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Photoprotective effect of kinetin on pigment content and photochemical activities of wheat chloroplasts aging *in vitro*

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Abstract

The effects of kinetin (Kn) on pigment content and electron transport activities (ETA) in wheat leaves *in vivo* and chloroplasts *in vitro* aging in light was investigated. Excised wheat leaves were infiltrated with Kn for 3 h under irradiation. The treatment increased zeaxanthin (Zx) content by 40 % and also increased chlorophyll (Chl *a*, Chl *b*) and major carotenoid (Car) contents in the leaves (per fresh mass unit). Chloroplasts isolated from Kn treated leaves, when incubated in light for 4 h showed relatively lower pigment loss and slower loss of ETA compared to the chloroplasts of untreated leaves. These observations suggest photoprotective action of Kn. The photoprotection was more prominent when Kn was applied directly to the irradiated chloroplasts *in vitro*. Moreover, chloroplasts aging *in vitro* under irradiation without Kn treatment lost pigments and ETA. Within 3 h of irradiation, both whole chain (H_2O to methylviologen) electron transport as well as photosystem (PS) 2 activity were completely lost. However, in the chloroplasts treated with Kn, the loss of pigments was slow and even after 4 h of irradiation the chloroplasts retained 15 % of PS 2 and 9 % of whole chain ETA. In the untreated chloroplasts, the loss of Zx after 4 h of irradiation was 49 % whereas in Kn treated samples its level was 1.3 times higher than that of control. Since a higher level of Zx was maintained in Kn treated chloroplasts, photoprotective action of Kn is possibly mediated through Zx.

Additional key words: antheraxanthin, carotenoid, chlorophyll, lutein, neoxanthin, taraxanthin, violaxanthin, zeaxanthin.

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Abbreviations: Ax - antheraxanthin; β-car - β-carotene; β-car' - β-carotene isomer; Car - carotenoids; Chl - chlorophyll; Chl *a*'/Chl *b*' - chlorophyll *a*/*b* isomers; DCPIP - 2,6-dichlorophenol indophenol; ETA - electron transport activities; Kn - kinetin; LHC2 - light harvesting complex of photosystem 2; Lu - lutein; MV - methylviologen; Nx - neoxanthin; PS - photosystem; qE - non-photochemical fluorescence quenching dependent on pH; Tx - taraxanthin; Vx - violaxanthin; Zx - zeaxanthin.

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Introduction

Carotenoids are the most widely distributed plant pigments. Their role in photosynthetic system is two-fold: *1*) Car act as accessory light harvesting pigments, trapping radiant energy and passing it on to Chl for photosynthesis and *2*) Car protect chloroplasts from light mediated stress. The photoprotective role of Car in plants has been well documented (*e.g.* Siefermann-Harms 1987, Demmig-Adams 1990, Young 1991).

Demmig-Adams and co-workers (see review Demmig-Adams and Adams 1992) have suggested that Car are used in an additional protective mechanism which involves Zx, a pigment of the xanthophyll cycle. Their observation is based on the relationship of Zx content and non-radiative energy dissipation in pigment bed in intact leaves under photoinhibition. On the other hand, studies have indicated that a near maximum level of nonphotochemical quenching (qE) can be formed in complete absence of Zx (Noctor *et al.* 1991) and in a number of species there is no simple relationship between Zx content and radiationless energy dissipation (Johnson *et al.* 1993, Richter *et al.* 1994, Schindler *et al.* 1994). However, based on some recent observations Ruban *et al.* (1994) have suggested that a simple model can be made for qE in which Zx, formed in the minor LHC 2 component, will fit as the energy trap. This suggests a possible photoprotective role of Zx in chloroplasts under light stress. In our previous communications (Choudhury *et al.* 1993, 1994), we have shown that when Zx content of chloroplasts is increased by high irradiance or ascorbate treatment, pigment contents and ETA are protected during *in vitro* irradiation showing a photoprotective role of Zx.

Kinetin is well known as a senescence retarding growth regulator. It helps in maintaining membrane organisation and function of cellular organelles under stress (Thomas and Stoddart 1980, Biswal and Biswal 1988). Chloroplasts aging *in vivo* or *in vitro* lose thylakoid membrane organisation and composition leading to a loss in photochemical functions. Hence, Kn treatment is expected to protect chloroplasts under radiation stress.

During *in vitro* aging of chloroplasts radiation becomes the main stress factor causing photooxidation of pigments and damage to photosynthetic system (Choe and Whang 1986, Siefermann-Harm 1987, Panda and Biswal 1989). Since Zx has been suggested as the possible agent associated with photoprotection of chloroplasts, the main objective of the present study was to examine if the protective action of Kn (if any) against photoinhibition could be explained in terms of increasing/maintaining a higher level of Zx during *in vitro* aging of chloroplasts.

Materials and methods

Plants: Wheat (*Triticum aestivum* L.) seedlings were grown in plastic pots filled with vermiculite in a growth chamber at 25 °C and 60 % relative humidity under continuous irradiation. The pots were irrigated with distilled water. The photon flux density (400-700 nm) at the leaf surface was 400 μmol m^{-2} s^{-1} and was provided by

incandescent and cool white fluorescent lamps. Apical 5 cm of primary leaves of 7-d-old seedlings were used. For Kn treatment, the bases of the excised leaves were immersed in 100 cm^3 of Kn (0.2 mM) solution in a 150 cm^3 glass beaker and irradiated (400 μmol m^{-2} s^{-1}) in a growth chamber for 3 h. The solution was slowly stirred by a magnetic stirrer. A set of leaves was also incubated in distilled water as a control. When treatment was done with isolated chloroplasts, 100 μM (final concentration) Kn solution was added to the irradiated (400 μmol m^{-2} s^{-1}) chloroplast suspension.

Chloroplast isolation: 10 g of Kn treated and control leaves were homogenized in a Waring blender for 30 s in 100 cm^3 of isolation medium (0.4 M saccharose, 10 mM NaCl and 50 mM Tris-HCl buffer, pH 7.8) following the procedure of Swain *et al.* (1990). After centrifugation, the pellet (with a mixed type of chloroplasts) was suspended in the same medium with a Chl concentration of approximately 0.5 mg cm^{-3}. This suspension was incubated in growth chamber under irradiation (400 μmol m^{-2} s^{-1}) or in darkness.

Measurement of ETA: Ferricyanide-DCPIP supported PS 2 dependent O_2 evolution, whole chain (H_2O to MV) and partial chain (ascorbate-DCPIP to MV) electron transport activities were measured polarographically according to Choudhury *et al.* (1993) using a Clark-type O_2 electrode (*Yellow Spring Instrument Co.*, Yellow Spring, USA). For measurement of PS 2 activity, the 3 cm^3 of reaction mixture consisted of 10 mM Tris-HCl buffer (pH 7.5), 3 mM $MgCl_2$, 10 mM NaCl, 1 mM NH_4Cl, 1 mM $K_3Fe(CN)_6$ and 15 μM DCPIP and chloroplasts equivalent to 40 μg of Chl were exposed to saturating "white light" coming through 10 cm of water bath and exposing the cuvette from two opposite sides. The cuvette was housed in a custom made circulating water bath maintained at 25 °C, which also acted as a water filter to cut off infrared radiation. When whole chain (H_2O to MV) ETA was measured, instead of $K_3Fe(CN)_6$ and DCPIP, MV (0.5 mM) was taken in the reaction mixture and when PS 1 (Asc-DCPIP to MV) activity was measured, DCMU (10 mM), MV (0.5 mM) and ascorbate (1 mM) couple with DCPIP (10 mM) were taken in the reaction mixture.

Pigment extraction and estimation: Immediately after the treatments, the leaves (0.5 g) were ground in chilled methanol with mortar and pestle using acid washed sand and centrifuged at 8000 *g* for 5 min. The residue was washed once. When chloroplasts were used, 0.1 cm^3 of chloroplast suspension was taken up in the same volume of chilled methanol and centrifuged in a microfuge centrifuge for 2 min. These samples were used for pigment analysis by HPLC.

Pigment analyses by HPLC: Chromatography of leaf or chloroplast pigments was performed on a *Shimadzu* HPLC system consisting of two pumps (*LC-6A*), a system controller (*SCL-6A*), a UV/Vis detector (*SPD-6AV*) and an integrator (*Chromatopac, C-R5A*). The samples extracted in methanol were injected manually using an injector valve (*Rheodyne*, Hewlett-Packard Co., Delaware, USA) through a 50 mm^3 sample loop. Separation was carried out using a *C-18, ODS* reversed phase column

(*Econosphere*, 250 mm, 4.6 mm, 5 μm particles, *Alltech Associates*, Deerfield, USA). An aqueous (gradient) elution system was used for the pigment analyses as described elsewhere (Choudhury *et al.* 1993).

Results and discussion

There was no qualitative difference in the pigment profile (not shown) between control and Kn treated leaves, although there were quantitative changes between the two samples. Contents of Chl *a*, Chl *b* and the major Car such as Nx, Vx, Lu and β-carotene generally increased in the Kn treated leaves over the control (Fig. 1, Table 1). The stimulation of pigment accumulation by Kn treatment during leaf/cotyledon development is well know (Thomas and Stoddart 1980, Biswal 1985, Behera and Choudhury 1990).

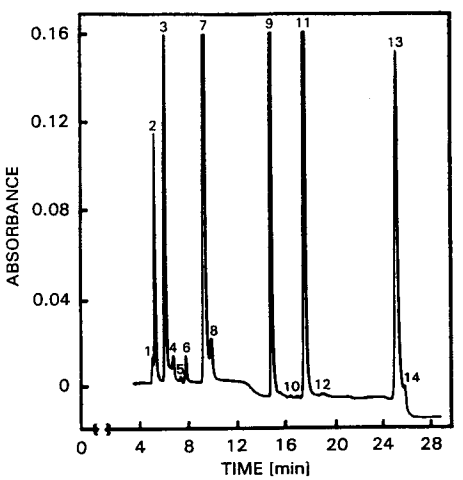

Fig. 1. Chromatogram of total pigment extract of the excised primary leaves of 7-d-old wheat seedlings. The peaks are: *1* - neoxanthin (*trans*), *2* - neoxanthin (*cis*), *3* - violaxanthin (*trans*), *4* - violaxanthin (*cis*), 5 - taraxanthin, *6* - antheraxanthin, *7* - lutein, *8* - zeaxanthin, *9* - chlorophyll *b*, *10* - chlorophyll *b* isomer, *11* - chlorophyll *a*, *12* - chlorophyll *a* isomer, *13* - β-carotene, *14* - β-carotene isomer. Although the peaks *10* and *12* are not prominent in the leaf chromatogram, they were prominent in the chromatogram of chloroplast samples (see Table 1). For other details, see Materials and methods.

The higher total Car and pigment contents of xanthophyll cycle (Vx+Ax+Zx), the higher Chl *a*/Chl *b* and the lower Chl/Car ratios (Table 2) and relatively higher Zx content (Table 1) in Kn treated leaves are characteristics of leaves exposed to, or grown at high irradiance (Thayer and Björkman 1990). When exposed to high irradiance, these leaves are better protected from surplus radiant energy (Demmig-Adams 1990). Except in the Chl/Car ratio, which did not show any significant change, similar characteristics in pigment content in the chloroplasts of Kn treated

leaves were observed (Tables 1 and 2). Therefore, it is expected that if chloroplasts isolated from Kn treated leaves are irradiated *in vitro*, the photodamage will be relatively less, compared to the untreated samples.

Table 1. Pigment content of leaves [μg g^{-1}(f.m.)] of wheat seedlings treated with or without kinetin (Kn) for 3 h and of chloroplasts isolated from leaves [μg cm^{-3}(suspension)] of control or Kn treated leaves irradiated for 4 h. Leaves incubated in water for 3 h did not show any change in pigment content. ND: not detected.

Pigments	Leaf		Chloroplasts			
	control	Kn	control		Kn	
	0 h	3 h	0 h	4 h	0 h	4 h
Nx(*trans*)	7.70	4.92	1.53	1.23	ND	ND
Nx(*cis*)	55.90	62.04	9.76	5.22	13.70	9.04
Vx(*trans*)	121.8	137.8	20.62	10.12	24.80	15.42
Vx(*cis*)	9.82	3.32	1.61	0.96	ND	ND
Tx	0.97	0.98	0.21	0.37	0.05	0.37
Ax	8.81	5.90	1.13	0.78	0.72	0.74
Lu	218.8	273.8	41.3	29.3	59.73	44.73
Zx	14.82	20.80	2.2	1.12	3.02	1.35
Chl *b*	736.8	816.5	166.5	144.5	230.6	219.7
Chl *b'*	ND	2.11	ND	5.55	1.32	3.30
Chl *a*	2043.5	2465.0	432.9	284.9	651.2	535.4
Chl *a'*	ND	5.84	1.9	11.1	2.82	9.12
β-car	141.0	243.6	22.7	10.97	45.80	29.32
β-car'	0.52	2.5	ND	ND	ND	ND

The chloroplasts isolated from control leaves when irradiated for 4 h lost Chl and all of the major Car. Among major pigments, Chl *b* showed minimum loss (13 %) and β-car maximum (52 %). The loss of Zx was 49 % compared to 0 h sample (Table 1). On the other hand, when chloroplasts isolated from Kn treated leaves were irradiated for 4 h, the losses of different pigments were signifcantly less ($P < 0.01$) compared to the chloroplasts of the control leaves during the same period of irradiation. Zx is associated with photoprotection of chloroplasts when the leaves are exposed to high irradiance (Bilger and Björkman 1990, Young 1991, Choudhury *et al.* 1994). Since radiant energy is the main stress factor for chloroplasts aging *in vitro* causing loss of pigment content (Siefermann-Harms 1987), a relatively higher level of Zx (37 %) in the chloroplasts of Kn treated leaves (Table 1) might have protected the organelles from radiant stress which is reflected in higher pigment levels in the Kn treated sample compared to the control.

The protective action of Kn on pigment content during aging of chloroplasts *in vitro* under irradiation is more pronounced when Kn is directly applied to the chloroplast suspension and irradiated (Table 3). Kn addition to the chloroplast suspension significantly ($P < 0.01$) prevented loss of Chl *a*, Chl *b* and major Car. Zx showed 49 % loss after 4 h of irradiation in the untreated control (Table 1). On the other hand, its level in the Kn treated chloroplasts after 4 h of irradiation was

30 % higher than the 0 h control and 2.6 times higher than the 4 h irradiated sample. The higher level of Zx maintained in the presence of Kn was possibly responsible for protecting the pigments contents from photooxidation.

Table 2. Pigment ratios, total pigments of xanthophyll cycle and total chlorophyll and carotenoid contents of wheat leaves [μg g^{-1}(f.m.)] treated with or without Kn for 3 h and chloroplasts [μg cm^{-3}(suspension)] isolated from control or Kn treated leaves and irradiated for 4 h.

	Leaf		Chloroplasts			
	control	Kn	control		Kn	
	0 h	3 h	0 h	4 h	0 h	4 h
Chl *a*/Chl *b*	2.77	3.01	2.61	1.97	2.82	2.44
Chl/Car	4.79	4.35	6.01	7.52	5.99	7.60
Vx+Ax+Zx	155.2	167.8	24.56	12.20	28.54	17.51
Total Car	580.1	755.6	100.0	59.29	147.8	100.9
Total Chl	2780.3	3289.4	601.3	446.0	885.9	767.5

Table 3. Pigment content of chloroplasts [μg cm^{-3}(suspension)] isolated from wheat leaves and irradiated for 4 h in suspension with Kn or in dark without Kn. Chloroplast incubated for 4 h in dark with Kn did not show any significant change in pigment content from that of the sample incubated in dark without Kn. For pigment content of 0 h (control) and 4 h irradiated samples (without Kn), see Table 1 column 3 and 4, respectively. ND: not detected.

Pigments	Light + Kn	Dark	Pigments	Light + Kn	Dark
Nx(*trans*)	1.42	1.32	Zx	2.87	1.85
Nx(*cis*)	9.16	9.36	Chl *b*	158.3	186.4
Vx(*trans*)	15.07	19.92	Chl *b'*	1.33	ND
Vx(*cis*)	0.93	0.58	Chl *a*	364.2	405.2
Tx	0.37	0.30	Chl *a'*	3.67	4.50
Ax	1.03	1.19	β-car	15.97	17.61
Lu	33.90	44.0	β-car'	ND	ND

The ratios of Chl *a*/Chl *b* and Chl/Car and total Chl, total Car and pigment contents of the xanthophyll cycle in Kn treated samples (Table 4) after 4 h of irradiation are closer to the corresponding values of fresh (0 h) chloroplasts compared to the chloroplasts without Kn (4 h irradiation) treatment (Table 2). The little changes of these parameters in Kn treated chloroplasts suggest that Kn has protected the organelles from photoinhibition which was possibly mediated through Zx.

The PS 2 (H_2O to DCPIP) as well as the whole chain (H_2O to MV) ETA were totally lost within 3 h of irradiation of chloroplasts in the control samples. However, when chloroplasts with Kn were irradiated even after 4 h, 15 % of PS 2, 9 % of whole chain, and 34 % of PS 1 activities were still observed (Table 5). In dark incubated chloroplasts with or without Kn, the loss of ETA was relatively slow

compared to the corresponding irradiated counterparts (Table 5). Contrary to the partial protection of ETA, a significant protection of pigment content by Kn indicates that its effect on ETA during *in vitro* irradiation of chloroplasts may operate under different mechanism. It has been suggested that under radiant stress the ETA loss is due to degradation of D1 protein (Shipton and Barber 1992, Long and Humphries 1994). Loss of the oxidizing side of PS 2 during plastid aging has also been suggested (Powles 1984, Biswal and Biswal 1988, Choudhury and Imaseki 1990). Chloroplasts incubated in dark with or without Kn also showed considerable loss of PS 2 and whole chain electron transport but not PS 1 activities. This suggests that a part from photoinhibition there could be factor(s) causing loss of ETA during *in vitro* aging of chloroplasts. This could be the reason that although a higher level of Zx was maintained in the Kn treated sample which efficiently protected the pigment content (Table 3), it was unable to effectively protect ETA (Table 5).

Table 4. Contents of chlorophyll, total carotenoids and pigments of xanthophyll cycle [μg cm^{-3}(suspension)] and pigment ratios of chloroplasts isolated from wheat leaves and irradiated for 4 h with kinetin (Kn) or in dark without Kn. For pigment contents and pigment ratio of 0 h (control) and 4 h irradiated (without Kn) samples, see Table 2.

	Light + Kn	Dark
Chl *a*/Chl *b*	2.30	2.19
Chl/Car	6.53	6.20
Vx+Ax+Zx	19.9	23.54
Total Car	80.72	96.13
Total Chl	527.5	596.1

Table 5. Changes in the electron transport activities of chloroplasts isolated from wheat leaves and incubated under irradiation or in dark for 4 h with or without kinetin (Kn). The activities at 0 h are taken as 100 %. These activities were: 50.5 mmol (DCPIP) kg^{-1}(Chl) s^{-1}, 47.4 mmol(MV) kg^{-1}(Chl) s^{-1}, 237.0 mmol(MV) kg^{-1}(Chl) s^{-1}, respectively. The data are means ± SD of 5 independent experiments.

Time [h]	H_2O to DCPIP		H_2O to MV		Ascorbate-DCPIP to MV	
	control	Kn	control	Kn	control	Kn
Light						
0	100 ± 7	100 ± 7	100 ± 7	100 ± 7	100 ± 5	100 ± 5
1	37 ± 4	46 ± 4	31 ± 4	49 ± 5	67 ± 5	76 ± 4
2	17 ± 2	26 ± 3	13 ± 2	29 ± 3	49 ± 3	52 ± 3
3	0	16 ± 2	0	10 ± 1	32 ± 2	34 ± 2
4	-	15 ± 2	-	9 ± 1	22 ± 1	34 ± 2
Dark						
2	67 ± 4	86 ± 6	67 ± 4	82 ± 6	104 ± 5	105 ± 4
4	45 ± 3	68 ± 3	46 ± 2	64 ± 4	98 ± 3	103 ± 4

Zx quenches the excitation energy in the pigment bed consequently causing a decrease in ETA of chloroplasts (Demmig *et al.* 1988, Bilger and Björkman 1990) when the leaves are under radiant stress. Therefore, the quantitative results of ETA measurement of chloroplasts under radiant stress with relatively a higher level of Zx (as in Kn treated sample) do not reflect the photoprotective action of Zx in terms of protecting ETA. This may be the reason for the low level of ETA in Kn treated chloroplasts (Table 5). Nevertheless, the slower rate of loss of ETA in Kn treated sample compared to the untreated control indicates that Kn has protected ETA. Since Kn treatment to the chloroplasts maintained a higher level of Zx compared to the untreated one, the photoprotective action of Kn during irradiation of chloroplasts in *in vitro* conditions could be due to maintaining a higher level of Zx under irradiation.

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