

BRIEF COMMUNICATION

Effect of endophyte infection on chlorophyll *a* fluorescence in salinity stressed rice

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Abstract

We have earlier reported that the endophyte infection can enhance photosynthetic capacity and antioxidant enzyme activities in rice exposed to salinity stress. Now, the changes in primary photochemistry of photosystem (PS) II induced by Na₂CO₃ stress in endophyte-infected (E+) and endophyte-uninfected (E-) rice seedlings were studied using chlorophyll *a* fluorescence (OJIP-test). Performance indices (PI_{ABS} and PI_{Total}) of E- and E+ rice seedlings revealed the inhibitory effects of Na₂CO₃ on PS II connectivity (occurrence of an L-band), oxygen evolving complex (occurrence of a K-band), and on the J step of the induction curves, associated with an inhibition of electron transport from plastoquinone A (Q_A) to plastoquinone B (Q_B). In E+ seedlings, Na₂CO₃ effects on L and K bands were much smaller, or even negligible, and also there was no pronounced effect on the J step. Furthermore, the OJIP parameters indicated that 20 mM Na₂CO₃ had a greater influence on the photosystem (PS) II electron transport chain than did the 10 mM Na₂CO₃, and that changes were greater in E- than in E+. Endophyte infection was therefore deemed to enhance the photosynthetic mechanism of *Oryza sativa* exposed to salinity stress.

Additional key words: electron transport chain, Na₂CO₃, OJIP test, *Oryza sativa*, photosystem.

Rice, one of the most popular cereals in Asia, is sensitive to saline and alkali environments, which often lead to significant reductions in yield (Tuteja 2007, Sengupta and Majumder 2010). This sensitivity is more pronounced during the early seedling period than at the reproductive stage (Sahi *et al.* 2006, Hakim *et al.* 2010).

It has long been known that endophytic fungi increase in host plant tolerance to biotic and abiotic stresses (Malinowski and Belesky 2000, Rodriguez and Redman 2008, Soleimani *et al.* 2010) including salinity (Wang *et al.* 2009), water deficit (Kane 2011, Ren *et al.* 2011), and zinc (Monnet *et al.* 2001). Previously, we identified an isolate of endophytic fungi from *Suaeda salsa*, known as EF0801, which promote growth of rice under Na₂CO₃ and Pb stress, primarily *via* enhancement of photosynthesis and antioxidant capacity (Bu *et al.* 2012, Li

et al. 2012). Unfortunately, the mechanism is not elucidated yet.

Recently, changes in the chlorophyll *a* (Chl *a*) fluorescence OJIP transient (OJIP transient) have been used as indicators of damage to photosynthetic apparatus resulting from several environmental stresses. For example, measurement of OJIP transients is a sensitive and reliable method for the detection and quantification of changes of photosystem (PS) II and PS I photochemistry induced by temperature (Zushi *et al.* 2012), salinity (Venkatesh *et al.* 2012), drought (Jedmowski *et al.* 2013), and heavy metals (Yusuf *et al.* 2010, Xue *et al.* 2013). The shape of the OJIP curve can be modified by application of glycine betaine and proline (Oukarroum *et al.* 2012) or a cytokinin (Shao *et al.* 2010). It has already been used to identify

Submitted 22 September 2013, last revision 22 January 2014, accepted 24 January 2014.

Abbreviations: see Table 1 Suppl. for detailed list of abbreviations

Acknowledgments: This research was financially supported by the National Natural Science Foundation of China (Grant Nos. 31270369 and 31070285), the Director Foundation of Eco-Environmental Research Center at Shenyang Normal University (EERC-K-201302), the Director Foundation of Experimental Center at Shenyang Normal University (SY201102 and SY201104).

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stress tolerance using the change of performance index (PIabs), a multicomponent fluorescence parameter derived from fast chlorophyll induction curves, and for describing the PS II-dependent first phase of the induction curve (Strasser *et al.* 2004).

With the aim to illustrate the endophyte effect on the primary photosynthetic reactions under Na₂CO₃ stress, the OJIP-test was determined on endophyte-infected (E+) and uninfected (E-) Na₂CO₃ stressed detached rice leaves.

An isolate of the endophytic fungus (EF0801) was obtained from leaves of *Suaeda salsa*. The molecular identification of this fungus was based on internally transcribed spacer regions that showed that it is congeneric to *Sordariomyces* sp. (99 % similarity; Li *et al.* 2012). Fungal isolates were maintained on potato dextrose agar (PDA) plates in refrigerator (4 °C). The initial medium pH was 7.0 - 7.5, and the strain was inoculated at the 3rd instar stage for 5 % into 75 cm³ PDA culture solution, and cultured in a 150 cm³ shaker flask for 12 d at 180 rpm, and temperature of 24 ± 1 °C. This fermentation broth was used for seedling treatments.

Rice seeds were surface sterilized in 2.65 % sodium hypochlorite, rinsed with distilled water, and transferred to Petri dishes for germination. After 2 d, germinated seeds (100 grains) were cultivated in a 500 cm³ beaker containing full Hoagland's nutrient solution. The seedlings were grown in a growth chamber (day/night temperatures of 27/20 °C, a 16-h photoperiod, photosynthetic photon flux density of 600 μmol m⁻² s⁻¹, and relative humidity of 80 %) for two days.

On the second day in the growth chamber, the Na₂CO₃ and endophyte treatments were initiated (Bu *et al.* 2012). Briefly, seedlings were divided into two groups: 1) inoculated with the fermentation broth by planting in full Hoagland's solution with 5 % fermentation broth (E+, endophyte-infected seedlings), and 2) the control group (not inoculated) planted in full Hoagland's solution alone (E-, endophyte-uninfected seedlings). Each group was randomly assigned to three Na₂CO₃ treatments (0, 10, and 20 mM Na₂CO₃ in Hoagland's solution).

Chlorophyll *a* fluorescence was measured after exposure to Na₂CO₃ stress for one week. OJIP transients were recorded using a *Plant Efficiency Analyzer (Pocket-PEA, Hansatech, Norfolk, UK)*. Before measurement, leaves were dark-acclimated for 20 min with plastic clips. During measurement, the irradiance of 2 - 3 μmol m⁻² s⁻¹ was used. A saturating pulse of 3 500 μmol m⁻² s⁻¹ with a peak wave length of 627 nm was provided by a single light emitting diode for 1 s. The collected data were processed using the program *PEA Plus 1.0.0.1*. The fluorescence OJIP transients were analyzed according to the equations of the JIP-test (Strasser *et al.* 2004). The following fluorescence characteristics were used: 1) minimal fluorescence after 20 μs, when all PS II reaction centers (RC) are open (the O step); 2) fluorescence after 300 μs used for calculation of the

initial slope (M₀), defined as the net ratio of reaction center (RC) closure; 3) fluorescence after 2 ms (J step); 4) fluorescence after 30 ms (I step), and lastly 5) maximum fluorescence when all PS II RCs are closed (P step = F_m). The formulae and glossary of parameters used by the JIP-test are presented in Table 1 Suppl. (Strasser *et al.* 2004). Data are presented as spider plots, which show the means of the relative change of the selected parameters in relation to the E-0 values.

The significant differences in JIP test parameters between infected and uninfected plants and under different Na₂CO₃ concentrations were determined using two-way analysis of variance (*ANOVA*) followed by LSD's multiple-range test for multiple comparisons. All analyses were made using the *SPSS* statistical software package (*v.13.0, SPSS, Chicago, IL, USA*).

The fluorescence of individual points of the OJIP curve in the leaves of E- was higher than that of E+ (Fig. 1A). The fluorescence in J, I, and P steps in the E+ leaves decreased at 20 mM Na₂CO₃ but increased at 10 mM Na₂CO₃. The fluorescence in J, I, and P steps in the leaves of E- decreased due to Na₂CO₃ application. Lastly, the fluorescence in J and I steps in the leaves of E+ was lower than in the leaves of E-.

Analyses of the OJIP-test parameters and deployment of the whole fluorescence transients allowed for a comparative study of photosynthetic responses of E- and E+ leaves to Na₂CO₃. To illustrate the variable fluorescence (V_{OP}), fluorescence curves were double normalized between F₀ and F_p. Differences in variable fluorescence (V_{OP}) are presented on a logarithmic time scale. The relative V_{OP} of the 20 mM Na₂CO₃ treated E+ was lower than that of the 20 mM Na₂CO₃ treated E-, and both were higher than V_{OP} of the untreated E- (Fig. 1B). In contrast, the 10 mM Na₂CO₃ treated E-, and E+ had lower fluorescence than the untreated E-. The untreated E+ produced the lowest fluorescence. At 20 mM Na₂CO₃, the ΔV_{OP} of E+ was lower than that of E-. The ΔV_{OP} of E- was similar to that of E+ at 10 mM Na₂CO₃, but the untreated E+ had the lowest ΔV_{OP}.

To evaluate if there were any differences between E- and E+ Na₂CO₃ treated samples and non-treated samples, further normalizations and corresponding subtractions on linear time scales were employed (Fig. 1C,D). The difference kinetics, ΔV_{OK} or ΔV_{OJ} of stress treatments *versus* control (E-0), reveals bands concealed between the steps O, J, I, and P of the normalized transients. Fluorescence data were double normalized between the steps O (50 μs) and K (300 μs), as V_{OK}, and plotted with the difference kinetics ΔV_{OK} in the time range 50 - 300 μs revealing a L-band (Fig. 1C). This L-band revealed by such a subtraction is an indicator of the energetic connectivity (grouping) of the PS II units. The subtraction of the E- plant treated with 10 and 20 mM Na₂CO₃ from E- samples exhibited higher L-bands, indicating lower connectivity, or a poor utilization of the excitation energy of the system.

Whereas the appearance of a lower L-band in E+ samples indicates the higher connectivity of this system.

The fluorescence data were also double normalized between the steps O and J (2 ms), as V_{OJ} , and plotted with

the difference kinetics, ΔV_{OJ} in the 50 μ s - 2 ms time range (Fig. 1D). The E+ plants treated with 0 and 10 mM Na_2CO_3 produced a negative K-band, suggesting better photosynthetic performance when compared with

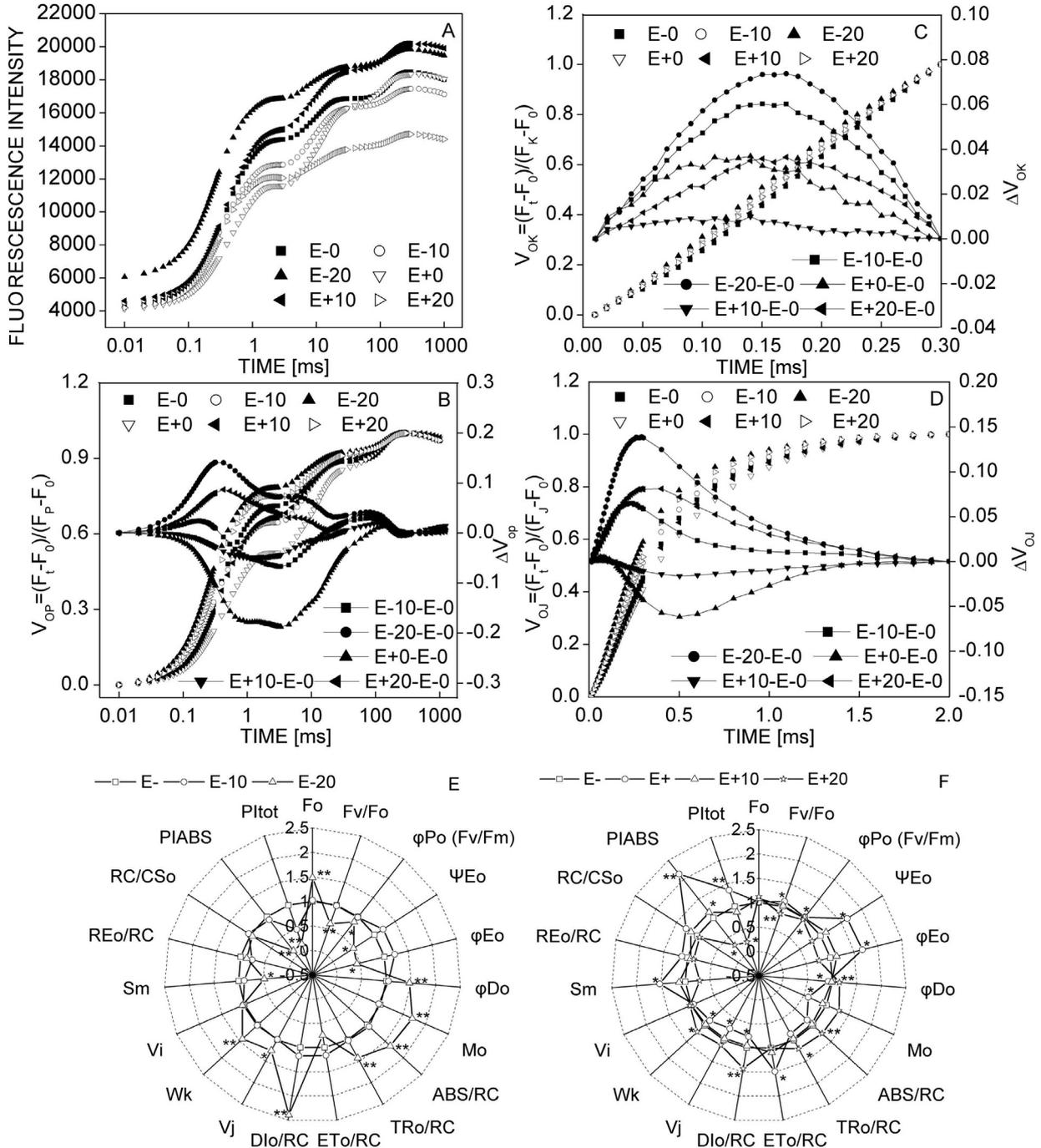


Fig. 1. The Chl *a* fluorescence kinetics OJIP (A), average kinetics (B-D) and spider plots (E,F) of JIP parameters of dark-adapted leaves of endophyte-uninfected (E-) and endophyte-infected (E+) rice after a week treatment with 0, 10, and 20 mM Na_2CO_3 in Hoagland's solution. *Open symbol* are used for E- and *closed symbol* curves for their corresponding E+ samples. B - $V_{OP} = (F_t - F_0)/(F_P - F_0)$ on the logarithmic scale; C - $V_{OK} = (F_t - F_0)/(F_K - F_0)$; D - $V_{OJ} = (F_t - F_0)/(F_J - F_0)$. In B-D, *open symbol* curves (left y-axis) and *closed symbol* curves to the difference kinetics, ΔV_{OP} , ΔV_{OK} and ΔV_{OJ} stand for stress treatments *versus* control (E-0) (right y-axis). ΔV_{OK} and ΔV_{OJ} revealing the L-band and K-band, respectively. E,F - for each parameter, the value of control (E-) is set as 1. One and two *asterisks* indicate significance of differences at $P < 0.05$ and $P < 0.01$, respectively (*t*-test).

E- samples. However, E- plants treated with 10 and 20 mM Na₂CO₃, and E+ plants treated with 20 mM Na₂CO₃ produced a positive K-band, suggesting these plants were severely affected by the Na₂CO₃ treatment.

The relative values (relative to the control E-) of JIP test parameters are presented as a spider plot (Fig. 2). All parameters in the E- plants treated with 10 mM Na₂CO₃ were not affected. Energy fluxes per active reaction center (ABS/RC and TR₀/RC), DI₀/RC, φD₀, M₀, V_j, W_k and F₀ were higher, whereas the parameters of F_v/F₀, φP₀, ψE₀, φE₀ and S_m were lower in E- plants treated with 20 mM Na₂CO₃ when compared with the control (Fig. 1E).

Compared with E- plants, F_v/F₀, φP₀, ψE₀, φE₀, S_m, φR₀, and ET₀/RC were higher, but DI₀/RC, φD₀, M₀ and V_j were lower in E+ plants (Fig. 1F). All parameters in the E+ plants were not affected by 10 mM Na₂CO₃. In E+ plants treated with 20 mM Na₂CO₃, the ABS/RC, TR₀/RC, DI₀/RC, φD₀, and W_k were higher, whereas, F_v/F₀, φP₀ and φR₀ were lower when compared with the control (Fig. 1F). The F₀, M₀, V_j, ψE₀, φE₀, and S_m in E+ plants treated with 20 mM Na₂CO₃ were nearly the same as those observed in the control (Fig. 1F).

The performance index describes the energy conservation between photons absorbed by PS II, the reduction of intersystem electron acceptors (PI_{ABS}), and the reduction of PSI end acceptors (PI_{Total}) (Strasser *et al.* 2010). In this study, the PI_{ABS} and PI_{Total} values decreased with increasing Na₂CO₃ concentrations (Fig. 1). With or without Na₂CO₃ all E+ seedlings had significantly higher PI_{ABS} and PI_{Total} values when compared to E- seedlings.

In the best of our knowledge, we were the first who studied the effects of endophyte infection on the photosynthetic apparatus in terms of Chl *a* fluorescence transients increase. The OJIP-test was able to distinguish between leaves of E- and E+ treated with Na₂CO₃, and reflected the effect of Na₂CO₃ on the photosynthetic electron transport. The OJ phase reflected the photochemical phase leading to the reduction of plastoquinone (Q_A) to Q_A⁻ (Strasser and Srivastava 1995). The O and J steps in the leaves of E- at 20 mM Na₂CO₃ increased significantly. This increase very likely is a result of the combination of the detachment of LHC from PS II, and an enhanced reduction state of the plastoquinone pool leading to closure of PS II centers by back electron flow (Havaux 1996). The decrease at phase J, I, and P in the leaves of E+ at 20 mM Na₂CO₃ can be explained as the inhibition of electron transport at the donor side of PS II, and subsequent accumulation of P680⁺, a strong fluorescence quencher (Schmidt *et al.* 2013).

Change in fluorescence occurs as a result of variation in the redox state of the reaction center complex of PS II (Haldimann and Strasser 1999). In the present study, the relative variable fluorescence (V_{OP}) in the 20 mM Na₂CO₃ treated E+ seedlings was lower than in

corresponding Na₂CO₃ treated E- seedlings, and in untreated E+ seedlings was the lowest (Fig. 1B). Strasser *et al.* (1995) reported that high fluorescence occurs as a result of decreased electron transport beyond Q_A⁻, which results from the accumulation of a fraction of reduced Q_A⁻. The difference kinetics, ΔV_{OP} also revealed that E+ seedlings produced lower fluorescence than E- seedlings, suggesting less biochemical inhibition, and energy loss beyond the redox couple of Q_A/Q_A⁻ in E+ seedlings.

The difference kinetics, ΔV_{OK} represented by the L-band allowed a comparative study of energetic connectivity (grouping) of PS II, being higher when connectivity is lower (Stirbet 1998). Therefore, all treatments resulted in a decrease of the energetic connectivity (positive L-bands), with the strongest effect exerted by the 20 mM Na₂CO₃ treatment (Fig. 1C). We also show that the E+ seedlings had higher connectivity than the E- seedlings (positive L-band). These data are similar to that reported by Yusuf *et al.* (2010), and Venkatesh *et al.* (2012). A higher connectivity results in a better utilization of the excitation energy and a higher stability of the system (Strasser *et al.* 2004).

The difference kinetics (ΔV_{OK}) is represented by the K-band. A positive K-band reflects either an inactivation of the oxygen evolving complex (OEC), and/or an increase of the functional PS II antenna size (Strasser *et al.* 2004), whereas a negative K-band has previously been demonstrated as a indication of better performance of plants under stress conditions (Venkatesh *et al.* 2012). In our study, E- plants treated with Na₂CO₃, and E+ plants treated with 20 mM Na₂CO₃ resulted in a positive K-band. E+ plants treated with 0 and 10 mM Na₂CO₃ resulted in a negative K-band. These data suggest that the infection of endophyte may in part alleviate OEC damage in rice plants exposed to Na₂CO₃.

Calculated OJIP parameters may serve to identify sensitive functions such as energy absorption, energy trapping, and electron transport (Strasser *et al.* 2000). In our study, E- seedlings, exposed to 20 mM Na₂CO₃ had a significant increase in the net rate of RC closure, as indicated by the increase in M₀. This increased M₀ suggested that the reduction of Q_A to Q_A⁻ was lower in E+ than E- at 20 mM Na₂CO₃. Previous reports showed that M₀ increased under heat stress (Chen and Cheng 2009).

In the specific fluxes “per RC”, ABS/RC, TR₀/RC, and DI₀/RC were lower in the E+ seedlings than in the E- at 20 mM Na₂CO₃. These results indicated that the average absorption (ABS/RC), and trapping (TR₀/RC) per active RC increased, likely due to the inactivation of some RCs, and that the ratio of total dissipation to the amount of active RCs (DI₀/RC) increased as a result of high dissipation of the inactive RCs (Zushi *et al.* 2012). The decrease of φP₀ in E- seedlings at 20 mM Na₂CO₃ suggested an inhibitory effect of salinity on the donor side of PS II. This led to the decrease in φE₀ and ψE₀.

Similar results have been observed in plants exposed to UV-B stress (Wang *et al.* 2010) and heat stress (Zushi *et al.* 2012). In E+ seedlings, a decrease in ϕP_0 only was observed, but no significant change in ϕE_0 and ψE_0 at 20 mM Na₂CO₃ was observed. These data suggest that 20 mM Na₂CO₃ significantly inhibited the primary charge separation photosynthetic apparatus within the E- seedlings, but not in that of E+ seedlings.

The PI_{ABS} and PI_{Total} are indices of photosynthetic performance as determined by absorption of radiation. A strong positive correlation was found between the performance index and physiological parameters, (e.g. growth, or survival rate) in stressed plants (Zubek *et al.* 2009, Venkatesh *et al.* 2012). Our results showed that the performance index reflected the effect of endophyte infection on the vitality of detached E- and E+ rice leaves. The performance index of E- dropped extensively showing loss, which means there was gradual increase of a negative "strain" on the system with increased Na₂CO₃ stress, whereas in E+ seedling restrained the negative

strain imposed by Na₂CO₃ stress as indicated by the gain in the performance index. These results are similar to that observed in Yusuf *et al.* (2010), and Venkatesh *et al.* (2012). It is apparent that the structural modifications induced in E+ seedlings enable the photosynthetic machinery of those same seedlings to perform better when exposed to Na₂CO₃ treatment (Fig. 2).

In conclusion, the use of the JIP-test to evaluate the performance of Na₂CO₃ treated rice seedlings enabled us to compare the photosynthetic responses of E- and E+. The seedlings of E+ exhibited efficient energy conservation when subjected to Na₂CO₃ stress. These results are also in agreement with those obtained by normalization/subtraction of fluorescence transients. The PI_{Total} value of the E+ samples increased more than that of the E- under stress conditions. These data collectively suggest that endophyte infection may indeed alleviate salinity stress as evidenced by photosynthetic apparatus performance within endophyte-infected rice.

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