Possible function of ABA in protection against photodamage by stimulating xanthophyll cycle in sorghum seedlings

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The effect of high light at 30°C on chlorophyll fluorescence, xanthophyll cycle and abscisic acid (ABA) was determined to study the relationship of xanthophyll cycle and ABA in protection against photodamage. Sorghum seedlings of 8 days were grown on Hoagland medium supplemented with ABA (10^{-5} M) and photoinhibited at 2200 and 3600 µmol m^{-2} s^{-1} PAR light intensities, and measurement of chlorophyll fluorescence (F_v/F_m ratio, *qN*, *f*-PS II and *f*-exc), xanthophyll cycle content and ABA content were made. The results obtained show that seedlings grown on ABA-supplemented medium had better energy dissipation (greater *qN*) and much greater level of de-epoxidation (high level of *Z*) than control seedlings. The level of endogenous ABA in seedlings grown on ABA-supplemented medium or otherwise remained the same. The results indicate a possible role of ABA in energy dissipation, probably by stimulating the xanthophyll cycle.

EXPOSURE of leaves to light levels where the excitation energy exceeds the capacity for orderly dissipation by the photosynthetic system can lead to photoinhibition. Acclimation of plants to different light environment has profound influence on the structure and function of photosynthetic apparatus. Plants grown under full sunlight have much greater photosynthetic capacity, which saturates at higher photon flux density (PFD), than plants grown in shade conditions. Shade leaves are considered to be more prone to photoinhibition than sun leaves. Photoinhibition is characterized by a sustained decrease in the efficiency of photon utilization by photosystem II (PS II) photochemistry, due to absorbing more excitation energy than could be dissipated by photochemical processes[1–3].

Under normal conditions, the light energy absorbed by the pigments in chlorophyll protein complexes of PS II and photosystem I (PS I) is utilized in a controlled manner leading to generation of NADPH and ATP. However, under photoinhibitory conditions, this process is not able to dissipate all the available energy resulting from the decrease in efficiency of the photosynthetic system in utilizing the energy. There are several built-in mechanisms of energy dissipation such as carotenoids, photorespiration, Mehler reaction, reducing substances, SOD, etc. which play a role in providing some protection against oxidative damage to the PS II reaction centre against photoinhibition[4]. Xanthophylls (violaxanthin, antheraxanthin and zeaxanthin) present in the light-harvesting complex II are supposed to play an important role in the photoprotection of plants. A well-known function of zeaxanthin is to promote rapid thermal energy dissipation, as revealed by energy-dependent fluorescence quenching (*qE*, which reflects the mechanism associated with both the PS II reaction centre and the light-harvesting antennae of the PS II) which allows rapid down-regulation of PS II energy capture under excess irradiance[5]. Studies using time-resolved chlorophyll *a* fluorescence also suggest that non-photochemical energy dissipation is related to the binding of de-epoxidized xanthophyll (A + Z) to the inner PS II antenna[6].

Recent reports[7–10] suggest that synthesis of ABA from the oxidative cleavage of zeaxanthin is of great interest as ABA is a plant hormone involved in many physiological and developmental processes such as transpiration, germination, dormancy and also with the adaptation of plants to environmental stresses (e.g. drought, chilling and pathogen attack).

The proposed biosynthetic pathway of ABA in higher plants[1] shows close relationship between xanthophyll-cycle components and ABA. Here we have studied the effect of ABA treatment on the xanthophyll cycle and thus, on the process of energy dissipation and photoprotection in sorghum seedlings.

Seeds of *Sorghum bicolor* L. cv MSH-51 were obtained from Mahyco Seeds Corporation, Jalna and were stored in a desiccator at room temperature.

The seeds were soaked in tap water for one hour and were surface sterilized with $HgCl_2$ (0.1%). They were germinated in glass petri plates (10 cm diameter) containing moist cotton pad and blotting paper, soaked in half strength Hoagland solution. The control plants were irrigated with the solution daily. ABA treatment (10^{-5} M) in 0.1 mM KOH was given to 4-day-old seedlings of normal growth. Seedlings were allowed to grow up to 8 days of age in a growth chamber illuminated with incandescent and fluorescence light (200 µmol m^{-2} s^{-1}) measured using radiometer model LI-189, Licor, USA. The day/night temperature was maintained at 30 ± 2°C and relative humidity at 50%.

Experiments were carried out with intact leaves of 8-day-old seedlings. Leaves were exposed to high irradiance of 3600 and 2200 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) using Kl-1500 electronic cold light source. Temperature during the treatment was maintained at 30°C.

Chlorophyll fluorescence was measured using a pulse amplitude modulation fluorometer (PAM 101 & 102 from Walz, Germany), according to Schrieber *et al.*[11]. Prior to the measurements, leaves were dark-adapted for

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10 min. The 1 cm^{-2} portion of the leaves was exposed to modulated light (4 μmol m^{-2} s^{-1}) to measure initial fluorescence (F_o) followed by the exposure of the leaves to a saturating pulse of white light (4000 μmol m^{-2} s^{-1}), to measure maximum fluorescence (F_m). After the measurement of F_m, the leaf was exposed to an actinic light of 800 μmol m^{-2} s^{-1} to allow it to reach a steady state of fluorescence (F_s). Another burst of saturating light was used to measure secondary maximum chlorophyll fluorescence (F_m'). F_o' was measured by exposing the leaves to infra-red radiation. The photochemical and non photochemical quenching coefficients were calculated according to Schreiber *et al.*[11] and fPS II and $f$exc were calculated according to Habash *et al.*[12].

The exposed portion of the treated leaves was cut and weighed accurately. The leaf tissue was thoroughly extracted in 0.5 ml acetone (containing 0.1% butylhydroxytoludene, BHT) in 1.5 ml eppendorf tube. The extract was centrifuged at 5000 *g* for 5 min at 4°C. The supernatant was filtered through 30 μm syringe filter and 10 μl of filtrate was used for HPLC analysis for carotenoids as well as for ABA.

Identification and separation of carotenoids was carried out according to Sharma and Hall[13] using HPLC (Spectra Physics, UK), with C-18 reverse-phase column (ET-250/4 nucleosil 100-5 C-18 ODS) Spectra Physics SP-ternery HPLC pump with SP-4270 integrator and Spectra 100 variable wave length detector. Ten μl of the filtered tissue extract was injected onto the HPLC column at 25°C. The separation was carried out using linear gradient of ethyl acetate and acetonitrile : water (9 : 1) over 25 min with 1.2 ml min^{-1} flow rate, at 445 nm. Identification of carotenoid was carried out on the basis of retention time and spectral characteristics of the standards.

Part of the leaf extract (see above) was used for the ABA analysis. The separation and quantitative estimation was carried out according to Rodrigues *et al.*[14] using HPLC (for details of the machine and specification, see above). The separation was carried out using a linear gradient of 0–100% methanol, containing 1% acetic acid throughout and quantified with peak integration after calibration with external standard ABA.

The effect of photoinhibition treatment (2200 and 3600 μmol m^{-2} s^{-1} PAR) at 30°C on the F_v/F_m ratio in sorghum seedlings grown with and without exogenously supplemented ABA is presented in Figure 1. Six hours of high light treatment (2200 μmol m^{-2} s^{-1} PAR) of sorghum seedlings grown without ABA resulted in a decrease of 69% in F_v/F_m ratio compared to only 27% decrease observed in ABA-grown seedlings treated at the same light level and the duration (Figure 1 *a*). Similarly, photoinhibition treatment at 3600 μmol m^{-2} s^{-1} PAR for six hours resulted in a decrease of 75% in F_v/F_m ratio for (–) ABA-grown seedlings compared to only 49% decrease in the seedlings grown with ABA and treated to the same high light level and the duration of the treatment (Figure 1 *a*).

Recovery of the photosynthesis (F_v/F_m ratio) following the photoinhibition treatment at 3600 μmol m^{-2} s^{-1} PAR for 6 h was also greater (76% recovery) in ABA-grown seedlings than observed in seedlings grown without ABA (63% recovery; Figure 1 *b*).

Relative quantum yield of PS II electron transport (fPSII; Figure 2 *a* and *b*) and the efficiency of excitation capture by open PSII centre, ($f$exc; Figure 2 *c* and *d*) calculated according to Habash *et al.*[12] from fluorescence measurements, was also decreased as a result of photoinhibition. The fPSII was decreased to 71% due to the photoinhibition of (–) ABA-grown seedlings when photoinhibited at 3600 μmol m^{-2} s^{-1} PAR for 6 h compared to a decrease of only 20% as a result of the same photoinhibition treatment of (+) ABA-grown seedlings (Figure 2 *a* and *b*). Similar results were obtained for $f$exc. The $f$exc gradually decreased to 80% in (–) ABA-grown seedlings over the six hour exposure to high light compared to control. Though $f$exc changed

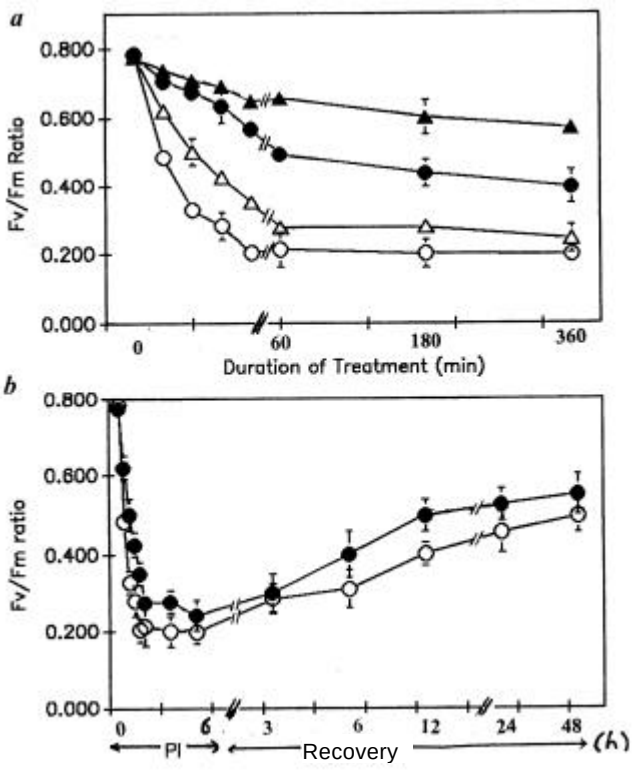

Figure 1. ***a***, Effect of photoinhibition at 2200 μmol m^{-2} s^{-1} on F_v/F_m ratio of sorghum seedlings grown with ABA (—▲—) and without ABA (—●—) and at 3600 μmol m^{-2} s^{-1} of sorghum seedlings grown with ABA (—Δ—) and without ABA (—○—). The points represent 5–6 separate sets of experiments. Vertical bars show standard deviation. ***b***, Effect of photoinhibition at 3600 μmol m^{-2} s^{-1} on F_v/F_m ratio in plants grown without ABA (—○—) and with ABA (—●—) for 6 h and thereafter recovery up to 48 h under growth condition. The points represent 5–6 separate sets of experiments. Vertical bars show standard deviation.

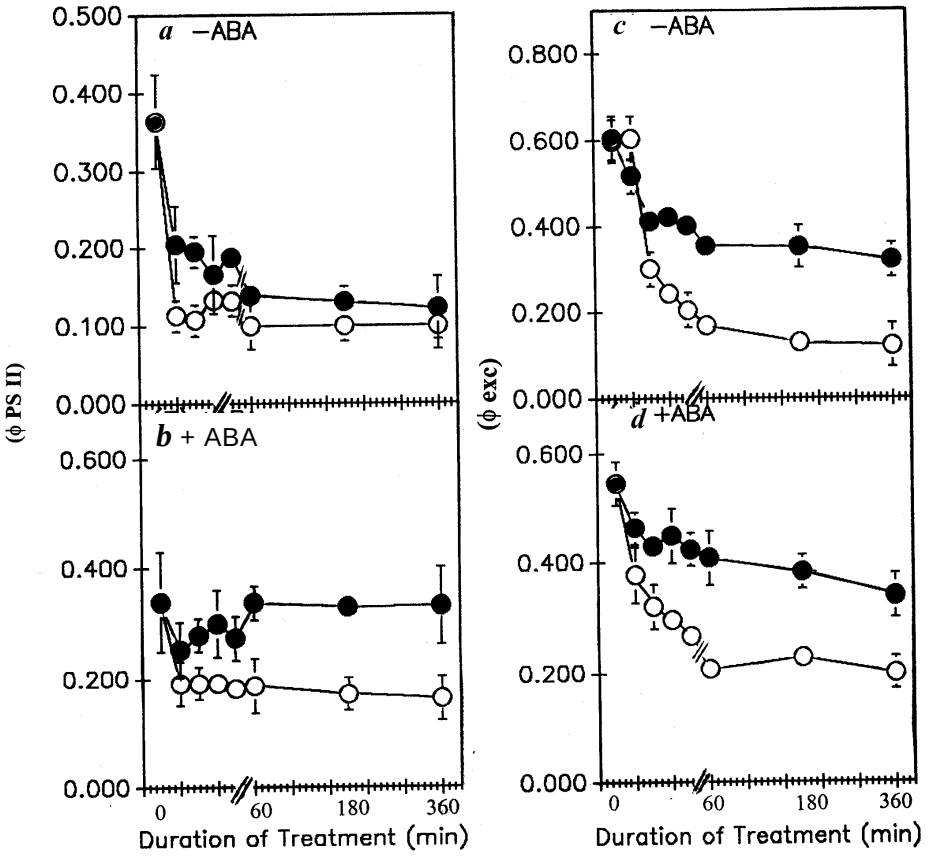

Figure 2. Effect of photoinhibition at 2200 µmol m^{-2} s^{-1} (—●—) and 3600 µmol m^{-2} s^{-1} (—○—) at 30°C on *f*-PS II (***a*** and ***b***) and *f*-exc (***c*** and ***d***) in sorghum seedlings grown without ABA (***a*** and ***c***) and with ABA (***b*** and ***d***). The points represent 5–6 separate sets of experiments. Vertical bars show standard deviation.

Table 1. Carotenoid composition of sorghum seedlings grown with and without ABA and photoinhibited at 2200 and 3600 µmol m^{-2} s^{-1} at 30°C for 6 h. Results are presented as µg pigments g FW^{-1} using *b*-carotene as an external standard. Data presented here are average of four repetitions

	Without ABA			With ABA		
Carotenoid	Control	2200 PAR	3600 PAR	Control	2200 PAR	3600 PAR
Neoxanthin	4.80	5.00	5.30	4.40	5.30	5.80
Violaxanthin	19.50	14.50	13.00	23.50	12.90	11.00
Antheraxanthin	0.30	0.70	0.50	0.80	0.70	0.70
Lutein	45.20	46.80	44.80	46.50	43.20	43.20
Zeaxanthin	0.80	6.10	7.60	0.00	14.10	17.70
Chlorophyll *b*	43.20	43.50	42.10	41.50	40.20	38.90
Chlorophyll *a*	60.50	60.30	62.20	65.70	63.50	63.80
V + A + Z Pool	20.60	28.50	28.50	37.50	27.70	29.40
% Epoxidation	95.00	70.00	63.00	24.30	29.10	23.50

in a similar fashion in ABA-grown seedlings, the extent of decrease in *f*exc was only 63% (Figure 2 *c* and *d*).

Table 1 shows a comparative HPLC analysis of the quantitative changes in the carotenoid composition and content in (–) ABA and (+) ABA grown sorghum seedlings. Sorghum seedlings grown with ABA showed a greater level of de-epoxidation (conversion of violaxanthin to zeaxanthin). A six-hour photoinhibition treatment of (+) ABA-grown seedlings at 2200 and 3600 µmol m^{-2} s^{-1} PAR resulted in 71% and 76% de-epoxidation level, respectively compared to 30% and 37% de-epoxidation in seedlings grown without ABA and photoinhibited at 2200 and 3600 µmol m^{-2} s^{-1} PAR. The V + A + Z pool was higher in ABA-grown control plants and it further increased as a result of high light treatment. However, the V + A + Z pool in control of (–) ABA-grown seedlings was 15% less than that seen in control of ABA-grown seedlings. The photoinhibition of (–) ABA-grown seedlings did not result in an increase in the V + A + Z pool, as observed in the (+)

ABA-grown seedlings. Chlorophyll to carotenoids ratio was slightly greater in (–) ABA-grown seedlings compared to the seedlings grown with ABA and did not change significantly as a result of high light treatment in either case, (–) ABA and (+) ABA-grown seedlings.

Table 2 shows the effect of photoinhibition on non-photochemical quenching (*qN*) in (–)ABA and (+)ABA grown plants and its relationship with the xanthophyll cycle. The results show that photoinhibition of plants grown with exogenously supplemented ABA exhibited a greater level of *qN* than observed in plants grown without ABA. Also, the greater increase in *qN* was matched with the greater level of de-epoxidation of violaxanthin to zeaxanthin, showing a relationship between the two.

Table 3 shows the level of endogenous ABA contents in (–)ABA and (+)ABA grown plants. The endogenous ABA contents of seedlings grown without exogenously supplemented ABA showed a slightly lower level of endogenous ABA in comparison to that of seedlings grown with exogenously supplemented ABA medium. However, photoinhibition treatment of both types of seedlings (grown with exogenously supplemented ABA or without exogenously supplemented ABA) showed more or less the same level of endogenous ABA.

Chlorophyll fluorescence gives indication about the health of the photosynthetic system in leaves. In this study, we observed that photoinhibition of sorghum seedlings at 2200 and 3600 $\mu mol\ m^{-2}\ s^{-1}$ PAR at 30°C resulted in a decrease in F_v/F_m ratio, which suggests that the efficiency of PS II electron transfer declined as a result of photoinhibition. This decline in the efficiency of the PS II was much greater in seedlings grown without ABA than in seedlings grown with exogenously supplied ABA, suggesting relatively less photoinhibitory damage to PS II in ABA-fed plants. The F_v/F_m level recovered, following the photoinhibition treatment, to a much greater level in plants grown with ABA than in those which were grown without ABA (Figure 1 *b*), indicating that ABA provided some protection to PS II damage under high light condition. The results shown in Table 2 further suggest that the protection to PS II provided by the application of exogenous ABA is due to better energy dissipation, as *qN* (which indicates dissipation of excessive energy in the pigment bed, thereby preventing over-energization of reaction centre under high light conditions) was much higher in plants grown with ABA than in plants grown without ABA. The increase in *qN* was also matched by greater level of de-epoxidation of violaxanthin to zeaxanthin, showing the relationship between *qN* and the xanthophyll cycle. These results were further substantiated with measurements of *f*PS II (relative quantum yield of PS II indicating PS II activity) as the photoinhibition of sorghum seedlings grown without ABA resulted in greater decrease in the *f*PS II than seen in seedlings grown with exogenously supplied ABA (Figure 2 *a* and *b*). Similarly *f*exc (efficiency of excitation capture by open PSII) was also decreased to a greater extent in seedlings grown without exogenously supplied ABA than in seedlings grown with ABA, indicating higher efficiency of energy transduction in open PS II reaction centres. These data clearly indicate certain protective roles of ABA on the PS II (as room temperature fluorescence is largely generated from PS II only) under high light conditions. Thus, the increased resistance to photoinhibition in ABA-grown seedlings could be ascribed to an increased capacity of the photosynthetic machinery to maintain an increased portion of the PS II reaction centre in an open state by efficient dissipation of absorbed excitation (seen as higher *qN* and *f*PS II).

Ivanov *et al.*[15] have also shown ABA-induced protection against photoinhibition of PS II in barley seedlings. They observed that exogenous application of ABA resulted in partial protection of PS II under high light condition at low temperature. They also observed 122% increase in the amount of xanthophyll in seedlings subjected to ABA. The results presented here are in confirmation with the work published by Ivanov *et al.*[15].

The radiation-less dissipation of excess excitation energy in the chlorophyll pigment bed has been proposed to be one of the major protective mechanisms against photoinhibitory damage of PS II which is supposed to be regulated by the xanthophyll cycle[16–18]. The ABA-grown seedlings upon photoinhibition showed much greater level of de-epoxidation; thus better energy dissipation in ABA-grown plants. The increase in the V + A + Z pool was largely on account of greater amount of V present in ABA-grown seedlings than in seedlings grown without ABA (Table 1).

Schwartz *et al.*[7] and others[9,19–21] have reported that violaxanthin also acts as precursor to the ABA biosynthetic pathway. Thus higher V + A + Z pool in ABA-grown plants could be explained, as exogenously supplied ABA would reduce the requirement of violaxanthin for the biosynthesis of ABA resulting in an increase of the xanthophyll pool size in plants grown with ABA-supplemented medium, largely on account of less conversion of violaxanthin to ABA in ABA-grown seedlings compared to that seen in seedlings grown without ABA. Another interesting fact is that ABA is also proposed to induce increase in LHC II level[22], a site where most (80%) of the V, A and Z is localized; this may also result in an increase in the level of carotenoids (V + A + Z) in ABA-grown seedlings as observed in this study.

Our results also show that the endogenous level of ABA in both type of seedlings after photoinhibition remains more or less constant (Table 3). As discussed earlier, a common pool of violaxanthin acts as precursor to ABA as well as zeaxanthin formation; thus violaxanthin would have to convert either to ABA or to zeaxanthin or both depending on environmental factors. If the violaxanthin pool is also used for ABA synthesis, then it

Table 2. Non-photochemical quenching and xanthophyll cycle (violaxanthin and zeaxanthin) content in sorghum seedlings grown without and with ABA and photoinhibited at 2200 and 3600 μmol m^{-2} s^{-1} PAR at 30°C for 6 h. Xanthophyll contents are given as μg pigments FW^{-1} using *b*-carotene as external standard

	Without ABA			With ABA		
	Control (0 h)	2200 PAR	3600 PAR	Control (0 h)	2200 PAR	3600 PAR
Non-photochemical quenching (qN)						
	0.366	0.443	0.509	0.564	0.648	0.654
Xanthophylls						
Violaxanthin	19.33	17.56	15.68	24.28	21.1	20.8
Zeaxanthin	0.0	1.9	4.6	0.0	4.9	6.32

Table 3. Effect of photoinhibition on ABA content measured using HPLC in 8-day-old sorghum seedlings grown with or without exogenous ABA (10^{-5} M) supplemented Hoagland's solution. Values of ABA are presented as μg mg FW^{-1} calculated using ABA as external standard. Each reading represents average of two repetitions

Duration of treatment (h)	Without ABA		With ABA	
	2200 μmol m^{-2} s^{-1}	3600 μmol m^{-2} s^{-1}	2200 μmol m^{-2} s^{-1}	3600 μmol m^{-2} s^{-1}
0	0.104 ± 0.0	0.104 ± 0.02	0.120 ± 0.03	0.120 ± 0.03
1	0.202 ± 0.05	0.219 ± 0.05	0.303 ± 0.04	0.217 ± 0.05
3	0.289 ± 0.06	0.283 ± 0.05	0.265 ± 0.05	0.263 ± 0.04
6	0.278 ± 0.07	0.210 ± 0.04	0.256 ± 0.05	0.216 ± 0.04

may result in limitation of zeaxanthin formation (as seems to be happening in seedlings grown without ABA) and thereby less efficient energy dissipation under excess light conditions (low *qN*), which probably is the reason of relatively greater photodamage to photosynthesis observed in seedlings grown without ABA in this study. When plants are grown with exogenously supplied ABA, the violaxanthin pool need not be diverted towards the ABA synthesis as it was made available exogenously. Therefore, during the high light treatment most of the violaxanthin could be available for conversion to zeaxanthin (observed in this study), thus resulting in higher *qN* (efficient energy dissipation) and provided better protection to PS II against photoinhibition in ABA-grown seedlings.

To summarize, the experimental data presented here clearly indicate that the seedlings grown with exogenously ABA-supplemented medium showed greater resistance to photoinhibitory damage. Although the exact mechanism of this ABA protective action has to be clarified more precisely, it is evident from this study that it could be related to increased activity of xanthophyll cycle (greater de-epoxidation of violaxanthin to zeaxanthin) resulting in better dissipation of absorbed energy (higher *qN* and *f*-PS II), thus providing greater protection against photodamage.

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