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EVIDENCE FOR DIFFERENTIAL PHOTO-REGULATION OF *IN VIVO* NITRATE ASSIMILATION IN BARLEY (C_3) AND CORN (C_4) SEEDLINGS

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SUMMARY

Effect of exposing barley and corn seedlings to light during the *in vivo* Nitrate (NO_3^-) assimilation assay and/or during the period preceding the assay, was studied in 9-day old intact seedlings. The effect of exogenously supplied sucrose was also studied in order to separate the specific (direct) effects of light from those of carbohydrates. The seedlings used had not been exposed to any exogenous nitrogen during germination and growth and hence, were uninduced for nitrate reduction. Interestingly, light conditions during the assay period (current) determined the NO_3^- assimilation in corn whereas in barely light conditions preceding as well as during the assay, influenced NO_3^- assimilation. In other words, light did not have any residual effect on NO^-_3 assimilation in corn whereas in barley a residual effect of light appeared to exist. Under continuous light (light present prior to, and during assay), 1% surcose in the ambient medium increased NO_3^- assimilation similarly in both barely and corn (89% and 77%, respectively), but the corresponding increase due to sucrose in continuous darkness was much greater in barley (967%) than in corn (51%). The results strongly suggest that in barley the total carbohydrate pool, rather than light *per se*, plays a dominant role in NO_3^- assimilation, whereas specific/direct light effects and/or current photosynthates may be more important in corn. Since both species were able to assimilate some NO_3^- in darkness, light did not appear to be an absolute requirement for the assimilation of NO_3^- either in barley or in corn.

Key words : **Barley, corn, darkness, light, NO_3^- assimilation**

INTRODUCTION

The process of NO_3^- assimilation in plants which is catalyzed by nitrate reductase (a substrate inducible and reductant requiring enzyme), has been known to be largely affected by light (Kessler 1964, Beevers and Hageman 1969, 1972, Canvin and Atkins 1974). Li and Oaks (1994) observed that the transfer of corn plants to darkness resulted in an immediate decline of nitrate reductase activity (NRA) and NR messenger RNA in shoots and after a lag period of 4 hours in roots. Huber *et al.* (1994) showed that changes in NRA in mature corn leaves involved reversible protein phosphorylation in response to light to dark shift. These reports indicate that light is obligatory for NO_3^- assimilation. However, Aslam *et al.* (1979) observed NO_3^- assimilation during darkness in intact barley seedlings. Goyal and Huffaker (1981) showed that the light-grown excised barley leaves were able to assimilate the supplied NO_3^- in darkness, even though slower than in light. Sugars have been reported to stimulate the expression of *nia gene* (Cheng *et al.* 1992, Krapp *et al.* 1993, Krapp and Stitt 1995) and post-translational activation of NR (Kaiser and Forster 1989, Kaiser and Huber 1994, Huber *et al.* 1996). There are marked diurnal

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changes in the transcriptional (Hoff *et al.* 1994) and post translational regulation (Moorhead *et al.* 1996) of NR, and changes in sugars play a major role in generating these diurnal changes of NR expression and activity (Hoff *et al.* 1994, Ferrario *et al.* 1995, Nussaume *et al.* 1995, Scheible *et al.* 1997). A clear distinction between direct effects of light *per se* and those exerted via CO_2 fixation has not been achieved.

The plants with C_4 pathway of photosynthesis are believed to be more efficient in using nitrogen in addition to being superior in carbon fixation, as compared to the ones with C_3 pathway (Brown 1978, Oaks 1994). Hence, differences in NO_3 assimilation commensurate with photosynthetic efficiency could be expected in plants possessing the two types of photosynthetic pathways. Higher NO_3^- uptake, tissue NO_3^- concentrations, and NRA in barley (C_3) as compared to those in corn (C_4) were reported by Oaks *et al.* (1990). However, the nitrogen incorporated into the protein fraction was higher in corn. Hocking and Meyer (1991) showed that when wheat (C_3) plants were grown in elveated level of CO_2, the flow of nitrate-N to protein was enhanced. These reports suggest that the metabolic use of NO_3^- may be more efficient in C_4 plants, perhaps due to higher photosynthetic carbon fixation. Whether the carbohydrate supply alone limits the efficiency of NO_3^- assimilation in plants has not been resolved.

It appears that more studies reporting specific and direct effects of light on NRA (or nitrate assimilation) come from corn (C_4) whereas those showing little or no direct effect of light on nitrate assimilation come from barely or wheat (C_3). It is, therefore, likely that the photo-regulatory mechanisms of nitrate assimilation in barley and corn are different and need to be examined along the species lines. The objective of this study was to investigate the comparative photo-regulation of *in vivo* NO_3^- assimilation in intact seedlings of barley and corn seedlings.

MATERIALS AND METHODS

Caryopsis of barley (*Hordeum vulgare* L. cv. UC 337) and corn (*Zen mays* L. cv. Pioneer 3162) were surface sterilized and germinated as described earlier (Goyal and Huffaker 1981). The germinated seeds were spread on cheese cloth supported on a stainless steel screen suspended above 4.5 L of aerated 0.5 mM $CaSO_4$. After 6 days in darkness, the seedlings were transferred to N-free, one-fourth strength Hoagland solution (Hoagland and Arnon 1950) in continuous light (500 µmol photon. $m^{-2}s^{-1}$) for another 3 days. The continuous light conditions during the growth were used in order to avoid changes due to diurnal variations and to fully load the seedlings with photosynthates. The temperature and relative humidity during growth were 25°C and 70% to 75%, respectively. The NO_3^- assimilation assay was conducted either in light or in darkness. Prior to assay, the seedlings were treated either with darkness or light for 24 h. Accordingly, four sets of seedlings, i.e., (1) pretreated with light and assayed in light (LL, continuous light), (2) pretreated with darkness and assayed in darkness (DD, continuous darkness), (3) pretreated with light and assayed in darkness (LD, light to darkness shift for assay), and (4) pretreated with darkness and assayed in light (DL, darkness to light shift for assay), were utilized. When corn seedlings without the residual endosperm were used, the endosperm was carefully removed by using a sharp razor blade, at the time when seedlings were shifted from darkness to light (3 days prior to assimilation assay).

In vivo nitrate assimilation

Nitrate assimilation was determined by subtracting the total amount of NO^-_3 accumulated in the roots and shoots from that disappeared from the assay medium during the allowed uptake peirod. This method represents the true amount of NO^-_3 assimilated *in vivo* under the conditions and hence is a preferred method. Methods that utilize only *in vitro* nitrate reductase activities indicate the potential rather than the actual NO_3^- assimilated. Preliminary experiments using $^{15}NO_3^-$ had validated the results of the methodology used. The results were expressed as µmol NO_3^- assimilated/g seedling fresh weight. Nitrate assimilation efficiency was also computed and is defined as percent NO_3^- assimilated of that taken up.

Nitrate uptake was measured as the amount disappearing from the external medium. Ten seedlings were incubated in 50 to 350 mL of nutrient medium (volume depending upon the treatment and duration of assay) containing 0.7 mM KNO_3. The nutrient solution volume was adjusted such that the final NO_3^- concentration did not decrease below 0.25 mM. Previous studies have

shown that the NO_3^- uptake rate is relatively constant in the concentration range of 0.25-0.7 mM (Goyal and Huffaker 1986). The assay nutrient medium was one-quarter strength Hoagland solution (Hoagland and Arnon 1950) buffered with 1.0 mM MES (2,5-morpholino-ethane sulphonic acid) at pH 5.75 ± 0.1. Sucrose was included in the medium at a concentration of 1% where specified. Chloramphenicol was used uniformly in all treatments at a concentration of 20 μM to retard bacterial growth. No loss of NO_3^- was noticed from controls without plants which shows that the NO_3^- disappeared from solutions only due to plant uptake. After the desired uptake duration, the seedlings were removed from the solutions, roots rinsed with running deionized water, and the seedlings separated into roots and shoots at scutellar node, weighed and frozen in liquid N_2 immediately. The tissue was then ground in a mortar and pestle with deionized water at below 4°C and centrifuged at 10,000 g for 10 min. The supernatant was analyzed for NO_3^- content.

Nitrate assay

Nitrate was assayed by using the High Performance Liquid Chromatography (HPLC) method of Thayer and Huffaker (1980). The HPLC system used in this study comprised of a pump (model LC-6A), a UV Spectrophotometric detector (model SPD-6A), a data system (model CR-5A) (all Shimadzu Scientific Instruments, Inc. Columbia, MD) and an injector (Rheodyne Inc, Cotati, CA). The HPLC column used was packed in our laboratory with Partisil-10 SAX (Whatman Inc., Clifton, NJ) and 30 mM KPO_4, pH 3.0 was used as the eluant.

RESULTS

Effect of light on *in vivo* nitrate assimilation

The in vivo NO_3^- assimilation during continuous light (LL) was almost similar in barley and corn seedlings; about 12 μmol NO_3^- was assimilated per g fresh weight of seedlings in 12 h (Fig. 1). However, in seedlings pretreated and assayed in darkness (DD), it was lower in barley as compared to corn. Continuous darkness (DD) decreased NO_3^- assimilation by 88% and 49% in barley and corn seedlings, respectively, as compared to those in continuous light (LL). Under the conditions of light to darkness shift

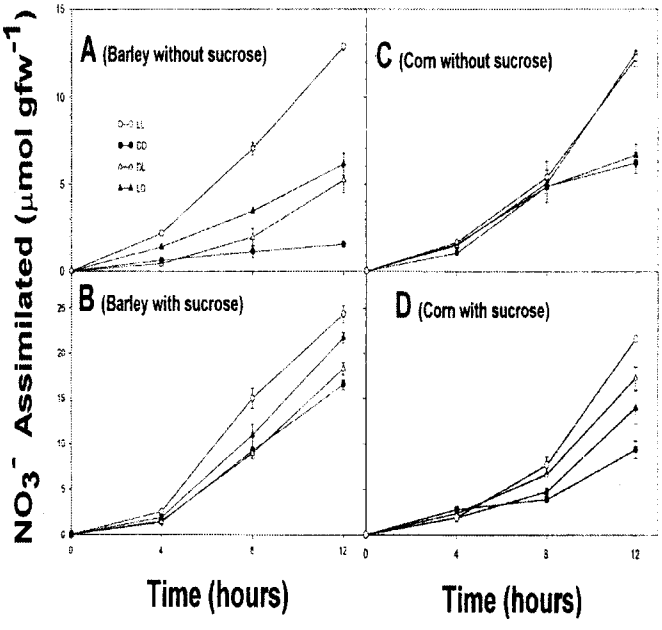

Fig. 1. Time course of *in vivo* NO_3^- assimilation by intact barley (A and B) and Corn (C and D) seedlings in the absence (A and C) and presence (B and D) of 1% sucrose in the assimilation medium. LL, DD, DL, and LD denote light conditions during the pretreatment and assay periods; LL: seedlings grown in light and assayed in light, DD: pretreated with darkness for 24 h and assayed in darkness, DL: pretreated with darkness for 24 h and assayed in light, LD: grown in light and assayed in darkness. The seedlings used were uninduced for nitrate reductase and grown as described in material and methods. The results are mean of three independent assays±S.E.

(LD) or darkness to light shift (DL), the *in vivo* NO_3^- assimilation in barley was midway of those in DD and LL treatments. In other words, in barley, darkness either during the assay or prior to the assay (DL or LD), decreased NO_3^- assimilation relative to continuous light. In contrast, LD and DL conditions in corn caused NO_3^- assimilation similar to that under DD and LL, respectively, resulting in essentially no influence of light on NO_3^- assimilation during the period preceding the assay (Fig 1). In general, corn seedlings assimilated a significantly greater percentage of the NO_3^- taken up, as compared to barley, in light as well as in darkness; the difference being more pronounced in darkness than in light (Fig. 2). Consequently, NO_3^- accumulation was lower in corn than that in barley seedlings. Only 9% of the NO_3^- taken up was assimilated during continuous darkness (DD) by barley seedlings as compared to about 73% by corn under the similar conditions (Fig. 2). In continuous light (LL),

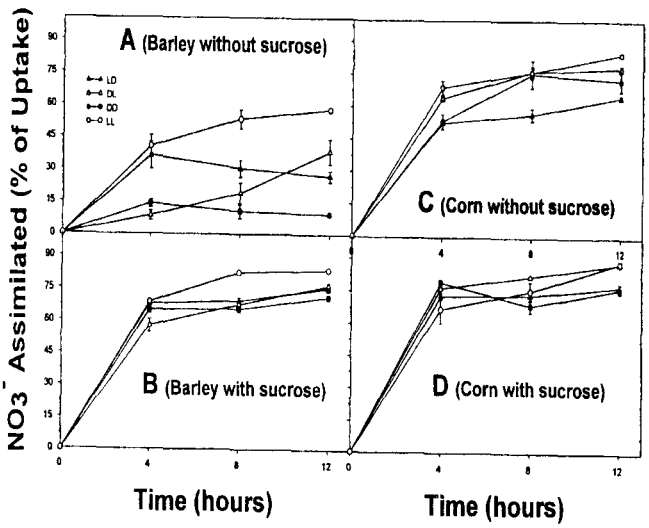

Fig. 2. Time course *in vivo* NO_3^- assimilation (expressed as percentage of uptake) by intact barley (A and B) and corn (C and D) seedlings in the absence (A and C) and presence (B and D) of 1% sucrose in the assimilation medium. LL, DD, DL, and LD denote light conditions during the pretreatment and assay periods, as described in Fig. 1. Other conditions same as in Fig. 1

barley and corn seedlings assimilated about 58% and 85% of the NO_3^- taken up in 12 h, respectively. Barley seedlings pretreated with darkness but assayed in light (DL) assimilated only about 8% of the NO_3^- taken up during the first 4 h, which was similar to that under continuous dark (DD) conditions. However, the percent NO_3^- assimilated, progressively increased as the assay time in light increased; about 38% of the NO_3^- taken up was assimilated during the entire 12 h of assay duration. During the same period (first 4h of assay), barley seedlings grown in light but assayed in darkness (LD), assimilated a higher percentage of NO_3^- (about 36%) than those under DD or DL treatments. However, in this treatment (LD), the percentage of NO_3^- assimilated progressively decreased as the assay time increased and only about 25% of the NO_3^- taken up was assimilated during the entire 12 h period (Fig. 2).

Effect of sucrose in assay medium on *in vivo* nitrate assimilation

Exogenously supplied sucrose in the medium increased NO_3^- assimilation in both species during light as well as darkness. In continuous light (LL), sucrose increased NO_3^- assimilation by about 89% (12.8 μmol/g to 24.3 μmol/g in 12 h) in barley and 77% in corn (12.2 umol/g to 21.6 μmol/g in 12 h). However, in continuous darkness (DD), a dramatic increase of 967% was observed in barley (1.54 μmol/g to 16.47 μmol/g in 12 h) but corn recorded only a modest increase of 51% (6.2 μmol/g to 9.35 μmol/g in 12 h), in response to the supplied sucrose. In barley seedlings, exogenously supplied sucrose greatly enhanced NO_3^- assimilation efficiency (as per cent of uptake) in all treatments and seemed to overcome the effects of dark pretreatment as well as darkness during the assay period. As compared to barley, corn seedlings assimilated a substantially greater percentage of the NO_3^- taken up and the relative effects of darkness or exogenously supplied sucrose were also much less in corn than those in barley (Fig. 2).

Effect of residual endosperm on nitrate assimilation in corn seedlings

Removal of endosperm from green corn seedlings decreased NO_3^- assimilation by 75% when pretreated and

Table 1. Effect of endosperm removal on NO_3^- assimilation in darkness by dark-pretreated intact corn seedlings.

Species	Nitrate assimilation (μmol/gfw*. 12 h)		Nitrate assimilation (% of uptake)	
	Without sucrose	With sucrose	Without sucrose	With sucrose
Corn without endosperm	1.9±0.4	10.4±0.7	19.5±4.8	53.3±1.1
Corn with endosperm	7.9±0.3	14.6±0.7	46.7±0.6	58.4±1.2

* gram fresh weight seedling

Nine days old corn seedlings with or without residual endosperm and NO_3^- uptake system uninduced (having never seen nitrogen during germination and growth) were incubated in 0.7 mmol NO_3^- with or without 1% sucrose for 12 h in darkness. NO_3^- assimilation calculated as the difference of amount of NO_3^- disappeared from incubation medium and the amount accumulated in the tissue. Residual endosperm was removed prior to shifting the seedlings to light. Seedlings used were dark pretreated for 24 h to unload stored carbohydrates of photosynthesis.

assayed in darkness (DD). Under these conditions, exogenously supplied sucrose substituted the endosperm for NO_3^- assimilation. When both endosperm and sucrose were present simultaneously, the assimilation was about 50% higher as compared to when either one of the two was present (Table 1).

DISCUSSION

Seedlings of both barley and corn previously uninduced for nitrate reductase system but grown and assayed in light, were able to assimilate similar amounts of NO_3^- (about 12 μmol per g in 12 h). However, photo-regulation of NO_3^- assimilation was strongly impacted by exposure of seedlings to light prior to the assay. Shifting the seedlings from light to darkness or darkness to light affected the *in vivo* NO_3^- assimilation differently in barley and corn (Fig 1.) Corn seedlings when shifted from darkness to light (DL) for assay, assimilated similar amounts of NO_3^- as those held continuously in light (LL) and vice-versa. On the other hand, in barley such shifting resulted in NO_3^- assimilation that was somewhat in the middle (average) of those held continuously in light (LL) or darkness (DD) (Fig 1). Such an observation and analysis of NO_3^- assimilation response with respect to light has not been reported before.

Clearly, NO_3^- assimilation in corn depended on the light conditions only during the assay period, regardless of light conditions during the period preceding the assay (Fig 1) whereas, in barley light conditions during both periods influenced NO_3^- assimilation. It is noteworthy that barley seedlings pretreated with 24 h light assimilated about the same amount of NO_3^- in darkness as did those in light but pretreated with darkness for 24 h (Fig. 1). This is a significant observation which suggests two distinct possible photoregulatory mechanisms for the two species: (1) Since light was no more important during the assay than during the pretreatment, NO_3^- assimilation in barley was perhaps driven by the overall carbohydrate pool of the seedlings and the role of light was simply to add carbohydrates in that pool via photosynthetic CO_2 fixation. Had the light activation of NR played a role in barley, NO_3^- assimilation during light would always be higher than that in darkness which was not the case (Fig 1). (2) In corn, NR was activated by light probably via dephosphorylation process (as suggested by Kaiser *et al.* 1993 and Huber *et al.* 1994) and/or the products of current CO_2 fixation played a pivotal role in driving NO_3^- assimilation. The current photosynthates in corn could be important because NO_3^- assimilation occurs only in mesophyll cells which are not capable of carbon fixation and are dependent on bundle sheath cells for the needed carbon skeleton. Products of current CO_2 fixation may also stimulate the post-translational activation of NR in corn.

The very fact that the seedlings of both barley and corn were able to assimilate NO_3^- in darkness and the exogenously supplied sucrose greatly enhanced the assimilation and its efficiency, shows that light was not an absolute requirement for NO_3^- assimilation, its induction, or activation. These findings rule out the involvement of phytochrome system as suggested by Jones and Sheard (1972) and Rajashekhar *et al.* (1988) for induction of NR. Rapid decrease in NRA of corn seedlings (Lillo 1991), and decay of NR-m RNA in corn shoots (Li and Oaks 1994), and phosphorylation of NR enzyme in corn leaves (Huber *et al.* 1994) upon light to dark shift could possibly have been due to a signal generated by cessation of recent photosynthate supply upon such shift. Kaiser *et al.* (1993) reported decrease in NRA even in continuous light when CO_2 was removed and reactivated when CO_2 was added. Also, significant NO_3^- assimilation by excised barley leaves in darkness has been reported (Aslam *et al.* 1979, Goyal and Huffaker 1981, Aslam and Huffaker 1982). Sugars have been reported to stimulate the expression of nia (Cheng *et al.* 1992, Krapp *et al.* 1993, Krapp and Stitt 1995), and post-translational activation of NR (Kaiser and Forster 1989, Kaiser and Huber 1994, Huber *et al.* 1996). There are marked diurnal changes in the transcriptional (Hoff *et al.* 1994) and post-translational regulation (Moorhead *et al.* 1996) of NR, and changes in sugars play a major role in generating these diurnal changes of NR expression and activity (Hoff *et al.* 1994, Ferrario *et al.* 1995, Nussaume *et al.* 1995, Scheible *et al.* 1997). Most of the reports showing rapid decrease in NR activity in darkness have used seedlings preinduced for NR in light (Lillo 1991, Reins and Heldt 1992, Kaiser *et al.* 1993, Li and Oaks 1994). However, throughout this study, seedlings uninduced for NR were used and still significant NO_3^- assimilation in darkness was observed (Fig. 1 and 2). Moreover, the exogenously supplied sucrose enhanced NO_3^- assimilation in darkness to the same level

as that in light without the exogenous sucrose (Fig. 1). These observations strongly suggest that at least in intact seedling system, sucrose may be able to replace light for NO_3^- assimilation, including its induction.

Exogenously supplied sucrose increased NO_3^- assimilation by 89% and 77% in barley and corn seedlings grown and assayed in continuous light, respectively (Fig. 1). Apparently even under the conditions of continuous light, the energy and/or carbon skeleton supply limited the NO_3^- assimilation in both species. Geiger *et al.* (1998) have shown that enhanced CO_2 led to a 2-fold increase in NRA in the roots but not in the leaves. The increase of root NRA in enhanced CO_2 was preceded by a transient increase of sugars followed by its decrease, indicating that sugars were being consumed to provide energy and carbon skeletons for NO_3^- assimilation. But the root NRA was only 10% and 20% of the leaf activity in air and enhanced CO_2, respectively (Geiger *et al.* 1998). Hence, the stimulation of NRA in the roots could make only a small contribution to the overall NO_3^- assimilation in the plants. Therefore, the increase in root NRA in response to exogenously supplied sucrose cannot account for the large increase in NO_3^- assimilation efficiency observed in this study. Instead, sucrose was probably transported to shoots where it enhanced NO_3^- assimilation by providing energy and carbon skeletons. Sucrose feeding leads to increased synthesis of amino acids, particularly the glutamine (Morcuende *et al.* 1998). Exogenously supplied sucrose also increased NO_3^- uptake by seedlings of barley and corn (Sehtiya and Goyal 2000). Since roots were submerged in the solution containing sucrose, its effect on uptake may be more readily understood than on assimilation. Therefore, the increase in NO_3^- assimilation in response to exogenously supplied sucrose could have been an indirect effect due to increased uptake. However, NO_3^- assimilation efficiency (per cent of uptake) also increased in both species in response to sucrose feeding (Fig. 2). This shows that increased NO_3^- assimilation, due to exogenously supplied sucrose, was not only due to increased NO_3^- uptake but may be an integrated effect of sucrose due to availability of additional energy and carbon skeletons. Sucrose may have also served as an activating factor of NR as discussed above.

When pretreated and assayed in darkness, barley and corn seedlings assimilated about 9.2% and 73% of the NO_3^- taken up in 12 h, respectively (Fig. 2). The higher efficiency of corn seedlings during darkness may have been due to the availability of carbohydrates from the residual endosperm that was still attached to the seedlings. This explanation was further supported by two observations: (1) a smaller response of NO_3^- assimilation to exogenously supplied sucrose in corn as compared to barley (Figs. 1 and 2) the removal of endosperm from the corn seedlings pirior to the assay, dramatically decreased the NO_3^- assimilation in darkness which increased again when sucrose was supplied exogenously (Table 1). During darkness, corn without the residual endosperm (Table 1) and barley (Fig. 1, DD) assimilated comparable amounts of NO_3^-. This shows that there are no differences in the two species during darkness but they differ in the mechanism by which they respond to light with respect to NO_3^- assimilation.

In summary, the research presented in this paper and the foregoing discussion provides a reasonable basis to conclude that: (1) Corn seedlings have an efficient control on NO_3^- assimilation linked directly to light. It is not fully clear from these data whether the influence of light in corn is exerted via the supply of recent photosynthates or via the activation of NO_3^- reduction system or both. On the other hand, in barley, NO_3^- assimilation is supported by the overall carbohydrate pool of shoots and the role of light is to add carbohydrates to that pool. These findings may be extrapolated as differences between C_3 and C_4 species but further confirmation using other species in both categories is needed. (2) In intact seedling system, light is not an absolute requirement for the assimilation of some quantities of NO_3^- and the carbohydrate supply may replace light.

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