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Short Communication

JIP Analysis on Rice (*Oryza sativa* cv Nipponbare) Grown under Limited Nitrogen Conditions

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Nitrogen deficiency significantly reduces the CO_2 assimilation capacity of plants and the quantum yield of photosynthesis. Here, we employed the JIP test to determine the effects of nitrogen-deficiency on the plant's photosysnthetic ability on the basis of chlorophyll fluorescence. Nitrogen-deficient and nitrogen-replete rice plants were analyzed for the fluorescence transients of the plant leaves in comparison with the nitrogen-sufficient controls. Results showed that 7 day-replete plants behaved normally while 5, 3, and 1 day-replete plants were significantly affected from nitrogen starvation. More specifically, nitrogen starvation of plants resulted in an inactivation of photosystem II (PS II) reaction centers and a decline in electron transport beyond the reduced plastoquinone (Q_A^-), and a decrease in both the pool size and the reduction of end electron acceptors at the PS I. The affected plants were fully recovered from the deficiency after 7 days of nitrogen repletion, as evidenced by the similar level of fluorescence transients to the positive controls. Thus, our results demonstrated that the movement of electron carriers leading to the reduction of end electron acceptors was affected by nitrogen limitation leading to a more pronounced decrease in the reduction of end electron acceptors. Together with the fact that nitrogen-deficiency limits the CO_2 assimilation of plants, this study indicates that nitrogen metabolism is tightly coupled with photosynthetic ability.

Key words: chlorophyll fluorescence, JIP test, nitrogen, *Oryza sativa*

Nitrogen is one of the essential macronutrients required for proper plant growth. It plays a major role in nutrition because of its importance in protein and nucleic acid synthesis. Excess nitrogen can cause shoot elongation and lodging, lower yield, and increases susceptibility to pest and fungal attacks. On the other hand, deficiency in nitrogen leads to loss of green color in the leaves, decreased leaf area, intensity of photosynthesis resulting to growth retardation, lower yield and death of plants [Amtmann and Armengaud, 2009]. Nitrogen content in the leaf is correlated with net photosynthesis and the amounts of photosynthetic components [Nakano *et al.*, 1997] particularly the size and morphology of chloroplasts [Sivasankar *et al.*, 1998]. Nitrogen deficiency significantly reduces the CO_2 assimilation capacity of plants [Terashima and Evans, 1988] and the quantum yield of photosynthesis [Nunes *et al.*, 1993]. This leads to a reduced CO_2 concentration that ultimately limits photosynthetic activity by direct inhibition of the photosynthetic enzyme Rubisco [Haup-Herting and Fock, 2000] or ATP synthase [Tezara *et al*., 1999].

In this study, the effects of nitrogen were evaluated using the JIP test based on the "Theory of Energy Fluxes in Biomembranes" by Strasser [1981]. This follows the dogma that, when plastoquinone (Q_A) in a reaction center (RC) is reduced to Q_A^-, the RC is closed and the chlorophyll fluorescence of the antenna is high, whereas when Q_A is in the oxidized state, the RC is open and the fluorescence of the antenna is quenched, i.e. oxidized Q_A quenches fluorescence. Fluorescence kinetics reflects the photosynthetic efficiency of plants and provides wealth of information on the relationship between structure and function of photosystem II (PS II) RCs and core complexes [Krause and Weis, 1991]. The sequential events reflected in the fluorescence rise proceed with different rates and concomitantly, the rise is polyphasic

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designated by the letters O-J-I-P [Strasser, 2004] hence, the term JIP test. The JIP test, here, is to determine the effects of nitrogen-deficiency on the plant's photosysnthetic ability on the basis of chlorophyll fluorescence.

Materials and Methods

Plant preparation. Rice (*Oryza sativa* cv Nipponbare) seeds were germinated and grown in nursery soil for 5 days inside a greenhouse at 28-30°C. On the 6th day, the soil was removed and the plants were grown in tap water for another 3 days before it was transferred into a Yoshida nutrient solution. Positive control plants were fed with the nutrient solution while NH_4NO_3 was excluded from the nitrogen-starved and negative control plants. The solution contains the following (in mg/L): NH_4NO_3 (40), $NaH_2PO_2 \cdot 2H_2O$ (10), K_2SO_4 (40), $CaCl_2$ (40), $MgSO_4 \cdot 7H_2O$ (40), $MnCl_2 \cdot 4H_2O$ (0.5), $(NH_4)_6 \cdot MO_7O_{24} \cdot 4H_2O$ (0.05), H_3BO_3 (0.2), $ZnSO_4 \cdot 7H_2O$ (0.01), $CuSO_4 \cdot 5H_2O$ (0.01), $FeCl_3 \cdot 6H_2O$ (2). Nitrogen-starved plants were grown for 10 days until the effects of nitrogen starvation were visible. Complete Yoshida solution was then supplied in a sequential time course. Plants supplied with nitrogen for 7 days until the time of JIP analysis (25th day) was labeled 7 day-replete plants while plants supplied with nitrogen for 5 days was labeled 5 day-replete plants. The same process was applied for 3 days- and 1 day-plants. Solutions were regularly replenished every three days to avoid algal growth and loss of nutrients.

Measurement of the fast chlorophyll *a* transients. Chlorophyll *a* fluorescence was measured using the Pocket-PEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments Ltd., King's Lynn Norfolk, PE 32 1JL, UK) on plants that were dark adapted for at least 30 min. The tallest and the visually healthy-looking leaves were selected for each plant. One leaf per seedling and for a total of 10 seedlings per treatment was used as samples. The readings were averaged using the Handy PEA Software (ver. 1.31). The Pocket-PEA fluorimeter was set in the following program: the initial fluorescence was at O (50 μs), J (2 ms) and I (30 ms) are intermediates and P (500 ms-s) as the peak. The transients were induced by red light at 627 nm of 3,500 μmol photons $m^{-2}s^{-1}$ provided by single light - emitting diodes (LED) positioned vertically above the sample and recorded for 1 s with 12 bit resolution. Data acquisition was set at every 10 μs (from 10 to 300 μs), every 0.1 ms (from 300 to 3 ms), every 1 ms (from 3 to 30 ms), every 10 ms (from 30 to 300 ms), every 100 ms (from 300 ms to 1 s) and downloaded to a PC through a Bluetooth wireless connection. To deduce information from the O-J-I-P transients normalizations and computations were performed using the Biolyzer 4HP software (ver. 4.0.30.03.02) while OriginPro 8 SR0 v9.0724 (B724) was used for the graphical illustrations. The O-J-I-P transients were analyzed according to the equations of the JIP-test [Strasser *et al.,* 2004]. The difference kinetics computed for the O-K phase (ΔW_{OK}) was performed by subtracting the normalized data of the samples ($V_{OKsample}$) with the positive controls ($V_{OKcontrol}$). Normalization for each data set was performed from the following equation $V_{OK} = (F_t - F_O)/(F_K - F_O)$. Student's t-test was used for the computation of significant differences at 0.05 level ($p < 0.05$).

Results and Discussion

The raw fluorescence of the plants exhibited the typical polyphasic O-J-I-P rise with similar variable fluorescence ($F_v = F_m - F_o$) indicating that the photosynthetic units of the plants are active even under nitrogen deficiency (Fig. 1A). Further analysis of the transients revealed more information regarding the effects of nitrogen-deficiency on the photosynthetic units of the plants. To be able to elaborate differences on the J phase, double normalization at the O and P phase (W_{OP}) was performed. Fig. 1B shows that at 2 ms (emphasized by a box) the positive controls and 7 day-replete plants had the lowest fluorescence while negative control plants were the highest. On the other hand, fluorescence of the plants with time for nitrogen recovery (5, 3, and 1 day-replete plants) were in between the two controls confirming the effects of nitrogen-starvation on their photosynthetic units. This increase in the fluorescence could be due to a decrease in the electron transport beyond Q_A^- [Haldiman and Strasser, 1999] which leads to the accumulation of a fraction of Q_A^- [Munday and Govindjee, 1969] consequently increasing the fluorescence at the J phase.

Difference kinetics at the O to J phase also revealed the so called K-band which, when positive, indicates either damage in the oxygen evolving complex (OEC) or increase in functional antenna size. As shown in Fig. 2A, 5, 3, 1 day-replete and negative control plants had positive K-bands while 7 day-replete plants behaved similarly with the positive control plants. The formation of the positive K-bands in the nitrogen-starved plants is more likely due to a decrease in the active RCs (which contain the OEC in PS II) rather than the increase in functional antenna size. This is because an increase in antenna size is coupled with the formation of a positive L-band through the difference kinetics of the O and K phase (V_{OK}). Figure 2B shows that all the L-bands behaved similarly with positive control suggesting that the functional antenna size was not affected by nitrogen

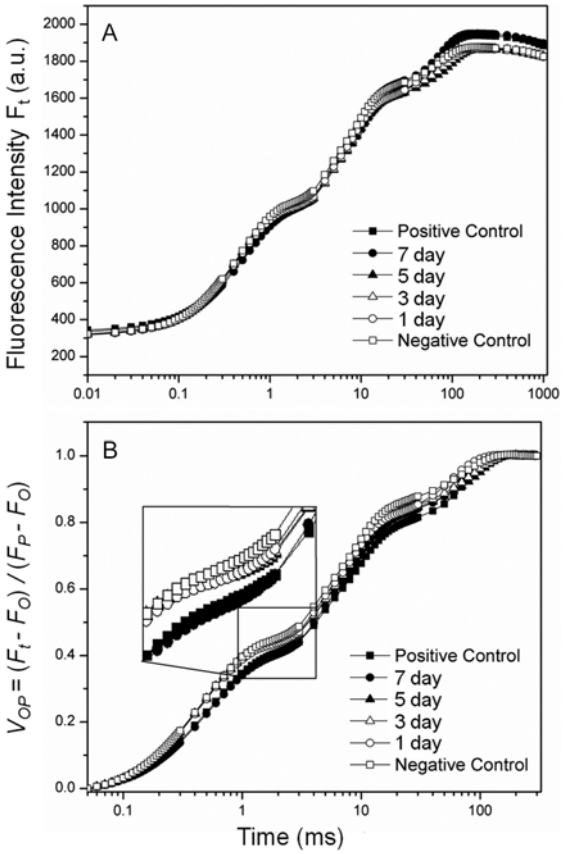

Fig. 1. Polyphasic chlorophyll *a* fluoresence of the plants. (A) Raw fluorescence of the plants showing the O (50 μs), J (2 ms), I (300 ms) and P (500 ms) phases. (B) Double normalization at the O-P phase, $V_{OP}=(F_t-F_o)/(F_p-F_o)$, elaborating the fluorescence change at the J phase (2 ms).

deficiency. Interestingly, drought-treated rice plants overexpressing *OsNAC10* [Redillas *et al*., 2011] showed L-band formation with no K-bands present. These suggest that plants react to drought through a decrease in apparent antenna size but not under nitrogen-deficient conditions.

Further analysis of the transients through double normalization at the O and I phase (W_{OI}) revealed that nitrogen-starved plants exhibited changes in the pool size of end electron acceptors. Fig. 2C illustrates only the transients above 1.0 ($V_{OI}\geq 1.0$) since it reflects the reduction of electron acceptors at the PS I. The maximal amplitude of the transients reflects the pool size of the end electron acceptors. Nitrogen-replete and negative control plants showed a decrease in pool size with the latter showing the most drastic effect compared to the positive controls. The reduction rate for the end electron acceptors, however, showed no correlation with the change in pool size, suggesting that the regulations of the two parameters are independent. This response was also similar to the chemically-treated *Brassica juncea* plants overexpressing α-tocopherol methyl transferase gene to simulate abiotic stress [Yusuf *et al.*, 2010].

The structural and functional parameters, deduced from the OJIP transients, were also evaluated. Structural parameters are the ratio of rate constants and/or the energy flux ratios expressed per absorption (ABS) e.g. as quantum yields (trapping per absorption, TR_0/ABS), or per electron transport between the two photosystems (electron transport per absorption, ET_0/ABS), or per electron transport reducing the end electron acceptors (RE_0/ABS) of photosystem I (PS I). Functional parameters on the other hand is a measure of specific energy fluxes expressed per active RC, i.e., RCs capable of reducing Q_A to Q_A^-. All of the plants were normalized to the positive controls and the degrees of deviation from the control plants illustrate the affected parameters by nitrogen limitations. The structural parameters of the 7 day plants behaved similarly with the positive controls except for the reduction of end electron transport per absorption (REo/ABS). On the other hand, 5, 3, 1 day replete and negative control plants showed similar reductions in RC/ABS, TRo/ABS, ETo/ABS, and REo/ABS. The negative control plants however, exhibited a more pronounced reduction in REo/ABS compared to the nitrogen-replete plants suggesting that the most affected event is the reduction of the end electron acceptors at the PS I acceptor side. The changes in the functional parameters of 7 day-replete plants also behaved similarly to the positive control plants except for the electron flux for reducing end electron acceptors at the PS I acceptor side per RC (REo/RC) indicating that this event is affected by nitrogen deficiency. Furthermore, the absorption flux (of antenna Chlorophylls) per RC (ABS/RC), trapping flux (leading to Q_A reduction) per RC (TRo/RC), and electron transport flux (further than Q_A^-) per RC (ETo/RC) increased in 5, 3, 1 day-replete and negative control plants. The observed increase in these parameters was due to the inactivation of a fraction of active RCs by being transformed into non-Q_A-reducing centers rather than an increase in apparent antenna size which coincided with the increased fluorescence in the J phase of the transients (Fig. 2B). ABS/RC is a measure of the apparent antenna size (total absorption or total chlorophyll per active RC) and an increase in this parameter means that either a fraction of RCs is inactivated or the apparent antenna size increased. An increase in antenna size, however, would be reflected in the formation of positive L-bands which were absent in all of the plants, suggesting that nitrogen-deficiency did not affect the antenna of the photosynthetic units. The inactivation of a fraction RCs might be a plant's response to avoid overproduction of reduced Q_A^- which can lead to

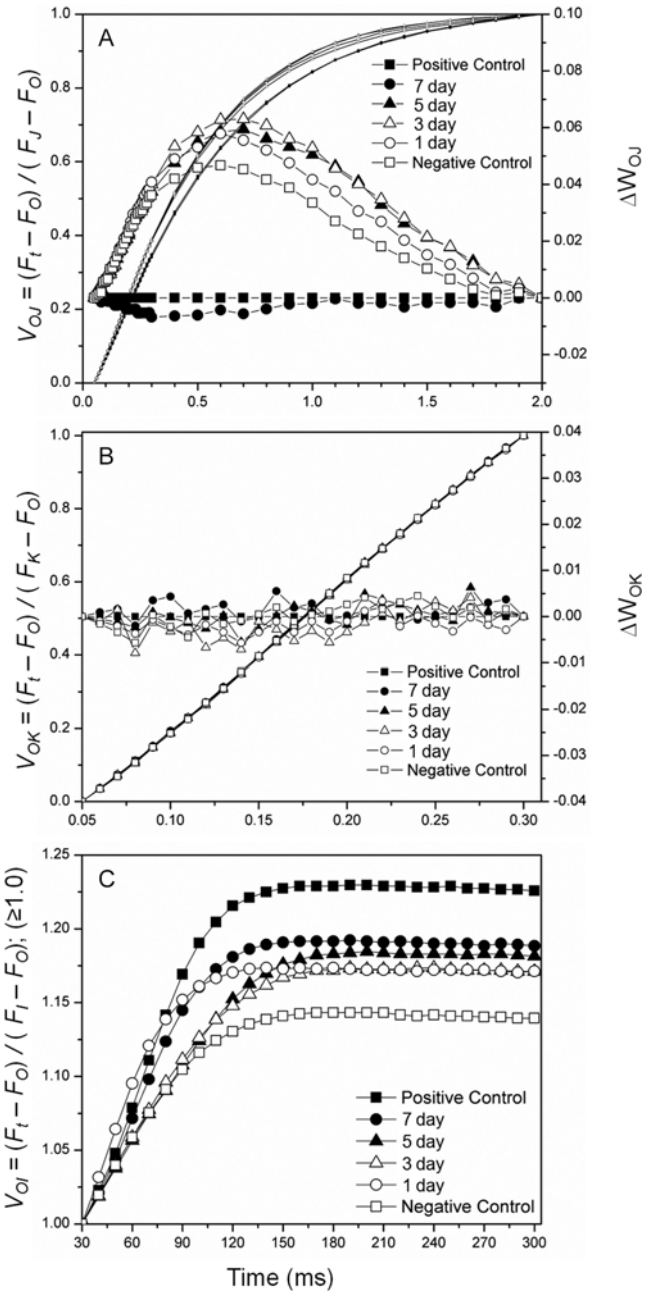

Fig. 2. Fluorescence kinetics from the OJIP transients. (A) Double normalization at the O-J phase, $V_{OJ}=(F_t-F_o)/(F_J-F_o)$; left axis and the difference kinetics was computed through the equation $W_{OJ}=V_{OJsample}-V_{OJcontrol}$, showing the K-band around 500 μs; right axis. (B) Double normalization at the O-K phase, $V_{OK}=(F_t-F_o)/(F_K-F_o)$; left axis and the difference kinetics, $W_{OK}=V_{OKsample}-V_{OKcontrol}$, for the L-band; right axis. (C) Double normalization at the O-I phase $V_{OI}=(F_t-F_o)/(F_I-F_o)$ showing only the transients V_{OI} 1.0 which illustrates the differences in the pool size of end electron acceptors.

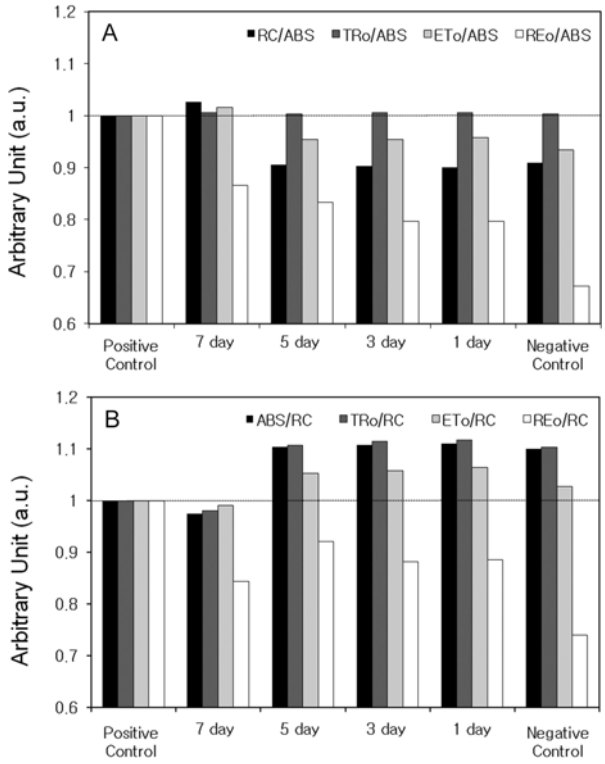

Fig. 3. Photosynthetic parameters derived from the JIP analysis relative to the positive control plants. (A) Ratio of energy fluxes representing the structural parameters; quantum yield for primary photochemistry (TRo/ABS), the quantum yield for the conversion of excitation energy to electron transport (ETo/ABS) and the quantum yield for the reduction of end acceptors (REo/ABS). (B) Specific energy fluxes (per RC) considered as functional parameters: absorption flux (ABS/RC), trapping flux (TRo/RC) electron transport flux (ETo/RC) and electron flux for reducing end electron acceptors at PS I side (REo/RC).

photoinhibition.

Along the energy cascade, the movement of energy starting from ABS followed by trapping (TRo), electron transport (ETo) and up to the reduction of end electron transport (REo), it is the reduction of end electron acceptor that is mostly affected by nitrogen deficiency due to the decrease in the pool size of end electron acceptors (Fig. 2C). Nitrogen-deficiency has been reported to reduce CO_2 assimilation which is coupled with the decrease in Rubisco content as well as other key enzymes in the Calvin cycle. This leads to the down-regulation of electron transport and a decline in the requirement to reduce the end electron acceptors $NADP^+$ and Fd. Because of this, the production of ATP and NADPH would be in equilibrium in the nitrogen cycle of nitrogen-deficient plants [Lu and Zhang, 2000]. Indeed, the efficiency or probability for electron transport beyond Q_A^- (ETo/TRo) and the efficiency or probability that the intersystem electron carriers move to reduce the end electron acceptors (REo/ETo) were also reduced in the nitrogen-deficient plants (Fig. 4A). Results showed that while the ETo/TRo of 7 day-replete plants behaved similarly with positive controls, the ETo/TRo of 5, 3, 1

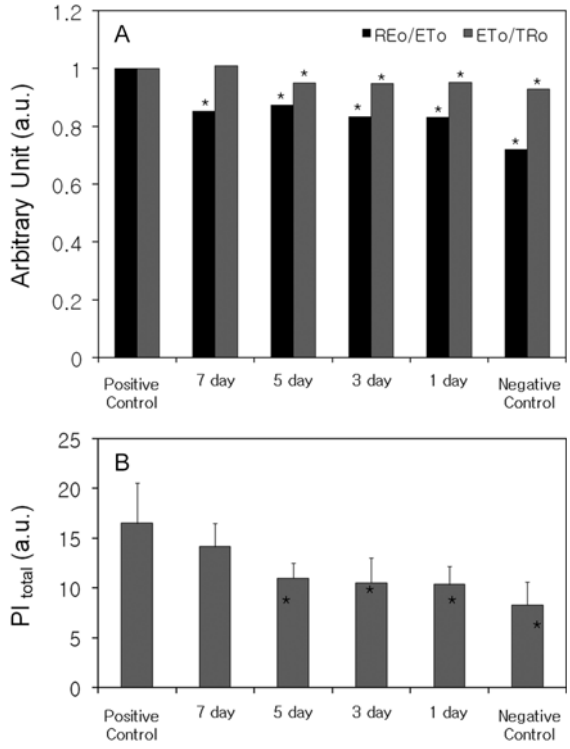

Fig. 4. Electron transport probabilities and partial forces of the plants. (A) Efficiency or probability for electron transport beyond Q_A^- (ETo/TRo) and the efficiency or probability that the intersystem electron carriers move to reduce the end electron acceptors (REo/ETo). (B) Summary of all partial forces represented by single parameter Total Performance Index (PI_{total}) for each plant. Asterisks denote significant difference with the positive control plants (p <0.05).

day-replete and negative control plants were significantly reduced (p <0.05). Also, the REo/ETo was reduced for all nitrogen-starved plants with the NT controls having the lowest REo/EoT. Verhoeven *et al.* [1997] stated that nitrogen-deficiency decreases the quantum yield of PSII electron transport and the maximal efficiency of PSII photochemistry suggesting that N deficiency induces some damage to PSII. However, this change in electron transport is thought to be a plant response to be able to decrease the utilization of ATP and NADPH generated from the primary photochemical reactions to match the demand for ATP and NADPH by carbon metabolism [Lu and Zhang, 2000] since nitrogen-deficiency limits the CO_2 assimilation of the plants. Collectively, the results confirmed that the movement of electron carriers leading to the reduction of end electron acceptors was affected by nitrogen limitation leading to a more pronounced decrease in the reduction of end electron acceptors.

The parameter PI_{total} summarizes all the partial driving forces and its individual effects on the component parameters such as the RC-density in the chlorophyll bed (RC/ABS), the performance due to the quantum efficiency of primary photochemistry ($\varphi_{Po}/(1-\varphi_{Po})$), the performance due to the quantum efficiency of the conversion of excitation energy to electron transport ($\psi_{Eo}/(1-\psi_{Eo})$) and the performance due to the quantum efficiency of the reduction of end acceptors ($\delta_{Ro}/(1-\delta_{Ro})$) [Smit *et al*., 2009]. Statistical analysis revealed that the PI_{total} of 5, 3, 1 day-replete and negative control plants were significantly reduced (p <0.05) compared to the positive control plants (Fig. 4B). This suggests that 25-day old rice seedlings, starved with nitrogen for 10 days, require at least 7 days of nitrogen repletion to restore its photosynthetic ability similar to those of nitrogen-sufficient plants. In summary, the JIP test revealed several events affected by nitrogen-deficiency in plants. These include the inactivation of PS II reaction centers, decline in electron transport beyond Q_A^-, decrease in the pool size of end electron acceptors, and a decline in the reduction of end electron acceptors at the PS I.

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