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Expression of aquaporin gene (*Os PIP1-3*) in salt-stressed rice (*Oryzasativa* L.) plants pre-treated with the neurotransmitter (dopamine)

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Abstract

Rice *(Oryza sativa* L.) plants are influenced by salinity, which is a global soil problem. Plants utilise different mechanisms to regulate solute homeostasis and to balance cellular water transport. The objective of the current study was to investigate the presence of any potential role of dopamine (D, a natural product synthesised in the catecholamine pathway from tyrosine) in enhancing salinity tolerance in two-month-old rice (*Oryza sativa* L.) plants through modulating the plasma membrane intrinsic proteins (*PIPs*). Using RT-PCR technique, the level of *OsPIP 1-3* expression was up-regulated in response to mild salt treatment (0.15 M NaCl), but gene expression was considerably down regulated in response to dopamine, indicating the possible regulatory role of dopamine in water permeation. Relative water content in stressed rice plants was highly retrieved in response to 0.2 µgml^{-1} dopamine. The content of pigment and proline was regulated significantly when dopamine was administered to plants before their exposure to salt stress. Fluorescence emission spectra showed non-significant changes in 0.2 µgml^{-1}D plants and slight intensity increase in 0.4 µgml^{-1}D plants, whereas no change was detected in 0.6 µgml^{-1}D plants. Decreased sodium uptake in response to 0.4 µgml^{-1} D is explained by the low expression of *PIP1-3* and, hence related to the possible block of aquaporins. A high detection of membrane stability index in stressed-rice plants pre-treated with dopamine was associated with low membrane leakage. We concluded two facts; (1) the participation of *OsPIP1-3*gene in water permeation in salinity-stressed rice and, (2) the role of exogenous dopamine in *OsPIP1-3* gene expression regulation, which was shown to be concentration-dependent. Subsequently, dopamine applications in a few doses are recommended as cheap and potential material which ameliorates salt stress in rice plants through its effect on plasma membrane aquaporins.

Keywords: Aquaporins; dopamine; fluorescence emission spectra; membrane leakage; plasma membrane intrinsic proteins; relative water content; rice; proline; salinity; stability index.
Abbreviations: Chl_chlorophyll; D_dopamine; PIP_plasma membrane intrinsic protein; MI_Membrane stability index; ML_Membrane leakage; *OsPIP1-3_Oryza sativa* plasma intrinsic protein; RWC_ relative water content.

Introduction

Salinity is a global soil problem that existed before mankind and increased with today's improper practices in agriculture, irrigation and drainage. Reduction of leaf water potential is the main salinity symptom that leads to reduced stomatal conductance and inhibits photosynthetic metabolism and, subsequently, causes wilting; the typical drought symptom (Baker and Rosenqvist, 2004). The salinity hazard effect on membrane integrity, nutrient transport, enzyme synthesis and photosynthetic apparatus has previously been reported by many authors (Zhu, 2001). A consensus concerning disruption of cellular homeostasis as an important key in salinity damage of crop plants has been reached yet. Accordingly, a conceivable solution of the salinity problem was suggested on three levels: (1) to avoid plants' exposure to salinity, (2) to regulate plants' homeostasis within the unfavourable environmental conditions, and (3) to resume plants' growth (Zhu, 2001; Munns and Tester, 2008). Many strategies were discovered so far to enhance salinity tolerance in wheat and sugarcane plants (Abdelkader et al., 2010; Patade et al., 2012).

Dopamine (D) is a catecholamine pathway natural product, identified in a number of plants and their organs. A total number of 29 of these plants were found to be edible (Kimura, 1968). Dopamine has hormonal activity-functions as antioxidant and is efficient during photosynthesis due to its reduction power (Kanazawa and Sakakibara, 2000) which ends with free radical scavenging. Thus, it can protect plant growth and productivity under different stress conditions (Endress et al., 1984). Dopamine also plays a role in sugar metabolism and is coordinated with phytohormones in regulating plant growth (Kulma and Szopa, 2007). As salinity leads to ionic imbalance and toxicity, it also causes drought stress (Lambers et al., 1998). Therefore, it is necessary to perform studies focusing on water uptake and translocation via water channels. Plasma membrane intrinsic proteins (*PIPs*), notably aquaporins, are integrated proteins in both plasma membranes and tonoplasts (King et al., 2004). Co-expression of PIP1 and PIP2 has expanded our knowledge as both had participated in regulating water permeation and reducing water uptake through the porins (Bellati et al.,

2010). Aquaporins are organ-specific and previous studies on plasma membrane intrinsic protein of tobacco plants suggested the presence of a gating mechanism influencing conductivity of plant aquaporin and protein phosphorylation under drought stress, as well as protonation following cytosolic acidification during flooding (Fischer and Kaldenhoff, 2008). Rice (*Oryza sativa*) is an important crop plant with a fully sequenced genome. Rice aquaporins were subdivided into four subfamilies with 33 gene members (Sakurai et al., 2005). Members of rice (*OsPIP*) family located on the mRNA exhibited significant abundance differences (Guo et al., 2006). The presence of one leaf-specific *OsPIP* gene *(OsPIP2-6*) and three root-specific genes (*OsPIP1-3*, *OsPIP2-2*, and *OsPIP2-7*) in rice plants was reported (Guo et al., 2006). In the present investigation, we conducted a series of experiments of both SDS-PAGE-Electrophoresis and Reverse Transcriptase (RT-PCR) techniques to study the effect of low dopamine concentrations (0.2, 0.4 and 0.6 µgml^{-1}) on plasma membrane intrinsic proteins (PIP) and on the expression pattern of aquaporine gene (*OsPIP1-3*) in rice plants, respectively. The status of *OsPIP1-3* in exposed rice plants to salinity through irrigation using 0.15 M NaCl for two weeks is also discussed. Possible salt tolerance acquisition in response to dopamine applications was underlined via physiological analyses (i.e. relative water content; RWC, pigment content, fluorescence emission spectra, proline, mineral content, membrane stability index; MI and membrane leakage; ML).

Results

Effect of dopamine on relative water content (RWC)

Water status of two-month-old rice plants was reported in Fig 1. The RWC was higher in this order: control plants (C, 99%), 0.2+S (96%), 0.6D (94%), 0.2D (92%), 0.4+S and 0.6+S (86%), 0.4D (80%) and salinity-stressed plants (S, 78%).

Photosynthetic pigment content in response to dopamine pre-treatment in rice plants

The efficiency of the photosynthetic apparatus was studied via chl *a*, *b* and carotenoids determination in rice plants under different dopamine treatments (0.2D, 0.4D, 0.6D, 0.2+S, 0.4+S and 0.6+S) besides untreated plants with dopamine (C, S). As shown in Fig. 2, values of chl *a* decreased with salinity stress, were higher in dopamine plants under stress than in dopamine plants. The proportion of chl *a* was inversely related to dopamine concentration. The highest chl *a* value was 18.14 µg ml^{-1} estimated in 0.2+S plants, followed by 16.87 µg ml^{-1} in 0.4+S plants and finally 13.8 µg.ml^{-1} in 0.6+S plants. The values of chl *a* in dopamine plants were: 6.67 µgml^{-1} in 0.2D plants, 10.9 µg.ml^{-1} in 0.4D plants and 7.93 µg ml^{-1} in 0.6D plants. The content of chl *a* from salinity in the recovered plants was similar to the control plant (8.2 µg.ml^{-1}). The content of chl *b* was higher in the control than in dopamine-treated plants (Fig 2). The values of chl *b* in µgml^{-1} were: 0.49 (0.2D), 1.49 (S), 2.8 (0.6D), 3.34 (R), 3.94 (0.4D) and 4.93 (c). A considerable increase in chl *b* values was detected in dopamine-stressed plants as follows: 0.2+S (6.0), 0.6+S (10.11), whereas, the lowest value was determined in 0.4+S (1.08). The ratio of chl *a/b* (data not illustrated) was expressed from lower to higher as follows: 0.6+S (1.36), C (1.7), R (2.44), 0.4D (2.77), 0.6D (2.83), S (2.87), 0.2+S(3.02), 0.2D (13.62) and 0.4+S (15.6). The following values of carotenoid were measured in µg/ml: 0.2+S (10.0), 0.4D (6.8), 0.4+S (5.3), R (5.2), C (4.5), 0.6D (4.2). In addition, low values of carotenoids were determined in stressed plants without dopamine (S, 3.69) and in 0.2D plants (2.36) and the lowest value of carotenoid was detected in 0.6+S (1.44).

Table 1. Effects of 0.15 M NaCl (S), dopamine (0.2, 0.4 and 0.6 µgml^{-1}) and their interactions on membrane stability index (MSI) and membrane leakage (ML) in *Oryza sativa* plant.

Treatment	MSI (%)	ML (%)
C	87.7	30.0
S	65.5	35.0
0.2	89.1	25.3
0.2+S	75.0	27.0
0.4	92.0 ^{a}	22.0
0.4+S	79.0 ^{a}	26.0
0.6	93.9 ^{b}	20.5
0.6+S	82.0 ^{b}	24.9
LSD at 0.05	0.0	0.0

Superscript letters expressed significant values according to Tukey's test

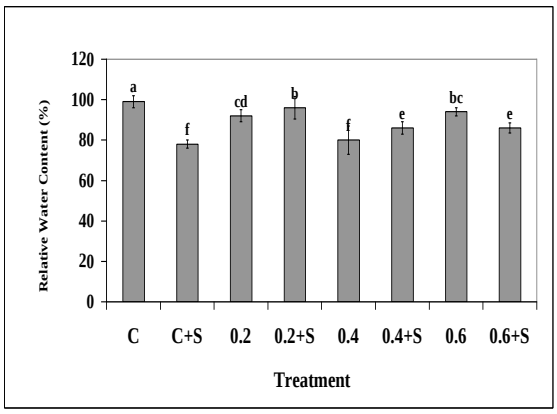

Fig 1. Relative water content (RWC) in pre-treated rice plants with dopamine concentrations (0.2, 0.4 and 0.6 µg.ml^{-1}) before being exposed to salt stress. Values are means of three replicates and significant differences are at p< 0.05 according to Duncan's test. Error bars represent standard deviations of the mean.

Proline level under the effect of dopamine gradient

Fig. 3 shows the amount of estimated proline in rice plants under different treatments. The highest proline value in µmol g^{-1}F.wt was determined in stressed rice plants lacking dopamine (1.85), followed by 1.5 in 0.6+S plants, 1.1 in 0.4+S plants and finally, 0.786 in 0.2+S plants.

Fluorescent emission spectra changes after dopamine pre-treatment in stressed rice

As shown in Figs 4-6, chlorophyll spectra from control plants presented main fluorescence emission peaks stabilised at 678.5 nm from S plants at 678nm and from 0.2D-plants at 675.5 nm. Whereas, a significant shift towards the red region was detected in 0.2+S plants (685.5 nm). A shoulder was also observed, localised around 655.5 nm in 0.2+S plants (Fig. 4).

Table 2. Accumulations of Mg, K and Na in *Oryza sativa* plant in response to treatments with different concentrations of dopamine (0.2, 0.4 and 0.6 µgml^{-1}), 0.15 M NaCl (S) and their interactions.

Treatments	Mineral content (mg.l^{-1})		
	Mg	K	Na
C	24.0 [a]	74.3	40.8
S	33.9	127.0	108.8
0.2	23.3	114.55	17.0
0.2+S	28.3	59.6	79.8
0.4	22.2	208.1	21.0
0.4+S	24.7	55.7	47.9
0.6	23.7[a]	107.4 [b]	25.7
0.6+S	34.8	117.4 [b]	87.4
LSD at 0.05	0.0	0.0	0.003

Superscript letters expressed significant values according to Tukey's test.

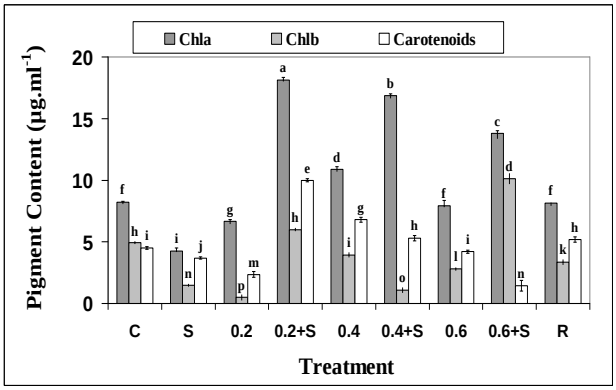

Fig 2. Pigment content of rice plants pre-treated with concentrations of dopamine (0.2, 0.4 and 0.6 µgml^{-1}) before being exposed to salt stress. Control (C), salt-stressed rice (S), recovered plants from salt stress (R). Values are means of three replicates and significant differences are at $p<0.05$ according to Duncan's test. Error bars represent standard deviations of the mean.

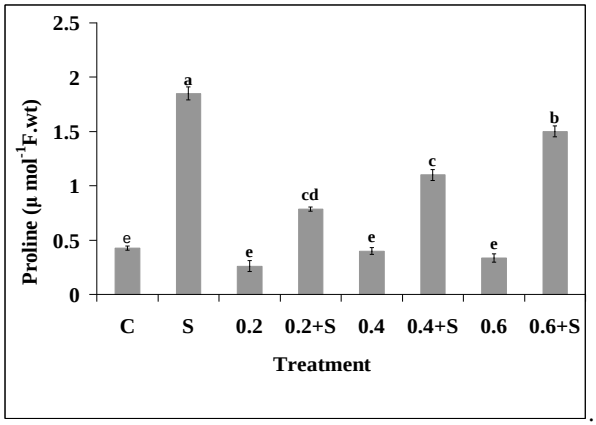

Fig 3. Proline content of rice plants treated with 0.2 µgml^{-1} dopamine, or pre-treated with dopamine before being exposed to salt stress; 0.2+S. Control (C), salt-stressed rice (S). Excitation wavelength 400 nm. Values are means of three replicates and significant differences are at $p<0.05$ according to Duncan's test. Error bars represent standard deviations of the mean**.**

Fluorescence emission spectra from 0.4D plants compared to 0.4+S plants showed main peaks located at 677.5 nm and 676.5 nm, respectively (Fig 5). In 0.4D plants, the fluorescence emission intensity had significantly increased by approximately 524 nm in response to 0.4 µgml^{-1} dopamine. Furthermore, spectra of 0.6D plants and 0.6+S plants were detected around 676.5 and 675.5, respectively (Fig. 6). No shoulders were observed within the spectra of the last four variances.

Changes in membrane stability index (MSI) and membrane leakage (ML)

The pattern of change in membrane stability index was opposite to that in membrane leakage (Table 1). In comparison with control plants, reduction in MSI in response to salinity stress was accompanied by an increase in ML. Plants protected with dopamine, prior to exposure to salinity stress had a successive increase in MSI in this order: 0.2D, 0.4D and 0.6D.

A reduction in MSI was significantly noticed when plants had been treated with solely NaCl. Such response was much more pronounced by the highest concentration of dopamine (0.6D). On the other hand, applications of the gradual concentrations of dopamine or their interactions with NaCl caused decrease in ML below that of salinised and control plants.

Nutritional value after dopamine application

Table 2 shows minerals (Na, K and Mg) accumulation values in rice plants. The accumulation of Mg was, generally, higher under the effect of salinity either in control or in dopamine plants. The values of Mg in mg.l^{-1} were: 33.9, 28.3, 24.7 and 34.8 in S, 0.2+S, 0.4+S and 0.6+S, respectively. Whereas, Mg values in unstressed plants were: 24.0, 23.3, 22.2 and 23.7, in C, 0.2D, 0.4D and 0.6D, respectively.

The accumulation of K in mg.l^{-1} was higher in stressed and unstressed samples, dependent upon the treatment (127.0, 59.6, 55.7 and 117.4, in S, 0.2+S, 0.4+S and 0.6+S, respectively). Alternatively, the values of K were: 74.3, 114.5, 208.1 and 107.4 in C, 0.2D, 0.4D and 0.6D, respectively. The highest value of K was estimated in 0.2+S plants. The accumulation of Na has increased in salinity stressed samples detecting: 108.8 in S pants, 79.8 in 0.2+S plants, 47.9 in 0.4+S plants and 87.4 in 0.6+S plants. Whereas, the values of Na which had been detected were: 17.0, 40.8, 21.0 and 25.7 in C, 0.2D, 0.4D and 0.6D plants, respectively.

Pattern of OsPIP1-3 gene expression using RT-PCR

To characterise the function of *OsPIP 1-3*gene, we examined the level of *OsPIP* gene expression using the semi-quantitative RT-PCR method in the leaves and roots of two-month-old rice seedlings. Interestingly, the result also showed that the expression of this gene is induced upon salt treatment (Figs. 7 & 8). Actin gene (Act2) was used as an internal housekeeping control. The expression of *OsPIP1-3* was down-regulated in response to treatments with different concentrations of dopamine in rice plants. Results showed that 0.4 µgml^{-1} dopamine irrigated to 0.4S plants caused a small rate of up-regulation in the gene expression, as compared with S plants. Dopamine administrated in 0.2D, 0.4D and 0.6D plants had no observable effect on changing the level of *OsPIP1-3* gene expression (Fig. 7).

***Changes of plasma membrane intrinsic proteins* (PIPs)**

Fig. 11 illustrates purity of membrane preparation as was estimated, firstly from enzymatic assay of ATPase (a known marker of plasma membrane) and by peptide profiles of different purification steps using SDS-PAGE gel (Fig. 12). The ATPase activity of final step (P2) of purification was 3.5–4.0 times compared with the microsomal fraction.

Discussion

The cultivation of rice over many areas is hampered by a number of stress factors. Salinity stress is a serious problem arising from an increased sodium proportion in the soil, which is usually a consequence of effluent irrigation (Balks et al., 1998). Scientists evolved methods to restore salinity tolerance in plants, based on the facts that dopamine is synthesised under saline conditions, and is used as a natural product to avoid grazers (Van Alstyne et al., 2006). In this study, we utilised dopamine for rice irrigation prior to exposure to salinity stress. Rice plants were irrigated 2-times per week using low doses of diluted concentration of the common neurotransmitter (dopamine) for two times per week before they were exposed to 0.15 M NaCl. The acquisition of salinity tolerance was discovered by analysing rice plants from physiological and molecular aspects. In the literature, dopamine influences the growth (in low doses) through its interaction with phytohormones, particularly exogenous or endogenous indole acetic acid (IAA) (Protacio et al., 1992). Since the subsequent role of IAA in raising the RWC was also revealed (Gadallah, 2000), we proposed that the indirect effects of dopamine in increasing the water balance of treated plants would occur for its interaction with IAA. The best water content was observed in untreated plants and the lowest water content was detected in salinity-stressed plants (Fig. 1). Dopamine treatments had differentially alleviated the effect of salinity stress.

The dopamine concentration which caused the best water retrieval in salinised plants was 0.2 $\mu g.ml^{-1}$ followed by 0.6 in salinity-free plants and 0.4 in salinised plants, respectively. Investigation of chl *a* level reflected the status of its fluorescence and also the changes which occurred in PSII photochemistry (Zribi et al., 2009). The value of chl *a* was raised, depends on dopamine concentration used in only stressed plants (Fig. 2). The investigated ratio of chl *a/b* exhibited reduction with increasing salinity level (Ashraf and Bhatti, 2000, Dhanapackiam and IIyas, 2010).

In another investigation, total chl content declined but chl *a/b* ratio was non-significantly changed under strong ionic stress (Parida et al., 2004). In the present study, chl *a/b* ratio in irrigated plants with 0.6D before being stressed was quite similar to control and recovered plants followed by 0.2+S, whereas *chl a/b* ratio in 0.4+S plants was relatively high (Fig. 2).

The decrease in chl content under salinity was reasoned either by changes in the lipid protein ratio of pigment–protein complexes or by the increased chlorophyllase activity (Iyengar and Reddy, 1996). Since high accumulation of chl *a* was observed in stressed plants, when only irrigated with dopamine, our data confirmed a crucial role of dopamine in maintenance of pigment–protein complexes and decrease of chlorophyllase activity. For example, the highest value/μgml^{-1} of total chl was: 24 (0.2+S), followed by 23.9 (0.6+S), 18 (0.4+S), 14.84 (0.4D), 13.12 (C), 11.5 (R), 10.74 (0.6D), 7.17 (0.2D) and, finally 5.76 in S (data are not illustrated). Carotenoid levels showed successive increases from 2.36 $\mu g.m^{-1}$ in 0.2D plants to 10.0 $\mu g.m^{-1}$ in 0.2+S plants (Fig. 2).

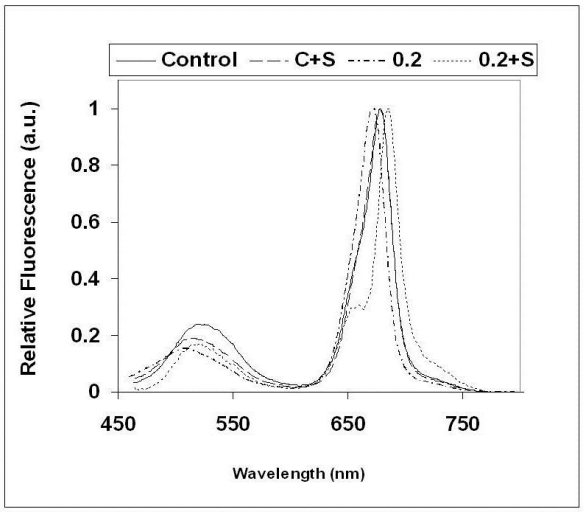

Fig 4. Fluorescence emission spectra of rice plants treated with 0.2 μgml^{-1} dopamine (0.2) or pre-treated with 0.2 µgml-1 dopamine before being exposed to salt stress (0.2+S). Control (C), salt-stressed rice (S). Excitation wavelength 400 nm.

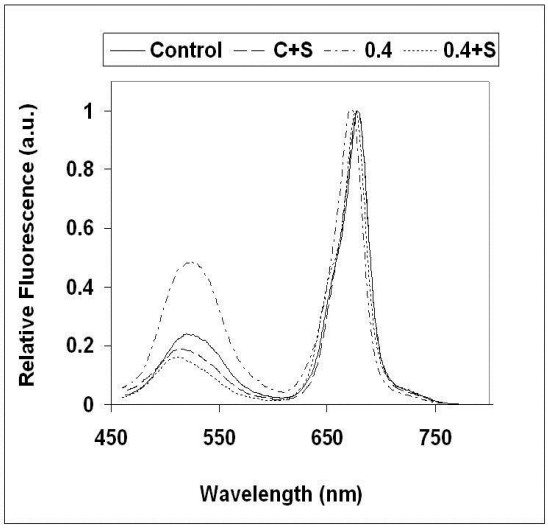

Fig 5. Fluorescence emission spectra of rice plants treated with 0.4 μgml^{-1} dopamine (0.4) or pre-treated with 0.4 μgml^{-1} dopamine prior being exposed to salt stress (0.4+S). Control (C), salt-stressed rice (S). Excitation wavelength at 400 nm.

This finding is in contradiction to the other dopamine-plants, where carotenoid levels decreased from 6.8 $\mu g.m^{-1}$ and 4.2 $\mu g.m^{-1}$ in 0.4D and 0.6D reaching 5.3 $\mu g.m^{-1}$ and 1.44 μgml^{-1} in 0.4+S and 0.6+S, respectively. The carotenoids located in the membranes are participating in the xanthophyll cycle, assisting with rapid trigger of light energy dissipation (Demmig-Adams and Adams, 1996) and, subsequently protecting the plasma membrane. Rice-plants irrigated with 0.2 μgm^{-1} dopamine had promoted carotenoids, both in synthesis and accumulation to confer salinity tolerance in these plants. The opposite was true in the case of 0.6-D plants. Salinity is known to influence the chlorophyll content of plant leaves (Kahn, 2003). Plants grown under high salinity conditions have chlorotic symptoms (Fedina et al.,

2003). Here, stressed rice plants appeared yellowish in colour due to chlorosis (Fig. 9).

Proline is an osmoticum used as stress indicator. Its accumulation level increases to balance the water and ionic homeostasis (Hasegawa et al., 2000). Plants protected with dopamine showed gradual decrease in proline accumulation level. Thus, the lowest proline value was determined in 0.2+S plants. Patel and Vora (2005) found a correlation between proline level and RWC, and this fact was also relevant in our study. The 0.2D rice plants have shown tolerance to salinity which was not observed when the dopamine concentration was increased. Thus, at 0.2 D, plants were not under potent stress. In addition, the lowest accumulation of proline (Fig. 3), which was detected in 0.2+S plants, was accompanied with the highest RWC (Fig. 1). It is known that PSII is sensitive to salt stress and could readily be impaired (Belkhodja et al., 1994; Corney et al., 2003; Fernandez et al., 1997). To precisely underline the dopamine irrigation effect on rice plants under environmental salinity stress, fluorescence emission spectra (excitation wavelength at 400 nm) were analysed in 70% methanolic extracts of rice leaves. Introduction of dopamine (0.2D) in irrigation water caused a blue-shift compared to control plants spectra. 0.2+S plants spectra were significantly red-shifted and a shoulder had appeared (Fig. 4). This spectra appearance could have contributed to the photosynthetic apparatus protection from stress. Dopamine (0.4D) had exhibited fluorescence emission increase at 415 nm.

The highest dopamine concentration (0.6 $\mu g.ml^{-1}$) led to spectra overlapping with control and with 0.6+S plants, which indicated the ruling out of dopamine effect as a hormone, when used in relatively high concentration on the photosynthetic apparatus, particularly PSII. Cell membrane stability technique was potentially used to screen plant tolerance under stress conditions to discover a factor that imparted membrane changes and to reveal the physiological and biochemical changes occurring under stress (Farooq and Azam, 2006). It is well known that the increase of sodium causes ion toxicity and membrane instability due to calcium displacement (Marschner, 1995). In Table 1, the raise of dopamine concentration during irrigation had reinforced the membrane stability in rice plants subjected to salinity stress. The stability of the membrane showed its highest level in 0.6 D plants followed by 0.4D plants and finally, 0.2D plants. As compared to salinised plants, these values revealed the role of dopamine with stress tolerance context.

The statistical analyses using t-test, showed a significant difference in membrane stability between 0.4D and 0.4+S plants and between 0.6D and 0.6+S plants. The difference was also significant in the case of membrane leakage between dopamine-free control and salinised rice plants (Table 1).

Salinity induced a nutrition disorder that could influence plant performance and productivity. It influenced mineral nutrition in plants via its physiological effects on nutrient availability, competitive uptake, transport or partitioning within the cell. Salinity also increased Na transport and accumulation at the expense of reduced K uptake. It also lowered both Ca availability and transport to growing parts (Grieve and Grayyan, 1998). Compared to control and salinised plants, Na seemed to have strongly been encountered by 0.2D action as its accumulation level has sharply declined in 0.2+S plants (Table 2). This effect has, likely, occurred due to the little water uptake determined in 0.2D plants for the very low expression of the *OsPIP 1-3* gene which will be further explained (Fig. 7). The Na decrease was the second in order in 0.4+S followed by 0.6+S plants. Both plants expressed varied levels of aquaporin

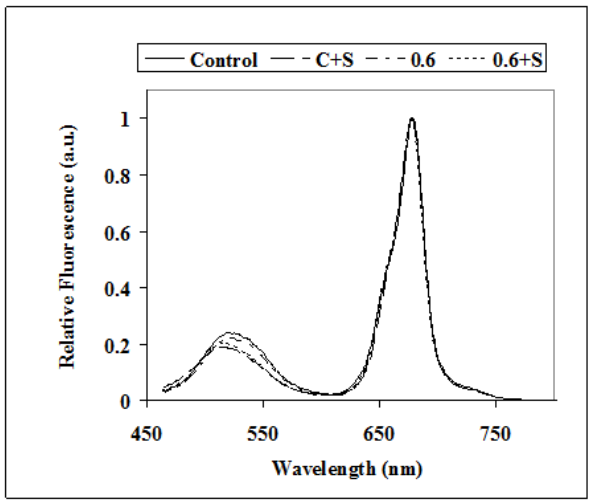

Fig 6. Fluorescence emission spectra of rice plants treated with 0.6 µgml-1 dopamine (0.6) or pre-treated with 0.6 µgml-1 dopamine prior being exposed to salt stress (0.6+S). Control (C), salt-stressed rice (S). Excitation wavelength at 400 nm.

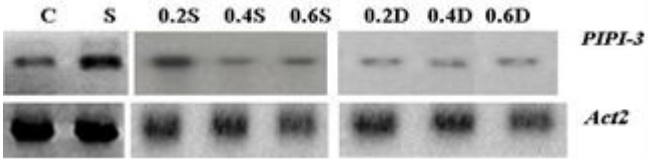

Fig 7. Expression of *PIP1-3* gene in two-month-old *Oryza sativa* plants treated with 0.15M NaCl (lane S) as compared to control plants (lane C) and to exposed plants to salt stress after treated with 0.2, 0.4 and 0.6 µgml-1 dopamine (lanes 0.2S,0.4S and 0.6 S respectively). RNA expression of the gene was also shown when plants were treated with only dopamine different concentrations (lanes 0.2D, 0.4D and 0.6D).

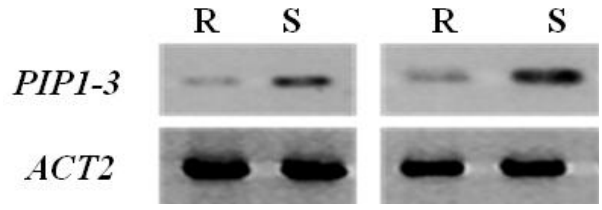

Fig 8. Expression of *PIP 1-3* gene in recovered rice (*Oryza sativa*) from salinity compared to control plants. cDNA of *Actint2* gene (*ACT2*) was used as internal control.

expression (Fig. 7). Magnesium was often transported under competition of Ca, especially in plasma membranes of roots where the high binding affinity of Ca has surpassed that of Mg (Marschner, 1995). Although NaCl was found to decrease Mg in citrus leaves (Ruiz et al., 1997), studies on a number of crop plants had recorded a decrease of Mg in plants different organs, but a stability level of Mg transport in leaves have been observed (Bernstein et al., 1974). The content of Mg has increased under the salinity effect, but the rate of Mg accumulation was relatively lower when dopamine was introduced into rice plants (Table 2). The t-test analyses

revealed a significant value differences between 0.4D and 0.4+S plants with respect to Mg level of accumulation, and between 0.6D and 0.6+S plants regarding K availability level (Table 2).

The expression pattern of *OsPIP 1-3* was studied under different irrigation conditions using dopamine (0.2, 0.4 and 0.6 μgml^{-1}), 0.15 M NaCl, water, in addition to the recovery from salinity. The maize homologue *ZmPIP1-5* was also detected only in roots (Chaumont et al., 2001). The organ-specific expression pattern of *OsPIP* genes suggested that they could be involved with water uptake and transport in a particular area. Plants frequently utilise different strategies under salt stress. Different research groups have reported different functions for the PIP protein family. It was previously reported that *OsPIP1-1* and *OsPIP 1-3* had the ability to transport water in the *Xenopus oocytes* (Lian et al., 2004), but other studies showed that only PIP2 proteins showed a rapid water transport activity, whereas PIP1s have no or a low water transport activity in the *Xenopus oocytes* (Martre et al., 2002).

The present RT-PCR data showed a variable level of *OsPIPs* induction under salinity stress either alone or in combination with dopamine treatments (Figs. 7 and 8). The level of this genes expression was the highest in salinity-stressed plants, whereas the expression of these genes was moderate in control plants. The induction of *OsPIPs* in rice plants irrigated with different concentrations of dopamine decreased below the control in a manner which mimics the rice plants recovered from salinity stress (Figs. 7 and 8). This suggests that the chemical action of dopamine, regardless of the dopamine concentration used, has led to suppression of aquaporin gene expression in unstressed-plants.

This action of dopamine had contrasted the effect of NaCl causing an up-regulation of Os*PIP1-3* gene expression. It might be also speculated that dopamine plays an important role in maintaining cellular turgidity via water permeation. Thus, water irrigation for a further two weeks in rice plants following dopamine treatments made it absolutely necessary to block porins and/or to suppress the *OsPIPs* (Fig. 7). The *OsPIPs* expression level was dopamine concentration-dependent in stressed rice plants (Fig. 7). The highest expression of *OsPIPs* was observed in 0.2D+S plants, followed by 0.6D+S plants and 0.4D+S plants. These results were consistent with the relative water content (RWC) values in the three variables. In terms of the lowest RWC value detected in salinity-stressed plants (S-plants) without dopamine, the overexpression of *OsPIPs* was not efficient, either in maintaining water balance or in decreasing the drought effect (Figs. 7 and 8). Since the typical symptoms reported of high osmotic potential were stomatal closure and wilting effects (Geissler et al., 2009), it is likely that dopamine had decreased these impacts in stressed plants. Thus, it could be suggested that dopamine might have ruled out the effects of stomata closure and drought, as evidenced from the green appearance of irrigated plants with dopamine before salinity exposure (Fig. 10). For further studies, we suggest the use of transgenic plants overexpressing individual PIP genes and also developing PIP knockout transgenic plants to reveal the function of this gene family in water transport in plants.

Fig. 11 shows ATPase activity of the final purification step (P2) that was 3.5–4.0 times compared with the microsomal fraction. In a parallel result, the isolation and purification of intrinsic proteins in Arabidopsis plasma membrane proteome

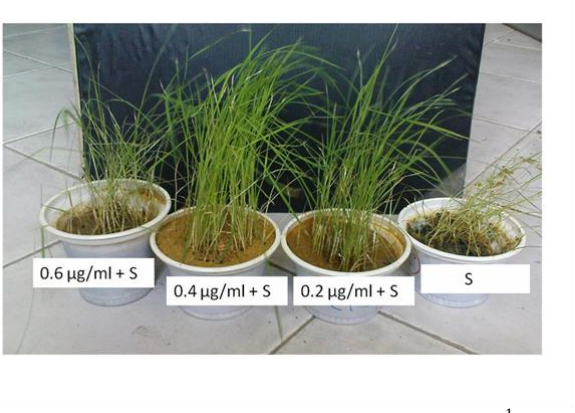

Fig 9. Effect of dopamine (0.2, 0.4 and 0.6 μgml^{-1}) pre-treatments on the morphology of two-month-old rice plants exposed to salt stress for two weeks. The picture shows the drastic salinity effect on dopamine-free seedlings exposed to salinity using 0.15 M NaCl (S).

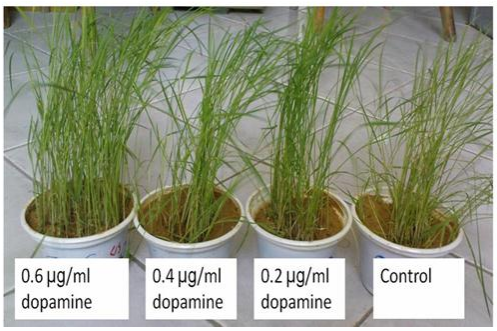

Fig 10. General effect of dopamine concentrations (0.2, 0.4 and 0.6 μgml^{-1}) on the morphology of two-month old rice plants.

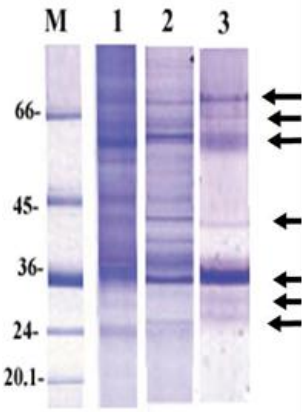

Fig 11. Purification steps of PM intrinsic proteins using coomassie blue-stained SDS-PAGE gel. (1) Microsomal fraction (2) PM fraction after partitioning in a two-phases partition system (3) PM intrinsic proteins after (C/M) extraction. The microsomal fraction (band 1) revealed a large number of proteins, the isolated plasma membrane was fractionated into 12 proteins (band 2), while in lane3 the intrinsic plasma membrane seven proteins (27, 32, 36, 40, 56, 66, 68 kDa) are marked with thick arrows.

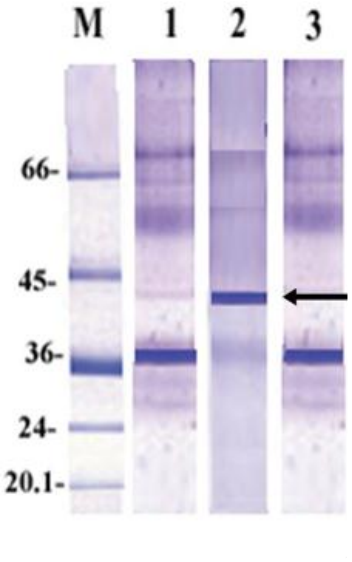

Fig 12. Coomassie blue-stained SDS-PAGE gel illustrated effect of salt stress on rice PM intrinsic proteins of plant leaf: (1) normal, (2) 0.15M NaCl treatment and, (3) recovery after salt stress. The arrow in lane 2 pointed to the over expression of protein band at 40 kDa in salt stressed plants compared to control (lane 1).

had the same ATPase activity result ratio in final step of purification compared with the microsomal fraction using also the two-phase partitioning method (Marmagne et al., 2004). This suggested that the PM contamination by tonoplast and other membranes displaying ATPase activities (endoplasmic reticulum, mitochondria and chloroplasts) was at most 8%. Thus, the present result revealed a high level of plasma membrane purity. As shown in Fig. 12, some PM intrinsic proteins were affected by treatment with salt (band 2). A protein band at 40kDa has been over-expressed compared to control (band1), in agreement with the previous result, in which *Osr40c1*protein (40-kDa protein) associated with salt tolerance in rice (Moons et al., 1995). *Osr40c1* plays a role in adaptive responses of roots to hyperosmotic environments and belongs to a novel plant protein family that most probably has structural functions (Moons et al., 1995) and is widespread in other species (Hochholdinger et al., 2005). The *Osr40c1* protein is rich in histidine residues (6%) constituting putative metal-binding domains and consists of a duplicated domain of 151 amino acids that can form amphiphilic a-helical structures that may be associated with membrane proteins. The PM intrinsic proteins retain their physical properties in the recovery step after removal of salt (band 3). It was proposed that the preferential interaction of anions with the flexible N-terminal part somehow "signals conformational changes in the structured region" resulting in a global destabilisation of the protein molecule (Apetri and Surewicz, 2003). These results revealed that plants can change their gene expression and protein accumulation to counteract salt stress.

Materials and methods

Plant material and stress treatment

Rice grains (*Oryza sativa* cv.Giza 79) were soaked overnight in water (25 °C). The next day, 15 grains were transferred to grow in chamber at 25 °C with a 16/18 h light/dark cycle. Growth took place in 24 pots (15 cm height × 15cm width) with 16 cm diameter each, filled with 1 kg of sandy soil. Plants were irrigated two times per week and left to grow for one month. Thirty-day-old plants were divided into eight groups and labelled according to the treatments, as follows: control (plants irrigated with water for 1 month), salinity-stressed (S) plants (irrigated with water for two weeks, followed with 0.15 M NaCl for another two weeks), 0.2D plants (irrigated with 0.2 $mg.ml^{-1}$ dopamine for two weeks, then with water for another two weeks), 0.4 D plants (irrigated with 0.4 $mg.ml^{-1}$ dopamine for two weeks, then with water for another two weeks), 0.6 D plants (irrigated with 0.6 $mg.ml^{-1}$ dopamine for two weeks, then with water for another two weeks), 0.2+S (irrigated with 0.2 $mg.ml^{-1}$ dopamine for two weeks, then with 0.15 M NaCl for another two weeks), 0.4+S plants (irrigated with 0.4 $mg.ml^{-1}$ dopamine for two weeks, then with 0.15 M NaCl for another two weeks), finally, 0.6+S plants (irrigated with 0.6 $mg.ml^{-1}$ dopamine for two weeks, then with 0.15 M NaCl for another two weeks). Five replicates from each group were elicited for physiological and molecular analyses.

Relative water content

Leaf relative water content (RWC) was estimated by recording the turgid weight of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in a hot air oven until constant weight was achieved (Weatherley, 1950). RWC = (Fresh wt:-Dry wt)/(Turgid wt:-Dry wt) X100

Pigment analysis and measurements

Pigment content from rice leaves was extracted in 80% acetone. The pigment amount was calculated according to Brouers & Michel-Wolwertz (1983).

Proline accumulation

Proline content was assayed according to the method described by Bates et al., (1973).

Fluorescence spectroscopy

In vivo fluorescence emission spectra were recorded using a Shimadzu RF5301 spectrofluorophotometer in the range (290–750 nm). The absorption spectra were recorded with a Unicam UV–visible double beam spectrophotometer from Helios Company Ltd (England). The emission was measured as photon emission per unit interval of wavelength. The fluorescence excitation spectra were electronically corrected for variations in the lamp emission spectrum. The fluorescence emission spectra were corrected for the spectral sensitivity of the photomultiplier. The spectra were normalised at the highest peak. The results were the average of at least five repetitions of each experiment. Emission spectra were measured with the excitation wavelength at 400 nm.

RNA isolation and cDNA synthesis

Total RNAs of the whole rice seedlings were isolated using the Plant RNeasy extraction kit (Qiagen, USA) and then digested with RNase-free DNaseI (Boeheringer Mannheim, Germany) according to the manufacture's instruction. RNA concentration was measured by spectrophotometer. Five micrograms of total RNA was used to synthesise cDNA using SuperScript TM II RNase H- Reverse Transcriptase Kit

(Invitrogen, USA) according to the manufacturer's instructions.

Primer design and cDNA cloning

The sequences of the forward and reverse primers for PCR amplification of full-length cDNAs of *OsPIP-3* gene for semi-quantitative RT-PCR are listed below: Forward: 5'-CGGCAAGAGAGCTAGTAGTG-3'; Forward: 5'-TAGAG-ACTTGATCCTCCATCAT-3'; Primers were synthesised at Sigma INC. DNA sequence comparisons were made to ensure that the pair of primers is specific to the corresponding *OsPIP* gene.

PCR amplification

PCR specific amplification was carried to amplify *PIP- 3* from rice (*Oryza sativa*). By optimising the number of PCR cycles, RT-PCR was set at 30 cycles in order to reflect a semi-quantitative way of estimating the abundance of mRNA. RNA was first reverse transcribed into cDNA using reverse transcriptase enzyme. Annealing temperature was 55°C. The resulting cDNA was diluted 1:10 and used as template for subsequent PCR amplification using gene-specific primers.

Membrane leakage

Membrane leakage (ML) was determined according to Vahala et al., (2003).

Mineral Analysis

Aliquots (50–500 mg) from each of the dried plant specimens were weighed and then wet-washed by refluxing overnight at 150 °C with 15 ml of concentrated HNO_3 and 2.0 ml of 70% $HClO_4$. The samples were taken to dryness at 120 °C and the residues dissolved in 10 ml of 4.0% HNO_3 –1% $HClO_4$ solution. The mineral content of each sample solution was determined by inductively coupled argon plasma atomic emission spectroscopy (ICP-AES, Jarrel-Ashas described by Yazzie et al., 1994). The samples were quantified against standard solutions of known concentration that were analysed concurrently. Each data sample was the mean of three replicates.

PM isolation and purification

The PMs of rice leaf were isolated according to the methods of Larsson et al. (1987), with a few modifications. Leaves were cut and washed three times with chilled, deionised water and ground in ice-cold homogenisation buffer using a mortar and pestle. The homogenisation buffer contained 250 mM sucrose, 250mM KI, 2mM EGTA, 10%v/v glycerol, 0.5%w/v BSA,2mM DTT, 1mM PMSF and 15mM b-mercaptoethanol, pH 7.8. The homogenate was centrifuged at 11,500g for 15 min at 41 °C to remove debris. The supernatants (whole cell lysate) were centrifuged further at 87,000g for 35min. The microsomal pellets were re-suspended in phase buffer (250 mM sucrose, 3mM KCl, and 5mM KH_2PO_4, pH 7.8). The microsomal membrane preparation was fractionated by two-phase partitioning in aqueous dextran T-500 and PEG, according to the methods of Yan et al., (2002), The PM fraction was again partitioned in a two-phase partition system consisting of 0.7 M PEG/K-PO4, pH 7 to eliminate contamination of the PM fraction with PEG (Busby and Ingham 1980). The PM was recovered as the pellet (P1) in the saline lower phase after an ultracentrifugation (110,000g) and the pellet was dissolved in 50 mM 4-morpholinepropanesulfonic acid (MOPS)/NaOH, pH 7.8, 1 mMdithiothreitol (DTT). Protein amounts were estimated using Bradford procedure (Bradford, 1976). For the analysis of PM purity, the activity of the Mg2+- ATPase activity was used as a marker of PM purification (Briskin et al., 1987) and by SDS-PAGE using 12% polyacrylamide gel.

Chloroform/Methanol (C/M) Extraction of intrinsic PM proteins

Hydrophobic proteins were extracted from the purified PM fraction using a C/M (v/v) treatment as described by Seigneurin-Berny et al. (1999).

Statistical analysis

Statistical analyses were performed using SPPS 17.0 for windows. The means were calculated from three to five separate experiments. The significance was calculated using t-test (2-tailed) ANOVA at p=0.05 and the data were evaluated according to Tukey's or Scheffe's tests. The means were separated by Duncan's multiple range test at 0.05 probability level.

Conclusion

The results of this study indicated that rice plants treated with dopamine and grown under salt stress conditions had achieved up-regulations in the pigment and relative water contents. Dopamine encountered sodium accumulation, proline and membrane leakage. On the other hand, increased pigment content and stability index of the membranes were observed after dopamine treatments. Dopamine caused down-regulations in *OsPIP1-3* expression level. The induction of *OsPIPs* in rice plants irrigated with dopamine decreased below the control in a manner which mimics the rice plants recovered from salinity stress. We point to the possible dopamine efficiency in maintaining cellular turgidity through water permeation. The water irrigation might lead to aquaporin blocks and/or low expression level of *OsPIPs*. We recommend the application of the neurotransmitter 'dopamine' for salinity tolerance purposes in rice pants.

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