Rapid determination of the damage to photosynthesis caused by salt and osmotic stresses using delayed fluorescence of chloroplasts

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Chloroplasts are one of the most susceptible systems to salt and osmotic stresses. Based on quantitative measurements of delayed fluorescence (DF) of the chloroplasts, we have investigated the damage to photosynthesis caused by these two kinds of stresses in *Arabidopsis* seedlings by using a custom-built multi-channel biosensor. Results showed that the DF intensity and net photosynthesis rate (Pn) decreased in a similar way with increasing NaCl or sorbitol concentration. Incubation of the seedlings in 200 mM NaCl induced a rapid and reversible decline and subsequent slow and irreversible loss in both the DF intensity and Pn. The rapid decline was dominantly related to osmotic stress, whereas the slow declines in the DF intensity and Pn were specific to ionic stress and could be reversed to a similar extent by a Na^+-channel blocker. The DF intensity and Pn also exhibited a similar response to irradiation light under NaCl or sorbitol stress. All results indicated that the DF intensity correlated well with Pn under salt and osmotic stresses. We thus conclude that DF is an excellent marker for detecting the damage to photosynthesis caused by these two stresses. The mechanism of the correlation between the DF intensity and Pn under salt and osmotic stresses was also analyzed in theory and investigated with experiments by measuring intercellular CO_2 concetration (*C*i), stomatal conductance (*G*s), chlorophyll fluorescence parameter, and chlorophyll content. This proposed DF technique holds the potential to be a useful means for analyzing the dynamics of salt and osmotic stresses *in vivo* and elucidating the mechanism by which plants respond to stress.

Introduction

Salt (NaCl) stress is a major factor limiting crop production.[1,2] Previous studies indicate that salt tolerance is a developmentally regulated, stage-specific phenomenon because tolerance at one stage of plant development is not necessarily correlated with tolerance at other stages.[3] Therefore, real-time detection of the physiological status of the plant under salt stress is important not only for identifying the salt-tolerant species but also for providing specific guidance for agricultural production.

Reductions in plant growth and production due to salt stress are often associated with a decrease in photosynthesis.[4,5] During salt stress, net photosynthesis rate (Pn) may decrease due to the restricted availability of CO_2 for carboxylation reactions caused by the reduction in stomatal conductance (*G*s), which is the speed of water evaporation or CO_2 uptake through the stomata in the plant's leaves.[2,6] For instance, in bean (*Phaseolus vulgaris* L.), a salt-sensitive species, and cotton (*Gossypium hirsutum* L.), a salt-tolerant species, the reduction in Pn under salt stress was found to be mostly due to stomatal limitation.[7] However, there are also increasing numbers of reports of nonstomatal inhibition of photosynthesis under salt stress.[8–10] Agastian *et al.* observed that Pn and *G*s declined in mulberry under salt stress, whereas intercellular CO_2 concentration (*C*i), that is the CO_2 concentration of intercellular air, increased.[10] This indicates that the reduction in *G*s is not a cause of the decrease in photosynthesis.

Some authors ascribed this nonstomatal inhibition to a reduced efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).[10,11] However, photosynthesis may decrease by the direct effect of salt stress on photosynthetic electron transport.[3] Salt stress involved the known components of osmotic stress and ionic toxicity.[12] It has been shown that reduction of chloroplast stromal volume due to osmotic stress and increase in Na^+ ion concentration in cytosol could inactivate photosynthetic electron transport.[13,14] Salt stress resulted in a decrease in not only photosystem (PS) II meidated electron transport activity in *Bruguiera parviflora* but also overall activity of the electron trasport chain in *Spirulina platensis*.[15,16] Experimental evidence obtained in *Synechococcus* sp. PCC 7942 demonstrated that osmotic stress induced a rapid and reversible inactivation of PSI and II, whereas ionic stress induced a slow and irreversible inactivation of PSI and II.[5] Nevertheless, it is still unclear whether this is also the case for higher plants.

Delayed fluorescence (DF) of chloroplasts, which is mainly emitted from PSII through inverse photochemistry reactions, is an intrinsic fluorescence label of the efficiency of charge separation at P680.[17–20] The mechanism of DF generation has been described in greater detail elsewhere.[21–23] DF as a sensitive indicator of many stress factors has many practical applications.[22] It has been widely used to detect the effects of heat stress, herbicide toxicity, UV-B radiation, and acid rain pollution on plant growth and development.[17,19,21,24–26] Investigation of DF invokes particular interest because its intensity depends directly on the rate of backward electron transport reactions in the reaction center of

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PSII. In their turn backward electron transport reactions are determined by the quantum efficiency of the primary processes of photosynthesis.[27,28] For example, in a recent report we have demonstrated that DF intensity could be used to ascertain the plant senescence process *in vivo* and quantitatively evaluate the plant senescence extent induced by age or hormones.[25]

To the best of our knowledge, in the past, one drawback of using DF was the lack of commercial fluorimeters that did not destroy the samples being tested and custom-made systems. Moreover, these instruments can only be used in the manicured segment of the detached leaves or the samples in liquid.[17,22,29,30] Nevertheless, it is necessary to detect DF emissions under the plants normal physiological conditions for an exact assessment of the effects of salt stress on their performance and the identification of salt-tolerant species. Recently, the development of a single-channel DF biosensor has made it possible to detect DF emissions *in vivo*.[24,25,31]

In this work, a multi-channel DF biosensor has been developed for detecting the damage to photosynthesis caused by salt and osmotic stresses. Compared with its primary single-channel version in our recent report,[25,31] this multi-channel biosensor can simultaneously analyze many samples. Moreover, each detection channel can automatically complete the measurements of light response curves of the DF intensity in a programmed mode. The applicability of this multi-channel biosensor for ascertaining the function of the photosynthetic apparatus under salt and osmotic stresses was investigated in *Arabidopsis thaliana* (Columbia gl1). Additionally, we also examined the mechanism of the correlation between the DF intensity and Pn under salt and osmotic stresses theoretically and experimentally.

Materials and methods

Plant material and stress treatments

Seeds of *Arabidopsis thaliana* (Columbia gl1) were surface sterilized, sown under aseptic conditions on Murashige and Shoog (MS) medium, and grown in a plant growth chamber (Conviron, model E7/2, Winnipeg, Canada) under a photoperiod of 14 h with a light intensity of 100 µmol photons m^{-2} s^{-1} at 25 °C. After 14 d, seedlings of uniform size were selected to be precultured in pots containing aerated nutrient solution (1 : 500 Hyponex 5–10^{-5}, Hyponex, Oosaka, Japan) in the plant growth chamber under the same growth conditions for another 14 d. After that time, the seedlings were subjected to various stress conditions. Different concentrations (final concentration) of NaCl (0, 100, 150, 200, 250, and 300 mM), LiCl (0, 100, 150, 200, 250, and 300 mM) or sorbitol (0, 200, 300, 400, 500, and 600 mM) were added to the aerated nutrient solution and the seedlings incubated for 18 h. 200 mM NaCl, 200 mM LiCl or 400 mM sorbitol (final concentration) were used to incubate seedlings for different times. All the experiments were carried out at 25 °C and repeated at least seven times.

Gas exchange measurements

Pn of the attached leaves of four-week-old *Arabidopsis* seedlings was measured directly using a commercially available system (LI-6400; LI-COR, Inc., USA) equipped with the 6400–15 Arabidopsis Chamber (1.0 cm in diameter) and artificial illumination (irradiated from a modulated tungsten lamp) in the morning (8:30–10:30). Pn of leaves of *Arabidopsis* seedlings treated with NaCl, sorbitol or LiCl was determined at a leaf chamber CO_2 concentration of 400 ppm after the leaves in the leaf cuvette were irradiated for about 10 min with a saturated irradiation of 500 µmol photons m^{-2} s^{-1}. The relative humidity (RH) and temperature of the leaf cuvette were about 85% and 25 °C, respectively. The responses of Pn to irradiation intensity were measured in a programmed mode under the same conditions as described above.

*G*s and *C*i were measured on attached leaves by using the LI-6400 system.[32] In these experiments, the LI-6400 was operated in the open mode, leaf temperature was controlled at 15 ± 0.2 °C, the vapor pressure deficit between the leaf and air was held at 0.8 ± 0.2 kPa, and the ambient CO_2 concentration was kept at 300 ± 10 ppm. O_2 concentration was controlled to be the same as that in normal air. The relatively low leaf temperature was chosen to increase the sensitivity of the LI-6400 system to small changes in *G*s. Steady-state *G*s at a saturated irradiation of 500 µmol photons m^{-2} s^{-1} was reached within 5–10 min. *C*i was automatically calculated from the values for Pn, *G*s to water vapor, and ambient CO_2 by the internal computer of the LI-6400 system.[32]

In vivo multi-channel DF biosensor system and simultaneous DF measurement

DF emission in the time window from 0.26 to 5.26 s after irradiation was recorded with a custom-built multi-channel biosensor system. The technical details of the single-channel system are described elsewhere.[31] Here a brief summary of the multi-channel biosensor will be presented (Fig. 1).

Eight dark sample chambers were coupled to eight ultra-high-sensitive single photon counting modules [SPCM (MP963, Perkin-Elmer, Wiesbaden, Germany)] through eight optical fibers, respectively. A 660 nm long-pass filter was placed in front of the optical fiber to protect the SPCM from scattered irradiation light. The chambers have been further improved for better controlling the environment conditions and increasing the ratio of signal to noise. Moreover, all chambers can simultaneously reach into the plant growth chamber to measure the DF emissions from plants *in vivo*. Each chamber had a set of super high light light-emitting diodes (LEDs) (λ = 628 nm, half wave width = 20 nm, single duct output luminous flux = 20 lm). All LEDs were uniformly arrayed along a circumference for homogeneous irradiation of the leaves. DF was monitored at an angle of 0° with respect to the incident LEDs light. The newly developed control program realized the automated adjustment of the light intensity of the LEDs in steps of at least 10 µmol photons m^{-2} s^{-1} within the range between 0 and 2000 µmol photons m^{-2} s^{-1}. So the response of DF to irradiation intensity can automatically be measured in the program mode for each detection channel. After Pn measurement, each sample was immediately placed inside the chambers of the biosensor to dark-adapt for 5 min before the irradiation source was turned on. Custom-built humidity, temperature, and CO_2 controllers controlled, respectively, the RH, temperature and CO_2 concentration of each chamber. Immediately after the illumination period, DF from the sample in each chamber was collected by an independent optical fiber bundle and transmitted to a corresponding SPCM with a wavelength detection range of 185–850 nm. The output signals from the independent sample,

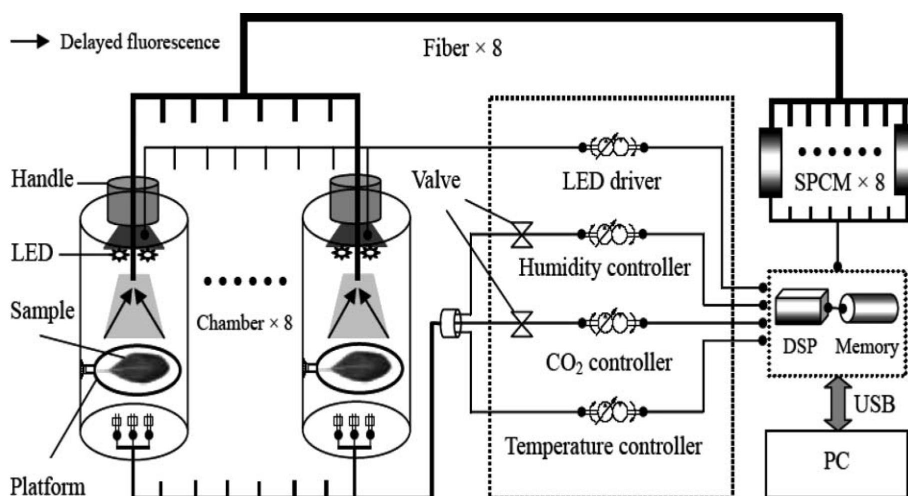

Fig. 1 A diagram of experimental setup for multi-channel DF measurement: LED, SPCM, and DSP represent the light-emitting diode, single photon counting module, and digital signal processor, respectively.

which had been amplified and discriminated by the SPCM, were further processed by a digital signal processor (DSP, TMS320C6416) in the local control mode. The processed signal could be recorded in a memorizer (AT29c020) before further data analysis using a PC in the remote control mode. The samples were irradiated by LED light for 0.2 s. The data collection started at 0.26 s upon the completion of the light irradiation and lasted for 5 s because the DF signal was stable at 0.26 s and decreased to nearly zero at 5.26 s.[31] The DF intensity was obtained by the integration between 0.26 and 5.26 s in the DF decay dynamics curve and presented by a display (local control mode) and a PC screen (remote control mode) in counts per second (cps). The DF intensity of seven independent replicates was simultaneously measured by opening seven channels of the multi-channel biosensor.

Measurement of chlorophyll fluorescence and pigment content

Chlorophyll fluorescence parameters were determined using a PAM fluorometer (Walz, Eichenring 6, Germany) according to the protocol described by Müller *et al.*[33] The quantum efficiency of the photochemistry in open PSII reaction centers (Φ_{PSII}) was calculated by an equation [$(Fm' - Fs)/Fm' = Fv'/Fm'$, where Fm' is the maximum fluorescence level at actinic light, and Fs is the fluorescence level in the steady state of photosynthesis].[27] Fm' is the fluorescence level induced by an 800 ms pulse of strong white light (>1500 µmol photons m^{-2} s^{-1}) in the actinic light (500 µmol photons m^{-2} s^{-1}) and Fs is the fluorescence level determined without the 800 ms pulse. Signals were accumulated 64 times and averaged with a digital oscilloscope (VP-5710A, Panasonic, Tokyo).

Chlorophyll was extracted from individual leaves by boiling them in 95% ethanol at 80 °C. The chlorophyll concentration per fresh weight (FW) of leaf was calculated as described by Lichtenthaler.[34]

Results

Effects of different concentrations of salt on DF intensity and Pn

We first investigated the effects of various concentrations of salt (NaCl and LiCl) on DF intensity and Pn of the attached leaves of *Arabidopsis* seedlings. As shown in Fig. 2a, after incubation for 18 h, DF intensity and Pn rapidly decreased in a similar way with increasing NaCl concentration. When the concentration of NaCl was above 150 mM, the decreases in DF intensity and Pn both became significantly different from the controls at $P < 0.01$, respectively. At 200 mM, DF intensity and Pn decreased to 51.8 and 49.5% of the control levels, respectively. Similar changes in

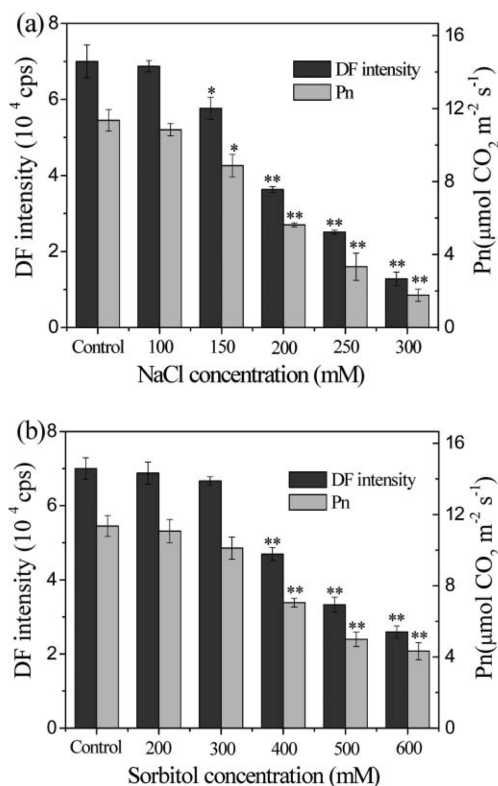

Fig. 2 Changes in DF intensity and Pn of leaves of *Arabidopsis* seedlings after exposure to various NaCl (a) or sorbitol (b) concentrations for 18 h. * and ** indicate that the treatment produced a significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively. Each value is the mean ± SE of seven independent leaves.

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DF intensity and Pn caused by LiCl treatment were also observed, although the effect of LiCl was greater than that of NaCl (data not shown).

To examine whether the effects of NaCl and LiCl might be related to osmotic effects, we further investigated the effects of sorbitol. It has been reported that when the concentration of sorbitol is twice that of NaCl or LiCl, sorbitol has approximately the same osmotic effect as NaCl or LiCl.[4] Thus, we used six various concentrations of sorbitol (0, 200, 300, 400, 500, and 600 mM) to incubate the *Arabidopsis* seedlings. After 18 h treatment, DF intensity and Pn also showed a consistent decrease with increasing sorbitol concentration (Fig. 2b). Moreover, the extent of the decrease of DF intensity and Pn caused by sorbitol resembled those caused by NaCl or LiCl, respectively. For example, when the concentration of sorbitol was 400 mM, DF intensity and Pn decreased to 63.4 and 67.1% of the control levels, respectively.

The dynamics of DF intensity and Pn during salt and osmotic stresses

Fig. 3 shows the changes in DF intensity and Pn of the attached leaves of *Arabidopsis* seedlings in the presence of NaCl or sorbitol for 108 h. DF intensity and Pn in the presence of 200 mM NaCl declined to about 34.6 and 37.7% of the original levels within 27 h, respectively. They then continued to decrease gradually until they disappeared almost completely at 108 h (Fig. 3a, b). Seedlings incubated in the presence of 200 mM LiCl gave similar results, although DF intensity and Pn declined more rapidly (data not shown). By contrast, DF intensity and Pn in the presence of 400 mM sorbitol declined to about 54.1 and 56.5% of the original levels within 27 h, respectively, and then they both remained almost unchanged for the subsequent 81 h (Fig. 3a, b).

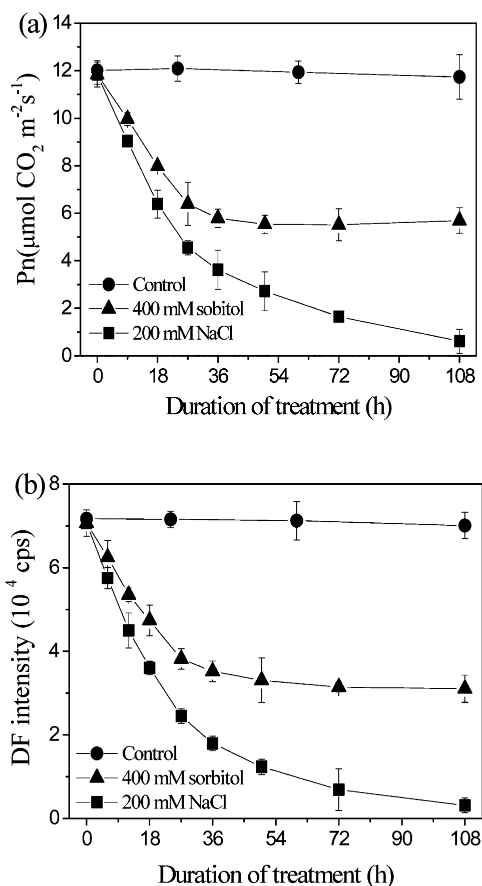

Fig. 3 Changes in Pn (a) and DF intensity (b) of leaves of *Arabidopsis* seedlings during incubation with 200 mM NaCl or 400 mM sorbitol. Each value is the mean ± SE of seven independent leaves.

Effects of Na^+-channel blocker on the NaCl-induced decline in DF intensity and Pn

The effects of Na^+-channel blocker on the NaCl-induced decline in DF intensity and Pn of the attached leaves of *Arabidopsis* seedlings were also investigated. The effects of 200 mM NaCl on DF intensity and Pn were both significantly suppressed by 50 μM phenytoin, which is a Na^+-channel blocker (Fig. 4a, b).[4] 50 μM phenytoin can reverse the slow decline in DF intensity and Pn caused by 200 mM NaCl to the level in the presence of 400 mM sorbitol, although it has no obvious effects on rapid decline.

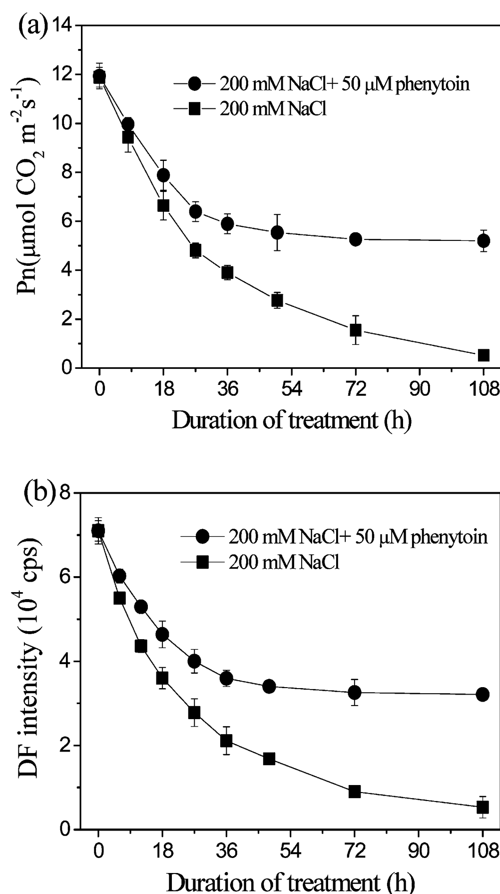

Fig. 4 Effects of a Na^+-channel blocker (phenytoin) on the NaCl-induced decline in Pn (a) and DF intensity (b) of leaves of *Arabidopsis* seedlings, which were incubated in the presence of 200 mM NaCl at 25 °C with or without 50 μM phenytoin. Each value is the mean ± SE of seven independent leaves.

Reversibility of the NaCl-induced inhibitory effects on DF intensity and Pn

To examine the reversibility of the NaCl-induced inhibitory effects on DF intensity and Pn, we released seedlings that had been

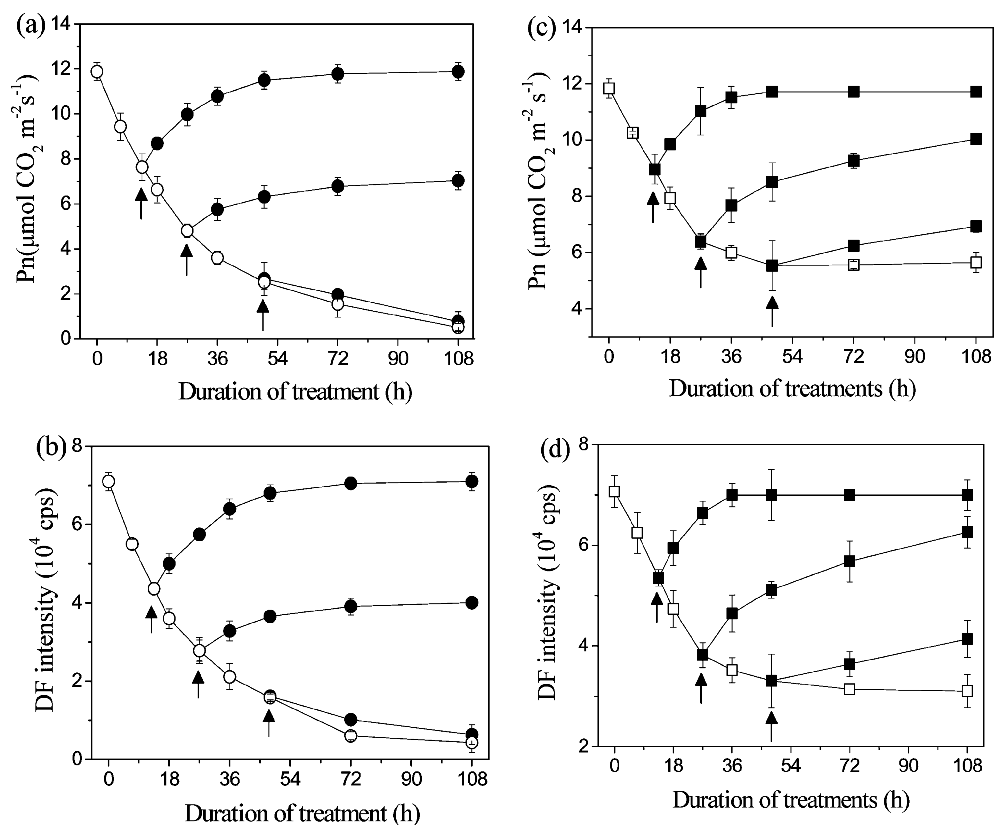

Fig. 5 Reversibility of the effects of NaCl on Pn (a) and DF intensity (b), and reversibility of the effects of sorbitol on Pn (c) and DF intensity (d) of leaves of *Arabidopsis* seedlings. Seedlings were incubated for 13.5, 27, 50, and 108 h with 200 mM NaCl at 25 °C. Seedlings were washed twice with fresh nutrient at times indicated by arrows and then incubated with fresh nutrient in the absence of NaCl and sorbitol at 25 °C. DF intensity and Pn were measured at indicated times. ○, incubation with 200 mM NaCl; ●, incubation without NaCl after washing; □, incubation with 400 mM sorbitol; ■, incubation without sorbitol after washing. Each value is the mean ± SE of seven independent leaves.

incubated with 200 mM NaCl from salt stress by washing them with fresh nutrient solution. DF intensity and Pn both recovered fully in fresh nutrient solution after 13.5 h incubation with NaCl, when only the rapid decline had been observed. When incubation with NaCl was extended to 27 h, DF intensity and Pn both recovered only partially in the absence of NaCl. When the initial incubation with NaCl was extended to 50 h, no recovery was observed (Fig. 5a, b).

We further investigated the reversibility of the sorbitol-induced inhibitory effects on DF intensity and Pn. Results showed that DF intensity and Pn both could quickly recover to the original levels in fresh nutrient solution after the 13.5 h incubation with sorbitol, respectively. Moreover, when incubation with sorbitol was extended to 27 h, DF intensity and Pn could also recover to 89.4 and 85.6% of the original levels after a subsequent 81 h in fresh nutrient solution, respectively. Even if the initial incubation with sorbitol was extended to 50 h, a marked recovery could still be observed (Fig. 5c, d).

The responses of DF intensity and Pn to irradiation intensity after 200 mM NaCl or 400 mM sorbitol treatment

To verify the usability of the multi-biosensor for automated measurement of the response of DF intensity to irradiation intensity, we further characterized the light responsiveness of DF intensity and Pn of the attached leaves of *Arabidopsis* seedlings exposed to 200 mM NaCl or 400 mM sorbitol for 18 h. It was clear that DF intensity and Pn at any given irradiation intensity were lower in seedlings with NaCl or sorbitol treatment than in the control, respectively (Fig. 6a, b). As irradiation intensity increased, both DF intensity and Pn initially increased linearly, then reached a plateau at the same irradiation intensity of 500 μmol photons m^{-2} s^{-1} and leveled off with a further rise in irradiation intensity in seedlings without stress. Whereas the seedlings incubated with 200 mM NaCl or 400 mM sorbitol, DF intensity and Pn both reached a plateau at the same irradiation intensity of 300 μmol photons m^{-2} s^{-1} (Fig. 6a, b). These results revealed that DF intensity from the biosensor and Pn from LI-6400 responded to irradiation intensity in a similar manner even under NaCl and sorbitol stress conditions.

Effects of salt and osmotic stresses on *G*s and *C*i

Because DF is an intrinsic fluorescence label of the photochemical efficiency of PSII,[17–20] the consistency of changes in the DF intensity and Pn suggested that the decline in photosynthesis induced by salt and osmotic stresses could be attributed to a decrease in the activity of the photosynthetic apparatus of the mesophyll cells, especially a decrease in the photochemical efficiency of PSII. To test the hypothesis, *G*s and *C*i were further investigated in *Arabidopsis* seedlings after exposure to 200 mM NaCl, 400 mM sorbitol or 200 mM LiCl for 18 h. As shown in

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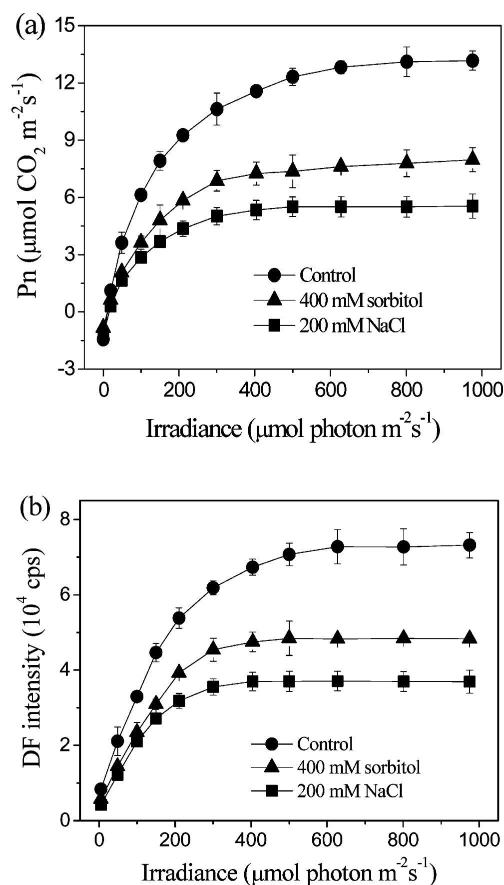

Fig. 6 Light response curves of Pn (a) and DF intensity (b) of leaves of *Arabidopsis* seedlings after exposure to 200 mM NaCl or 400 mM sorbitol for 18 h. Each value is the mean ± SE of seven independent leaves.

Fig. 7, after these kinds of salt treatments for 18 h, no obvious decrease in *G*s could be observed in stressed *Arabidopsis* seedlings relative to the control. By contrast, in the presence of NaCl, sorbitol or LiCl for 18 h, a significant increase at $P < 0.01$ in *C*i was observed in stressed *Arabidopsis* seedlings relative to the control (Fig. 7). The extension of the incubation of NaCl, sorbitol or LiCl did not result in further decrease and increase in *G*s and *C*i, respectively (data not shown). These results suggested that reduction in Pn caused by salt stress might not be limited by stomatal closure.[35]

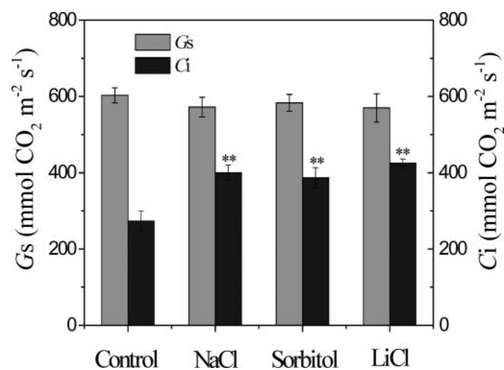

Fig. 7 Changes in *G*s and *C*i of leaves of *Arabidopsis* seedlings after exposure to 200 mM NaCl, 400 mM sorbitol, and 200 mM LiCl for 18 h. ** indicates that stress treatment produced a significant difference from the control at $P < 0.01$. Each value is the mean ± SE of seven independent leaves.

Changes in chlorophyll fluorescence parameter and chlorophyll content after salt and osmotic stresses

To further confirm whether the decline in photosynthesis was caused by the inactivation of the photosynthetic electron transport, we determined chlorophyll content and chlorophyll fluorescence parameters. The changes in chlorophyll fluorescence parameter Fv'/Fm' and chlorophyll content are shown in Fig. 8. Both Fv'/Fm' and chlorophyll content gave a fast decline and subsequent slow decline in a similar manner in the presence of 200 mM NaCl (Fig. 8a, b), which was in agreement with the changes in DF intensity and Pn (Fig. 3a, b). Moreover, upon treatment with 400 mM sorbitol, Fv'/Fm' and chlorophyll content also declined in a trend consistent with that in DF intensity and Pn, *e.g.* Fv'/Fm' and chlorophyll content in the presence of 400 mM sorbitol declined to about 53.1 and 54.5% of the original levels within 27 h, respectively, and then they both remained almost unchanged for a subsequent 81 h. Further statistical analyses showed that DF intensity positively correlated with not only chlorophyll content but also Fv'/Fm' even under salt and osmotic stress conditions (Fig. 8c, d).

Discussion

Slow decline in DF intensity and Pn caused by salt stress is irreversible and specific to ionic effects

Salt (NaCl) stress is the most serious threat to agricultural production in many parts of the world.[1–3,10] It has been well established that photosynthesis is the major target for salt stress in plants.[5,10] Since NaCl has both osmotic and ionic effects,[12,36] it is necessary to analyze these effects on photosynthesis separately. To mimic the osmotic effects of NaCl, we used sorbitol, whose concentration is twice that of NaCl, to incubate the *Arabidopsis* seedlings.[4] Measurements of gas exchange and DF signal showed that both NaCl and sorbitol stresses inhibited photosynthesis in a concentration-dependent manner, and the effects of NaCl on photosynthesis were dominantly related to osmotic effects (Fig. 2a, b).

Analysis of the dynamics of salt and osmotic stresses demonstrated that the decline in DF intensity and Pn induced by NaCl both involved rapid and slow phases, with the same one-half-decay times of about 13.5 and 50 h, respectively (Fig. 3a, b). The rapid phase of the NaCl-induced decline in DF intensity and Pn appeared to correspond to the time course of osmotic stress-induced decline,[37] suggesting that the rapid decline in photosynthesis might have been caused by osmotic pressure. The slow phase, which occurred in the presence of NaCl or LiCl but not of sorbitol, appeared to be specific to ionic effects, as verified with a specific channel blocker (Fig. 4a, b). The Na^+-channel blocker protected DF intensity and Pn against the NaCl-induced slow decline, but not against the rapid decline. In its presence, the NaCl-induced decline resembled the sorbitol-induced decline (Fig. 3 and 4). The findings presented here were in agreement with those obtained in *Synechococcus* sp. PCC 7942.[4,5,37]

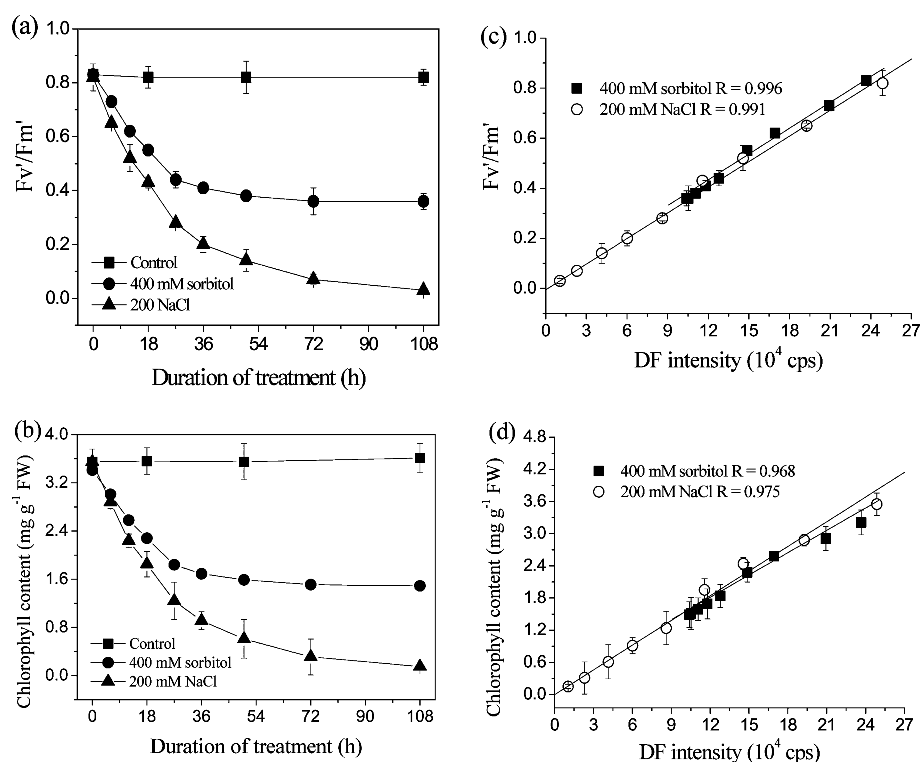

Fig. 8 Changes in Fv'/Fm' (a) and chlorophyll content (b) of leaves of *Arabidopsis* seedlings during incubation with 200 mM NaCl or 400 mM sorbitol as well as the relationships between DF intensity and these two parameters ((c) and (d)). Each value is the mean ± SE of seven independent leaves. The values of the DF intensity are taken from Fig. 3b.

The determination of recovery dynamics demonstrated that osmotic stress-induced declines in DF intensity and Pn were reversible but the ionic effects were irreversible (Fig. 5a, b, c and d). It is likely that Na^+ ions damage the photosynthetic machinery that is necessary for the recovery of seedlings from NaCl-induced damage. These findings were in line with the hypothesis that the rapid decline in oxygen-evolving activity of *Synechococcus* sp. resembled that caused by sorbitol (*i.e*. an osmotic effect) and was reversible, whereas slow inactivation was an irreversible process due to ionic effects.[4]

DF intensity correlates well with Pn under salt and osmotic stresses

Measurements of the DF signal showed that DF intensity of the attached leaves of the *Arabidopsis* seedlings decreased with increasing NaCl or sorbitol concentration in a manner consistent with Pn (Fig. 2a, b). Analysis of various dynamics also demonstrated that DF intensity exhibited a characteristic change quite similar to that in Pn under salt stress and sorbitol stress (Fig. 3–5). DF intensity and Pn responded to irradiation intensity also in a quite consistent way even under NaCl and sorbitol stress conditions (Fig. 6a, b). Taken together, the consistent changes in DF intensity and Pn under NaCl or sorbitol stress demonstrated that DF intensity from the multi-channel biosensor could determine the extent of the damage to photosynthesis and rapidly ascertain photosynthetic behavior under stress conditions (Fig. 2–6).

In a previous report we have shown that there is a linear correlation between DF intensity and Pn in detached leaves of spinach (*Spinacia oleracea*) in a bench study.[22] Recently, a lot of contrasting experiments have further demonstrated that DF intensity also correlates with Pn in attached leaves of many plant species of soybean (*Glycine max* (L.) Merr.), rice (*Oryza sativa* L.), and maize (*Zea mays* L.) even under field conditions.[31] Nevertheless, photosynthetic metabolism is an integrated and complex process and its efficiency depends on a sequence of metabolic events such as electron transport and the activity of Rubisco.[8] Moreover, salt stress also has complex effects on the photosynthetic process.[14–16] The question therefore arises why DF intensity correlates well with Pn under salt and osmotic stress conditions?

The mechanism of the correlation between DF intensity and Pn under salt and osmotic stresses

To address this question we analyze the relationships between DF intensity, Fv'/Fm', and the rate of electron transport. DF of chloroplasts, as a phenomenon of photon emission due to the inverse photochemistry reactions in the PSII, has many components and its total relaxation kinetics of DF can be defined as a sum of kinetic components:[17,18]

$$I_{\mathrm{DF}}(t) = \sum_i I_{\mathrm{DF}_i} e^{-t/\tau_i} \quad (1)$$

where $I_{\mathrm{DF}}(t)$ is the DF intensity at time t after illumination has ceased, $I_{\mathrm{DF}I}$ is the amplitude of the ith component, and τ_i is its

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lifetime. The DF intensity (I_{DF}) represents the integration under the decay dynamics curve:[27,28,31]

$$I_{DF} = \int_{t=0}^{+\infty} I_{DF}(t)\,dt \quad (2)$$

I_{DF} could be indirectly determined by the quantum efficiency of the primary processes of photosynthesis.[27,28] Therefore, I_{DF} can be estimated by:

$$I_{DF} \sim \alpha \times PFD_a \times \Phi_{PSII} \quad (3)$$

where PFD_a is absorbed light (μmol photons m^{-2} s^{-1}) and Φ_{PSII} is the quantum efficiency of the primary photochemical reaction in PSII reaction centers.[27] α is assumed to be 0.5 according to the partitioning of the energy between PSI and PSII.[38,39]

It has previously been demonstrated that Fv'/Fm' is a widely used chlorophyll fluorescence parameter that estimates the quantum efficiency of photochemistry in open PSII reaction centers.[27,38–41] Therefore, I_{DF} can also be estimated by:

$$I_{DF} \sim \alpha \times PFD_a \times Fv'/Fm' \quad (4)$$

The rate of PSII electron transport (R_e) in leaves under light was estimated using the following equation:[38–41]

$$R_e = \alpha \times PFD_a \times Fv'/Fm' \quad (5)$$

Considering eqn (3)–(5), we can obtain the following formula:

$$I_{DF} \sim R_e \sim Fv'/Fm' \quad (6)$$

From eqn (6), we thus conclude that I_{DF} positively correlates with not only Fv'/Fm' but also R_e.

In our experiments, salt and osmotic stresses led to a decline in Fv'/Fm' and chlorophyll content similar to those in Pn (Fig. 2–6 and 8a, b). The rapid decline in Fv'/Fm' was also reversible but the slow decline in Fv'/Fm' was not (data not shown). Moreover, incubation of the seedlings in NaCl, sorbitol or LiCl for 18 h had no effects on *G*s but produced a significant increase at $P < 0.01$ in *C*i (Fig. 7). These results demonstrated that osmotic stress induced inhibition in photosynthesis could be mostly due to reversible inactivation of photosynthetic electron transport. By contrast, the increase in the number of Na^+ ions in the cytoplasm under high salt conditions could irreversibly inactivate photosynthetic electron transport.[8,35] The inactivation of photosynthetic electron transport (indicated by the DF intensity and Fv'/Fm') under salt and osmotic stresses may be *via* the shrinkage of intercellular space due to the efflux of water through water channels in the plasma membrane.[4] However, the linear relationship between DF intensity and chlorophyll content suggested that the inactivation of photosynthetic electron transport also might be a direct result of a decrease in chlorophyll content (Fig. 8d). The mechanism of the inactivation of photosynthetic electron transport needs to be further explored. In summary, considering eqn (6) and the excellent correlation between DF intensity, Fv'/Fm', and chlorophyll content (Fig. 8c, d), we could thus conclude that DF intensity always displays a highly consistent trend with Pn under salt and osmotic stresses.

Conclusions

In this study, the effects of salt and osmotic stresses on photosynthesis and the effectiveness of the multi-channel DF biosensor for determination of the damage to photosynthesis caused by these two stresses were thoroughly investigated. All results suggested that the rapid decline in photosynthesis under NaCl stress is reversible and specific to osmotic effects, whereas the slow decline is irreversible and specific to ionic stress. Contrasting measurements demonstrated that DF intensity from the biosensor correlates well with Pn from LI-6400 under salt and osmotic stresses.

The DF biosensor technique reported here was non-invasive and in particular, was not prone to interference from environmental factors because of using intrinsic DF to probe the plant. To the best of our knowledge, the improved DF biosensor has, for the first time, realized synchronous measurements of DF emissions from multi-samples *in vivo*. In this regard, the DF biosensor has merit in terms of rapidity and reliability of measurements of intact leaves. For example, according to the previous report and practical operation, DF signals of seven replicates obtained by the DF biosensor take less than 10 s, while steady Pn values of seven replicates obtained by LI-6400 need about 35–70 min.[31] Thus, the DF biosensor would be extremely suitable for the simultaneous assessment of the photosynthetic behavior of a great number of samples and fast screening of potentially interesting mutants with genetic modifications that change plant salt tolerance. Moreover, each detection channel of the improved DF biosensor can automatically measure the light response curves of DF intensity. Therefore, it is very ease-to-use to ascertain the light response characteristics of photosynthesis. These aspects might be very important and useful for biotechnological applications, although field testing and further improvements of the reported biosensor must be performed both for portable considerations and economical reasons.

Abbreviations

Pn	Net photosynthesis rate
*G*s	Stomatal conductance
*C*i	Intercellular CO_2 concentration
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
PSII	Photosystem II
PSI	Photosystem I
DF	Delayed fluorescence
FW	Fresh weight

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