEK1-X1 and DsMEK1-X2 gene were performed in those lines, which confirmed that the related genes were overexpressed or knock down (Fig. 5C)[35].

MDA, proline, total sugar and so on are often used as indicators of salt tolerance in plants. To verify the function of DsMEK1-X1 and DsMEK1-X2, we detected some physiological indexes under salt stress in *D. salina*. Firstly, we analyzed the growth curve under the salt condition of these lines. The cell growth rate of DsMEK1-X2-oe lines increased and DsMEK1-X1-oe lines were not significantly different with that of control types, while DsMEK1-X2-RNAi lines were lower than that of the control lines (Fig. 6A). These results showed that DsMEK1-X2 can regulate salt stress and affect growth. The contents of total sugar content, proline content and MDA content were determined by the methods described previously. Under salt stress, the DsMEK1-X1-oe, DsMEK1-X2-oe, and DsMEK1-X2-RNAi lines were no difference with the control lines in total sugar content (Fig. 6B). On the other hand, both overexpression strains decreased MDA content compared with control, DsMEK1-X2-RNAi strains increased MDA content under salt stress (Fig. 6C). Furthermore, we also detected the proline content of the DsMEK1 transformants (Fig. 6D). The

proline content in DsMEK1-X1-oe and DsMEK1-X2-oe increase compared to the control. Interestingly, DsMEK1-X1-oe increase more than DsMEK1-X2-oe. The content of proline in DsMEK1-X2-RNAi was less than control (Fig. 6D). Previous reports that MDA and proline can reduce the damage of oxidative[2], so we speculate that DsMEK1-X1 involved in mitigating oxidative-stress damage under salt induced. We analyzed the expression level of DsMEK1-X1 and DsMEK1-X2 under oxidative stress. As shown in Fig. 7, DsMEK1-X1 can regulate oxidative stress, however, DsMEK1-X2 does not respond to oxidative stress, it means that DsMEK1-X1 is mainly involved in antioxidant defense in response to salt stress.

As we all know, *Dunaliella* can survive in a wide range of salt concentrations is attributed to its ability to adjust osmotic potential by changing intracellular glycerol concentration[36]. Given that DtMAPK was found to be involved in the regulation of glycerol synthesis in *D. tertiolecta* [9], we predicted that the DsMEK1 would regulate glycerol production in response to salt stress. To investigate this, we analysis the glycerol content under high salinity conditions (3.5 M NaCl concentration). As expected, glycerol content in DsMEK1-X1-oe, DsMEK1-X2-oe, and control could be enhanced significantly after salt stress. The glycerol content in DsMEK1-X1-oe has a similar increased rate compared with control, which both increased by about 40% after 30 min. Interesting, glycerol of DsMEK1-X2-oe increased almost 100% after 30 min of salt stress, it's 2.5 times than the control and DsMEK1-X1-oe lines. Then, DsMEK1-X1-oe, DsMEK1-X2-oe and the control have similar glycerol content after 2 h. DsMEK1-X2-RNAi strains have fewer changes in glycerol content under salt stress (Fig. 6E). These results indicating that overexpression of DsMEK1-X2 could enhance glycerol accumulation to mediate salt stress in *D. salina* cells.

The results reveal that DsMEK1 involved in the regulation of glycerol synthesis under salt stress (Fig. 4, Fig. 6). Glycerol-3-phosphate dehydrogenase (GPDH) is a rate-limiting enzyme in the glycerol synthesis pathway and intracellular glycerol concentration functions as the counterbalancing osmolyte in *D. salina*[37]. So we further analyzed the transcription levels of all DsGPDH (DsGPDH1-7)[38], which were reported to be involved in glycerol synthesis. The expression profile of these genes in the DsMEK1-X1-oe, DsMEK1-X2-oe, DsMEK1-X2-RNAi lines and control were analyzed by qRT-PCR under salt stress (Fig. 8). There was no difference in DsGPDH1/7 genes between the control strains and the DsMEK1-X1-oe, DsMEK1-X2-oe, DsMEK1-X2-RNAi lines under salt stress. The expression of DsGPDH4 was upregulated in all strains under salt stress. Those results mean that DsGPDH1/4/7 was not regulated by DsMEK1 under salt stress. DsGPDH2/3/5 genes were upregulated in the DsMEK1-X2-oe strains compared to control under salt stress. The expression of DsGPDH2 increased almost 4 times, the expression of DsGPDH3 was upregulated approximately 2.5 times and the expression of DsGPDH5 increased approximately 2 times in the DsMEK1-X2-oe strain compared to control. It is evidenced that DsMEK1-X2 can positively regulate DsGPDH2/3/5 expression and thus glycerol synthesis under salt stress. Furthermore, DsGPDH6 upregulated in the DsMEK1-X2-RNAi strains and control lines, it is speculated that DsGPDH6 was negatively regulated by DsMEK1-X2 under salt stress. Based on the data analysis, it was confirmed that DsMEK1-X2 can mediate DsGPDH2/3/5/6 and is essential for the regulation of glycerol synthesis under salt stress.

# Interaction of DsMEK1 Splice Variants with their Upstream and Downstream interactors

Alternative splicing could provide selective advantage for choosing upstream and downstream regulators [32, 39]. So we investigated the effect of AS on the regulation of the variants by performing Y2H assay. The Y2H results showed that DsMAPK1 and DsMAPKKK1/2/3/9/10/17 interacted with DsMEK1-X2 but not with DsMEK1-X1 (Fig. 9).

To further investigate the function of DsMEK1, we analyzed the expression of DsMAPKKK1/2/3/9/10/17 and DsMAPK1 in control, DsMEK1-X1-oe, DsMEK1-X2-oe and DsMEK1-X2-RNAi lines under salt stress (Fig. 10). Notably, DsMAPK1 and DsMAPKKK1/3/10/17 were up-regulated in DsMEK1-X2-oe lines under salt stress, DsMAPKKK2/10/17 were down-regulated in DsMEK1-X2-RNAi lines under salt stress. Combine with the results of Y2H, we confirmed that DsMAPKKK1/3/10/17-DsMEK1-X2-DsMAPK1 positively regulate salt stress, and DsMAPKKK2-DsMEK1-X2 negatively regulate it.

## Discussion

In an increasing number of species, alternative splicing is an important regulation mode under different stress conditions, allowing organisms to reprogram their regulatory networks [39, 40]. MAPK cascade pathway plays a pivotal role in plant development or stress response. In many species, the numbers of MAPKKK and MAPK are far more than MAPKK [29, 41, 42], the number of interaction pairs of MAPKKK-MAPKK and MAPKK-MAPK are also more than the number of MAPKK gene [42, 43]. This results indicate that the number of MAPKK genes does not match the functional diversity of the MAPK cascade pathway. The intriguing point is how the least number of MAPKK deal with this situation. The functional diversity of MAPK cascade pathway is related to the large number of MAPKKK and MAPK, and one of the reason for the high abundance of MAPKKK and MAPK is that they exist alternative splicing [32, 33], so we speculate that MAPKK also undergo alternative splicing. Thus increasing the number of MAPKK to cope with the functional diversity of MAPK cascade pathways. However, there are few reports about it. In the present study, we searched the *D. salina* transcriptome database and identified two alternative splicing genes, DsMEK1-X1 and DsMEK1-X2. The lengths of the DsMEK1-X1 and DsMEK1-X2 were 340 and 488 amino acids, respectively. This study is helpful for us to understand the regulation of DsMEK1 alternative splicing on stress response network.

Alternative splicing can produce proteins with different biological functions to help organisms deal with a variety of stresses[44]. In *Arabidopsis*, HAB1 encode HAB1.1 and HAB1.2. HAB1.1 switching the ABA signaling off, while, HAB1.2 keeping the ABA signaling, that play opposing roles in ABA-mediated seed germination and ABA-mediated post-germination developmental arrest[45]. Alternative splicing events that allow rapid adjustment of the abundance and function of key stress-response components. In our research, AS of DsMEK1 can produce two proteins with different biological functions in *D. salina* to deal with different effects caused by salt stress. We found that DsMEK1-X1 overexpression strains have a similar increase rate of glycerol with the control after salt stress (Fig. 6E). Hence it can be suggested that

DsMEK1-X1 response to salt stress independent with glycerol synthesis. In DsMEK1-X1-oe and DsMEK1-X2-oe lines, the content of MDA both less than control lines under salt stress (Fig. 6C). Furthermore, the proline content both increased in DsMEK1-X1-oe lines, and DsMEK1-X2-oe lines compared with control under salt stress. The increase rate of proline in DsMEK1-X1 lines was slightly higher than that in DsMEK1-X2 lines (Fig. 6D). Previous studies of plant have reported that proline can protect the plasma membrane by upregulating activities of various antioxidant systems to minimize membrane lipid and protein oxidation resulted from salinity-induced oxidative stress[46], MDA is the product of cell membrane lipid peroxidation and the content of MDA increased can leads to plant metabolic disorders[47]. Combining with the qRT-PCR assay shown that DsMEK1-X1 was induced under oxidative stress (Fig. 7). Based on our results, we speculated that DsMEK1-X1 can mediate various antioxidant systems to reduce the damage of oxidative and help *D. salina* adaptive to salt stress (Fig. 11). The glycerol accumulation rate of DsMEK1-X2-oe strains was 2.5 times than that of the control after 30 minutes of salt stress, however, in DsMEK1-X2-RNAi strains, there was less change in glycerol content under salt stress (Fig. 6E). Furthermore, DsGPDH2/3/5 were upregulated in DsMEK1-X2-oe strains and DsGPDH6 was induced in DsMEK1-X2-RNAi strains under salt stress (Fig. 7). These results revealed that DsMEK1-X2 is essential for the regulation of glycerol synthesis by activating the expression of DsGPDHs in response to salt stress. Our work provides insight into the alternative splicing of DsMEK1 responding to different process caused by salt stress.

The different functions of DsMEK1-X1 and DsMEK1-X2 are attributed to the change of regulatory network caused by alternative splicing. We found that there were differences in the interaction networks between DsMEK1-X1 and DsMEK1-X2 through in vitro, in vivo experiments. Yeast two-hybrid assay revealed that DsMEK1 variants displayed a difference in choosing upstream and downstream interactors, DsMAPKKKs and DsMAPK1. DsMEK1-X2 interacted with DsMAPKKK1/2/3/9/10/17 and DsMAPK1, however, DsMEK1-X1 interact with neither DsMAPKKK1/2/3/9/10/17 nor DsMAPK1 (Fig. 9). Under salt stress, the overexpression strains or knock down strains of DsMEK1-X2 could change the expression of DsMAPKKK1/2/3/10/17 and DsMAPK1, but the overexpression strains of DsMEK1-X1 did not affect the expression level of these genes, except for DsMAPKKK9 (Fig. 10). Summarizing the present knowledge on DsMEK1-X2, we put forward the hypothesis of the MAPK cascade pathway in *D. salina* (Fig. 11). DsMAPKKK1/2/3/10/17-DsMEK1-X2-DsMAPK1 cascade pathways were essential for the regulation of glycerol synthesis by activating the expression of DsGPDHs in response to salt stress. In Y2H and in vivo overexpression experiments, we did not find the interacting proteins of DsMEK1-X1, so we just constructed the overexpression strain of DsMEK1-X1, knock-down strain of DsMEK1-X1 were not constructed for further study. The difference between DsMEK1-X1 and DsMEK1-X2 interaction networks may be caused by protein structure. DsMEK1-X1 lacks a part of the NTF2 domain (Fig. 1C), and it has been reported that the knockout of NTF2 will lead to the weakening of protein interaction ability[48], so we speculate that the difference of NTF2 domain may lead to the change of DsMEK1-X1 and DsMEK1-X2 regulatory networks. There are two possible reasons for the interaction network of DSMEK1-X1 was not found in this study: 1. In the initial predicted interaction network, because of the high similarity between DsMEK1-X1 and DsMEK1-X2, the results of the interaction network predicted by STRING database were

the same. May have not hit the interaction network of DsMEK1-X1; 2. DsMEK1-X1 has evolved a MAPK-independent phosphorylation pathway, and can directly phosphorylate downstream transcription factors, just like OsMAPK5, directly phosphorylation by CPK18, instead of MKK-dependent phosphorylation pathway[49]. In this paper, the MAPK cascade pathway of *D. salina* was studied systematically for the first time, which is helpful for us to further study the salt tolerance mechanism of *D. salina*.

## Conclusion

In conclusion, DsMEK1-X1 and DsMEK1-X2 in *D. Salina* were successfully cloned and characterized. We showed that overexpression of DsMEK1-X2 enhanced cell growth under salt stress. Overexpression of DsMEK1-X2 in *D. Salina* resulted in glycerol accumulation and DsGPDHs expression level change, and DsMAPKKK1/2/3/10/17-DsMEK1-X2-DsMAPK1 cascade involved in glycerol synthesis under salt stress (Fig. 11). These results confirmed that a MAPK signaling pathway, similar to yeast HOG pathway[50], may be involved in the salt stress of *D. Salina*. Overexpression of DsMEK1-X1 increase the content of proline and decrease MDA, and DsMEK1-X1 was induced by H<sub>2</sub>O<sub>2</sub>. These findings revealed that DsMEK1-X1 can reduce the damage of oxidative caused by salt stress (Fig. 11). Totally, the existence of AS forms has regulatory functions or diversify the function of the DsMEK1 gene in *D. salina*.

## Materials And Methods

### Unialgal strains, culture condition and growth pattern

*D. salina* strain 435 was provided by Freshwater Algae Culture Collection of the Institute of Hydrobiology, Wuhan, China. The culture was maintained in De Walne's medium and cultivated at 25 ° C and 40 μmol m<sup>-2</sup> s<sup>-1</sup> provided by cool-white fluorescent lamps, under 16/8 h light/dark cycle with various NaCl concentrations (1.5 M and 3.5 M) and 0.4 mM H<sub>2</sub>O<sub>2</sub>[51]. For transformation and selection experiments solid and liquid TAP mediums (Tris-Acetate-Phosphate) were used, adjusted to contain a final concentration of 0.5 M NaCl. For solid medium 2.5% agar was added[52].

Ten milliliters (7 × 10<sup>6</sup> cells) unialgal culture was inoculated in triplicate into 100 ml of De Walne's culture media and was grown under controlled laboratory conditions to study the growth pattern. Growth was measured in terms of cell numbers using Neubaur haemocytometer[53].

### Gene cloning, DNA sequencing, bioinformatic analysis, and phylogenetic tree analysis

The full-length CDS of DsMEK1 was accessed in *D. salina* transcriptome database. The forward and reverse primers used to analyze the CDS of DsMEK1 genes (Table S1 No. 1–2). PCR products were analyzed on a 2% agarose gel, then combined with the pMD19-T Vector (TaKaRa, China) and target bands were sequenced by Tsingke Co., Ltd. (Beijing, China). The molecular weights (Mw) of the two DsMEK1 splice variants (DsMEK1-X1 and DsMEK1-X2) were calculated using the compute Pi/Mw tool online

(<http://isoelectric.ovh.org/>). The identified motifs were annotated based on CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The gene structures were analyzed with GSDS software (<http://gsds.cbi.pku.edu.cn/>).

The AtMAPKK sequences were obtained from The Arabidopsis Information Resource (TAIR). The *O. sativa* MAPKK and *Chlamydomonas reinhardtii* MAPKK1 sequences were obtained from the NCBI database. Multiple protein sequence alignments were performed using ClustalX 2.0, while a phylogenetic tree was constructed from the amino acid sequences using the neighbor-joining method with MEGA6 software. A bootstrap analysis was performed using 1000 replicates[54].

## Subcellular localization

For protein localization observation, DsMEK1-X1 and DsMEK1-X2 were inserted into the p1300-GFP expression vector using gene specific primers (primers No. 3–4 in Table S1)[38]. To transiently express the fusion proteins in protoplasts isolated from Arabidopsis leaves, PEG-mediated transformation was used to transfect the protoplasts with each DsMEK1::GFP construct. GFP was excited by 488 nm laser lines and detected with bandpass 498–543 nm filters[55].

## Yeast two-hybrid assays

Yeast two-hybrid assays were performed with the Y2H Gold-Gal4 system (Clontech). DsMEK1-X1/2 were inserted into the pGADT7 vectors and DsMAPK1 (NCBI, GenBank: EF186770.1), DsMAPKKK genes were inserted into the pGBKT7 vectors to form the bait and prey constructs (primers No. 5–43 in Table S1). The bait and prey constructs were transformed into yeast strain Y2H Gold according to the manufacturer's instructions (Clontech). The yeast cells were cultured on the SD/-Trp/-Leu/-His/-Ade medium containing X-gal at 28 ° C in the dark for three days.

## Construction of DsMEK1-X1/X2 overexpression strains and DsMEK1-X2 knock down strains

The pGreen-0029 binary vector was modified and used as the *D. salina* transformation vector. To generate the DsMEK1s transformation constructs, a 573 bp chloramphenicol resistance gene (Cmr) was amplified and inserted to the pGreen-0029 as a dominant selectable marker (primers No. 44–45 in Table S1). To construct pGreen-0029-Cmr-DsMEK1 overexpression strains (DsMEK1-X1-oe and DsMEK1-X2-oe), the full-length CDS of DsMEK1-X1 and DsMEK1-X2 were amplified by PCR (primers No. 46–47 in Table S1), then inserted them into the modified pGreen-0029-Cmr vector. To generate the DsMEK1-X2 knock-down construct DsMEK1-X2-RNAi strains, obtaining interference sequences by chemical synthesis (Sangon Biotech Co., Ltd), and inserted into the modified pGreen-0029-Cmr vector.

## Transformation of *D. salina*

Constructed plasmids were transformed into *D. salina* cells using the Agrobacterium-mediated transformation method as described previously with slight modifications[56]. The empty vector pGreen-

0029-Cmr (control), and constructed plasmids were transformed into *Agrobacterium tumefaciens* GV3101 via Freeze-thaw method.

Approximately  $10^7$  cells of *D. salina* were plated onto solid TAP medium. Algae cells were incubated one week until a lawn of cells was formed. Three replicates were maintained for each treatment. The transformed agrobacterium culture was supplemented with 100  $\mu\text{M}$  acetosyringone and incubated for 4 h at 28 ° C. *D. salina* cells were then infected using a solid infection medium. For solid infection, 500  $\mu\text{L}$  of induced *A. tumefaciens* was added onto the microalgae culture plate. The treatment plates were further incubated two days at 25 ° C in dark. After co-cultivation, cells were harvested and washed three times. Then the transformed *D. salina* was plated onto TAP medium agar plates containing 100  $\text{mg L}^{-1}$  Cefotaxime and 400  $\text{mg L}^{-1}$  chloramphenicol as the selection marker. Colonies that appeared within 3 weeks were subcultured in liquid selective medium. Individual DsMEK1-X1-oe, DsMEK1-X2-oe and DsMEK1-X2-RNAi colonies were subjected to genotyping PCR to confirm the presence of gene integration (primers No. 48–49 in Table S1).

## Total sugar content

Ten-milliliter culture was centrifuged at 5,000 rpm for 5 min and the pellet was resuspended in 4 ml distilled water and incubated at 100 ° C for 1 h. Homogenized cells were centrifuged for 10 min at 5,000 rpm and 2 ml upper aqueous solution was transferred in a fresh test tube. A measure of 1 ml phenol solution (5%) was added into the test tube, followed by the addition of 5 ml sulphuric acid. The test tube was kept at room temperature for 30 min. Upper color phase was taken out for the absorbance reading at 485 nm[53]. Reading was compared to find out the total sugar content of samples with a standard curve, which was drawn by the same method, using a known amount of glucose as standard.

## Proline content

The proline content of each sample subjected to 3.5 M salinity concentrations was measured with a proline measurement kit (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Each experiment was performed with 3 independent replicates. The proline content was divided by cell number to obtain the proline content per cell.

## MDA content

The MDA content of each sample subjected to 3.5 M salinity concentrations was measured with a Malondialdehyde (MDA) assay kit (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Each experiment was performed with 3 independent replicates. The MDA content was divided by cell number to obtain the MDA content per cell.

## Glycerol content

Intracellular glycerol content of *D. salina* was measured using the method of Prabhakar et al[57] with slight modifications. All samples (5 mL) were centrifuged (6000 g, 5 min at RT) and the pellets were washed with fresh culture medium. Those pellets were suspended in distilled water (1 mL). The

suspensions were freeze-thaw and then centrifuged (12000 g, 10 min, RT). The supernatant (200 $\mu$ L) of each sample was made up to 1 mL by adding distilled water. 1.2 ml sodium periodate reagent was added and mixed followed by the addition of 1.2 mL acetylacetone reagent. These samples were gently mixed and placed in a water bath (60 ° C, 10 min) and quickly cooled in an ice-bath. The absorbance of each sample was read at 413 nm after cooling at room temperature.

## RNA extraction and qRT-PCR

The total RNA of each sample was isolated with the TRIzol™ Reagent (Invitrogen) according to the manufacturer's instructions. The first cDNAs were synthesized using ExTaq™ PCR Kit following the manufacturer's protocol (Takara, China). qRT-PCR was carried out with BIORAD CF96 Real-Time PCR system using SYBR® Premix Ex Taq™ II (Takara, China). The primers used for qRT-PCR are listed in Table S2, and the  $\beta$ -tubulin gene was used as an internal reference. Each treatment was repeated three times independently. The  $2^{-\Delta\Delta C_t}$  method was used to analyze the relative expression of genes.

## Abbreviations

MAPK:Mitogen-activated protein kinase; MAPKK (MEK):Mitogen-activated protein kinase kinase; MAPKKK:Mitogen-activated protein kinase kinase kinase; MDA:Malondialdehyde; Cmr:chloramphenicol resistance gene; GPDH:Glycerol-3-phosphate dehydrogenase.

## Declarations

### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

### Acknowledgments

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## Contributions

ZYT designed the study, performed the experiments, analyzed the results and drafted the manuscript. YC and HX, as the corresponding author, developed the concept of this research, supervised the progress of this research, and commented on the manuscript. XYC assisted in the experiments. JJ, YPZ and DRQ participated in the design of the study and commented on the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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## Ethics declarations

## Ethical approval and consent to participate

Not applicable.

## Consent for publication

All authors give consent to publish the research in *Biotechnology for Biofuels*.

## Competing interests

The authors declare that they have no competing interests.

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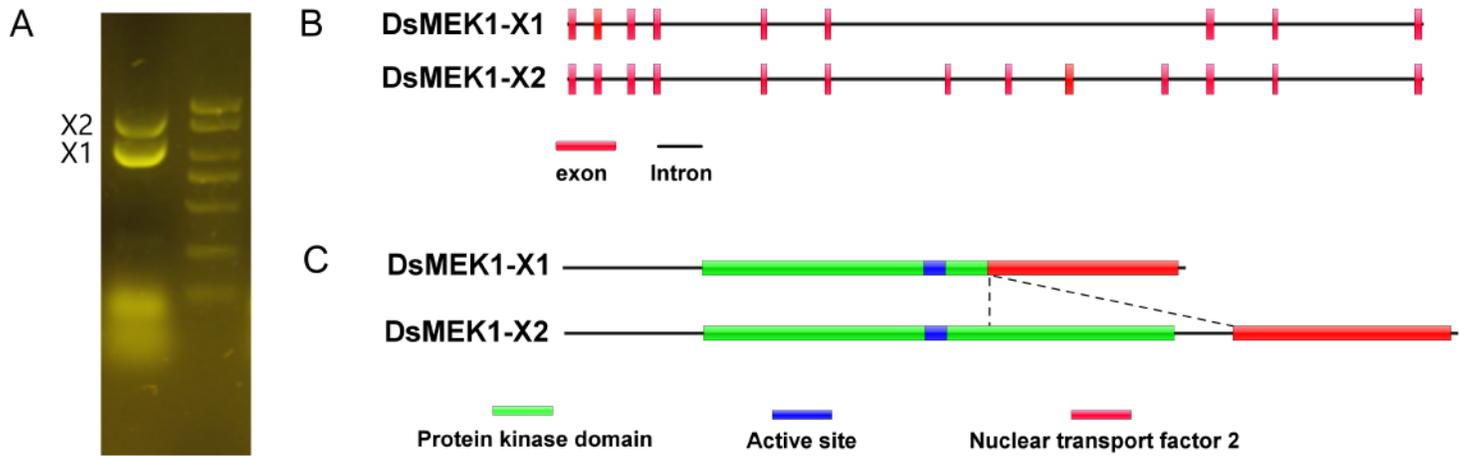
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## Additional Files

Table S1. Primers and antisense oligonucleotides used in this study.

Table. S2. List of primer sets used in qRT-PCR

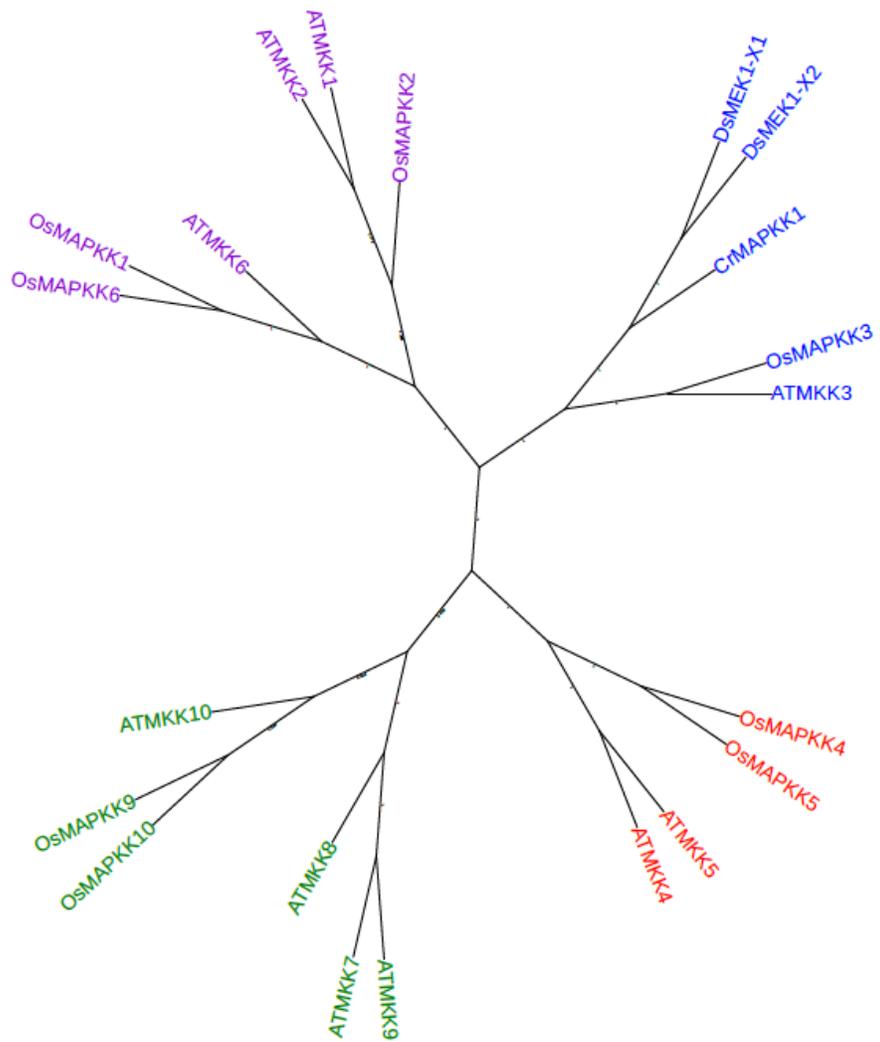
## Figures



**Figure 1**

Characteristics of alternatively spliced variants of DsMEK1. (A) Detection of alternative transcripts of DsMEK1 by PCR analysis. M, 2000+ DNA size markers. X1 and X2 indicate the DsMEK1-X1, and DsMEK1-X2 transcripts, respectively. (B) Genomic structures of alternatively spliced variants. Black lines indicate the introns and red boxes represent exons. (C) Domain structures of protein splice variants (Protein kinase domain, Active site, and Nuclear transport factor 2) indicated by the color boxes. Colored boxes mark the different regions in the proteins.

Subfamily	
	A
	B
	C
	D



**Figure 2**

Phylogenetic analyze of DsMEK1. The evolutionary tree was constructed from the full-length MEK1 proteins from *D. salina* and all MKK protein from *A. thaliana*, *O. sativa* and *C. reinhardtii*.

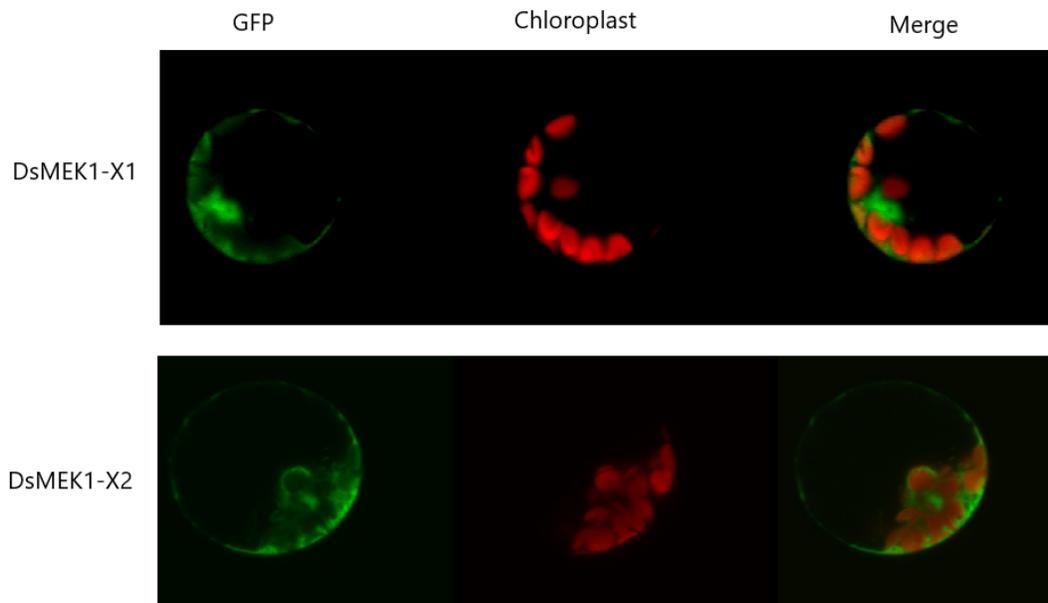


Figure 3

Subcellular localization of DsMEK1-X1-GFP and DsMEK1-X2-GFP fusion proteins in mesophyll protoplasts of *A. thaliana*.

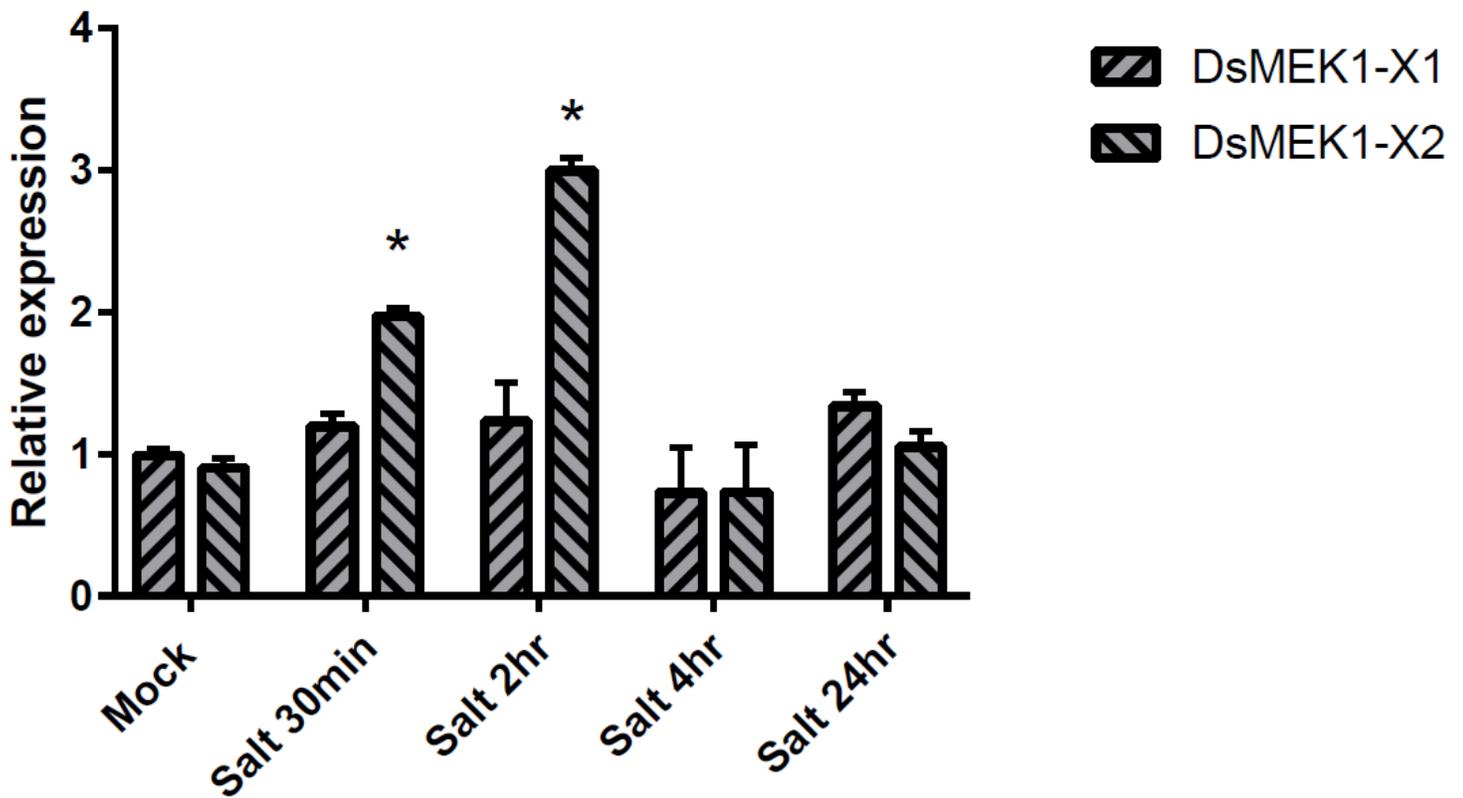
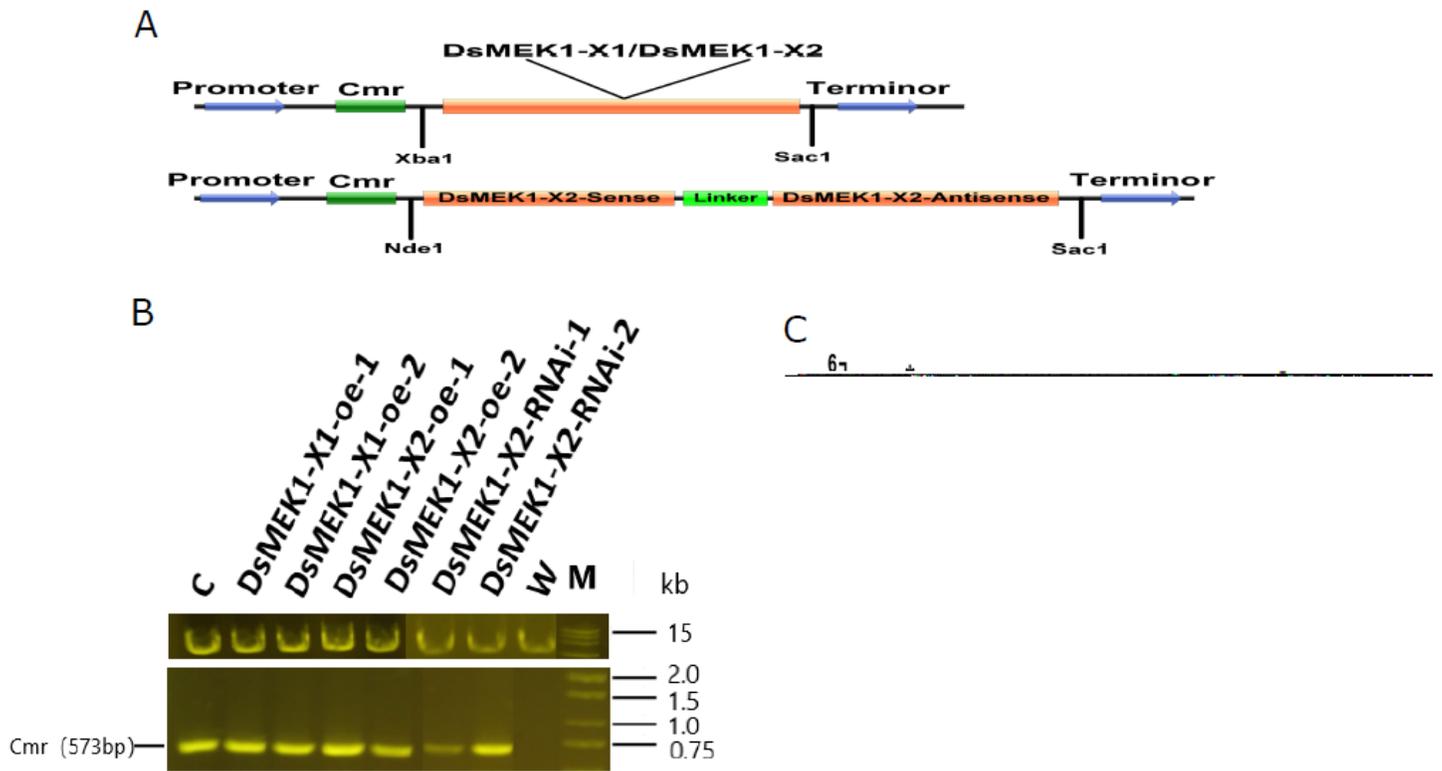


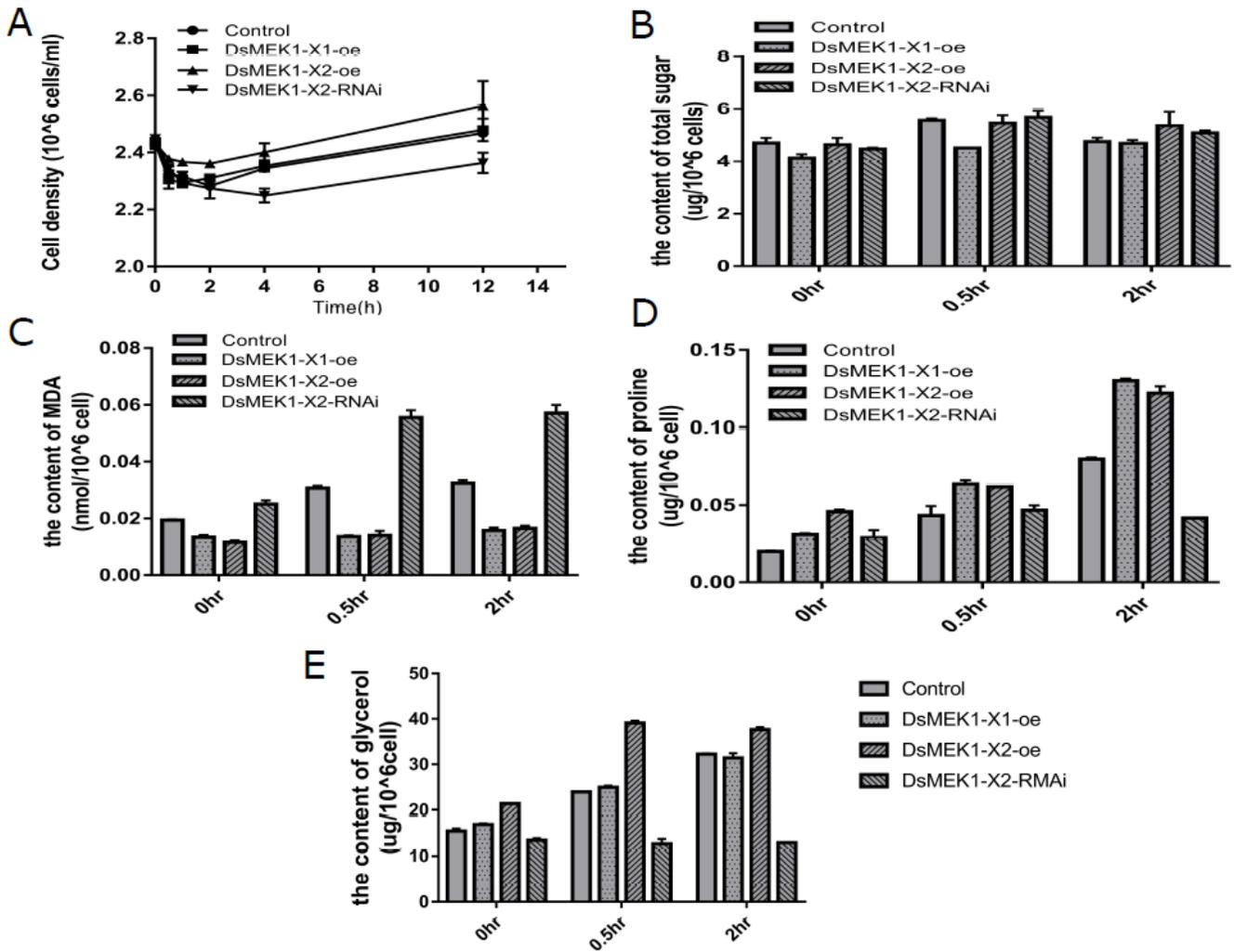
Figure 4

Expression profiling of DsMEK1 in *D.salina* under salt stress treatment. Data are presented as means ( $\pm$ SE, n = 3). The columns with "\*" had a statistical difference ( $p < .05$ , fold change  $> 2$ ).



**Figure 5**

The DsMEK1 transformation vector and molecular identification of DsMEK1 lines. (A) Schematic map of the DsMEK1 vector. (B) Verification of Cmr (573bp) PCR products in the WT strain, control strain and DsMEK1 lines by agarose gel electrophoresis. (C) The expression levels of *dsmeK1-x1* and *dsmeK1-x2* in both DsMEK1-X1-oe, DsMEK1-X2-oe and DsMEK1-X2-RNAi lines, respectively. Data are presented as means ( $\pm$ SE, n = 3). The columns with "\*" had a statistical difference ( $p < .05$ , fold change  $> 2$ ).



**Figure 6**

Phenotype analysis of DsMEK1-X1-oe, DsMEK1-X2-oe, and DsMEK1-X2-RNAi lines. (A) The growth rates of the control strains and the DsMEK1-X1-oe, DsMEK1-X2-oe and DsMEK1-X2-RNAi lines were measured according to the cell numbers. (B) Total sugar contents, (C) MDA and (D) proline (E) glycerol in the control strains, DsMEK1-X1-oe, DsMEK1-X2-oe and DsMEK1-X2-RNAi lines under salt stress. The results are shown as the mean expression  $\pm$  standard deviation (SD) of three independent experiments. Student's t-test,  $P < 0.05$ .

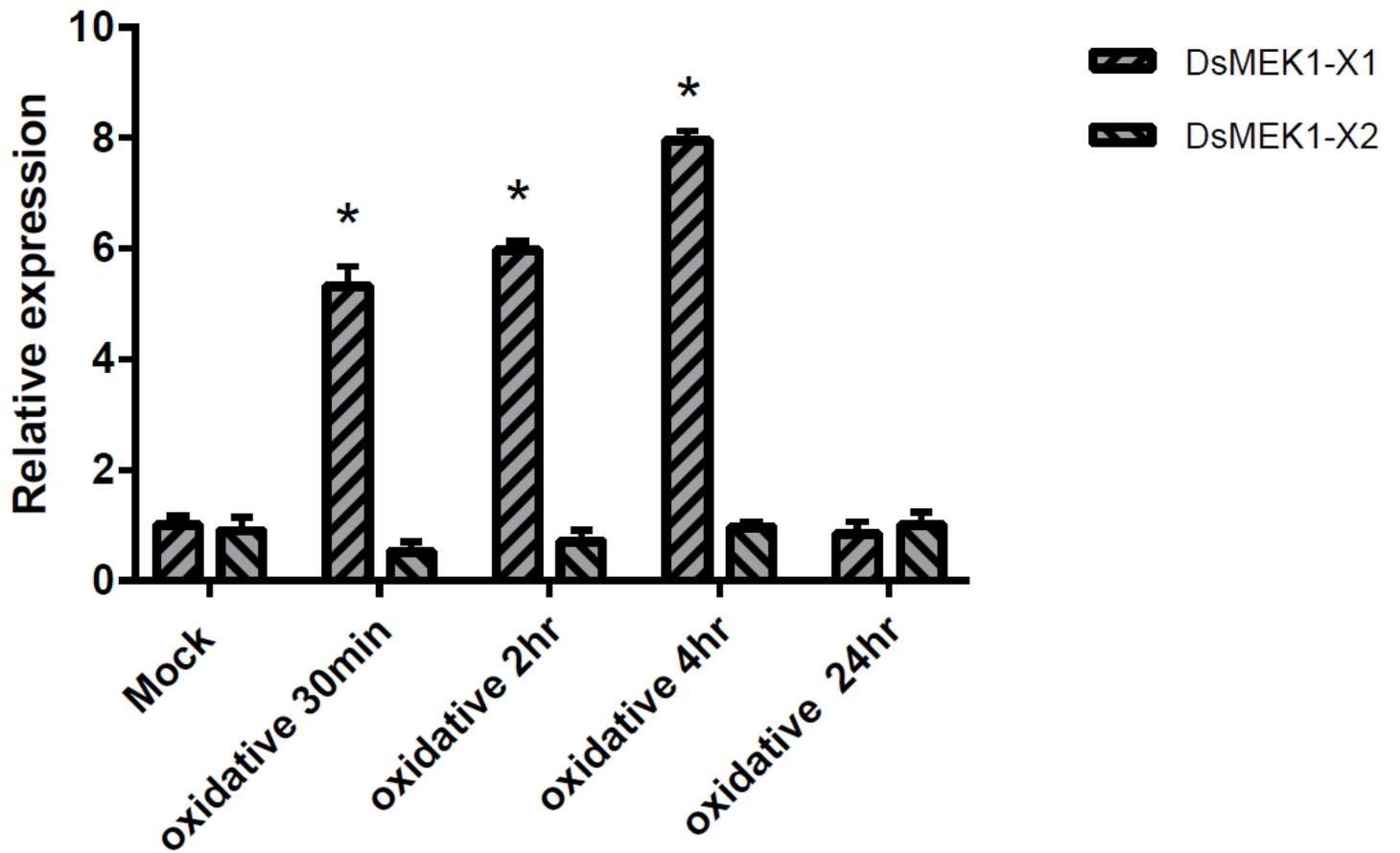
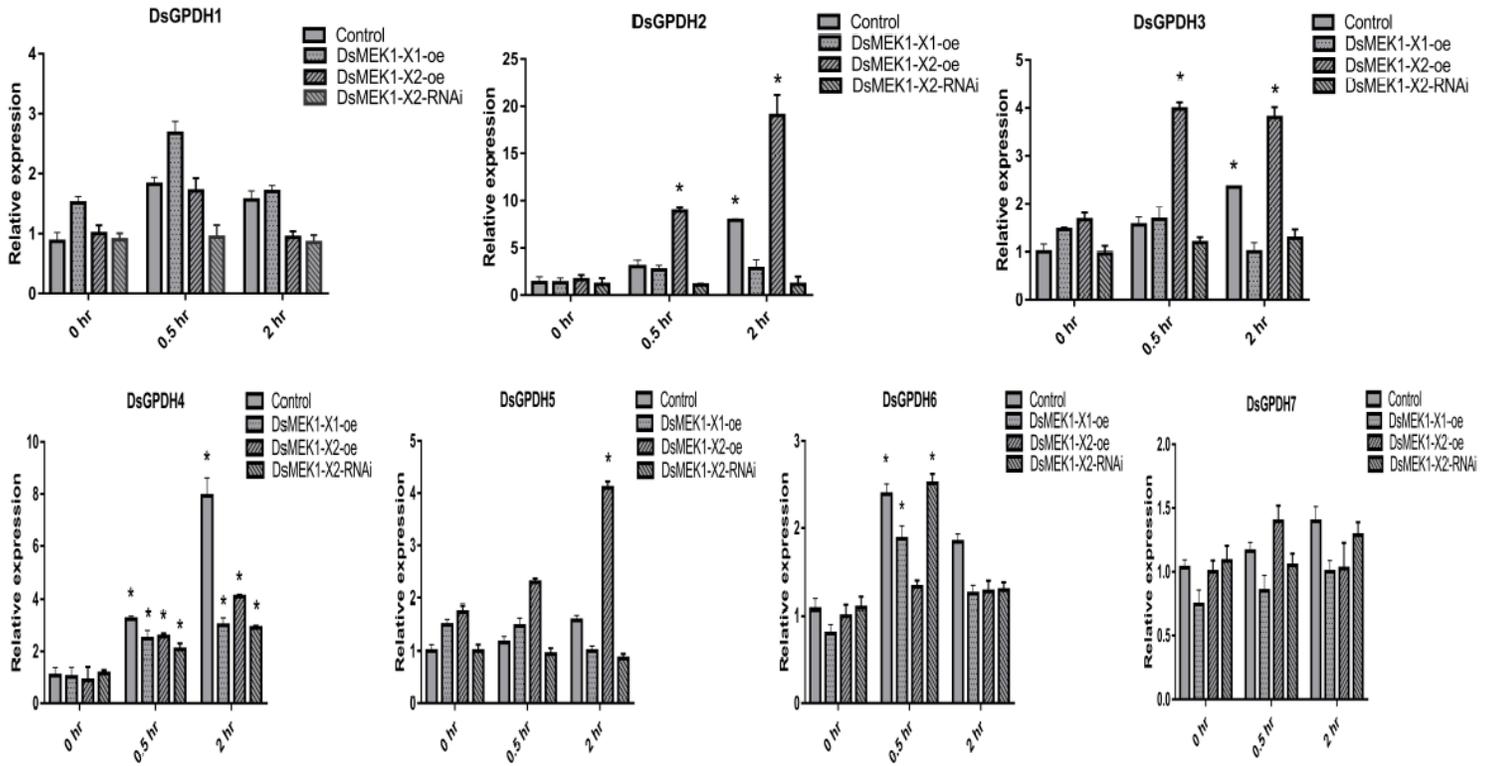


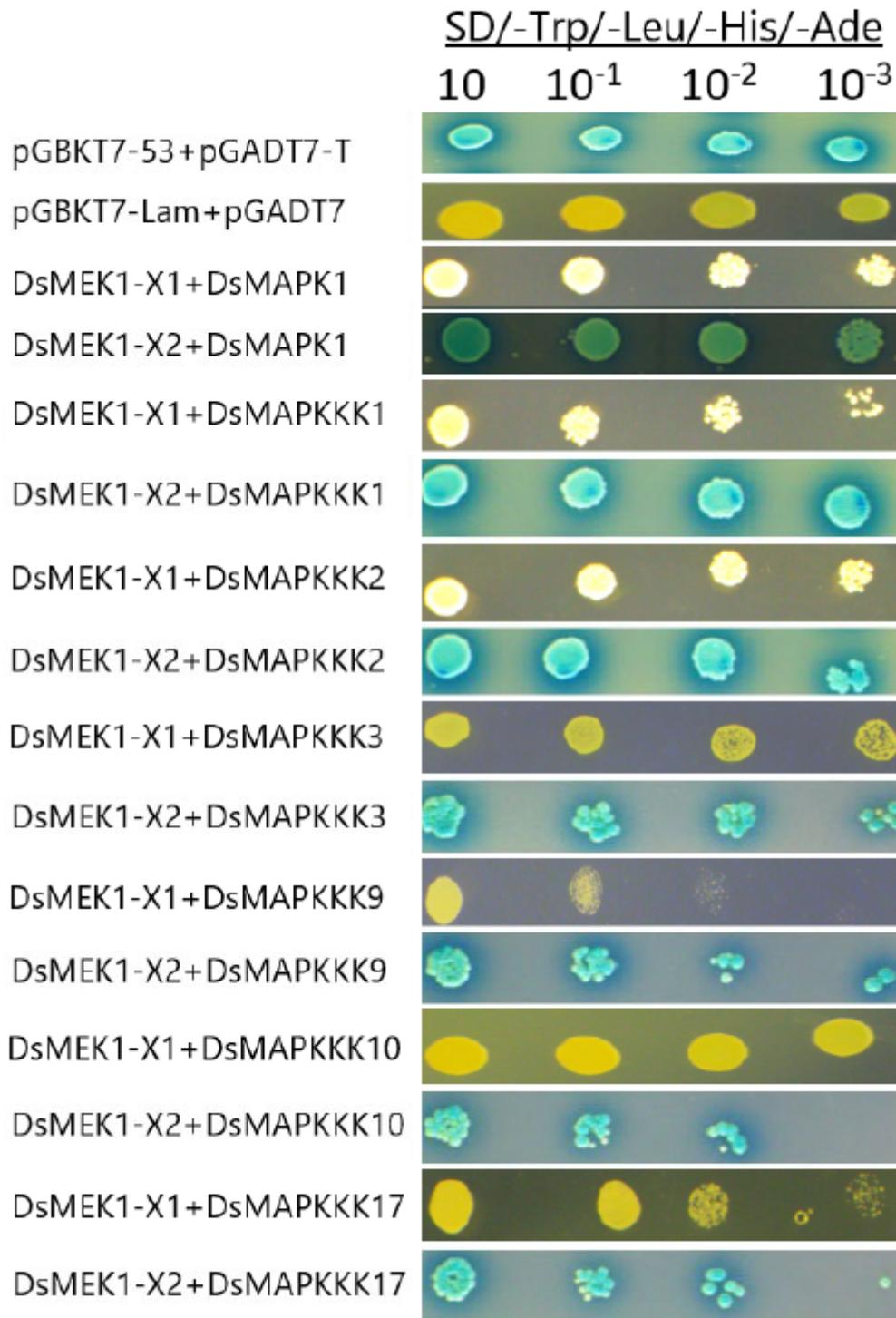
Figure 7

Expression profiling of DsMEK1 in *D.salina* under oxidative stress treatment. Data are presented as means ( $\pm$ SE, n = 3). The columns with "\*" had a statistical difference ( $p < .05$ , fold change > 2).



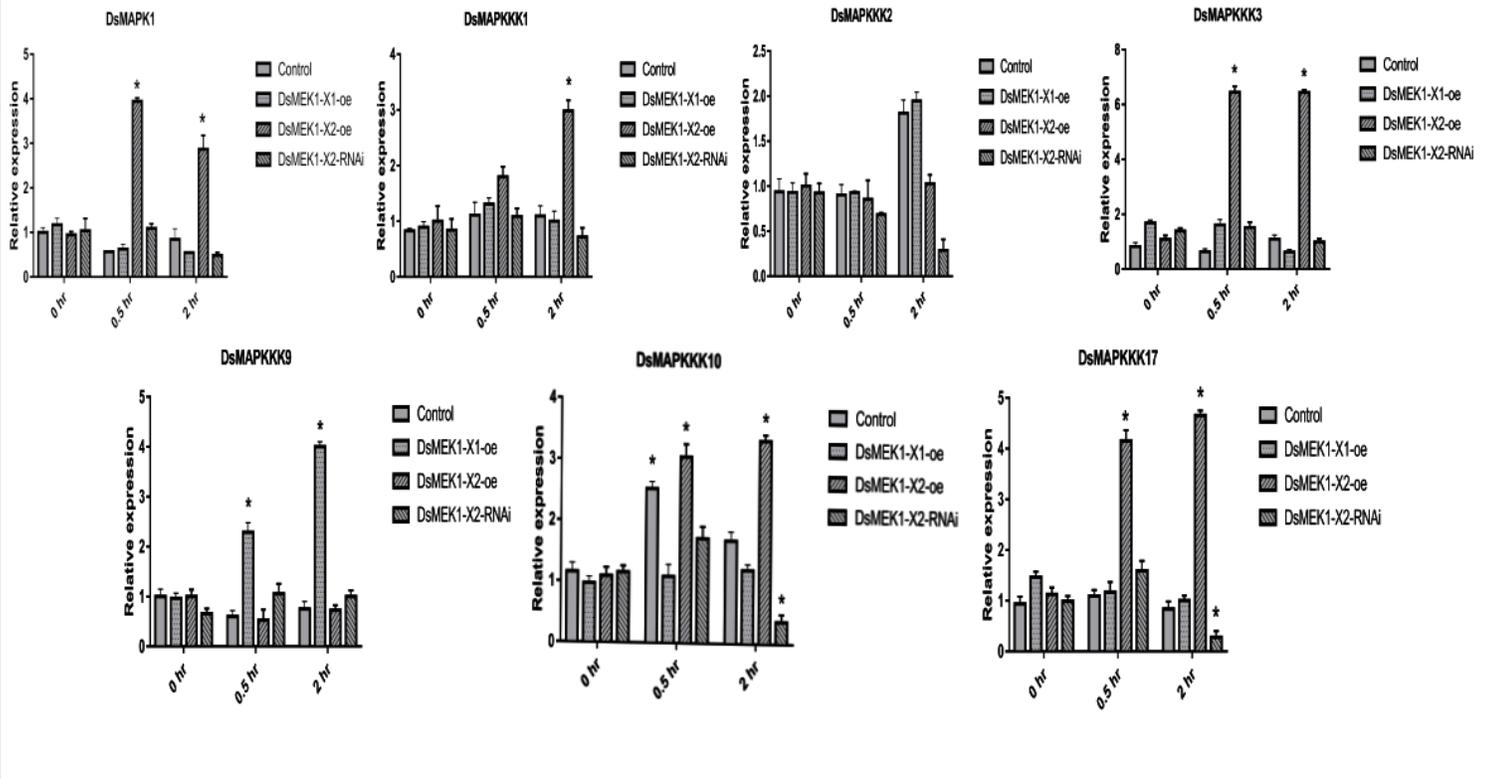
**Figure 8**

The expression levels of DsGPDH in DsMEK1-X1-oe, DsMEK1-X2-oe, and DsMEK1-X2-RNAi lines, were detected by qRT-PCR assays. Data are presented as means ( $\pm$ SE, n = 3). The columns with “\*” had a statistical difference (p < .05, fold change > 2).



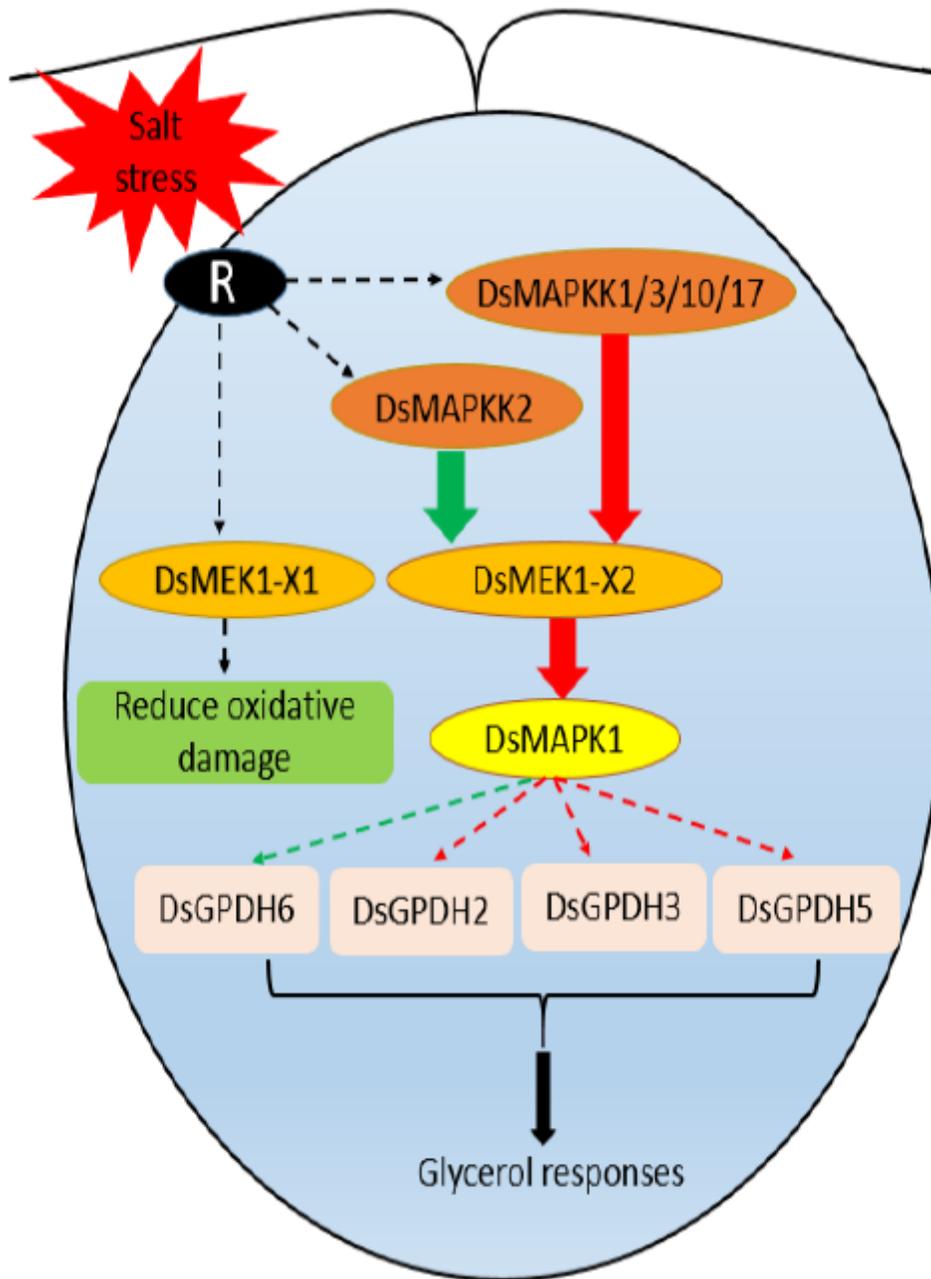
**Figure 9**

Interactions of DsMEK1 splice variants with six MAPKKs and one MAPK in yeast two-hybrid assay. We confirm that our bait does not autonomously activate the reporter genes in Y2H Gold in the absence of a prey protein.



**Figure 10**

The expression of DsMAPK1 and DsMAPKKK1/2/3/9/10/17 in DsMEK1-X1-oe, DsMEK1-X2-oe and DsMEK1-X2-RNAi lines under salt stress. Data are presented as means ( $\pm$ SE, n = 3). The columns with “\*” had a statistical difference ( $p < .05$ , fold change  $> 2$ ).



**Figure 11**

Hypothetical model depicting the functional role of DsMEK1 splice variants and upstream, downstream targets in salt stress signaling in *D. salina*. Solid arrows depict known pathways, while dashed arrows indicate proposed or unknown pathways. Red lines mean positively regulated, green lines suggest negative regulate.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.docx](#)