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High Light Acclimated Developing Primary Leaves of Wheat Seedlings Generate Signal for Stress Adaptation through Super Oxide Dismutase

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Abstract

Wheat seedlings grown in petri dishes were acclimated to high light during their development and subsequently were subjected to osmotic stress by application of polyethylene glycol (PEG). The photochemical efficiency of photosystem II (PS II) of the acclimated seedlings was found to be higher than that of non-acclimated ones under osmotic stress during senescence. The damage of the photosynthetic apparatus seems to be mediated by reactive oxygen species. The higher level of lutein (Lut) and superoxide dismutase (SOD) activity in acclimated seedlings suggest their involvement in the protective mechanism. The senescing leaves, although in a deteriorating phase, exhibit tolerance to osmotic stress due to the adaptation potential acquired earlier during high light (HL) acclimation.

1. Introduction

The study of damage, repair and adaptation of the photosynthetic apparatus of green plants under stress has been a major field of research (Demmig-Adams and Adams, 1994; Biswal, 1997, 2005; Maxwell et al., 1999; Zheng, et al., 2009; Ahmad et al., 2016; Wang et al., 2018). Quite a number of studies have been devoted to understand the effects of two stresses in combination like that of drought and high light (Cornic, 1994; Zhou et al., 2007), water and heat (Havaux, 1992; Silva et al., 2010), water and nutrition deficiency (Verhoeven et al. 1997), ultraviolet radiation and temperature (Nayak et al., 2003). But in nature plants, besides experiencing many stress factors in combination, experience a host of environmental stresses in a sequence. Although inadequate results of some of the studies make obvious that plants exhibit cross-adaptation by generating resistance against a stress in response to another stress experienced earlier. Demonstration of an increased thermostability of photosystem II (PS II) of wheat leaves in osmotic stressed plants as compared to their non-stressed counterparts (Lu and Zhang, 1999), or lesser susceptible to photoinhibition of cold-acclimated plants (Schöner and Krause, 1990) are but some examples. Further, most of these studies mentioned have been conducted in developing or mature leaves with scant regard to the senescence phase, an integral part of leaf development. Previously we have demonstrated that the adaptive potential of leaves induced by high light stress during developmental phase is retained till senescence to tolerate the damaging effect of osmotic stress (Behera et al., 2003).

The present study is in continuation of the previous work with a view to understanding the underlying mechanism of adaptation acquired during developmental phase and cross tolerance exhibited during senescence. Therefore, we have acclimated wheat seedlings to high light during their developmental phase and have examined the photosynthetic response as well as enzymatic and non-enzymatic modes of defense under PEG induced osmotic stress during senescence.

2. Materials and Methods

2.1. Plant materials and growth conditions

Wheat seeds (*Triticum aestivum* L. var. Sagarika) obtained from National Seeds Corporation, Sambalpur were surface sterilized with 30% ethanol and then kept in running water for six hours. These seeds were then rolled in wet blotting paper and kept in darkness for 24 h for germination. The well germinated seeds were grown in a culture room on sterilized cotton in petri dishes at 25 ± 2 ^{0}C. The seedlings were planted in three different sets of petri plates under three different conditions as described by Behera *et al.* (2003). The first set grown in continuous white fluorescent light of intensity 12 W m^{-2} until 15 day (d) (control) while the second set, grown under the same light intensity and transferred to 7% polyethylene glycol (PEG-4000) solution to impose osmotic stress from 6 to 15d

(non-acclimated). The third set grown in moderate light but exposed to high light of intensity 60 W m^{-2} for 4 h daily from 3d to 6d and subsequently subjected to osmotic stress from 6 to 15d (acclimated). The photosynthetic response of primary leaves to these stresses was examined on 15d. The schematic diagram of light and water stress treatment is as given in Figure 1.

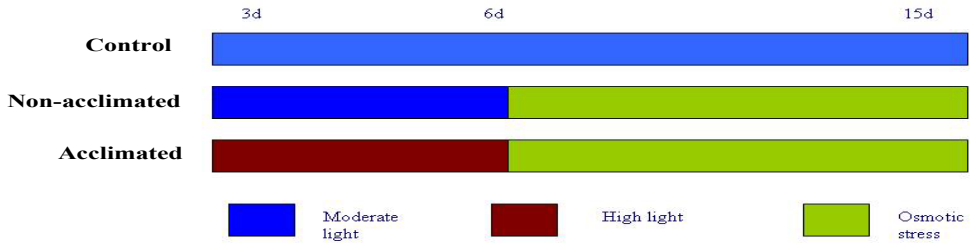

Fig. 1: Schematic diagram of light and water stress treatment

2.2. Measurement of relative water content of leaves

For measurement of relative water content (RWC), 100 leaves were harvested, weighed for fresh weight and immediately floated on distilled water at 25 ^{0}C. After 4 h, the leaves were weighed to get the (water) saturated weight and then dried in the oven at 80 ^{0}C for 48 h for the dry weight. The weight of the leaves was taken in a monopan balance (model AE 163, Mettler Instruments, Switzerland). The RWC was calculated using the formula of Turner (1981).

$$RWC\,(\%) = \frac{Fresh\ weight - Dry\ weight}{Saturated\ weight - Dry\ weight} \times 100$$

2.3. Pigment analysis by high performance liquid chromatography (HPLC)

Leaf samples were ground in 100% HPLC grade acetone under liquid nitrogen and centrifuged at 9000 g at 4°C for 10 minutes. The supernatant was stored and pellets extracted with 100% acetone until they were colorless. All the extraction steps were performed at low temp (0-4°C) and under a weak light intensity to avoid degradation of photosynthetic pigments. The pigment separations were carried out by reversed phase liquid chromatography (RPLC) as described by Darko et al. (2000) using HPLC consisting of Waters model (Water Corp, Milford, USA), a binary pump (Waters 515) equipped with a online degasser, Spherisor ODS2 column RP - 18 (250 x 4.6 mm, i.d., 5 µm pore size), guard column of same chemistry, a Waters PCM and Rheodyne injector with a 25 µl loop was used. Detection was done using 2996 PDA detector. The chromatograms were recorded using Waters Millennium software. HPLC finger print profile was recorded for the major photosynthetic pigments. Elution was carried out at a flow rate of 0.8 ml min^{-1}. The elution solvent A contained acetonitrile-methanol, 85:15 and solvent B contained 100% ethyl acetate. Prior to sample injection, the column was equilibrated for 10 min with solvent A. A 0.01 cm^3 sample was then injected, and solvent A was pumped for another 6 min. Next, solvent B was pumped for 13 min at 20% followed by 6 min at 50% [to elute β-Car, β-Carotene (β-Car)]. Within 1 min, solvent B was brought to 0%. The eluted pigments were detected and recorded at different wave lengths, but for the best resolution of xanthophylls the chromatograms were recorded at 437 nm by normalizing the higher value of chlorophyll a (Chl a) and chlorophyll b (Chl b). The pigments were identified by comparing their retention times with those of standards (Chl a, Chl b, Lut, β-Car, zeaxanthin (Z) and violaxanthin (V), obtained from Sigma Chemicals.

2.4. Measurement of Thiobarbituric acid reactive substances (TBARS) content

The level of lipid peroxidation was estimated following the method of Bartoli *et al.* (1995). Five hundred milligram of leaf tissue was homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) and 0.1% (w/v) butylated hydroxytoluene (BHT). The reaction mixture containing 0.7 ml of tissue homogenate, 0.2 ml of 8.1% (w/v) sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% (w/v) aqueous solution of TBA was heated at 95°C in water bath for 1h. After cooling in cold water, 4 ml of n-butanol was added, vortexed and centrifuged at 4,000 x g for 10 min. Absorbance of the upper organic layer was measured at 532 nm in UV/visible Spectrophotometer-Ultrospec 2000 (Pharmacia Biotech, Cambridge, England). tetramethoxypropane (TMP) was used as an external standard (Ohkawa *et al.,* 1979) and the level of lipid peroxides was expressed as nmol TBARS g^{-1} DW.

2.5. Isolation of chloroplasts

Chloroplasts were isolated by the method described by Choudhury and Biswal (1980). About 25 leaves were homogenized in a pre-chilled mortar and pestle with ice-cold isolation medium containing 0.4 M sucrose, 0.01 M EDTA-Na and 0.1 M phosphate buffer (pH 7.8). The homogenate was squeezed through cheese cloth and the filtrate was centrifuged at 500 g for 1 min. The supernatant was again centrifuged at 1000g for 10 min and the pellet was collected in a small volume of homogenizing medium.

2.6. Measurement of O_2 evolution

PS II mediated O_2 evolution was measured with a Clark

type electrode at 21^{0}C in rate saturating red light as described by Joshi et al. (1997). The basic assay buffer contained 30 mM Na/K phosphate buffer (pH 7.2), 30 mM NaCl, and 200 mM sucrose. The electron acceptors used were 0.3 mM dichlorobenzoquinone (DCQ) or 0.4 mM ferricyanide. Chloroplasts containing 40 µg Chl were placed in 2 ml reaction mixture. Gramicidin (2.5 µM) was used as uncoupler.

2.7. Superoxide dismutase (SOD) assay

Fresh leaf tissue (0.5 g) was homogenized in 1.5 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 2% (w/v) polyvinyl pyrrolidone (PVP) using chilled mortar and pestle. The homogenate was centrifuged at 15,000 x g at 4^{o}C for 30 min. Clear supernatant (enzyme extract) was used for all enzyme assays at 25°C. SOD (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay according to Beyer and Fridovich (1987). 20 µl of enzyme extract was added to 1 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 µM NBT, 0.025% triton-X-100 in a small glass tube. Soluble protein content was determined according to Bradford (1976) method with BSA as a standard using a UV/visible Spectrophotometer- Ultrospec 2000 (Pharmacia Biotech, Cambridge, England). SOD activity was expressed as U (unit) mg^{-1} protein.

2.8. Statistical Analysis

The experiment was performed in completely randomized block design. All the experiments were carried out in four replicates. All dataset obtained from the experiment were subjected to one way analysis of variance (ANOVA). Statistical significance was determined using a Duncan's Multiple range test (DMRT) for multi-comparisons of mean (Gomez and Gomez 1984). Significance levels were compared at p<0.05. Statistical analyses were performed using SPSS software.

3. Results

3.1. Alteration in pigment content

Table 1 describes the changes in the level of different components of xanthophylls, β-Car and Lut in the primary leaves of control, non-acclimated and acclimated wheat seedlings on 15d. In acclimated seedlings on 6d, the levels of both violaxanthin (V) and β-Car was lowered by 45% and 30% respectively while the levels of Lut and zeaxanthin (Z_x) increased by 17% and 30%, respectively.

The level of these components declined on 15d. The amount of Lut and β-Car in acclimated sample were more than those in the non-acclimated sample by14% each.

3.2. Changes in relative water content (RWC) of leaves

Figure 2 shows the RWC of primary leaves on 6 and 15d of wheat seedlings grown under different irradiances. On 6d the RWC of leaves of control was higher (94.27%) compared to acclimated seedlings (87.56%). On 15d the RWC of leaves of control seedlings was the highest (79.37%) compared to acclimated (53.5%) and non-acclimated seedlings (58.05%). This indicated that the RWC of primary leaves on 6d did not show significant difference when grown under different irradiances. However, on 15d PEG treatment significantly decreased the RWC both in acclimated and non- acclimated plants as compared to its control.

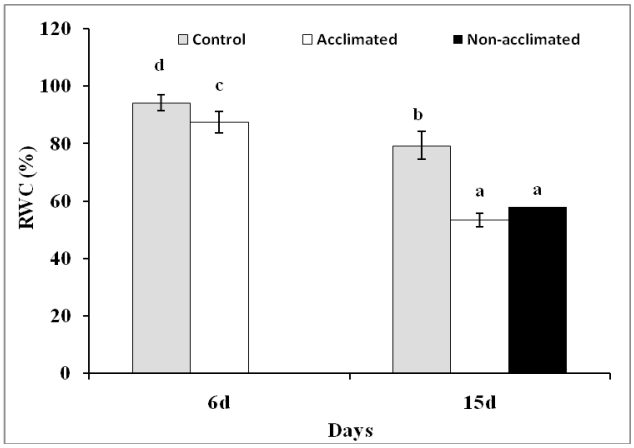

Fig. 2: Alteration in relative water content in control, acclimated and non-acclimated primary leaves of wheat seedlings. The seedlings were subjected to osmotic stress after 6th day. The data presented are means of three replicates ± SD. Significant changes are denoted by different alphabets (DMRT, p < 0.05)

Table 1: Pigment analyses by HPLC of control, acclimated and non-acclimated primary leaves of wheat seedlings on 6th day (without osmotic stress) and 15thDay (with osmotic stress). The values are in µg g^{-1} fresh weight).

Peak No.	6th Day			15th Day		
	Pigments	Control	Acclimated	Control	Acclimated	Non-acclimated
1.	V	38	21	36	15	9
2.	Lutein	214	258	196	132	116
3.	Z	19	43	14	26	32
4.	Chl b	358	436	234	222	210
5.	Chl a	1264	1472	967	832	768
6.	β- car	124	87	130	72	63

3.3. Loss of PS II mediated O_2 evolution

Figure 3 exhibits the result of PS II mediated O_2 evolution from the chloroplasts of control, acclimated and non-acclimated on 15d. The leaves of control plants show higher level of O_2 evolution than that of acclimated and non-acclimated, where the levels were significantly (p<0.05) different from each other. However, the level of O_2 evolution in leaves of acclimated plants was more by 25% as compared to that of non-acclimated.

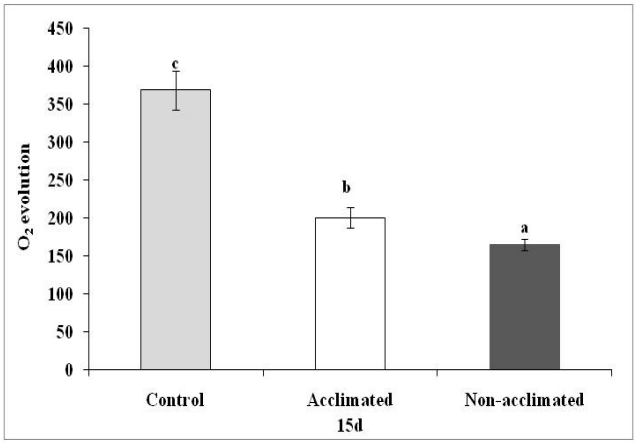

Fig. 3: Alteration in PSII mediated O_2 evolution in chloroplasts isolated from primary leaves of 15 day old wheat seedlings under osmotic stress. The data presented are means of three replicates ±SD. Significant changes are denoted by different alphabets (DMRT, $p < 0.05$).

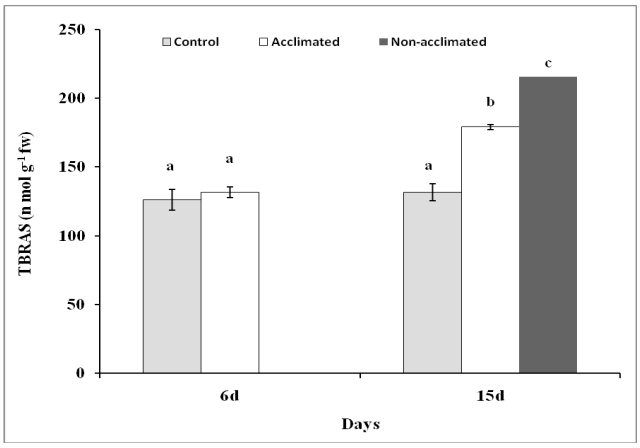

Fig. 5: Alteration in TBARS content in control (vertical faint line), acclimated (oblique faint line) and non-acclimated (crossed dark lines) primary leaves of wheat seedlings. The seedlings were subjected to osmotic stress after 6th day. The data presented are means of three replicates ±SD. Significant changes are denoted by different alphabets (DMRT, $p < 0.05$).

3.4. Changes in superoxide dismutase (SOD) activity

Figure 4 shows the activity of SOD in the primary leaves of wheat seedling on 6 and 15d with different treatments. During developing phase on 6d, the enzyme activities of SOD significantly ($p<0.05$) increased by 57% in the acclimated leaves. During senescence PEG induced water stress increased the SOD activity in acclimated seedlings by 214% while it decreased in non-acclimated seedlings by 24%.

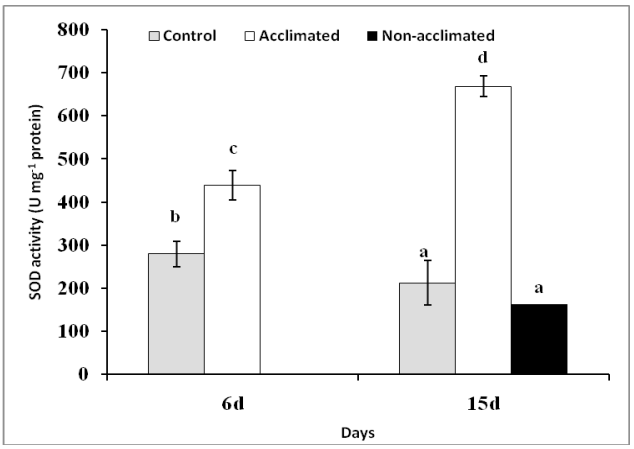

Fig. 4: Alteration in SOD activity in control (vertical faint line), acclimated (oblique faint line) and non-acclimated (crossed dark lines) primary leaves of wheat seedlings. The seedlings were subjected to osmotic stress after 6th day. The data presented are means of three replicates ± SD. Significant changes are denoted by different alphabets (DMRT, $p < 0.05$)

3.5. Alteration in TBARS content

Figure 5 depicts the level of peroxidation of thylakoid membrane lipid in terms of TBARS content in the primary leaves of wheat seedlings. On 6d the TBARS content in the acclimated seedlings was not significantly ($p < 0.05$) different from the control. However, there was a significant ($p < 0.05$) increase in the level of TBARS in both acclimated and non-acclimated as compared to the control on 15d by 31% and 62%, respectively.

4. Discussion

Different developmental phases of the seedlings have been characterized earlier on the basis of the levels of Chl and protein (Behera *et al.,* 2003). The primary leaves of wheat seedlings exhibit developmental phase up to 7d, steady phase from 7d to 11d followed by senescence phase. Therefore, the seedlings were acclimated to high light till 6d during the development phase and subjected to PEG induced osmotic stress thereafter. Application of PEG is known to create a condition of water deficit in plants (Deo and Biswal, 2001). In the present work the effectiveness of PEG was checked by measuring RWC, a reliable parameter for quantifying plant osmotic stress response (Sinclair and Ludlow, 1985). On 15d PEG treatment significantly decreased the RWC both in acclimated and non-acclimated plants as compared to that of control (Fig. 2).

In order to examine the PSII photochemistry, the rate of oxygen evolution has been measured and it is found that the acclimated seedlings retain higher level of oxygen evolution compared to non-acclimated ones during senescence (Fig. 3). This finding is in accordance with our previous data on PS II photochemistry (Behera *et al.*, 2003), demonstrating the retention of stress adaptive potential induced by high light stress during development of wheat leaves that helps to mitigate the osmotic stress effect operating in sequence during senescence.

The HPLC data (Table 1) show an increase in Lut in high light acclimated plants on 6d. Lut is known to play a fundamental role in the assembly and development of active PS II holocomplex (Humbeck *et al.,* 1989). The increased level of Lut may indicate that Lut is essential for development of light harvesting complexes (LHCs) and reaction centre (RC) of PS II as observed by earlier workers (Humbeck and Bishop, 1986; Bishop *et al.,* 1989; Senger *et al.,* 1993; Esteban *et al.,* 2008). Moreover, Lut quenches triplet Chl by triplet-triplet energy transfer mechanism (Valentin *et al.,* 2009). Degl'Innocenti *et al.* (2008) reported that in severely dehydrated leaves of *Ramonda serbica*, ROS formation is apparently better prevented by mechanisms that quench Chl triplet formation via Lut. Hence, relatively high level of Lut on 15d in acclimated plants supports the proposition that leaves exposed to high irradiance subsequently provide a relatively stable system.

On 6d, the leaves exposed to high light also exhibit a significant increase in the level of Z as compared to that in leaves exposed to moderate light intensity. Such a higher accumulation of Z under high irradiance has also been observed earlier (Thayer and Bjorkman, 1990; Demmig-Adams *et al.,* 1996). Z is viewed as the primary carotenoid responsible for prevention of photoinhibition through NPQ with antheraxanthin (A) functioning as transition state molecule in the xanthophylls cycle (Demmig-Adams *et al.,* 1989, 2006; Jung and Niyogi, 2009). Hence, the significant increase in Z with a decrease in violaxanthin (V) in the leaves (Table 1) of the acclimated wheat seedlings may suggest that Z formed by reversible de-epoxidation of V serves to dissipate excessive and potentially harmful excitation energy and plays a crucial role in photoprotection under high irradiance condition (Haripal *et al.,* 2006; Wang *et al.,* 2008a; Six *et al.,* 2009). But the level of Z subsequently declines more in the acclimated seedlings. Interestingly, on 15d (Table 1), the level of Z in acclimated is less than that in non-acclimated seedlings. Since the plant is already acclimated with relatively stable system, the level of Z which counters the stress induced damage is expected to remain low compared to the non-acclimated seedlings.

Environmental challenges are known to promote oxidative stress in plants (Halliwel and Gutteridge, 1989). An increase in the activity of antioxidant enzymes under osmotic stress is an indication of increased production of reactive oxygen species (ROS) and up-regulation of the protective mechanism to reduce oxidative damage in plants. It has been suggested that elevated level of antioxidant enzymes could be associated with stress tolerance of plants (Hernández *et al*., 1995). SODs constitute the first line of cellular defense against oxidative stress as its activity directly modulates the amount of $O_2^{\cdot-}$. It catalyses the chemical conversion of superoxide radicals into H_2O_2, which is in turn metabolized by the action of peroxidase (Scandalios, 1993; Khosravinejad *et al.,* 2008). On 6d during the developing phase the significant increase in SOD activity of high light acclimated seedlings over control one suggests that the ROS like $O_2^{\cdot-}$ and H_2O_2 produced due to high irradiance are scavenged immediately (Wang *et al.,* 2008b), which is also evident from the unchanged level of TBARS (Fig. 5). Imposition of stress led to a significant increase in SOD activity in acclimated plants during senescence. The decreased SOD activity under osmotic stress might have rendered the non-acclimated plants less efficient in scavenging $O_2^{\cdot-}$. Mayak *et al.* (1983) observed a reduction in the SOD activity and at the same time an increase in the free radical as well as lipid peroxidation levels during the senescence of carnation petals. This observation led them to conclude that $O_2^{\cdot-}$ induces the degradation of phospholipids and the polyunsaturated fatty acids released by this breakdown are then peroxidized; which may be due to the activity of lipoxygenase enzyme (Siedow, 1991). In the present work, the significantly higher TBARS content (Fig. 5) on 15d may be correlated with the sharp decline in SOD activity in non-acclimated plants (Fig. 4).

5. Conclusion

In course of the investigation we have observed two distinct modes of adaptation mechanism employed by plant to overcome the stress induced hazards. Firstly, an immediate push in the level of Z as observed here and NPQ (reported earlier, Behera *et al.,* 2003), usually linked to radiation decay (Govindjee, 2002; Havaux and Niyogi, 1999) was observed in high light acclimated plants on 6d and the difference in the levels of these parameters died down subsequently. It is a short term measure employed by leaf to escape the high light stress (Schumann *et al.,* 2017). On the other hand, the activity of SOD and the level of Lut were higher in acclimated than those in non-acclimated on 6d and continued to remain higher till 15d. The higher levels of SOD and Lut induced in response to HL may be a long-term adaptive measure providing cross tolerance to osmotic stress during senescence. Because the elimination of $O_2^{\cdot-}$ is highly and immediately required during a stress, a high level of SOD is expected at all times to provide adequate protection. It is surmised that high light acclimation of developing leaves putatively generates a signal that persists to provide tolerance to osmotic stress during senescence. The senescing leaves also tolerates to a stress which, however, depends on the adaptation potential already acquired during earlier stages of development.

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