RESEARCH PAPER | Agriculture | Volume : 3 | Issue : 4 | April 2013 | ISSN - 2249-555X

Lutein, Anthocyanin and Xanthophyll Cycle Components in Wheat (Triticum aestium) Flag Leaves Under high Light and high Temperature Conditions

KEYWORDS: Carotenoids, HPLC, TLC, Spectrophotometer, wheat, high light, high temperature

Santosh Kumari

Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi 110012. India

ABSTRACT *Non photochemical quenching (NPQ) decreased in wheat flag leaf under high light and at a temperature 5°C higher than normal growth conditions. The accumulation of the xanthophyll cycle carotenoids and zeaxanthin particularly indicated pH dependent energy dissipation (qE) a major component of NPQ. Neoxanthin and zeaxanthin served important functions as an antioxidant in the lipid phase of the membrane and are likely to act as key components in the memory of the chloroplast with respect to preceding photo-oxidative stress in next generation even under low light and normal temperature conditions.*

Introduction

Light is required for photosynthesis in plants, but the quantity of light in natural environments is highly variable. Within a certain range of relatively low incident light intensities, photosynthetic carbon fixation increases linearly with increases in photon flux density. However, above a certain threshold, photosynthetic capacity is saturated, and a plant absorbs more light than it can actually use. Absorption of excess light can lead to over excitation of chlorophyll and over reduction of the electron transport chain, which result in increased generation of reactive oxygen species and harmful by-products of photosynthesis (Niyogi, 1999). Over excitation of chlorophyll would result in an increase in the life time of singlet-excited chlorophyll (1Chl*), which consequently increases the production of triplet-excited Chl (3Chl*) via intersystem crossing. 3Chl* interacts with molecular oxygen to generate singlet O_2 (1O2*), which can damage proteins, pigments, and lipids in the photosynthetic apparatus (Niyogi, 1999; Asada, 2006). To avoid massive radical formation plants have evolved several mechanisms to prevent or to reduce oxidative damage during high light exposure. These include various alternative energy dissipation pathways and multiple antioxidant systems.

The excited chlorophyll can return to ground state by the emission of fluorescence (light of longer wavelength than the previously absorbed light). This process is however not apt to release large amounts of excess energy because only 5% of chlorophylls can emit fluorescence in a given time. In stress situations energy dissipation by conversion to heat is of far greater importance. This event is often referred as non photochemical quenching (NPQ) and is done by oxygenated carotenoids, called xanthophylls that are bound together with the chlorophylls in the antennae complex of the photysystems. These can take over the energy from chlorophylls by resonance transfer (Müller et al., 2001). Excited carotenoids mostly return to ground state by releasing excess energy as heat. Up to 75% of excitation energy can be dispelled this way (Demmig-Adams et al., 1996).

The plant carotenoid biosynthetic pathway branches at the cyclization reactions to produce carotenoids with either two β-rings or one β- and one ε-ring. Lycopene is either cyclized twice by lycopene β-cyclase to produce β-carotene or through the sequential action of lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) to produce α-carotene that is precursor to lutein. It has been hypothesized that partition of flux into the β- and α-branches of the pathway is controlled by the relative activities of LCYB and LCYE (Cunningham et al., 1996; Pogson et al., 1996; DellaPenna and Pogson, 2006). β, β-Xanthophyll biosynthesis requires hydroxylases belonging to the so-called 'non-heme di- iron'group while the biosynthesis of lutein involves enzymes belonging to the vast group of P450 monooxygenases with different enzymatic specificity due to the distinct rings of α-carotene. The very wide range of functions of plant carotenoids is due to the long system of conjugated double bonds which characterizes their molecular structure. This feature determines not only their light-absorbing properties but also other photochemical properties as well as their reactivity toward oxidizing agents and free radicals (Britton, 1995). The xanthophyll zeaxanthin is primarily responsible for the safe dissipation of excess light energy as heat, wheareas β-carotene, amongst others, is a potent antioxidant.

Materials and Methods

Plant material and growth conditions

Wheat cultivars C 306 (water stress tolerant), HD 2428 (water stress susceptible) were planted at different dates of sowing (15 November and 15 January (2009-2010-2011). Seeds harvested from November sown and January sown plants were planted next year in November and compared for pigment profiles under normal, late sown conditions. Plants were raised in earthen pots of 30 x 30cm size filled with sandy loam soil and farmyard manure in 3:1 under natural environment. Each pot was fertilized corresponding to 120, 90 and 60 kg ha-1 of N, P and K, respectively. Four plants were maintained in each pot. Plant protection measures were taken as per requirement. Flag leaf of late sown plants developed at 5°C higher temperature i.e. 29°C than normal plants i.e. 24°C.

Pigment Analysis

Leaf pigments were extracted from 50 mg of frozen plant material by addition of 1 ml ice-cold 100% acetone to ground tissue (Lichtenthaler, 1987). After centrifugation, 250 ml of the supernatant were spotted on precoated-silica gel 60 F254 TLC plate (Merck- Germany) and separated with petroleum ether: isopropanol: H2O (100: 15: 0.25) as running solvent. Pigment identification and separation was also performed on a 150 mm x 4.6 mm (particl size 5 µm) Zorbax XDB-C18, column at 440 nm wavelength with an Agilent 1200 System, HPLC consisted of a micro vacuum degasser, a binary pump, a diode array detector, an autosampler and Chem station software, Agilent Technologies, Agilent. Lutein, β-carotene, xanthophylls were scraped from TLC plate dissolved in 100% acetone and run separately to find the peaks in samples. All solvent were HPLC grade from Merck (India). Peak identification of lutein, violaxanthin, neoxanthin, zeaxanthin, and β-carotene was carried out through retention times and spectral properties of authentic standards following (Taylor et al., 2006, and Fraser et al., 2007). The samples were also analyzed for absorption spectra of different pigments in acetone following the literature using UV-VIS Spectrophotometer (Model ECIL UV 5704 ss).

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Chlorophyll Fluorescence
Chlorophyll Fluorescence was measured using Portable Photosynthesis system (IRGA Model LI-6400 XT, Nebraska, USA)

Light Microscopy
All samples for microscopy, were taken from fully developed flag leaves around 11.00 A.M. Flag leaf sections for ultra structural characterization were fixed overnight at 4 °C in 2% (v/v) para formaldehyde- 2% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2), post fixed in 2% (w/v) osmium tetra oxide (OsO4), embedded in a mixture of Epon and Araldite after a standard dehydration procedure. Semi-thin leaf cross-sections were used for light microscopy Light microscopy sections were stained with 1% (w/v) Toluidine Blue O in 1% (w/v) sodium borate.

Result and Discussion
Plate 1 displayed that no xanthophyll was detected in drought tolerant cultivar C306 whereas neoxanthin and violaxanthin were detected in drought sensitive wheat cultivar i.e. HD 2428 under normal growth conditions. Further, zeaxanthin and xanthophyll cycle component (violaxanthin + neoxanthin) appeared on TLC in reverse cytogenetic study in both cultivars i.e. HD 2428 +ABA and C306+ABA respectively. Violaxanthin de-epoxidation (zeaxanthin) to combined action of high temperature and high light implies that the protective role of the xanthophyll cycle may be important under heat and light stress. W hen a leaf is exposed to light exceeding the capacity of utilisation by photosynthesis from an inhibition of net carbon dioxide assimilation by drought (high temperature and high light conditions created water stress); the yield of chlorophyll fluorescence at maximum fluorescence level (Fm) i.e. non photochemical quenching (NPQ) is decreased (Table 1). Our data on rate of photosynthesis and chlorophyll fluorescence was in agreement with Krause and Weis (1991). NPQ monitor the non radiative energy dissipation process, in which part of the excitation energy is channeled away as heat from the photosystem II (PSII) reaction centres. The efficiency of PS II photochemistry decreased to approximately 65% in plants exposed to the interaction of high light and drought (Munne-Bosch and Alegre, 2000). The decreased F_v/F_m (Table 1) could be associated with an increase in energy dissipation in the PS II antennae (Demmig-Adams et al., 1995) and by the increase in proportion of closed PS II reaction centres and decrease in excitation energy capture (Lu et al., 2001). The specific rate of carbon metabolism needed to avoid photoinhibition will vary according to radiance maintained during the photoinhibitory treatment and the radiance under which the plant developed, as well as other genotypic, phenotypical, and physiological characteristics of the plant (Powles, 1984; Krause, 1988) as shown in Plate 1.

Table1. Chlorophyll fluorescence and photosynthetic rate in flag leaf of normal and late sown wheat cultivars

Wheat	Fm	Fv/Fm	Photosynthetic Rate ($\mu mole^{-2}$ sec^{-1})
HD2428 (November)	2374	0.81	18.75
HD2428 (January)	1884	0.78	7.0
C306 (November)	2181	0.81	19.72
C306 (January)	1882	0.76	6.0

A number of processes contribute to the induction and relaxation of NPQ over time. One component, termed qE, is turned on and off rapidly (seconds to minutes) and depends on the formation of the transthylakoid proton gradient (ΔpH). The development of qE is associated with the accumulation of the xanthophyll cycle carotenoid zeaxanthin (Demming-Adams, 1990). The xanthophyll cycle is a reversible interconversion of zeaxanthin and violaxanthin that is directly linked to the energization of the thylakoid membrane during the induction of photosynthesis in the light. As light saturation is reached, the rise in ΔpH increases the proton concentration within the thylakoid lumen. This has a number of effects, including the activation of the enzyme violaxanthin deepoxidase, which converts violaxanthin to zeaxanthin (Plate 1, Plate 2 & Fig 1) and increases the deepoxidation state of the xanthophyll cycle pool. The reverse reaction, converting zeaxanthin to violaxanthin, is catalyzed by zeaxanthin epoxidase. Although there is no doubt that zeaxanthin plays a key role in qE, there is still discussion over whether zeaxanthin is a direct quencher of singlet excited chlorophyll or an allosteric effector that alters the sensitivity of qE to the ΔpH and evidence exists for both mechanisms. A change in pH was evident in alterations in chloroplast ultrastructure (Electron micrograph) in flag leaf of wheat (Santosh Kumari, 2010). Analysis of Arabidopsis thaliana mutants that lack qE has been a very useful approach to define factors that are necessary for qE, including xanthophylls, the PsbS protein, and light harvesting complex (LHC) proteins (Niyogi, 2000).

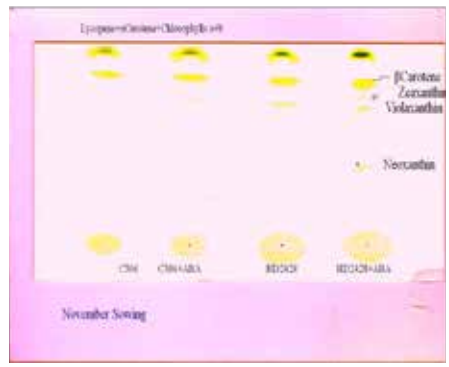

Plate1. Xanthophyll cycle pigments (Zeaxanthin+Violaxanthin+Neoxanthin) detected in pre stressed wheat flag grown under low light and low temperature conditions.

C306 –Drought tolerant wheat cultivar (Control) from normal sowing conditions i.e. November

HD2428 – Drought sensitive wheat cultivar

C306+ABA- and HD2428+ABA-Wheat plants with endogenous abscisic acid in seeds harvested from late sown condition i.e. January

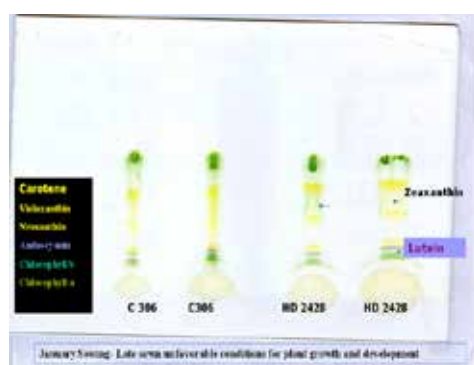

Plate 2. Pigment profile in flag leaf of drought tolerant (C306) and drought sensitive (HD 2428) wheat cultivars under high light and temperature conditions.

In photosynthetic tissues, in particular, the lutein level is mainly determined by the relative rate of Lcy-ε and Lcy-β transcription (Bai et al. 2009, Li et al. 2009). Li et al. (2009) found in Arabidopsis that a point mutation in the Lcy-b gene resulted in an increase in the leaf lutein content due to an impaired activity of the LCY-B enzyme and explains the existence of multiple, conserved gene products in the plant antenna system. Lutein biosynthesis and accumulation in wheat (Plate 2) indicated the conserved gene expression under unfavorable growth conditions of high light and high temperature. Anthocyanin granules were observed in light micrograph (Plate 3), and appeared on TLC (Plate 2) and in absorption spectra (Fig 2). The pigment had been associated with light screening effect under excess of light. Since radiation of 470 nm is

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well absorbed by the major antenna of PS II (the light harvesting complex II), anthocyanin screening of radiation can effectively reduce excitation energy in the PS II complex and, hence, prevent damage by reactive oxygen species, which may be formed during disassembly and degradation of the photosynthetic apparatus during autumn (Hoch et al., 2001, Steyn et al., 2002). Further, the presence of anthocyanins was found to lower the photoinhibitory effects of blue radiation (400–550 nm) in senescing leaves of Cornus stolonifera (Cornaceae) (Field et al., 2001). Specifically, Pfündel et al. (2007) suggest that the peak at 497 nm arises from excitation of light-harvesting carotenoids, which, at wavelengths of 490 nm and below, are reduced due to competing xanthophyll absorption and, in the range of 500–530 nm, drops to zero owing to the known absorption properties of carotenoids.

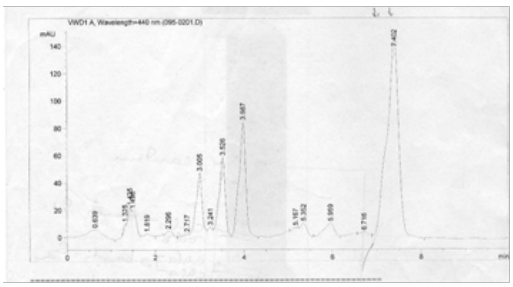

Fig 1. HPLC chromatogram of total pigments at 440 nm wavelength in wheat flag leaf under drought (High light and high temperature) conditions

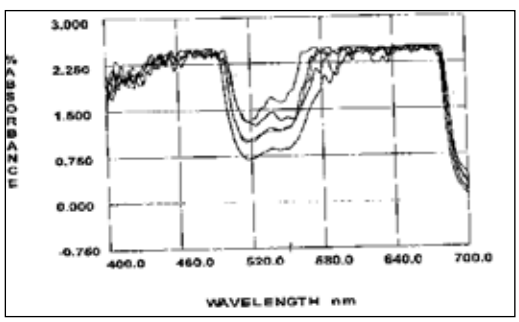

Fig 2. Absorption spectrum of total pigments (extracted in 100% cold acetone) of flag leaf from drought stressed plants (January sown) sowing absorption peak for carotenoids in 430-480 nm, anthocyanin in 520-550 nm and chlorophyll a and chlorophyll b in 663 nm 645 nm λ region

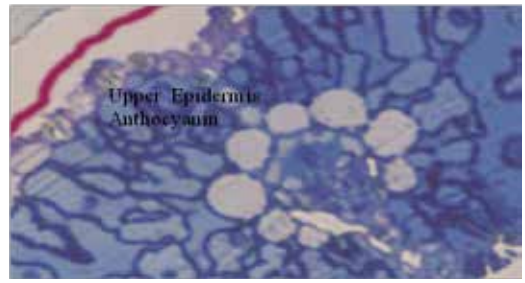

Plate 3. Anthocyanin granules detected in upper epidermis of wheat under high light and high temperature in late sown condition under light microscope

The current knowledge supports the view that the photoprotective role of Lutein (Plate 2) is predominantly restricted to its function in the deactivation of triplet excited chlorophyll, while zeaxanthin is the major player in the deactivation of excited singlet Chl and thus in non-photochemical quenching. Additionally, neoxanthin and zeaxanthin serve important functions as an antioxidant in the lipid phase of the membrane and are likely to act as key components in the memory of the chloroplast with respect to preceding photo-oxidative stress (Plate 1) even under low light and normal temperature conditions i.e. epigenetic changes (our unpublished data).

To conclude, it is clear that we can genetically manipulate and enhance a plant's capacity for photoprotection. This may have beneficial effects for crops in suboptimal environments, but it must be weighed against the evidence that such processes have a cost that could limit carbon gain in optimal conditions as carbon is channeled in secondary metabolism for adaptation and survival of the plant under stressed conditions.

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