Light-induced Inversion of Na+ Uptake in Rice (*Oryza sativa* L.) under Salt Stress

Imshik Lee (ilee@nankai.edu.cn)
Nankai University https://orcid.org/0000-0002-0098-392X

Yajun Sun
Northeast Forestry University

Xiaoxue Ye
Northeast Forestry University

Yipeng Cao
Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Deng Gao
Nankai University School of Physics

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Abstract

Background: Rice is a salt-sensitive crop, and salinity is a major negative constraint on rice production, while light has a major positive effect on plant growth and development. How plants integrate the external salinity and light signals remains poorly understood.

Results: Here, we demonstrated that light exposure enhanced the cation (Na^+, K^+, H^+, and Ca^{2+}) efflux from the rice (*Oryza sativa* L.) root for both salt-free and NaCl-treated rice. Surprisingly, net flux of Na^+, $J(Na^+)$, in NaCl-treated rice was inverted in the light-on state. This inverted light-inducible $J(Na^+)$ signal suggests crosstalk between salt stress and light signals, with salt stress interfering with Na^+ transport via down regulation of the light receptors. PPI network of this crosstalk suggested that there were key players, phytochrome A and B, phototropin 1A and 1B, and 2 at the bridging group and AKT1 potassium channel, monovalent cation/H^+ antiporter and Na^+/K^+ antiporter (SOS1) at the interface between bridging group and cation transport group.

Conclusions: Crosstalk between light and salt stress caused the inverted light-inducible $J(Na^+)$ signals. Phytochrome A and B, phototropin 1A and 1B, and 2 at the light-responding group and AKT1 potassium channel, monovalent cation/H^+ antiporter and Na^+/K^+ antiporter (SOS1) at salt-stress responding group play an important role in this unique inverted light-inducible $J(Na^+)$ switch, which offers a new paradigm to understand the crosstalk between salt-stress and light signals.

Background

Rice is the world's most important crop, as it is used as food for about half of the world's population (Kumar et al., 2013). However, rice is a salt-sensitive plant, and salinity is one of the main factors that lowers rice productivity. Thus, soil salinity is a major environmental issue, especially regarding sustainable agricultural development of irrigated land (Zhu, 2001; Rengasamy, 2010).

Salt-stress causes water deficiency and disruption of cellular ion homeostasis in plants (Niu et al., 1995). The cytosolic concentration of K^+ ($[K^+]_{cyt}$) is critical for the proper functioning of plant cells, maintaining appropriate enzyme activity and intracellular turgor (Apse and Blumwald,2007; Kronzucker and Britto, 2011). Excess cytosolic ions not only cause ion toxicity but also suppress cytosolic and organelle processes. Na^+ concentrations > 0.4M inhibit most enzymes due to disruption of the balance of the electrostatic and hydrophobic interactions required to maintain protein structure. A $[Na^+]_{cyt}$ of 0.1 M directly affects certain biochemical and physiological processes because Na^+ competitively binds to K^+ binding sites (Szczerba et al., 2009; Deinlein et al., 2014). Plants react to salt-stress by attempting to maintain a low $[Na^+]_{cyt}$ and high $[K^+]_{cyt}/[Na^+]_{cyt}$ ratio. Plants are equipped with Na^+ extrusion and/or Na^+ compartmentalization mechanisms to maintain a high $[K^+]_{cyt}/[Na^+]_{cyt}$ ratio. These mechanisms are activated by network signaling.

Light is one of the most important environmental factors for plant growth and development (Quail, 2002; Franklin et al., 2005), while salt in the soil inhibits the photosynthetic process (Liska et al., 2003; Baker 2008; Stepien and Johnson, 2009). The effects of light on plant development can be classified into three categories: the development of energy sources, the induction of developmental signals, and harmful effects. The energy sources promote growth by photosynthesis, the developmental signals regulate morphogenesis (such as transition to reproductive development), and excessive absorption of light promotes the formation of harmful compounds (such as free radicals). Light has a wavelength-dependent effect on plants (Smith, 1994; Lin, 2000; Xie, 2014). Typically, plants have phytochromes, which absorb red and far-infrared rays; cytochromes and phototropin, which absorb blue light; and tryptophans, which absorb ultraviolet-A/B light.

A molecular signaling network involves two or more signaling pathways that converge, diverge, and interact depending on specific environmental factors (Nakagami et al., 2005). Thus, there is crosstalk between the pathways influenced by environmental factors (Glombitza et al., 2004; Mahajan and Tuteja, 2005). Understanding the interactions between light and salt signals represents an important challenge that could improve rice productivity (Indorf et al., 2007; Porcel et al., 2015). In this study, real-time light-induced Na^+, K^+, H^+, and Ca^{2+} fluxes in rice with or without salt-stress were assessed. A salt-tolerant model plant, *Puccinellia tenuiflora* (Newman et al., 2012), was compared to rice. Surprisingly, real-time evidence of crosstalk between light and salt stress signals appeared to involve a light-inducible switch that altered the Na^+ signal only in rice roots under salt-stress and not in *P. tenuiflora*.

Results

Light-induced $J(Na^+)$

Weexamined the real-time crosstalk between light and salts-tress signals. $J(Na^+)$ under light exposure in the NaCl-free and NaCl-treated rice roots was recorded at $[NaCl]_m$ of 0, 50, 100, and 200 mM. The measurements were started in a5-min light-on state followed by a 5-min light-off state, and this operation was repeated twice more, as shown in Fig. 1. According to our replicates involving different plants, the scale of J was different for each plant. Thus, the set of results for each treatment (NaCl-free and NaCl-treated conditions) was obtained from a single plant (with the abovementioned three replicates), to allow comparative analysis.

Regarding the NaCl-free results, it is easily observed that $J(Na^+)$ differed between the light-on and light-off states. In the light-on state, $J(Na^+)_{on}$ decreased gradually, while in the light-off state, $J(Na^+)_{off}$ increased gradually. This pattern represents a diurnal rhythm, which is consistent with the expression pattern of members of the salt overly sensitive (SOS) pathway (Soni et al., 2013). The mean difference ($\Delta J(Na^+)$) between $J(Na^+)_{on}$ and $J(Na^+)_{off}$ was positive at $[NaCl]_m$=0–100 mM, indicating a remarkable enhancement of Na^+ efflux in the light-on state relative to the light-off state ($p < 0.05$). As $[NaCl]_m$ increased, the light-induced enhancement of Na^+ efflux faded (Fig. 1A), with no clear difference between

$J(Na^+)$ in the light-on and light-off states at $[NaCl]_m$=200 mM ($p > 0.05$), and signal difference wasn't bigger than noises. The mean of both $J(Na^+)_{on}$ and $J(Na^+)_{off}$ (averaged across the repeated measurements) was negative at $[NaCl]_m$=200 mM, indicating an influx of Na^+.

Interestingly, the $J(Na^+)$ pattern in the NaCl-treated rice was inconsistent with that in the NaCl-free rice. In terms of similarities, $J(Na^+)$ was significantly different between the light-on and light-off states in the NaCl-treated rice ($p < 0.001$) (as in the NaCl-free rice at $[NaCl]_m$=0–100 mM) and, at $[NaCl]_m$=0 mM, the $J(Na^+)$ pattern in the NaCl-treated rice was similar to that in the NaCl-free rice. However, $[NaCl]_m \geq 50$ mM, the $J(Na^+)$ pattern was completely inverted, as shown in the red box in Fig. 1B. More precisely, compared to in the NaCl-free rice, $J(Na^+)_{on}$ was more shift to be negative (indicating a relative increase in Na^+ influx) and $J(Na^+)_{off}$ was more shift to be positive (indicating a relative increase in Na^+ efflux). While $\Delta J(Na^+)$ in NaCl-free rice was positive at $[NaCl]_m$=0–100 mM, $\Delta J(Na^+)$ in NaCl-treated rice was inverted, becoming negative at $[NaCl]_m \geq 50$ mM. Thus, under salt-stress, a light-inducible switch caused inversion of the Na^+ signal the root of rice.

Inverted light-inducible switch regarding the $J(Na^+)$ signal at various $[NaCl]_m$ values

We assessed both $J(Na^+)_{on}$ and $J(Na^+)_{off}$ of NaCl-treated rice (during 5-min light-on and 5-min light-off states, respectively) in ascending $[NaCl]_m$ values from 0 to 100 mM (in increments of10 mM) and descending $[NaCl]_m$ values from 100 to 10 mM (in decrements of10 mM) (Fig. 2). A unique inverted light-inducible switch was observed at $[NaCl]_m \geq 10$ mM for the NaCl-treated rice. At $[NaCl]_m$=0 mM, $\Delta J(Na^+)$ was positive like in the salt-free samples. However, at $[NaCl]_m \geq 10$ mM, $\Delta J(Na^+)$ became negative, indicating the operation of an inverted light-inducible switch.

The $J(Na^+)$ profile showed that $J(Na^+)_{on}$ was positive at $[NaCl]_m$=0, but the inverted light-inducible switch made it become negative from $[NaCl]_m$=10 mM. The $J(Na^+)$ signal difference between at light-on and at light-off status was maximal at $[NaCl]_m$=40 mM, with $\Delta J(Na^+)$ = -2.87 ± 1.21 ($p < 1 \times 10^{-4}$) and − 1.45 ± 0.51 nM/cm^{2}s ($p < 1 \times 10^{-4}$) for the ascending and descending $[NaCl]_m$ processes, respectively.

$J(Na^+)_{on}$ was substantially negative at $[NaCl]_m \geq 10$ mM (indicating Na^+ influx into the roots under light exposure) until $[NaCl]_m$ reached 50 mM, at which point it was still negative but the magnitude was small. In other words, Na^+ continued to be substantially absorbed into the rice roots in the light-on state until the extracellular NaCl concentration reached 50 mM. However, as $[NaCl]_m$ increased > 50 mM, $J(Na^+)_{on}$ became positive (indicating Na^+ efflux out of the roots under light exposure), though $J(Na^+)_{on}$ was still significantly lower than $J(Na^+)_{off}$ ($p < 0.001$). Figure 2 shows that $\Delta J(Na^+)$ was not reversible in terms of $[NaCl]_m$, but $J(Na^+)_{off}$ appeared to be somewhat proportional to $[NaCl]_m$, with the slope of $J(Na^+)_{off}$ vs $[NaCl]_m$ being $\sim 2.24 \times 10^{-5} cm^{-2} s^{-1}$ in both the ascending and descending $[NaCl]_m$ processes.

Light-induced $J(K^+)$

The Na^+/K^+ ratio is critical for maintaining cell physiological functions, and the light-induced net flux of K^+, $J(K^+)_{on}$, was measured under the same conditions as $J(Na^+)$. Figure 3 shows that $J(K^+)_{on}$ decreased gradually(though the mean $J(K^+)_{on}$ tended to be positive) and $J(K^+)_{off}$ increased gradually (though the mean $J(K^+)_{off}$ tended to be negative), in both the NaCl-free and NaCl-treated rice. Thus, $J(K^+)$ generally demonstrated a diurnal rhythm at $[NaCl]_m$<200 mM. This indicated that light enhanced the K^+ efflux in NaCl-treated rice, except at the high extracellular NaCl concentration. No inverted light-inducible switch was observed for $J(K^+)$, unlike for $J(Na^+)$. Because K^+ efflux would be enhanced at the high NaCl concentration ($[NaCl]_m$=200 mM), it seemed that K efflux mechanism activated at both light-on and light-off not to observe significant.

Figure 3. $J(K^+)$ in rice experienced for 5 days in (**A**) NaCl-free and(**B**) 10 mM NaCl conditions. Real-time extracellular NaCl concentrations ($[NaCl]_m$) were set at 0, 50, 100, and 200 mM. *J* is the net ion flux (with positive indicating efflux and negative indicating influx). Light exposure was at 54 μmol/m^2s intensity.

Light-induced $J(H^+)$ and $J(Ca^{2+})$

As the cations H^+ and Ca^{2+} play very important roles in cellular Na^+ homeostasis, it is expected that they may be involved in crosstalk with the Na^+ signals. We investigated how H^+ and Ca^{2+} fluxes in NaCl-treated rice responded to light exposure at $[NaCl]_m$=0 and 20 mM.

The light-induced $J(H^+)$ pattern was similar to the $J(K^+)$ pattern (Fig. 4A). Light enhanced H^+ efflux. As $[NaCl]_m$ increased from 0 to 20 mM, $J(H^+)_{on}$ was ~ 2 pM/cm^2s at both concentrations, with no significant difference ($p > 0.1$). However, $J(H^+)_{off}$ was increased at 20 mM, which reduced $\Delta J(H^+)$ at this higher extracellular NaCl concentration. In other words, the H^+ efflux was similar at $[NaCl]_m$ of 0 and 20 mM in the light-on state, but increased as $[NaCl]_m$ increased in the light-off state.

The light-induced $J(Ca^{2+})$ response was a little different to the light-induced $J(H+)$ response (Fig. 4B). $J(Ca^{2+})$ tended to rise more slowly in the light-on state, though it also decreased in the light-off state. Curves were fitted using $J_{on}=A(1-e^{-t/to})+B1$ for the light-on state and $J_{off}=Ae^{-t/to}+B2$ for the light-off state, where *A* is amplitude, t_o is relaxation time, and *B1* and *B2* are base lines (standing for the initial signals after changing light state). The J_{on} baseline was higher than the J_{off} baseline (as for H^+ and K^+). The relaxation times of $J(Ca^{2+})$ were ~ 30 and ~ 60 s at $[NaCl]_m$=0 and 20 mM, respectively. In the presence of extracellular NaCl (20 mM), the light-induced changes in $J(Ca^{2+})$ were slower than at $[NaCl]_m$=0. In the presence of extracellular NaCl, the change in $J(Ca^{2+})$ from the baseline was increased and the light-inducedenhancedCa^{2+} efflux was somewhat lessened.

Light-induced $J(Na^+)$ in *P.tenuiflora*

The salt-tolerant model plant *P.tenuiflora* was compared to the salt-sensitive rice. Figure 5A shows that *P. tenuiflora* did not exhibit a similar pattern of $J(Na^+)_{on}$ and $J(Na^+)_{off}$ to that in Fig. 1 for rice. For NaCl-free *P. tenuiflora*, there was no obvious difference between $J(Na^+)_{on}$ and $J(Na^+)_{off}$. It seems that $J(Na^+)$ in *P. Tenuiflora* was not sensitive to light. Regarding NaCl-treated *P. tenuiflora* (10 mM NaCl for 7 days), $\Delta J(Na^+)$ was positive (indicating efflux) at $[NaCl]_m$ of 0, 50, 100, and 200 mM which was similar to the K^+, H^+, and Ca^{2+}efflux in the light-on state in NaCl-treated rice.

Discussion

Light-induced enhancement of cation efflux in rice

It has been reported that light can change ion signals in plants. Research showed that red and blue light improved the uptake of K^+ and Rb^+ in bean leaves (Blum et al., 1992). Illumination near the mesophyll and attached epidermis of bean also caused changes in H^+, K^+, Ca^{2+}, and Cl^- concentrations (Shabala and Newman, 1999). It was also shown that *Arabidopsis* hypocotyl cells exhibited light-induced changes in Cl^-, K^+, and Ca^{2+} fluxes; in particular, blue light illumination induced Cl^- and K^+ efflux from the hypocotyl cells (Babourina et al., 2002).

Homeostasis of Na^+, K^+, H^+, and $Ca^{2+}$is vital for cellular physiological processes and protein function, so we assessed these cations under salt stress and light exposure to identify possible crosstalk between salt stress and light signals. We measured real-time cation fluxes in the root. We found that the scale of *J* in the different replicates varied, though the cation flux patterns were consistent. The differences in scale might have been caused by differences in the sensitivity and measuring point on the root in each experiment. Therefore, to comparatively study the cation fluxes, we conducted experiments on the real-time light and extracellular NaCl conditions using the same plant for each treatment (i.e., the NaCl-free and NaCl-treated conditions).

There was light-induced enhancement of Na^+, K^+, H^+, and Ca^{2+}efflux in the NaCl-free rice in a NaCl-free environment ($[NaCl]_m$=0 mM); as $[NaCl]_m$ increased, this enhancement faded, e.g., there was no discernable difference in $J(Na^+)$ between the light-on and light-off states at $[NaCl]_m$=200 mM. In other words, the light-induced enhancement of cation efflux was strongest in the NaCl-free environment, with light-induced enhancement fading under high extracellular NaCl concentrations. Salt stress (10 mM NaCl) seemed to suppress the light-induced enhancement of cation efflux. The light-induced enhancement of cation efflux is likely related to light-induced changes in the plasma membrane potential. In other words, light-induced cation in flux causes plasma membrane depolarization (Shabala and Newman, 1999) and/or hyper-polarization (Roelfsema et al., 2001), leading to changes in ion channel activities. Although we didn't collect direct data on the relationship between the changes in the membrane potential and changes in trans-membrane ion transportation, we assumed that the light-induced changes in the membrane potential influenced the transition of the channels between conducting and non-conducting states. According to the Goldman–Hodgkin–Katz flux equation, ion flux is driven by diffusion down the

ion concentration gradient and membrane potential (Goldman, 1943; Hodgkin and Katz, 1949). It was assumed that the membrane potential that resulted from the light-induced depolarization and/or hyper-polarization played an important role in the enhanced cation efflux. However, long exposure to salt stress (such as in our experiments) will cause damage or allow time for new defense gene expression-related mechanisms to come into operation, and both changes in membrane potential and new gene expression leading maybe involved in the light-induced changes in ion signals.

Inverted light-inducible switch regarding $J(Na^+)$ in rice

Figure 1 shows that $J(Na^+)$ clearly changed between the light-on and light-off states. For the NaCl-treated rice (10 mM NaCl for 5 days), the various cation fluxes exhibited similar patterns except for $J(Na^+)$. In the case of $J(Na^+)$ at $[NaCl]_m$=10 mM, the pattern became inverted, as $J(Na^+)_{on}$ was negative while $J(Na^+)_{off}$ was positive. This pattern was designated the “inverted light-inducible switch.” Regarding the salt-tolerant *P. tenuiflora*, this switch was not observed. The presence of an inverted light-inducible switch was a unique characteristic of the salt-sensitive rice. In contrast to in rice, there appeared to be well-balanced crosstalk between light and Na^+ signals in the root of *P.tenuiflora*, with the $\Delta J(Na^+)$ enhancement in *P.tenuiflora* being similar to the$\Delta J(K^+)$, $\Delta J(H^+)$, and$\Delta J(Ca^{2+})$ in NaCl-free and NaCl-treated rice.

If the inverted light-inducible switch in the rice root was driven by the light-induced change in the membrane potential, other cation fluxes would have exhibited the same switch. As no similar switch was found for other cations, it was assumed that a change in gene expression caused this inverted $\Delta J(Na^+)$. When plants undergo longer-term salt-stress, they eventually respond better to the salt stress, as new salt regulation mechanisms are implemented (Botella et al., 2005). Thus, after the rice seedlings were grown under salt stress (10 mM NaCl) for 5 days, a new gene expression-related regulatory mechanism for Na^+ uptake and extrusion may have come into operation.

Ion signal pathways often regulate environmental stress response genes such as salt-stress response genes. These genes are divided into early and delayed response genes. The early response genes are activated at the early stage of salt stress but the activation is not long lasting. The delayed response genes are activated after several hours of salt stress, and once activated, they are activated for a long time (Xiong and Yan, 2003). Salt stress would be an example of a factor that can induce the regulation of the expression of upstream or downstream genes that defend the plant against adverse external factors, and the inverted $\Delta J(Na+)$ signals might be caused by the expression of delayed response genes and/or transient impairment of the regulatory mechanisms underlying the new gene expression.

Generally, Na^+ influx into the root involves passive transport, and the transporter types include high-affinity K^+ transporters (HKTs), low-affinity cation transporters (LCTs), and voltage-dependent nonselective cation channels (NSCCs). HKTs have been shown to function as Na^+/K^+ symporters and Na^+-selective uniporters (Horie and Schroeder, 2003). In rice, which is a salt-sensitive plant, OsHKT2;1 and OsHKT2;2 have been shown to be expressed in the root (Kader et al., 2006). LCTs may be responsible for

Ca^{2+}-insensitive Na^+ influx. At high external Na^+ concentrations, NSCCs may be the main pathway for Na^+ influx into the root. Na^+ efflux from the root is facilitated by Na^+/K^+ transporters, P-type ATPase, SOS1, and members of the cation/proton exchange (CHX) family. Na^+ efflux is also a competitor for uptake via plasma membrane K^+-inward rectifying channels (KIRC), such as AKT1 channels (serine/threonine-protein kinases) (Zhu, 2001).

To determine the possible causes underlying the inverted light-inducible switch regarding $\Delta J(Na^+)$ in NaCl-treated rice, a protein–protein interaction (PPI) network of salt tolerance-related and light-related proteins in rice was constructed (Franceschini et al., 2013).We identified 211 salt-tolerance-related proteins and light-related proteins using the UniProt database(Wu et al., 2006).The 211 protein names were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (which contains various types of evidence on PPIs, involving gene and protein analyses) and the PPIs with confidence scores > 0.4were selected of the 211 proteins, 166 (Table S1) had evidence of PPIs(Fig. 6). There were three PPI groups, which were characterized as the light-sensitive group, cation transporter group, and bridging group.

The bridging group contains blue-light receptors (phototropin1A, 1B, and 2) and red-light receptors (phytochrome A and B). Phytochrome A and B are negatively regulated in response to salt stress in salt-sensitive *Arabidopsis* (Franklin and Quail, 2010). In salt-tolerant plants under salt stress, phytochrome mediates an increase in the expression of endogenous abscisic acid (ABA) and jasmonic acid (JA) (Yang et al., 2018), which activate the reactive oxygen species (ROS)-scavenging enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD). In turn, these enzymes minimize damage by salt stress-induced ROS in salt-tolerant plants. *P.tenuiflora* is a salt-tolerant model plant with well-developed salt regulation mechanisms, and it didn't exhibit an inverted light-inducible switch regarding $J(Na^+)$ (Fig. 5). However, for salt-sensitive rice, phytochrome A and Bare down regulated under salt-stress (Wang et al., 2018) with no significant up-regulation of ABA and JA ($p > 0.05$) (Mizuno et al., 2010). It seems that, in rice, the ROS-scavenging system isn't enough to activate under salt stress, so ROS may have damaged the Na^+ transportation system, which may be associated with the mal function of the crosstalk between the light and Na^+ signals. It seemed that salt-stress induced changes in light receptor expression impaired Na^+ transporter, and this inverted light-inducible $J(Na^+)$ signal suggested crosstalk between salt stress and light signals, with salt-stress interfering with Na^+ transport via down-regulating the light receptors.

In the cation transporter group, there are five key players in genes (marked with red triangles in Fig. 6) that had evidence of more than five interactions with light receptors. The first gene (gene ID 4326245) encodes a K^+ channel (AKT1) that mediates K^+ uptake by plant roots. It is thought to open and close in response to the membrane potential, being activated by hyperpolarization, and it may be a major salt-sensitive K^+ channel in roots. Three of the genes (gene IDs 4337811, 4346626, and 4344217) encode monovalent cation/H + antiporters. The fifth gene (gene ID4352928) is SOS1, which encodes a plasma membrane Na^+/H^+ antiporter. SOS1, SOS2, and SOS3 contribute to salt tolerance in plants, with SOS1

serving as an extremely salt sensitive Na^+ efflux channel (Zhu, 2001; Qiu et al., 2002). SOS1 interacts directly with a multidrug-resistance protein (gene ID 4336042). The PPI network suggested that our empirical observation regarding the inverted $\Delta J(Na^+)$ may be associated with down-regulation of the light receptors in the bridging group, leading to decreases in SOS1 activity.

Relationships of net $J(K^+)$, $J(H^+)$, and $J(Ca^{2+})$ with light-induced Na^+ signal

Cytoplasmic Na^+ is closely related to K^+, H^+, and Ca^{2+}. K^+ and Na^+ are often transported by the same transporters, such as KIRC, KORC, VIC, K^+ uptake transporter-high-affinity K^+ transporter (KUP-HAK), and HKT1 (White and Lemtiri-Chlieh, 1995; Amtmann and Sanders, 1998; White, 1999; Ashraf, 2004). Additionally, in plants, the main mechanism for Na^+ efflux involves the plasma membrane H^+-ATPase (Amtmann, 1997), while Ca^{2+} alleviates Na^+ toxicity via various mechanisms, including controlling K^+/Na^+ selective accumulation (Mahajan and Tuteja, 2005). Therefore, we investigated K^+, H^+, and Ca^{2+}. Figures 3–5 show that light exposure enhanced the K^+, H^+, and Ca^{2+}efflux in NaCl-free and NaCl-treated rice.

In general, K^+ channels transport K^+ and Na^+ in opposite directions. Therefore, we expected that light-induced $J(K^+)$ and $J(Na^+)$ would be in opposite directions, but we did not observe evidence of Na^+/K^+ antiporter flux, because our observations were not from a single ion channel but involved net ion flux. According to our results, light was an enhancer of net cation efflux. As seen in Fig. 4A, light enhanced H^+ efflux and the absence of light led to weakened H^+ efflux. The plasma membrane H^+-ATPase, which mediates Na^+ efflux, uses energy from ATP hydrolysis to pump H^+ out of the cell, generating an electrochemical H^+ gradient. This proton-motive force operates the Na^+/H^+ antiporters, which couple the movement of H^+ into the cell (along its electrochemical gradient) to the extrusion of Na^+ (against its electrochemical gradient). If light could directly affect H^+-ATPase, which is an active pump involved in salt tolerance, light-induced change in $J(Na^+)_{on}$ would be observed even under harsh salt stress, the enhancement of the H^+ efflux by light might be due to some unknown reasons that require further investigation.

There is a Ca^{2+}-dependent SOS pathway involved in salt-stress signaling that controls ion homeostasis and salt tolerance. Ca^{2+} binds to SOS3, which interacts with and activates the kinase SOS2, which in turn phosphorylates and thereby regulates the plasma membrane Na^+/H^+ antiporter SOS1 (Zhu, 2001). The light-induced enhancement of Ca^{2+}efflux was slow compared to the enhancement of other cation fluxes, but the $J(Ca^{2+})$ pattern was rather similar to the $J(K^+)$ and $J(H^+)$ patterns.

The inverted light-inducible switch that appeared for $J(Na^+)$ in the NaCl-treated group was unique to $J(Na^+)$, only interrupting the Na^+ signal. According to the PPI network diagram (Fig. 6), salt stress might disrupt Na^+/Ca^{2+} and/or monovalent cation/H^+ exchangers, which may be involved in the unique light-inducible switch in the salt-sensitive rice.

Inverted $\Delta J(Na^+)$ signal

There is still uncertainty regarding the explanation for the appearance of the inverted $\Delta J(Na^+)$ (i.e., it became negative, indicating Na^+ influx into the roots) in the NaCl-treated rice. One possible explanation is that a Na^+-related ion channel in the plasma membrane exhibited reversal. The voltage-gated Na^+ channel has a wide funnel in the extracellular matrix, with an intracellular activation gate (McCusker et al., 2011; Payandeh et al., 2011). Statistically, the normal channel would be dominant, but reversal may be possible. However, there is currently no possible mechanism underlying potential channel reversal. Also, $\Delta J(Na^+)$ is not the result of a single channel but, rather, it is the net $J(Na^+)$, involving a diverse set of channels after crosstalk.

The inverted light-inducible J(Na+) signal indicated crosstalk between salt stress and light signals. For *A. thaliana* and *N. tobacum*, salt stress causes down-regulation or mutation of photoreceptors (phototropin and phytochrome A and B) (Indorf et al, 2009; Yang et al., 2018), which are associated with up-regulation of JA and ABA. However, for a salt sensitive plant, rice (Mizuno et al., 2010), no sufficient activity of the ROS-scavenging system leads to ROS-mediated damage to the Na^+ transportation system as suggested by the PPI network analysis. We believe that a change in the expression of delayed response genes and/or a transient impairment of the regulatory mechanisms underlying the new gene expression caused the inverted $\Delta J(Na^+)$. As the rice was under salt stress, new salt regulation mechanisms come into operation. The negative effects of salt stress regarding the down-regulation of the light receptors (phototropin and phytochrome A and B) should be investigated further and build a theoretical model of crosstalk among photoreceptors and Na^+ transports.

Conclusions

The crosstalk between light and salt stress signals in rice was studied by assessing ion-specific signals. The net cation fluxes, $J(Na^+)$, $J(K^+)$, $J(H^+)$, and $J(Ca^{2+})$, in NaCl-free or NaCl-treated rice, were monitored in real-time in response to light exposure. In general, light increased net cation efflux. Light-induced enhancement of cation efflux was observed in both NaCl-free rice and NaCl-free *P. tenuiflora*. However, surprisingly, for the NaCl-treated rice, the enhancement of net Na^+ efflux was inverted (with $\Delta J(Na^+)$ becoming negative at $[NaCl]_m \geq 10$ mM), indicating an inverted light-inducible switch. This may involve crosstalk between light and salt-stress signals, involving down-regulation of light receptors (phototropin and phytochrome A and B). PPI analysis suggested that there were also five key genes in the cross-talk. This finding of a unique inverted light-inducible switch regarding $J(Na^+)$ offers new perspectives on the crosstalk between light and salt stress signals.

Materials And Methods

Rice growth conditions

Rice seeds (*Oryza sativa* L.) were surface-sterilized with 0.1% mercuric chloride solution for 10 min and then rinsed three times with Milli-Q® ultrapure water. Then the seed were soaked in a solution containing mixed with Murashige and Skoog medium to allow the seed germination. To create Petri dishes containing 0.8% solidified agar, 1 L solution was created by mixing Murashige and Skoog medium and agar in distilled water (with the pH adjusted to 5.8–6.0) and then and poured into the Petri dishes in a laminar flow clean bench. The rice seeds were then aseptically inoculated into the Petri dishes and placed in an aerated incubator at 30°C for 48 h. After germination, the seedlings were allowed to grow until the three main leaves appeared. After three-leaved seedlings were transplanted to ~ 1 cm plugged holes in foam sheets to float over medium, the seedlings were divided into two groups: the NaCl-treated seedlings were transplanted into 10 mM NaCl medium, while the NaCl-free seedlings were transplanted into NaCl-free Milli-Q® ultrapure water. They were cultured at 28°C, with a 10/14 light/dark period, for 5 days. The light intensity was 55.4 μmol/m^2s, as measured using a photometer (TPJ-D; Zhejiang Top Instrument Co. Ltd, China).

P. tenuiflora growth conditions

The seed germination and seedling of *P.tenuiflora* were conducted as described above. The seeds of *P.tenuiflora* were surface-sterilized, and grown on half strength MS medium (1/2 MS) plates containing 1% sucrose, 0.8% agar, and 5 mM nitrate as the sole nitrogen source. After the first pair of leaves was fully expanded, seedlings of the salt-tolerant model plant *P. tenuiflora* were transplanted into 10 mM NaCl solution or NaCl-free Milli-Q® ultrapure water. They were cultured at 22°C, with an 8/16 light/dark period, for 10 days. Thereafter, for the experiment, 13–15-day-old plants were selected for assessment.

Preparation of non-invasive ion-selective microelectrodes

First, microelectrodes were filled with the filling solutions (250 mM NaCl for Na^+ and 100 mM KCl for K^+). Thereafter, the clean microelectrode tips were sealed with an ion-selective liquid ion exchanger (LIX) for Na^+, K^+, H^+, orCa^{2+} (Younger Co., Beijing, China), which was placed a few tens of micrometers from the tips by capillary force. Finally, the microelectrodes were examined and calibrated before mounting them on a non-invasive micro-test technology (NMT) 3D-micromanipulator system (NMT100-SIM; YoungerUSA, LLC, Amherst, MA, USA).

Ion flux measurements

NaCl-free and NaCl-treated rice seedlings were placed into the measuring chamber, which was filled with NaCl solutions set at 0, 50, 100, and 200 mM NaCl during the measurements ($[NaCl]_m$). The microelectrodes were then positioned 20 μm above the root surface. The chamber was then mounted on the NMT100-SIMsystem and left to equilibrate for ~ 10 min. During assessment, iFluxes® software (YoungerUSA, LLC) was used to control the NMT100-SIMsystem, moving the microelectrodes between two positions, which were 20 and 40 μm from the root surface, in a ~ 5-ssquare-wave manner.

One rice seedling from the NaCl-free group and one from the NaCl-treated group was assessed at the various extracellular NaCl concentrations ($[NaCl]_m$). At $[NaCl]_m$ of 0 50, 100, and 200 mM NaCl, we measured the Na^+ flux (*J(Na⁺)*) or K^+ flux (*J(K⁺)*), with and without light exposure (54 μmol/m^2s), switching between the light-on and light-off states every 5 min. We conducted the measurements a total of three times in the light-on state and three times in the light-off state. We also measured H^+ and Ca^{2+} fluxes in the root at $[NaCl]_m$ of 0 and 20 mM in both light-on and light-off states, in rice grown for 7 days in 10 mM NaCl-treated conditions.

J(Na+) measurement in *P. tenuiflora*

One *P. tenuiflora* seedling from the NaCl-free group and one from the NaCl-treated group was assessed, in terms of *J(Na⁺)* in the root at $[NaCl]_m$ of 0, 50, 100, and 200 mM with/without light exposure (54 μmol/m^2s), switching between the light-on and light-off states every 5 min. We conducted the measurements three times in both the light-on and light-off states.

Protein-protein interaction network

A protein–protein interaction (PPI) network of salt tolerance-related and light-related proteins in rice was constructed by using STRING database (Franceschini et al., 2013). We identified all salt tolerance-related proteins and light-related proteins using the UniProt database (Wu et al., 2006). The obtained protein names were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (which contains various types of evidence on PPIs, involving gene and protein analyses). The existing PPIs in STRING database were selected with confidence scores > 0.4.

Data analysis

The NMT100-SIM data were voltage differences between two measuring points (20 μm apart). The voltage difference (ΔV) was converted into ion flux (*J*) using the following equation(Newman, 2001): $J = cuF\frac{58}{Nernstslope}\frac{\Delta V}{\Delta r}$, where *c* is the ion concentration, *u* is the ion electrochemical potential, *F* is the Faraday constant, and the Nernst slope was extracted from the experimental calibration graph involving the following equation:$V = V_0 + (Nernstslope)\,log_{10}\,(c)$. We used JCal software version3.2 (YoungerUSA, LLC) to obtain the ion flux(*J*). Positive values indicate ion efflux from the root, while negative values indicate ion influx into the root.

All data were plotted and statistically analyzed using Excel (Microsoft). Student's t-tests were used to assess statistical significance.

List Of Abbreviations

ABA: Abscisic acid

APX: Ascorbate peroxidase

CAT: Catalase

CHX: Cation/proton exchange

HKT: High-affinity K^+ transporter

JA: Jasmonic acid

KIRC: K^+-inward rectifying channels

KORC: Outward-rectifying K+ channel

KUP-HKT: K^+ uptake transporter-high-affinity K^+ transporter

LCT: Low-affinity cation transporter

LIX: Liquid ion exchanger

NMT: Non-invasive micro-test technology

NSCC: Voltage-dependent nonselective cation channels

POD: Peroxidase

PPI: Protein-protein interaction

ROS: Reactive oxygen specie

SOD: Superoxide dismutase

SOS: Salt overly sensitive

VOC: Voltage-independent cation channel

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Supporting data.

The datasets supporting the conclusions of this article are included within the article and its additional file. Table S1 : Salt tolerance-related and light-related proteins in rice. Descriptions of the salt tolerance-related and light-related proteins used in the protein–protein interaction (PPI) network and cation transporters in rice (*Oryza sativa* L.).

Competing Interests

The authors declare they have no competing interests.

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Authors' contributions

IL designed the experiments. YS and XY prepared the samples. YS, XY, and IL performed the experiments for cation flux measurement. YC and IL carried out the protein–protein interaction network analysis. DG and IL carried out the data analysis. IL wrote the paper.

Authors Information

Institute of Physics, Nankai University, Tianjin, 300071, China
Imshik Lee (ilee@nankai.edu.cn) and Deng Gao (2120190200@mail.nankai.edu.cn)

SAVER, Northeast Forestry University, Harbin, Heilongjiang, China
Yajun Sun (767324555@qq.com) and Xiaoxue Ye (xiaoxueyexx@gmail.com)

National Clinical Research Center for Cancer, Tianjin Medical University Cancer Institute and Hospital, Tianjin, China
Yipeng Cao (vgsplayer1@163.com)

Corresponding Authors

Imshik Lee

ilee@nankai.edu.cn

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Figures

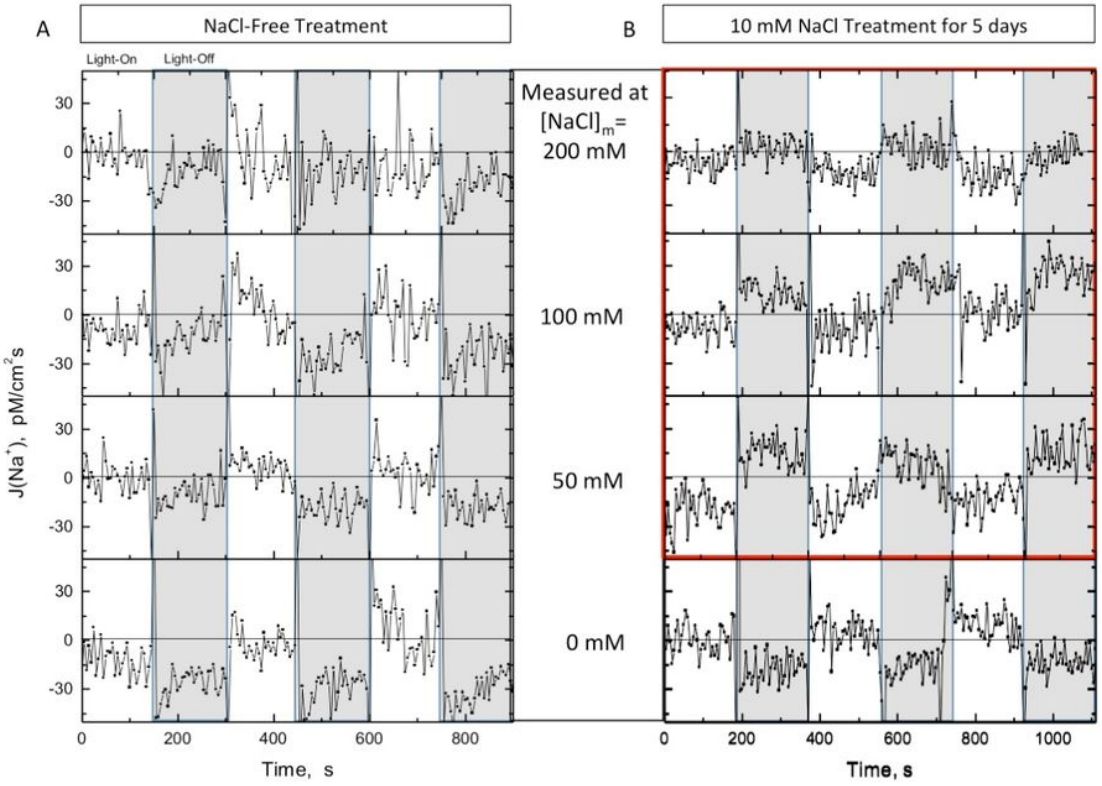

Figure 1

Net J(Na+) in rice experienced for 5 days in (A) NaCl-free or (B) 10 mM NaCl-treated conditions. Light exposure was at 54 µmol/m2s intensity. Real-time extracellular NaCl concentrations ([NaCl]m) were set at 0, 50, 100, and 200 mM. J is the net ion flux. (with positive indicating efflux and negative indicating influx)

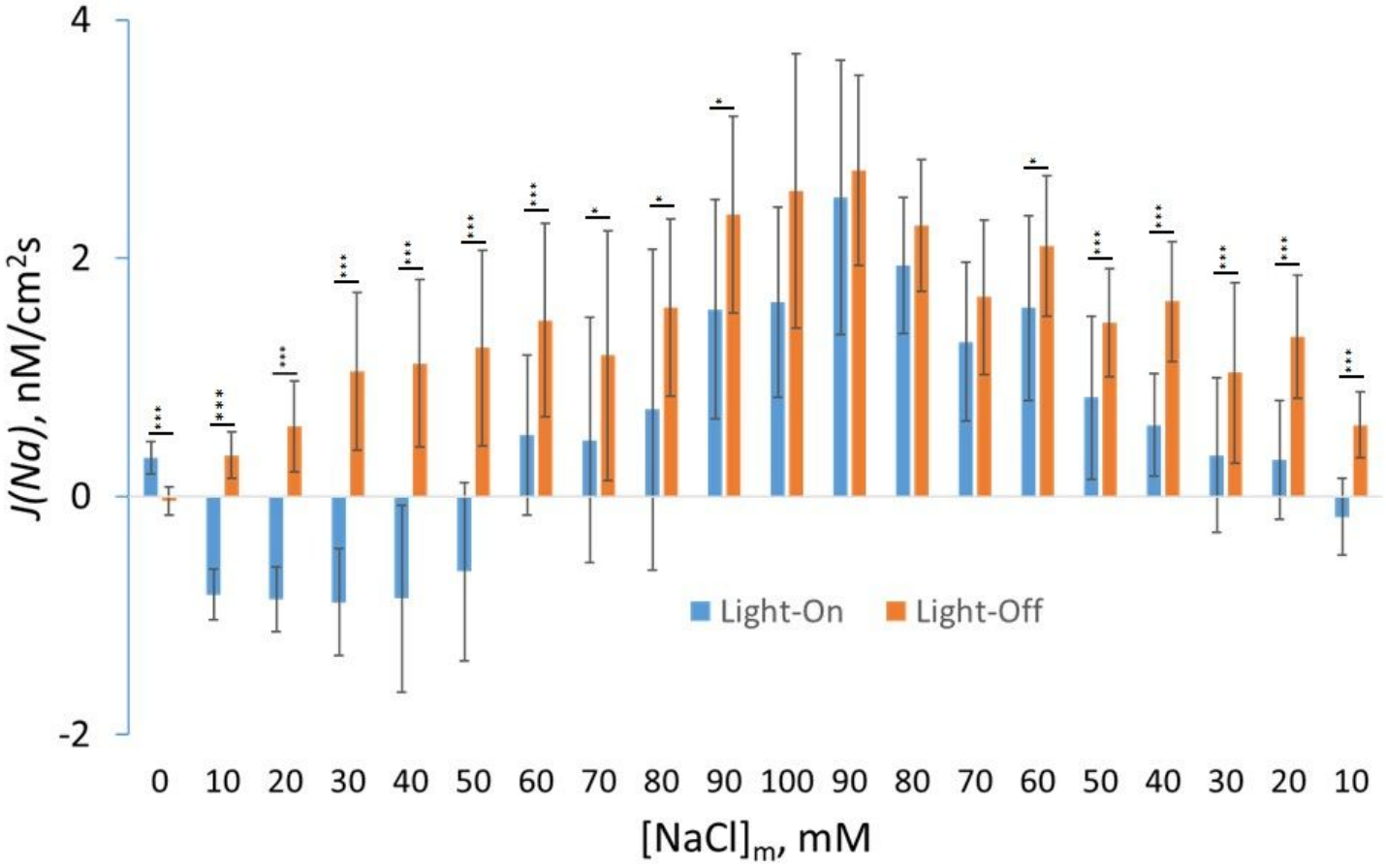

Figure 2

J(Na+) profile in 10 mM NaCl-treated rice at ascending real-time extracellular NaCl concentrations ([NaCl]m) from 0 to 100 mM and at descending [NaCl]m back to 10 mM. J is the net ion flux (with positive indicating efflux and negative indicating influx). Where *** is for p<0.0001 and * is for p<0.01.

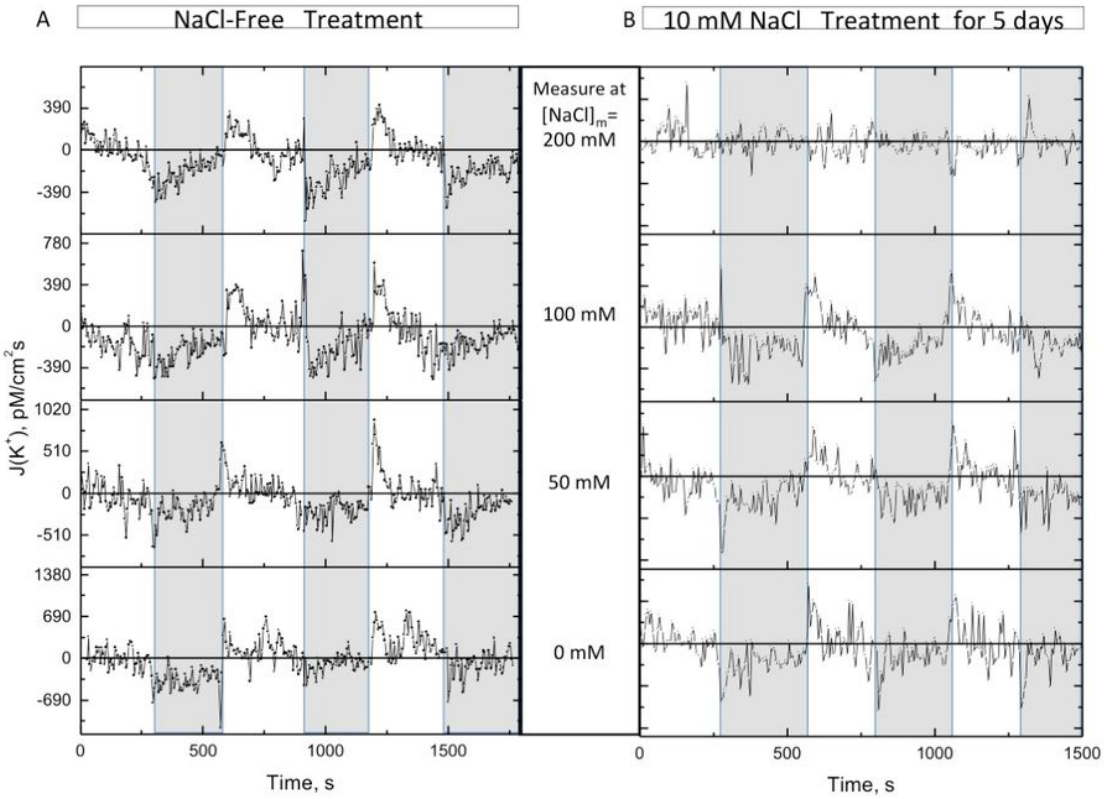

Figure 3

J(K+) in rice experienced for 5 days in (A) NaCl-free and(B) 10 mM NaCl conditions. Real-time extracellular NaCl concentrations ([NaCl]m) were set at 0, 50, 100, and 200 mM. J is the net ion flux (with positive indicating efflux and negative indicating influx). Light exposure was at 54 μmol/m2s intensity.

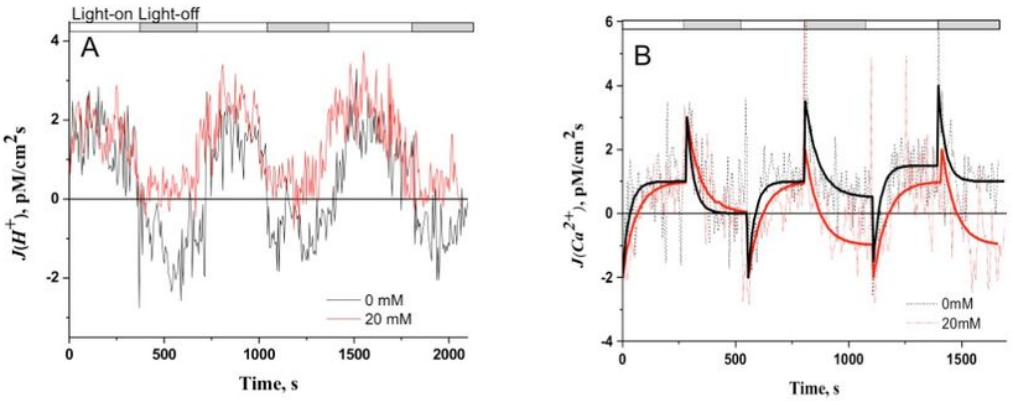

Figure 4

(A) J(H+) and (B) J(Ca2+) in rice experienced for 7 days in 10 mM NaCl-treated conditions. Real-time extracellular NaCl concentrations ([NaCl]m) were set at 0 and 20mM. Thick solid lines in (B) were fitted to the data using Jon=A(1-e-t/to)+B1 for the light-on state and Joff=Ae-t/to+B2 for the light-off state, where J is the net ion flux (with positive indicating efflux and negative indicating influx), A is amplitude, to is relaxation time, and B1 and B2 are baselines. Light exposure was at 54 μmol/m2s intensity.

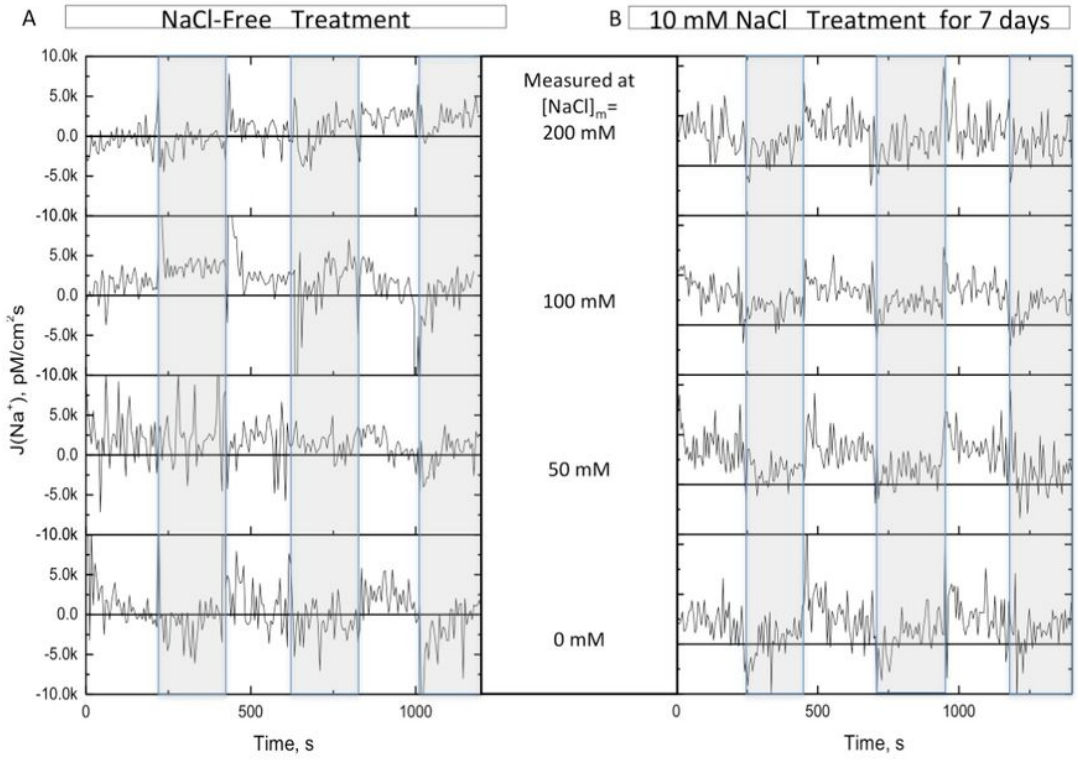

Figure 5

J(Na+) measurement of Puccinellia tenuiflora. (A) NaCl-free and (B) 0 mM NaCl-treated conditions for 7 days. Light exposure was at 54 µmol/m2s intensity. Real-time extracellular NaCl concentrations ([NaCl]m) were set at 0, 50, 100, and 200 mM. J is the net ion flux (with positive indicating efflux and negative indicating influx).

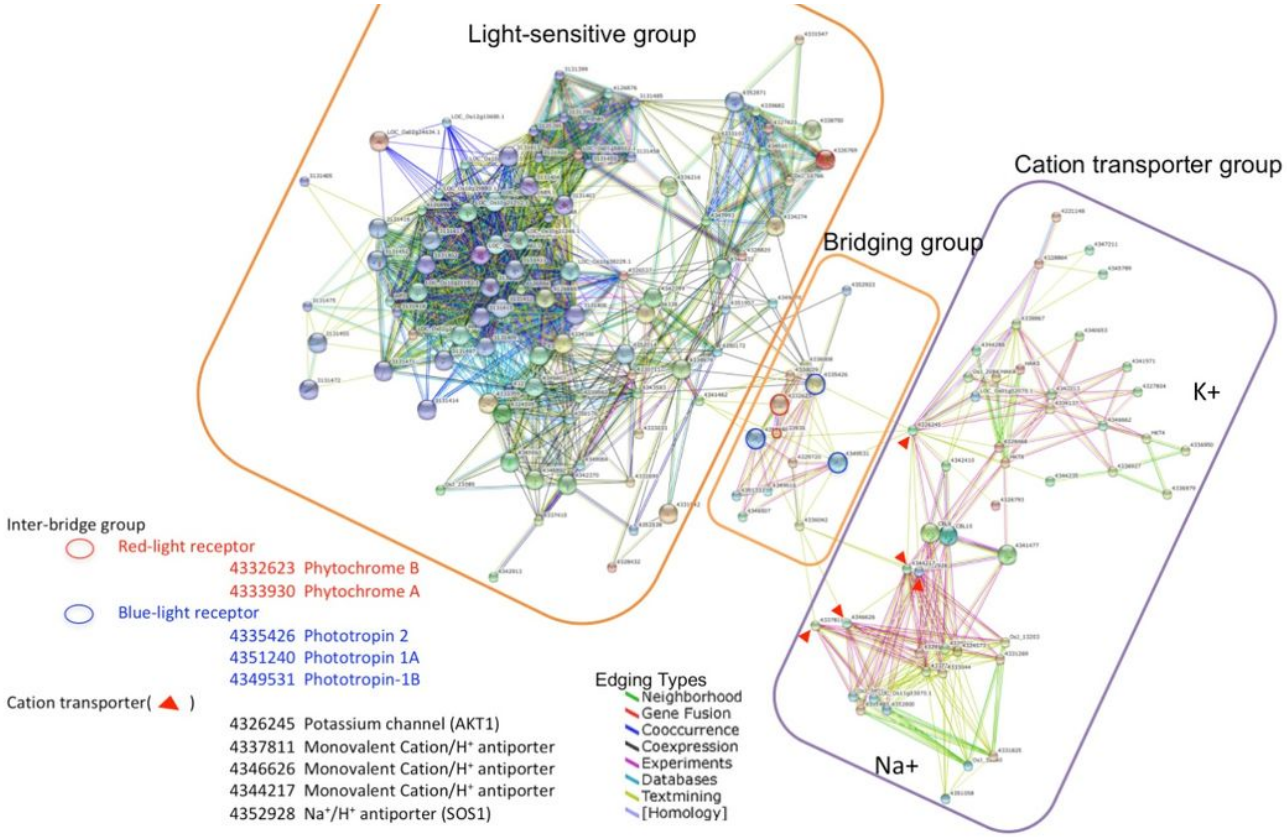

Figure 6

Protein–protein interaction (PPI) network of light-related and salt tolerance-related proteins (including Na+ and K+ transporters), with gene IDs. The three groups were characterized as the light-sensitive group, cation transporter group, and bridging group. The bridging group contains red-light receptors (circled in red) and blue-light receptors (circled in blue). The cation transporters marked with red triangles are crucial players in the crosstalk between the cation transporters and the light receptors.

Supplementary Files

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- SuplTableS1.XLSX