Studies on some biomarkers for submergence tolerance in rice cultivars

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Abstract

In the present study, three rice varieties namely Swarna, Swarna Sub1A, and FR13A were evaluated on the basis of their antioxidative responses to screen submergence tolerance capabilities. Fourteen-day- old seedlings were completely submerged for 7 days and were observed to have a significant variation in reactive oxygen species like H_2O_2 and O_2^-. The variety Swarna recorded maximum accumulation of H_2O_2 and O_2^- and showed more susceptibility to submergence. On the other hand, chlorophyll content did not show highly significant variation among the varieties thus proving its uncertainty to be a trait under submergence. The activity of superoxide dismutase (SOD) had declined throughout the submergence period irrespective of varieties, being maximum in Swarna Sub1A and minimum in Swarna. Almost similar phenomenon was recorded in other two varieties showing their submergence susceptibility however with insignificant variation in terms of APX and CAT. The results from the present experiment point at the fact of submergence tolerance with the view of oxidative sensitivity in rice varieties and finally their possible roles as screening indices for submergence tolerance are discussed.

Keywords: rice (*Oryza sativa*); biomarkers; Reactive Oxygen Species

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Introduction

Out of a number of abiotic stresses, plants predominantly suffer from water deficits that are manifested into a number of anomalous activities. Submergence, a common facet in rice cultivation with various depth and duration has been detrimental in a number of ways. Though, there is an abundance of water under submerged condition, still plants suffer from some sort of osmotic stress under anoxic condition mediated through lower root hydraulic activities (Fukao et al., 2011). Apart from this, anoxia or/and hypoxia also appear as a source of oxidative burst in tissues under submergence and more under re-aeration on recede of water level.

Rice varieties variably respond to different physiological and cellular activities for the submergence response of reaction in plants. It is predominantly the Sub1 gene that in a complicated pathway modulates ethylene metabolism and thus results in every dimension of physiological responses (Fukao and Bailey-Serres, 2008). So, antioxidation pathways may be a key for the plants to avoid oxidative injuries under submerged condition. Rice varieties are displayed with many facets of antioxidation pathways mostly by the application of different chemical

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elicitors, plant hormone derivatives and synthetic analogues of plant metabolites like proline, glycine betaine, polyamines, salicylic acid etc. (Goufo and Trindade, 2014). Interestingly, all of them could modulate the plant responses at submergence and coordinate with effective physiological traits or even set as biomarkers. Accumulation of different ROS is the adhered phenomena to degenerate the submerged tissues in different degrees in rice cultivars earlier (Sharma et al., 2012).

Ethylene sensitivity is also interconnected to rice varieties at different stages of submergence response with regard to antioxidation pathway. However, both antioxidation and ethylene are yet to be deciphered fully in regards to submergence tolerance. It is the antioxidation and its allied peripheral attributes that characterize the submergence tolerance in post anoxic stages when the plants are re-aerated. In the present experiment, we hypothesize that the rice varieties could variably be displayed with antioxidation paths according to genotypic potentials under the said condition. These may characterize the varietal potentials under submergence and thus may also be screening indices for efficient submergence tolerance. With this, we discuss in reference to few antioxidative enzymes and reactive oxygen species what may regulate the submergence resistance in three rice varieties as being thought of improved submergence tolerant traits.

Materials and Methods

In order to fulfill the aforesaid objectives, an experiment was conducted in the laboratory of Plant Physiology and Plant Molecular Biology Research Unit, Department of Botany, University of Kalyani, Kalyani- 741235, West Bengal, India. Seeds of three rice varieties (namely Swarna, Swarna Sub1A, and FR13A) were collected from Rice Research Institute, Chinsurah, West Bengal, India. Out of these three varieties, Swarna is submergence sensitive; FR13A is a naturally occurring submergence tolerant variety whereas Swarna Sub1A has been made submergence tolerant by the introgression of Sub1 allele from FR13A.

Thus, these three varieties may prove their efficacies in distinguishing different physiological parameters under inundation. The seeds were first soaked in 0.1 % mercuric chloride for 15 minutes, followed by rinsing them in distilled water thrice. The seeds were then kept for germination on plastic trays at 37° C for 5 days. Germinated seedlings of uniform appearance were selected and transplanted to earthen pots containing 5 kg of alluvial soil (containing 58.9, 4.5 and 64.7 mg kg-1 of N, P, and K, respectively in the form of urea, single super phosphate and muriate of potash, respectively) with 7 seedlings per pot. Plants were grown in a green house and subjected to natural conditions of temperature (34-37° C), photoperiod (13/11 hour light/dark) with 70-75% relative humidity. The entire work was carried out during the summer season (April-June 2013).

Fourteen-day-old seedlings were submerged for 7 days in a cemented tank under 90 cm depth of water from the floor in order to submerge the plants completely (Panda and Sarkar, 2011). Inundation of the seedlings at this stage beyond 7 days may affect their lives. After completion of 7 days of submergence, plants were taken out of the water, separated into roots and shoots and the estimation of different metabolites were done according to standard referred protocols.

Generation of H_2O_2

This was followed as described by Loreto and Velikova (2001). One g fresh leaf tissue was thoroughly homogenized in liquid nitrogen and extracted with 3 ml of 1 % (w/v) trichloro acetic acid (TCA). Then, it was centrifuged at 10,000 x g, for 15 min at 4° C and the supernatant was collected. In an assay mixture containing 10 mM potassium phosphate buffer (pH 7) and 1 M potassium iodide (KI), solution and appropriate volume (to maintain equal amount of protein) of the supernatant was added, followed by incubation in dark for 15 min. The absorbance was measured at 390 nm. The content of H_2O_2 was determined using the standard curve and H_2O_2 concentration was expressed as µg g^{-1} of fresh weight (FW).

Generation of O_2^-

The assay of O_2^- was done following Achary et al. (2008). 1 g of tissue sample was homogenized in liquid nitrogen and extracted by addition of 65 mM phosphate buffer (pH 7.8). The homogenate was centrifuged at 6,000 x g for 15 min at 4° C. The supernatant was collected and assayed in a mixture consisting of 65 mM phosphate buffer (pH 7.8) and 10 mM of hydroxylamine hydrochloride, then incubated at 25° C for 30 min. Following incubation, a mixture of 10 mM sulphanilamide and 7 mM α-napthyl amine was added and again incubated at 25° C for 20 min. The change in absorbance against a reagent blank was recorded spectrophotometrically at 530 nm (Ghosh et al., 2012). The concentration of O_2^- was calculated using the extinction coefficient of 12.8 mM^{-1} cm^{-1} and expressed as µM NO_2 min^{-1} g^{-1} FW.

Assay of SOD

The SOD activity was done by following the method of Nakano and Asada (1981). Fresh sample was crushed in liquid nitrogen and homogenized in ice cold 50 mM phosphate buffer (pH 7.0) followed by centrifugation at 20,000 x g for 30 min at 4° C. An aliquot of supernatant equivalent to 50 µg proteins was incubated in an assay mixture containing 50 mM sodium phosphate buffer (pH 7.0), 15 mM methionine, 75 µM NBT, 2 µM riboflavin, and 100 mM EDTA, then kept under fluorescent light for 10 min. The absorbance was read at 560 nm and expressed as U g^{-1}FW.

Assay of APX

Plant sample was crushed in liquid nitrogen and homogenized in 100 mM Tris-Cl (pH 7.8) buffer containing 10 mM $MgCl_2$, 1 mM PMSF, 100 mM EDTA, 10 mM DTT, and 2 % PVP and centrifuged at 17,000 x g for 25 min at 4° C. For in vitro assay of APX, equivalent amount of protein from enzyme source was added into the reaction mixture, containing 100 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 0.2 mM H_2O_2, and absorbance was read at 290 nm (Davletova et al., 2005). The activity was calculated using extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as µM of enzyme consumed $(30\ min)^{-1}$ g^{-1} FW.

Assay of Catalase

Catalase activity was assayed according to Nahakpam and Shah (2011). The sample was crushed in liquid nitrogen and homogenized in 50 mM Tris buffer (pH 8) containing 0.5 mM EDTA, 2 % (w/v) PVP, and 0.5 % (v/v) Triton X-100. The homogenate was centrifuged at 22000 x g for 15 min at 4° C. The supernatant was incubated in a reaction mixture containing 200 mM potassium phosphate buffer (pH 7) and 200 mM H_2O_2. The activity was determined by reading the decreasing absorbance at 240 nm and it was detected using the extinction coefficient of 0.036 mM^{-1} cm^{-1} as suggested by Aebi (1983) and enzyme specific activity was expressed as µmol of H_2O_2 oxidized min^{-1} g^{-1} FW.

Estimation of Chlorophyll

The total chlorophyll content was estimated from the Salvinia plants according to Ghosh et al. (2011). Fresh samples from each treatment were homogenized thoroughly with 80 % acetone and centrifuged at 3,500 x g for 10 min at 4° C. Then the supernatant was taken as the source of chlorophyll, which was measured by reading the absorbance at 645 nm and 663 nm with a UV–VIS spectrophotometer and expressed as mg g^{-1} FW.

Results

In quest of rice varieties to respond under submergence in relation to oxidative stress, significant variations ($p \leq 0.05$) were recorded as compared to control condition. Initially, 14-day-old rice seedlings were subjected to complete submergence for 7 days. The leaf samples were collected and evaluated for the accumulation of H_2O_2 and O_2^-, as described in materials and methods section. The results showed an overexpression of H_2O_2 and O_2^- with the range of 1.53 to 1.91 and 1.69 to 2.11 as compared to control, respectively. However, on the average, the changes of H_2O_2 and O_2^- were varied 1.74 fold and 1.85 fold regardless of varieties under submergence. Interestingly, FR13A appeared to be more prone for the accumulation of H_2O_2 than other varieties. Similarly, for O_2^-, it was Swarna

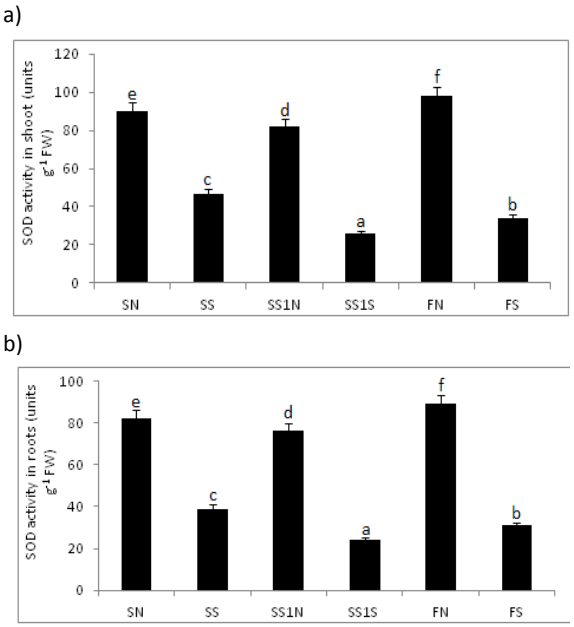

Fig. II. Changes in SOD activity in shoot (a) and in root b) under 7 days of complete submergence of rice genotypes; the data are presented herein with the mean ± S.E. of three replicates (n=3). The different letters on each bar denotes the significant differences (p≤0.05); SN-Swarna under normal, SS- Swarna under submergence, SS1N- Swarna Sub1A under normal, SS1S- Swarna Sub1 under submergence, FN- FR13A under normal, and FS- FR13A under submergence.

which recorded maximum accumulation. On account of enzymatic lysis of superoxide, plants also varied significantly (p≤0.05) for all enzymes. Likewise, for SOD, which causes non reversible one electron reduction of $O_2^{\cdot-}$, was maximally scored under control than complete submergence. This probably denotes the plant's inability to cope with accumulated superoxide so generated under oxidative condition of submergence. The SOD activity in shoot was decreased by 47%, 65.3% and 68.2% in Swarna, FR13A and Swarna Sub1A, respectively under submergence. Likewise, when the activity was concerned with roots, an almost similar trend was recorded. However, SOD activity in root also got declined and on the average, it was 61.9% less under submergence as compared to control irrespective of varieties. For roots, the decreased activity of SOD was found maximum (68.4%) in Swarna Sub1A and minimum (52.4%) in Swarna under submergence, and with FR13A as intermediate (65.1%). The activity of APX, the enzyme, which is more simultaneous to the lysis of

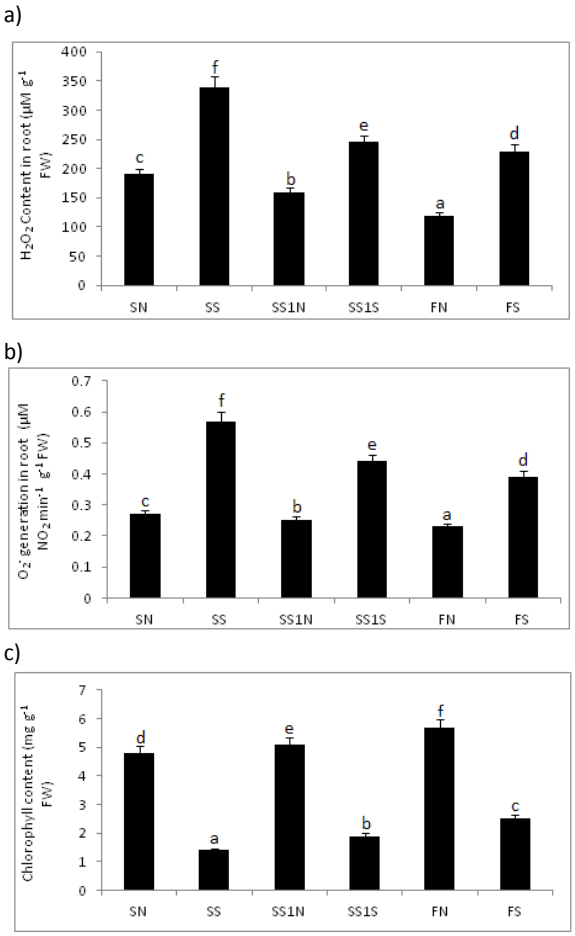

Fig. I. Changes in Hydrogen Peroxide content (a), Superoxide generation in shoots (b) and chlorophyll content in roots (c) under 7 days of complete submergence of rice genotypes; the data are presented herein with the mean ± S. E. of three replicates (n=3). The different letters on each bar denotes the significant differences (p≤0.05); SN-Swarna under normal, SS- Swarna under submergence, SS1N- Swarna Sub1A under normal, SS1S- Swarna Sub1 under submergence, FN- FR13A under normal, and FS- FR13A under submergence.

H_2O_2 in chloroplast, mitochondria and other subcellular fractions, was monitored both in shoot and root. A declining trend was recorded for APX activity in the varieties.

On the average, it was found that the plants were affected more in shoot (24.7% over control) than root (17.7% over control) under submergence. The varietal performances for the decrease in activity was maximum in FR13A (32.4%) and minimum in Swarna (20.0%) in shoots. For roots, activity was maximally decreased (20.1%) in FR13A and minimally (15.7%) in Swarna. For catalase, which is the enzyme requiring no phenolic residues as electron donor had the same

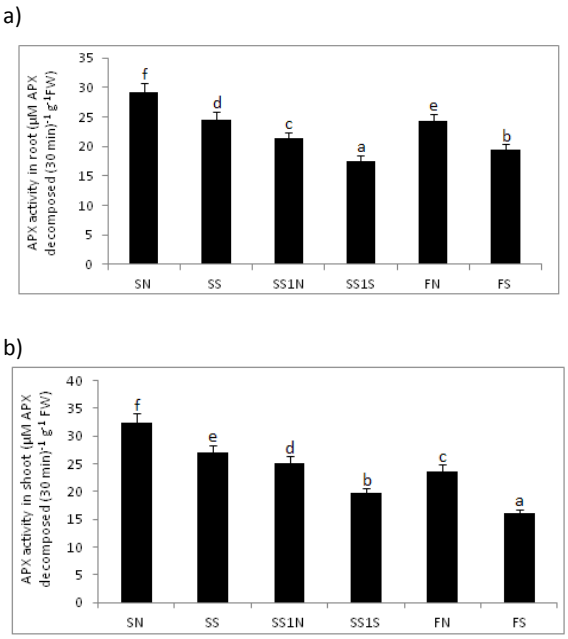

Fig. III. Changes in APX activity in shoot (a) and root (b) under 7 days of complete submergence of rice genotypes; the data are presented herein with the mean ± S.E. of three replicates (n=3). The different letters on each bar denotes the significant differences (p≤0.05). SN-Swarna under normal, SS- Swarna under submergence, SS1N- Swarna Sub1A under normal, SS1S- Swarna Sub1 under submergence, FN- FR13A under normal, and FS- FR13A under submergence.

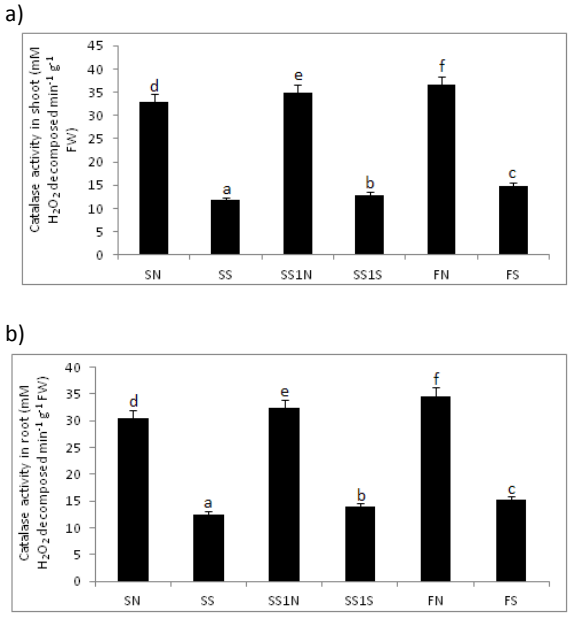

Fig. IV. Changes in Catalase activity in shoot (a) and root (b) under 7 days of complete submergence of rice genotypes; the data are presented herein with the mean ± S.E. of three replicates (n=3). The different letters on each bar denotes the significant differences (p≤0.05). SN-Swarna under normal, SS- Swarna under submergence, SS1N- Swarna Sub1A under normal, SS1S- Swarna Sub1 under submergence, FN- FR13A under normal, and FS- FR13A under submergence.

kind of resources when the plants were put under submergence. The decline in CAT activity in shoots irrespective of the varieties was 62.2% over the control whereas in roots, the activity was curtailed by 57.3% in average suggesting that the shoots are more affected than the roots. Descending in activity was in the order of Swarna, Swarna Sub1A and FR13A. In roots, the declining trend was recorded with the same order of varieties.

The chlorophyll content in shoots also got reduced irrespective of varieties. Maximum reduction in chlorophyll content was found in Swarna (70.8%) followed by Swarna Sub1A (62.7%) and FR13A (56.1%). Therefore, it is clearly recorded that plants under submergence are more or less similarly induced for the antioxidation pathways to regulate either of ROS generation or diminish the activities of ROS. Still, whatever the cases might be, it appears that the genotypic potential of plants are almost tuned with the overexpression of some genes that may collectively broaden the submergence induced damage possibly through antioxidation pathways for one of the resistance mechanism.

Discussion

Plants responding to excess water are mostly focused on the impairment of the growth and development like linear leaf area and its duration, allocation of dry matter in different plant parts as well as photosynthetic activities. However, under condition of submergence, rice plants recorded some alterations of cellular activities particularly, when dealt with oxidative stress (Sarkar et al., 2001). Thus, in the present experiment, regardless of rice varieties, the establishment of oxidative stress was monitored with ROS accumulation in leaves. H_2O_2 accumulation and its concomitant effects in the plants resulting oxidative degradation have also been documented in plants including rice and other cereals (Vijayalakshmi et al., 2014). H_2O_2, though not a free radical, also possess the

characteristic phenomena to lyse the tissue beyond its cellular threshold concentration. In the present experiment, irrespective of the varieties, maximum H_2O_2 content was recorded compared to control. Swarna, the variety under submergence has registered maximum H_2O_2 in the leaves as compared to other varieties.

O_2^-, the most transient but effective ROS had also featured almost in a similar manner to that of H_2O_2 among the rice varieties in earlier studies. Thereby, both these ROS could be ascertained as indices for the cellular oxidative stress under submergence. In our earlier findings, the effects of ROS had indirectly been shown on lipid peroxidation and protein oxidation pathways with submergence as stressor to culminate the deleterious effects of anoxia under flooding. These results were also corroborated with other rice varieties showing varying degrees of submergence tolerance with the effects of oxidative stress. Following the oxidative stress under submergence with the elevation of ROS, rice varieties were also found to modulate the antioxidative responses. In this context, the evaluation of SOD, APX, and CAT activities *in vitro* were monitored under submerged condition of rice varieties. In connection to our earlier finding with other antioxidative enzymes, regulation of SOD was recorded regardless of varieties in both shoot and root. SOD is marked as the first line of defense which lyses O_2^- into H_2O_2 and O_2 (Sarkar et al., 2001). It is nteresting to note that the rice varieties are not tolerant to O_2^-. Also, their defense to this ROS was also not enough and thereby becomes more prone to oxidative damages. Moreover, the variation in SOD activity as compared to control is almost alike in both shoots and roots. Thus, in cells, a number of reactions involving O_2^- are featured for elevation of oxidative deterioration finally leading to tissue death (Sarkar et al., 2001). Submergence being an important facet of oxidative stress is of no exception for ROS. In a number of cases, the activity of SOD are found to be minimal or intermediate to experience the shock of oxidative burst in tissues. A number of tolerant species may differ from susceptible members to vary in SOD activity during the exposure of submergence after entering the post anoxic condition on receding of water level. It is being well acclimatized for tolerant varieties to other courses of ROS activities in succession, particularly, which causes many serious oxidative damages at post submergence period (Steffens et al., 2013). In the present experiment, the downstream oxidation pathways of SOD were also reflected in APX and CAT activities. In connection with the SOD, both APX and CAT also played an important role in the lysis of H_2O_2. However, for CAT, activities are also connected with the generation of H_2O_2 both under stress as well as normal metabolic courses (such as photorespiration) (Dhindsa et al., 1981). The involvement of APX is important for its activity mostly offered in chloroplast, mitochondria and other subcellular fractions than cytosol (Caverzan et al., 2012). In the present experiment, roots were found more affected with APX activity than shoots. On the other hand, activity of CAT also recorded down regulatory trend irrespective of rice varieties in both shoots and roots. In many instances, the fall in CAT activity have been recorded and justified mostly in excess load of H_2O_2 as overburden for the affected tissues. The chlorophyll content and its variation is the initial marking for the plants' responses to any kind of stress. In case of submergence, the attenuation of chlorophyll both with its content and biophysical activities has also been referred in rice in many instances. The loss of chlorophyll could be granted both as inhibition of its biosynthesis as well as rapid degradation of the biomolecules. In case of later, the possibility is more arisen due to over-accumulation of ROS in the submerged tissues and its associated peroxidation phenomena of metabolites (Sagi and Fluhr, 2006). Chlorophyll degradation with ROS through oxidative reactions has also been documented earlier in rice (Jamil et al., 2012). In fact, oxidative stress in plants in any forms is experienced in intermediate or later period of growth for photosynthetic activities. In earlier seedling condition, plants have an inbuilt strategy to escape the ROS exposure from the chloroplast by existing antioxidative defense. In extreme cases, the proneness of the ROS may trail the chloroplast bearing antioxidative enzyme to act properly. With this, chlorophyll attenuation becomes a stable and consistent as well as reliable trait adhered to submergence sensitivity in rice and also strengthened with the biophysical properties of chlorophyll like fluorescence, none

photochemical quenching and others (Panda and Sharma, 2007).

Conclusion

From the present experiment, submergence is clearly ascertained as a possible oxidative stress for rice plants. The varietal differentiation in relation to submergence tolerance is modulated by a number of ways in many plants including rice also. In most of the cases, the inbuilt strategies of plants being induced under submergence become the selective parameters. In the present experiment also, the antioxidative enzymes like APX, CAT, and SOD have featured their respective mode to be influenced under submergence. The activities of the enzymes were also concomitant with the types of ROS (H_2O_2 and O_2^-) accumulated in the tissues. This possibly suggests those attributes so revealed from the sensitivity of submergence as stressors in rice plants. However, more insights are required to establish biomarkers for rice plants responding to submergence in breeding programs.

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