Changes in PS II heterogeneity in response to osmotic and ionic stress in wheat leaves (Triticum aestivum)

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Abstract High salt stress involves ionic stress as well as osmotic stress. In this work we have tried to differentiate between the ionic and osmotic effects of salt stress on the basis of their ability to cause changes in PS II heterogeneity. PS II heterogeneity is found to vary with environmental conditions. Osmotic stress caused no change in the Q_B reducing side heterogeneity and a reversible change in antenna heterogeneity. The number of Q_B non-reducing centers increased under ionic stress but were unaffected by osmotic stress. On the other hand ionic stress led to a partially irreversible change in Q_B reducing side heterogeneity and a reversible change in antenna heterogeneity. In response to both ionic and osmotic effect, there is conversion of active PS IIα centres to inactive PSIIβ and γ centres.

Keywords Heterogeneity · High salt · Ionic stress · Osmotic stress · Photosystem II · Wheat (*Triticum aestivum*)

Abbreviations

Chl	chlorophyll
DCMU	3-(3, 4-dichlorophenyl)-1, 1-dimethylurea
Fm	maximal Chl *a* fluorescence
Fo	minimal Chl *a* fluorescence
FR	fluorescence rise
Fv	variable fluorescence
K, J, I	intermediate steps of Chl *a* fluorescence rise between Fo and P
LHC	light harvesting complex
OEC	oxygen evolving complex
PEA	plant efficiency analyzer
PS II	Photosystem II
PQ	plastoquinone
Q_A	primary plastoquinone
Q_B	secondary plastoquinone
RC	reaction center

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Introduction

Photosystem II (PS II) is a multi-subunit complex consisting of several different types of chlorophyll binding components whose function is to organize the chlorophylls for light harvesting and harbor the electron transport cofactors needed for the oxidation of water (Andersson and Melis 1983). It responds to several environmental stresses like photoinhibition, high temperature and high salt by production of ROS, suppression of protein synthesis de novo, impairment of the PS II repair cycle etc. (Murata et al. 2007).

As compared to other protein pigment complexes participating in light reaction, PS II shows diverse nature in certain aspects and this is termed as PS II heterogeneity. The idea originated in order to explain the biphasic nature of the kinetics of primary photochemistry of PS II. Most important observation was that the extent and nature of PS II heterogeneity varies under different physiological conditions (Lavergne and Briantais 1996) i.e. salinity, temperature and pH stress (Mathur et al. 2011; Mehta et al. 2010a;

Tongra et al. 2011). Two major types of PS II heterogeneity have been identified: reducing side heterogeneity and antenna heterogeneity. The details of different types of PS II heterogeneity are mentioned below.

Reducing side heterogeneity

The first type of PS II heterogeneity is related with electron flow to the plastoquinone pool i.e. the reducing side of Q_A^-. It has been proposed that a number of PS II centers, though photochemically competent, are unable to transfer electrons efficiently from electron acceptor Q_A^- to secondary electron acceptor Q_B and Q_A^- can only be reoxidized by a back reaction with the donor side of PS II (Lavergne 1982; Thielen and van Gorkom 1981; Melis 1985; Graan and Ort 1986; Guenther et al. 1988; Schansker and Strasser 2005). These centers were called as PS II Q_B-non-reducing centres and were incapable of reducing the PQ pool. Q_B-non-reducing centres are located in the non-appressed regions while Q_B-reducing centres are localized in the appressed regions of the thylakoids (Tyystjarvi and Aro 1990).

Antenna heterogeneity

The second type of PS II heterogeneity is related to heterogeneity in the antenna size as well as their energetic connectivity. Electron transport in PS II can easily be probed by fluorescence measurements. It was difficult to explain the fluorescence rise measured with DCMU treated plant material by a single exponential increase (Doschek and Kok 1972). Analysis of the biphasic data so obtained suggested the presence of three distinct populations of PS II centers in the chloroplast, termed as PS IIα, PS IIβ and PS IIγ (Melis and Homann 1976; Melis and Duysens 1979; Black et al. 1986). In a stepwise process during development, the addition of LHC II-inner portion first augments the antenna of PS IIγ (contains ~50 Chl) by about 80 Chl to yield PS IIβ (~130 Chl) and addition of LHC II-peripheral part increases the antenna size by another 80 Chl to yield PS IIα (Melis 1991). Amongst the three centers, PS II α is the dominant form, has its localization in grana partition regions (Andersson and Melis 1983), with a higher absorption cross section area, large light harvesting antenna and is responsible for the majority of the water oxidation activity and plastoquinone reduction. In other words PS IIα is the major 'normal' PS II center. Chl a core complex, an accessory Chl a–b light harvesting inner antenna (LHC II-inner), and a peripheral antenna (LHC II-peripheral) containing a combined total of about 210–250 Chl a and Chl b molecules (Morrissey et al. 1989) are the major components of PS IIα. PS IIα is distinctly characterized by the possibility of excited states transfer between PS II units and is the only phase that can respond to depletion of Mg^{2+} (Hsu and Lee 1991).

PS IIβ is found in the stromal region of thylakoid membranes. They have a light harvesting antenna about 2.5 times smaller compared to PS IIα with an impossibility of the excited states transfer between PS IIs. The absence of peripheral LHC II in PS II is considered as one of the important reasons for smaller antenna size of PS IIβ. PS IIγ marks its localization in the stroma lamellae region (Hsu and Lee 1995) with smallest antenna size and maximum lifetime among the three components. PS IIβ and PS IIγ represent two minor groups of 'abnormal' PS II centers with low quantum efficiencies due to their slow electron donation systems. The slow rate of β and γ centers might be due to slow electron donation to their reaction center, which might undergo many turnovers via back reaction under continuous excitation, until their reduced primary acceptors are stabilized by the electron donation into the system (Hsu and Lee 1995). The PS IIβ and PS IIγ centers also have defects in their quinone-iron complexes and thus are unable to reduce plastoquinone (Hsu and Lee 1991). In spinach, the pools of PS IIβ and Q_B-non-reducing centers were found to be identical (Melis 1985). However in *Duneliella salina*, Q_B-non-reducing centers were found to be a subpopulation of PS IIβ (Guenther and Melis 1990). Several other points of difference between PS IIα and PS IIβ are in terms of their reopening rate, kinetic properties, connectivity to the plastoquinone pool, as well as their DCMU sensitivity (Thielen and van Gorkom 1981; Sundby et al. 1986).

High salt stress is a major environmental stress encountered by terrestrial plants. The detrimental effects of it can be observed at the whole-plant level by adversely affecting plant growth and metabolism resulting in decreased productivity. Many crop plants including wheat are susceptible to high salt. Salt stress involves both osmotic stress and ionic stress. In other words, high salt stress inhibits plant growth by osmotic stress as well as ion imbalance (Tester and Davenport 2003). In cyanobacteria, NaCl (0.5 M) (exhibiting both ionic and osmotic stress) and sorbitol (1.0 M) (exhibiting only osmotic stress) were found to inactivate both PS II and PS I (Allakhverdiev et al. 2000a; Mehta et al. 2010b).

In several earlier works, ionic and osmotic effects have been studied together. Osmotic stress was found to reversibly inactivate photosynthetic electron transport via shrinkage of the intracellular space. By contrast ionic stress inactivated both photosynthetic and respiratory machinery due to leakage of Na^+ (Allakhverdiev et al. 2000b). However, to our knowledge, there is no report to explain and differentiate between the effects caused by ionic and osmotic stress on the heterogeneity of PS II. In the present study we have determined the changes in different types of PS II heterogeneity in response to osmotic and ionic stress simultaneously in wheat leaves (*Triticum aestivum*). Sorbitol was used to give osmotic stress while high salt (NaCl)

concentration was used to give osmotic as well as ionic stress. A comparison of the effects observed in these two cases will help us to differentiate between the effects caused by osmotic and ionic components of the high salt stress. It was thought to be important to study these stresses separately but on the same crop in order to differentiate between the changes caused by each stress (osmotic and ionic) distinctly. Interestingly, PS II was found to undergo changes in its structural and functional heterogeneity differently in response to ionic and osmotic stress.

Materials and methods

Plant material: wheat (*Triticum aestivum*)

Lok-1 cultivar of wheat was used. Wheat seeds were allowed to germinate and then transferred to petriplates containing 1/10th strength Knop solution with a photosynthetically active photon flux density (PPFD) of 300 μmol m^{-2} s^{-1} at 20 °C. The plantlets were grown up to two leaf stages and then osmotic and ionic treatments were given in light. For measurement of control leaves, the leaves were dipped in distilled water.

Osmotic stress treatment

The detached wheat leaves were immersed in various concentration of sorbitol (0.5 M, 1.0 M, 1.5 M, 2.0 M) for 2 h in light.

High salt treatment

In order to give ionic and osmotic stress both, the detached wheat leaves were immersed in various concentration of NaCl (0.25 M, 0.5 M, 0.75 M, 1.0 M) for 2 h in light.

Measurement of fluorescence induction kinetics

The chlorophyll *a* (Chl *a*) fluorescence induction kinetics was measured at room temperature using a Plant Efficiency Analyser (PEA), (Hansatech, England). Excitation light of 650 nm (peak wavelength) from array of three light-emitting diodes is focused on the surface of the leaf to provide a homogenous illumination. Light intensity reaching the leaf was 3000 μmol m^{-2} s^{-1} which was sufficient to generate maximal fluorescence for all the treatments. The fluorescence signal is received by the sensor head during recording and is digitized in the control unit using a fast digital converter. Control leaves exhibit a polyphasic rise called O–J–I–P Chl *a* fluorescence transient; the O to J phase (ends at ~2 ms), the J to I phase (ends at ~30 ms) and I to P phase (ends at ~500 ms). The JIP test is named after the basic steps

in the fluorescence transient when plotted on a logarithmic time scale (Force et al. 2003). The measurements were performed two inches away from the tip and the base i.e. in the middle portion on the ventral surface of the leaves i.e. the abaxial surface. Leaves were given dark adaptation of 5 mins before each measurement.

Determination of Q_B-reducing and Q_B-non-reducing centers

Q_B-reducing and Q_B-non-reducing centers were calculated using a double hit (pulse) method of Strasser and Tsimilli-Michael (1998). According to this method, two fluorescence transients were induced by two subsequent pulses (each of 1 s duration). The first pulse was conducted after a dark period long enough to ensure the reopening of all reaction centers, followed by a second pulse. The duration of the dark interval between the two pulses was 500 ms. The dark interval between the two pulses is short enough to allow only the reopening of the Q_B-reducing centers (fast opening centers). Closed centers that do not open within about 500 ms were considered as Q_B-non-reducing centers (slow opening centers).

$$Fv = Fm - Fo; Fv^* = Fm^* - Fo^*$$

Fv: variable fluorescence of 1st pulse, Fv * variable fluorescence of 2nd pulse

Fm: maximal fluorescence of 1st pulse, Fm *: maximal fluorescence of 2nd pulse

Fo: minimal fluorescence of 1st pulse; Fo*: minimal fluorescence of 2nd pulse.

Q_B-non-reducing centers were calculated by the following equation:

$$Bo = \frac{[(Fv/Fm) - (Fv^*/Fm^*)]}{(Fv/Fm)} \times 100$$

where Bo = Relative amount of Q_B-non-reducing PS II centers

Determination of antenna size heterogeneity

Determination of PS II heterogeneity from fluorescence rise (FR) curve measured with DCMU was first introduced by Melis and Homann (Melis and Homann 1976; Melis and Homann 1975). For calculation of antenna heterogeneity the DCMU poisoning method was used (Strasser 1981; Hsu et al. 1989). The method is as described below. The detached leaves were put into a small tray filled with 100 ml DCMU solution (the DCMU concentration was 200 μM dissolved in 1 % ethanol) (Toth et al. 2005) overnight in light. The leaves were removed from the DCMU solution, wiped and left

in the air for ~1 h to avoid possible effects of anaerobiosis. Following this, osmotic and ionic stress was given to the leaves in light and then parameters were recorded. Alpha (α), beta (β) and gamma (γ) centers were calculated from the complementary area growth curve (Melis 1985; Melis and Homann 1976; Melis and Homann 1975). It involved the calculation of growth of the normalized complementary area, defined by the fluorescence induction curve and the line parallel with the maximum level of fluorescence (Fm), with time. The kinetics of Q_A accumulation was obtained by the calculation of the kinetics of complementary area $\left[B = \int (Fm - Ft)dt \right]$, where B is the double normalized (between 0 and 1) kinetics of the complementary area (Strasser et al. 2000) and the B kinetics of the first light pulse were fitted with three exponentials that correspond to α, β and γ type centers (Toth and Strasser 2005) . Their contribution to the total amplitude (A) of the kinetics of the complementary area has been indicated as percentage of α, β and γ centers (Strasser 1981; Hsu et al. 1989).

The connectivity for different centers was calculated according to Lazar et al. (2001). The curves are presented by means of relative variable fluorescence (rFv(t)),which is defined as (F(t)−Fo)/(Fm−Fo), where Fo, Fm, and F(t) are the minimal and maximal measured fluorescence intensity at time (t) respectively.

Results and discussion

The fluorescence induction curve in wheat leaves treated with sorbitol and NaCl stress are shown in Fig. 1a and 1b respectively. When dark-adapted plants are illuminated by strong actinic light, fluorescence rises from its minimal level (Fo or O) to its maximal level (Fm or P) in three distinct phases separated by two shoulders named J and I. The shape of the J-I-P fluorescence rise has been related to a major change in the photosynthetic electron transport where J-I and particularly I-P rise emphasizes the complexity of the PS II acceptor side reduction kinetics (Joly and Carpentier 2009). The O-J phase corresponds to the photochemical reduction of the primary quinone electron acceptor of PS II (Q_A) and thus accumulation of Q_A^- (Strasser and Govindjee 1992; Boisvert et al. 2006; Joly and Carpentier 2007). The I-P phase is directly related to the reduction of the PQ pool, while J-I seems to be related to the full closure of PS II RC or to a Q_B-quenching mechanism (Boisvert et al. 2006). Thus according to the recent view, the O-J phase is related to the accumulation of Q_A^-, I-P is associated with the PQ pool reduction while J-I is not related with reduction in the PQ pool (Gauthier et al. 2010). The I-P amplitude in the transient has also been related to the relative size of the pools of final PS I electron acceptors (Tsimilli-Michael and Strasser 2008; Kalachanis and Manetas 2010). An increased J step was
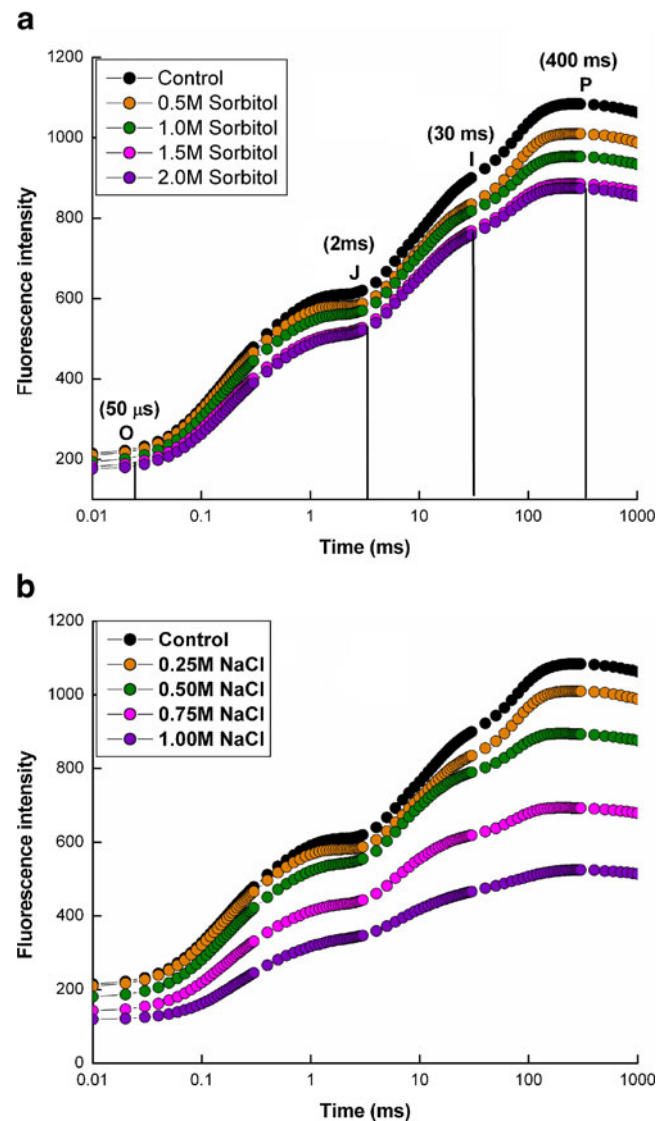

Fig. 1 The OJIP Chl *a* fluorescence transient curve (log time scale) in wheat leaves exposed to various concentrations of sorbitol (**a**) and NaCl (**b**) for 2 h

observed in 1 M NaCl treated leaves as compared to 2 M sorbitol. The increased J step in 1 M NaCl may be due to an increased number of inactive centers (Fig. 2). It has been proposed that J-I is largely dependent on events on the donor side of PS II (Schreiber 2002). The study of the I-P phase (corresponding to the oxidation state of Q_A) in fluorescence induction curves after the 1st pulse shows that there is no significant change in the I-P phase in sorbitol treated leaves (Fig. 1a) while a decline in this phase is observed in NaCl treated leaves (Fig. 1b). The decline of the I–P phase at 1 M NaCl may occur if the electron transfer has been partially blocked from Q_A to Q_B as a result of considerable increase in the number of Q_B-non-reducing centers. The number of inactive PS II centers also display heterogeneity on the reducing side of PS II with respect to electron transfer from Q_A to

Fig. 2 Fluorescence transient curves (log time scale) double normalized at Fo and Fm showing a distinctive J step
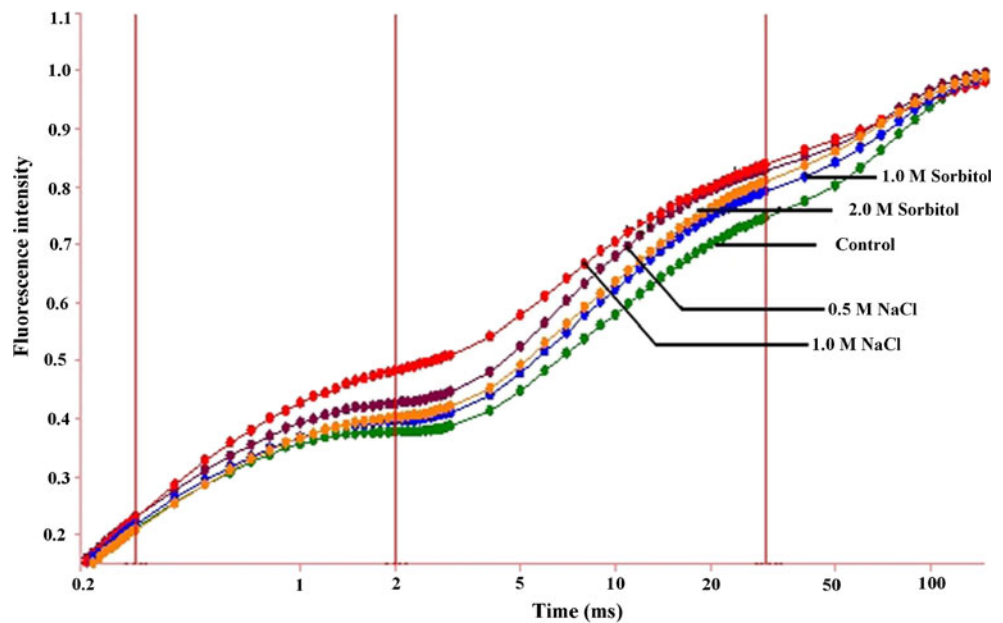
Q_B (Lavergne 1982; Thielen and van Gorkom 1981; Melis 1985; Graan and Ort 1986; Guenther et al. 1988). The Fo to F_I rise seen in the Chl a fluorescence induction has been suggested to be due to the presence of 'PS II inactive centers' (Cao and Govindjee 1990). In case of NaCl treated leaves, this rise is relatively lower than sorbitol treated leaves again suggesting an increase in the number of inactive centres in NaCl treated leaves. As compared to osmotic stress a prominent decline in the reduction of PQ pool was observed in leaves treated with 1 M NaCl which may be because of the retardation of the Q_A oxidation step.

Effect of osmotic and high salt stress on reducing side heterogeneity

Reducing side heterogeneity was estimated by measuring relative amounts of Q_B-reducing and Q_B-non-reducing centers as described in Materials and Methods. From the fluorescence induction curves obtained after the 2nd pulse in osmotic and high salt stressed leaves (curves not shown), the population of Q_B-non-reducing centers was quantified. As shown in Table 1, in control leaves about 18–20 % of total PS II was found to be Q_B-non-reducing centers while around 80–82 % PS II centres were Q_B-reducing centres. Exposure of leaves to varying concentrations of sorbitol did not affect the relative fractions of Q_B-non-reducing PS II centers while in the case of NaCl, a gradual change in the fractions of Q_B-non-reducing PS II centers was observed (Table 1). The increased fractions of Q_B-non-reducing centres at higher NaCl concentration implied that these centers were unable to reduce Q_A to PQ pool and also that the active Q_B-reducing centers were converted into inactive Q_B-non-reducing centers. Based on these results we have

concluded that osmotic stress is not related with electron transfer beyond Q_A while ionic stress affects electron transfer between Q_A and Q_B. The increased fraction of Q_B-non-reducing centers at high NaCl corresponds with the reduced rate of Q_A oxidation which is consistent with the results shown in a recent report (Zaghdoudi et al. 2011). Moreover in case of wheat, it seems that Q_B-non-reducing centers form a sub population of PS IIβ. However more research needs to be done in order to confirm it.

Table 1 Changes on relative amounts (normalized) of Q_B-reducing and Q_B-non-reducing centers as a result of osmotic and ionic treatment in wheat leaves. For recovery studies, stressed leaves were kept in distilled water for 2 h in light. Five repeats were done for each treatment

Treatment	% of Q_B non-reducing centers	% of Q_B reducing centers
Control	20±1	80±1
0.5 M Sorbitol	22±1	78±2
1.0 M Sorbitol	22±1	78±1
1.5 M Sorbitol	22±1	78±1
2.0 M Sorbitol	22±1	78±1
0.25 M NaCl	24±1	76±2
0. 5 M NaCl	27±1	73±1
0.75 M NaCl	28±1	72±2
1.0 M NaCl	30±1	70±1
Recovery		
0.25 M NaCl	20±1	80±2
0. 5 M NaCl	24±1	76±1
0.75 M NaCl	26±1	74±1
1.0 M NaCl	26±1	74±1

Recovery of the stressed leaves was also studied in order to investigate whether the changes in PS II heterogeneity are temporary or permanent. To measure recovery, stressed leaves were put in distilled water for 2 h in the light. The leaves exposed to osmotic stress did not cause any change in the relative fractions of Q_B-non-reducing centers. Therefore no recovery study was performed for osmotic stressed leaves. In case of high salt stressed leaves, complete recovery was observed for 0.25 M NaCl, while partial recovery was observed for 0.5 M NaCl to 1 M NaCl (Table 1). These results are different from the earlier report (Mehta et al. 2010a) where no recovery in reducing side heterogeneity was observed in NaCl treated samples. However, those experiments were performed in the dark and the stress was given gradually.
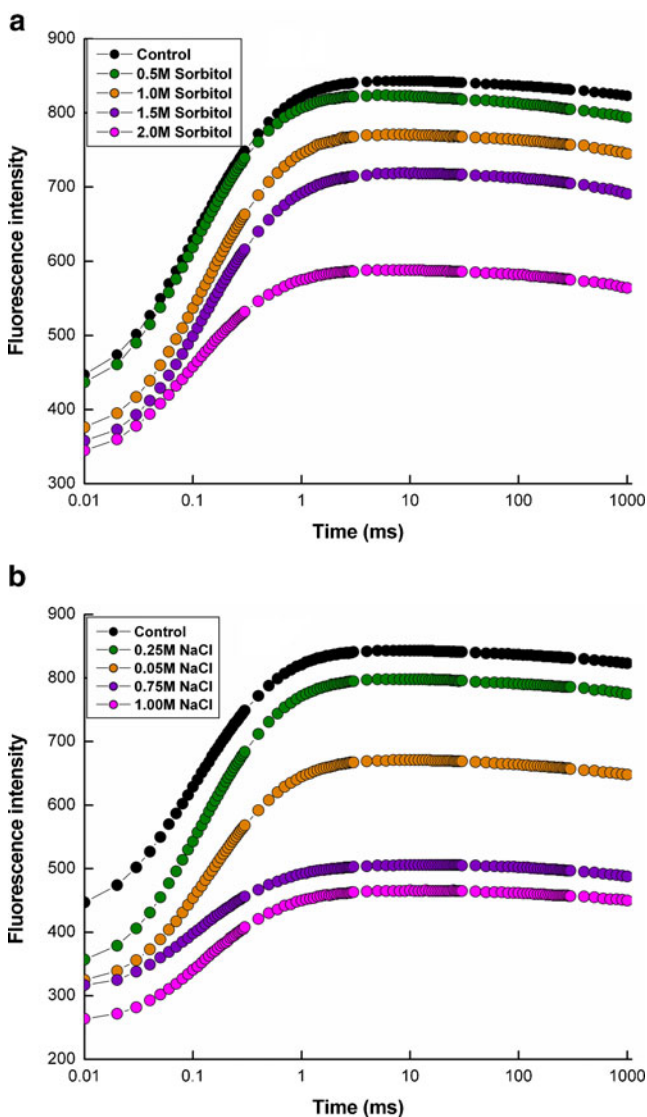

Fig. 4 Representative complementary area curves (linear time scale) showing percentage and life times (τ) of alpha (α), beta (β), and gamma (γ) centers in control wheat leaves

Effect of osmotic and high salt stress on antenna heterogeneity

The antenna heterogeneity involves antenna size heterogeneity (relative amounts of α, β and γ centers) as well as energetic connectivity between PS IIs (Lazar et al. 2001). The fluorescence induction curves after DCMU treatment

Fig. 3 The OJIP Chl a fluorescence transient curve (log time scale) in DCMU treated wheat leaves exposed to **a** osmotic stress, **b** ionic stress for 2 h
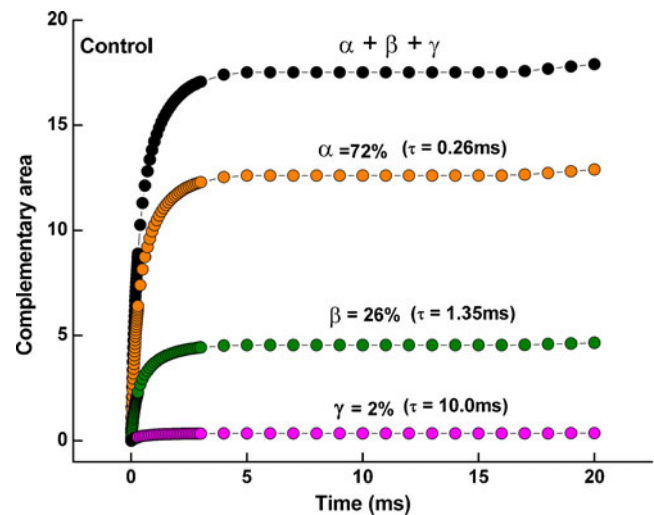
Table 2 Change in the amounts (in %) of α, β and γ centers in response to osmotic and ionic stress in wheat leaves. For recovery studies, stressed leaves were kept in distilled water for 2 h in light. Five repeats were done for each treatment

Treatment	α	β	γ
Control	72±1	26±1	2±1
0.5 M Sorbitol	69±2	30±2	1±1
1.0 M Sorbitol	66±1	31±1	3±1
1.5 M Sorbitol	54±1	36±1	10±1
2.0 M Sorbitol	49±2	40±2	11±1
0.25 M NaCl	66±2	31±2	3±1
0. 5 M NaCl	63±2	32±2	5±1
0.75 M NaCl	52±1	36±1	12±1
1.0 M NaCl	49±2	39±1	12±1
Recovery			
0.5 M Sorbitol	70±2	27±1	3±1
1.0 M Sorbitol	69±2	26±1	5±1
1.5 M Sorbitol	55±1	35±1	10±2
2.0 M Sorbitol	55±2	36±1	9±1
0.25 M NaCl	70±1	26±1	4±1
0. 5 M NaCl	71±2	25±2	4±1
0.75 M NaCl	55±1	34±1	11±1
1.0 M NaCl	54±2	35±1	11±1

are shown in Fig. 3 a and b and antenna size heterogeneity was determined from these curves. It gave information about the relative proportions of α, β and γ centers in osmotic and high salt stressed treated wheat leaves. In control leaves (Fig. 4), the fastest α component (lifetime ~0.26 ms) represents 72 % of the total amplitude. The β component (lifetime ~1.35 ms) was responsible for ~26 % of the total amplitude and the γ component being slowest (lifetime ~10.0 ms) represents ~2 % of the total amplitude. Subsequent increase in osmotic and ionic concentration from 0.5 to 2 M sorbitol and 0.25 M NaCl to 1 M NaCl led to a decrease in the proportion of α centers while β and γ centers showed an increase (Table 2). These components are probably interconvertable depending on the environmental conditions, and the active centers seem to get converted into inactive centers. PS IIα may dissociate into free LHC II and PS IIβ and the latter may migrate from the grana to the non-appressed thylakoid membranes (Sundby et al. 1986; Sinclair and Spence 1988) as a result of changes in the antenna organization following osmotic and ionic stress. Another possibility is that since PS IIβ are located in stroma lamellae, they may come in contact with the PS I reaction center leading to spillover. However it needs further investigations.

Another component of antenna heterogeneity is concerned not with the size of antenna but with the connectivity between antenna molecules. According to the concept of connectivity (also called grouping) closed PS II reaction centers (RC) may transfer their excitation energy to the open neighbouring PS II units that results in a sigmoidal fluorescence rise instead of an exponential rise (Strasser et al. 2004). It was suggested that the three populations of PS II units (α, β and γ) may be different in their connectivity properties i.e. the α-centers are supposed to be grouped. These centers are capable of exchanging excitation energy with each other (Joliot and Joliot 1964), whereas the two others are not, and the trapping efficiency of the γ-centers is thought to be lower. PS IIβ was characterized by an exponential rise of the time course of complementary area (CA), whereas PS IIα showed a non-exponential (sigmoidal) rise (Melis and Homann 1976). The exponential shape of this rise for PS IIβ was suggested to reflect mutual energetic separation of these PS II which are unable to exchange excitation energy (Joliot and Joliot 1964). On the other hand, the non-exponential fluorescence rise of PS IIα is generally believed to reflect energetic connectivity between these PS IIs. The fluorescence rise (FR) curves measured in osmotic and high salt stressed treated wheat leaves with DCMU was measured and no significant difference was observed in antenna connectivity for both the type of stresses (data not shown). This result is in contention with an earlier report where no change in the connectivity of the PS II antenna was observed in case of NaCl treated samples (Mehta et al. 2011).

In recovery studies, almost complete recovery of antenna size heterogeneity was observed with 0.5 M, 1.0 M sorbitol while only partial recovery was obtained with 1.5 M and 2.0 M sorbitol. In the same way, complete recovery was obtained with 0.25 M and 0.5 M NaCl while only partial recovery was obtained with 0.75 M and 1.0 M NaCl (Table 2).

Conclusions

The changes in different types of heterogeneity of PS II in response to osmotic and ionic stress in wheat leaves have been investigated in order to understand the physiological basis of stress tolerance. In case of osmotic stress only antenna size heterogeneity was affected and no change in reducing side heterogeneity was observed. Compared to osmotic stress, ionic stress caused changes in both antenna size and reducing side heterogeneity. Also the recovery process seems to depend on light. However, it is yet to be investigated in more details. Thus, osmotic and ionic stress led to changes in PS II heterogeneity which may be one of the adaptive mechanisms to cope with high salt induced changes in the environment.

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