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Ontogenic changes in epicuticular wax and chloroplast integrity of a cotton (*Gossypium hirsutum* L.) leaf

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Abstract

The progressive decline in cotton leaf photosynthesis with season could be accounted for by gaining an insight into ontogenic changes in chloroplast integrity and epicuticular wax ultrastructure. Therefore, the sequence of ultrastructural changes in chloroplast and epicuticular wax morphology were probed in 10-, 20-, 40-, and 60-d-old cotton (*Gossypium hirsutum* L.) leaves using electron microscopy. Scanning electron microscopy illustrated that the epicuticular wax on the periclinal walls of the convex epidermal cells occurred as striations and persisted as such during the course of leaf aging. The degree of wax spread, however, increased as the leaf progressed towards senescence. Transmission electron microscopy revealed that a 20-d-old photosynthetically active leaf possessed healthy chloroplasts (6.8 μm long and an area of 9.7 μm^2) with absolute membrane integrity depicted by large appressed grana stacks of thylakoids interconnected by non-appressed stroma lamellae. The thylakoid membrane network was oriented parallel to the long axis of the chloroplast and a few small plastoglobuli (1.85 μm^2) scattered in the stroma. Conversely, membrane integrity was lost with leaf age after 20 d as evidenced by disruption of the grana and stroma lamellae. Concurrent with the membrane damage, extensive occlusion of chloroplast by several large spherical plastoglobuli (5.68 μm^2) occurred, the rate of occlusion increased with leaf age distending the chloroplast as evidenced by proliferation of its cross-sectional area (12.8 μm^2). Of particular interest was the finding that the plastoglobuli ensued through the chloroplast envelope into the cytoplasm. The progressive loss of chloroplast membrane integrity coupled with increased leaf waxiness may have limited photosynthetic activities of cotton leaves during senescence.

Additional key words: grana; microscopy; plastoglobuli; stroma lamellae.

Introduction

Cotton produces large number of fruits which rely exclusively on the leaves for their growth and development since the leaf tissue houses the chloroplasts, the chemical machinery equipped with functional components required to transmute radiant energy into chemical energy (Wullschleger and Oosterhuis 1991). The young developing bolls avail the transduced energy *via* conducting tissues in the form of carbon compounds (Oosterhuis and Wullschleger 1992). The major suppliers of carbon for the development of cotton fruit are the leaf subtending the boll, the leaf subtending the adjacent fruiting position, and the leaf subtending the sympodia (Ashley 1972, Wullschleger and Oosterhuis 1990). In addition to feeding the bolls, leaves also contribute to further carbon assimilation by translocating their assimilates and nutrients to the new expanding leaves. Apart from leaves, other organs of cotton plants such as the bracts and the capsule wall are also photosynthetically active, but their contribution to the developing boll and further carbon assimilation is small (Morris 1965, Wullschleger and Oosterhuis 1990). This is caused by the absence of pertinent anatomical and ultrastructural features essential for optimizing photosynthesis (Bondada *et al.* 1994). Therefore, the leaves constitute the mainstay of cotton production (Oosterhuis and Wullschleger 1992).

One of the leaf factors that modulate the photosynthetic capacity of a leaf includes its age. It is profoundly important to fathom how leaf age influences photosynthetic capacities since such knowledge aids in estimating the long-term carbon budget of a leaf and of the whole canopy (Kitajima *et al.* 1997). Hence, many studies have been devoted specifically to consider the effect of leaf age

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on photosynthetic capacity. These studies elucidated that the photosynthetic rate maximized in young leaves, generally after the full expansion of a leaf, and thereafter the maximum photosynthetic capacity weakens which is best characterized by a monotonic decline (*e.g.* Šesták *et al.* 1985). In cotton, the maximum photosynthetic rate of leaves on sympodial branches is achieved about 20 d after leaf initiation, thereafter, the photosynthetic function deteriorates with leaf age (Constable and Rawson 1980, Wullschleger and Oosterhuis 1990, Peng and Krieg 1991, Bondada and Oosterhuis 1998). Although a redistribution of resources to young leaves for optimization of the whole photosynthetic income has been linked to photosynthetic declines with leaf age (Field and Mooney 1983), the underlying mechanism leading to such phenomenon, however, is not clearly understood. We hypothesize that the decline in photosynthetic activity with leaf age is related to alterations in chloroplast ultrastructure and leaf surface morphology such as epicuticular wax. Such assertion is based on the premise that the chloroplasts carry out light and dark reactions of photosynthesis (Salisbury and Ross 1992) and the PPFD requirement for light reactions is determined by the distribution of epicuticular wax on the leaf surfaces (Martin *et al.* 1991). The sequential ultrastructural changes in chloroplast and epicuticular wax morphology during the life span of a single leaf are yet to be described in cotton. A study of such nature may furnish a decisive ultrastructural substantiation for the decline in photosynthesis with leaf age. The aim of this study is to provide a comprehensive description of the changes in chloroplast ultrastructure and epicuticular wax morphology of cotton leaves of different ages.

Materials and methods

Field-crop management: Seeds of "Stoneville 506" cotton (*Gossypium hirsutum* L.) were planted on May 15, 1990 and May 20, 1991, in small plots consisting of six 5-m rows spaced 0.95 m apart in a moderately well-drained Captina (Typic Fragiudults) silt loam at the Agricultural Experiment Station, Fayetteville, AR. The daily PPFD equaled or exceeded 1 200 μmol m^{-2} s^{-1}. All the plots were hand-thinned to a stand density of approximately 7.2 plants per m^2 (72 000 plants per ha). Fertilizer consisted of 3.2-1.4-2.6 g m^{-2} (32-14-26 kg ha^{-1}) of N-P-K incorporated before planting and an additional side-dressing of 3 g(N) m^{-2} at the pinhead (floral bud) square stage. The plots received supplemental furrow irrigation throughout the season to provide a well-watered environment to maintain optimal growth. The customary cultural practices pertaining to herbicides and insecticides were used as needed. The experiment was a completely randomized design with three replications. Treatments consisted of sympodial leaves of different ages, 10-, 20-, 40-, and 60-d-old leaves.

Leaf age was determined by tagging nearly all the plants in the plots with white jeweler's tags at main-stem node 10, sympodial fruiting branch position 1 (Oosterhuis 1990). The day when leaves first unfolded was considered as day 1. Ultrastructural alterations were examined in four different leaf age groups viz. 10-, 20-, 40-, and 60-d-old leaves at main-stem node 10, fruiting position 1.

Scanning electron microscopy: Tissue samples were taken from 10-, 20-, 40-, and 60-d-old leaves. Leaf segments (15 mm^2) from a single leaf of each replication belonging to each leaf-age group were vacuum-infiltrated in 30 % ethanol for 1 h or until the pieces sank, indicating the replacement of cellular air spaces with ethanol. The tissues were then dehydrated in a graded ethanol series and held in 95 % ethanol for 2 h. The segments were subsequently critical point dried, coated with gold, and viewed with an *International Scientific Instrument 60* scanning electron microscope using an accelerating voltage of 15 kV.

Transmission electron microscopy: Leaf segments (<2 mm^2) from each leaf-age group (one leaf per replication from each treatment) were fixed at room temperature under weak vacuum for 2 h in a modified Karnovsky's fixative consisting of 2 % paraformaldehyde and 2 % glutaraldehyde, buffered in 0.05 M cacodylate buffer at pH 7.2. The specimens were rinsed twice in the same buffer and post-fixed for 2 h in a solution of 1 % osmium tetroxide in the same cacodylate buffer. Specimens were then rinsed in distilled water and pre-stained overnight in 0.5 % aqueous uranyl acetate at 4 °C. Specimens were dehydrated in a graded ethanol series followed by two changes of propylene oxide to further dehydrate the tissue and prepare for infiltration. Samples were sequentially infiltrated in 50 : 50 and 25 : 75 propylene oxide-Spurr's medium (Spurr 1969) for 24 h and then transferred to 100 % Spurr's medium and left for four days at room temperature in a desiccator. Tissue samples were subsequently embedded in Spurr's epoxy resin, thin-sectioned (70-90 nm) with a glass knife, double-stained in 2 % uranyl acetate and lead citrate (Reynold 1963), and then examined under a *Siemens Elmiskop 1A* transmission electron microscope at 75 kV.

Measurement of chloroplast characteristics: Eight to ten chloroplasts representing different mesophyll cells in one representative section from each of the three replications were photographed at a primary magnification of 13 000×. Contact prints (8×10) were used to determine the length of chloroplast (the longest dimension) and

plastoglobuli and chloroplast profile (visible cross-sectional) areas were quantified with an image analysis system (*Image-Pro Plus*, *Media Cybernetics*, Silver Spring, MD, USA).

Results and discussion

Epicuticular waxes: They are a mixture of diverse long chain hydrocarbons which, upon crystallization, form a hydrophobic boundary layer at the surface of the aerial portions of higher plants (Fisher and Bayer 1972). Scanning electron microscopy (SEM) revealed that the epicuticular wax on cotton leaves appeared as fine slender striations analogous to epicuticular wax morphology found in cotton bracts and capsule wall (Bondada *et al.* 1996) and fruit crops such as apple (Leece 1976) and peach (Bondada *et al.* 2000). The patterns of the wax striations were wavy and straight that ran parallel or random on the periclinal walls of the epidermal cells on both adaxial and abaxial leaf surfaces. The wax morphology as striations was similar in all leaf age classes and, for this reason, only the wax morphology of abaxial surfaces from each leaf age class is shown in Fig. 1. Although the wax morphology was similar in all leaf age classes, the magnitude of wax covering varied with leaf age. In the 10-d-old leaf, the wax striations were hardly discernible on the periclinal walls of the epidermal cells, while the wax striations increased as the leaves advanced towards senescence (Fig. 1). The wax striations were densely distributed in 40-d-old leaves and were very prominent in 60-d-old leaves in which the entire periclinal walls of epidermal cells were eclipsed by wax striations (Fig. 1*D*). The progressive dense spread of wax on the epidermal cells is reflective of wax content to increase with leaf age as elucidated by quantitative analysis of wax content of leaves of different ages using gravimetric method (Bondada *et al.* 1997).

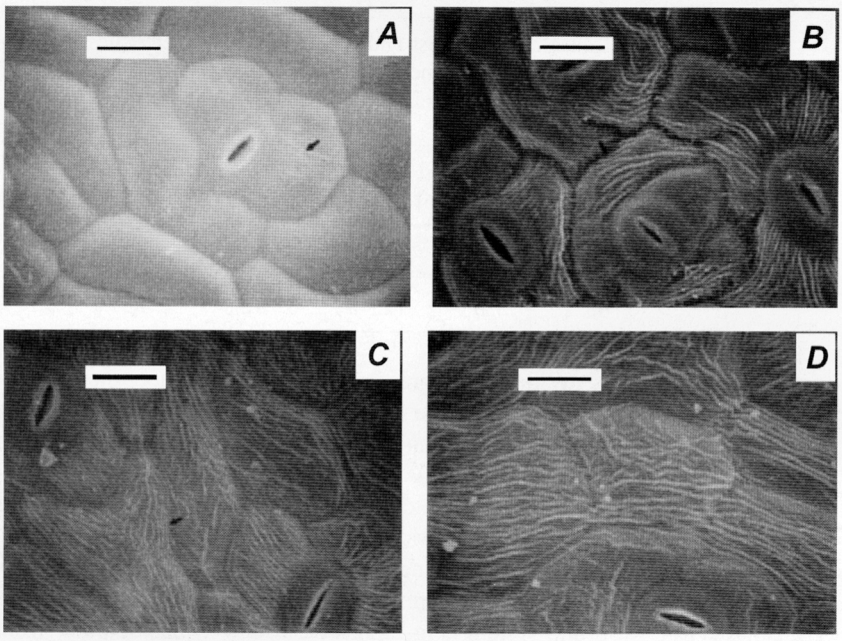

Fig. 1. Scanning electron micrographs of different leaf age groups (*A*) 10-d-old, (*B*) 20-d-old, (*C*) 40-d-old, and (*D*) 60-d-old cotton leaves showing epicuticular wax in the form of striations (*arrow*) which increased with leaf age. Bar = 10 μm.

Cotton leaves normally begin to diminish their photosynthetic rates approximately 20 d after leaf unfolding (Constable and Rawson 1980, Wullschleger and Oosterhuis 1990). Such age-induced repression could be accounted for partially by the profusion of wax covering on the surface of old leaves. The photosynthetic process commences with the interception of radiation determined by the leaf optical characteristics (Smith 1964, Mooney and Gulmon 1982) which varies substantially depending upon the spread of epicuticular wax layer on the periclinal walls (Martin *et al.* 1991, Vogelmann 1994). The increased waxiness with leaf age perhaps reflected most of the incident radiation from the surface of old leaves (> 20-d). Thereby it reduced PPFD for utilization in the light-driven reactions, as a consequence, the enzymatic process of CO_2 assimilation failed to operate fully, and ultimately, these impediments contributed to the recorded reductions in photosynthetic rates. The increased load of epicuticular wax endowed the old leaves with additional inefficacies, for instance, leaves older than 20 d were inept at absorbing leaf-applied nitrogen (N), a production practice adopted to supplement the high N demand of the

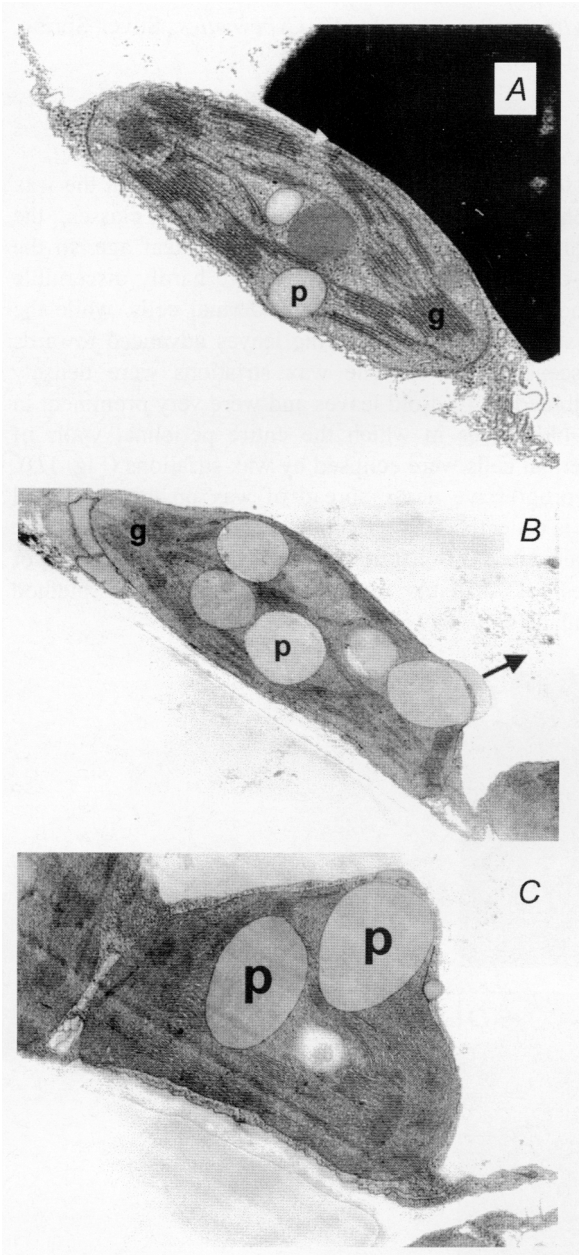

Fig. 2. Transmission electron micrographs of (*A*) a 20-d-old leaf chloroplast with well developed grana (g), stroma lamellae (*arrow*), and small plastoglobuli (p), (*B*) a 40-d-old leaf chloroplast showing disintegration of the membrane system and large plastglobuli (p), the *arrow* indicates passage of plastoglobuli into the cytoplasm, and (*C*) a 60-d-old leaf chloroplast showing distortion of chloroplast by large plastoglobuli (p) following disintegration of the membrane system.

developing bolls (Bondada *et al.* 1997).

Chloroplast ultrastructure: Since the symptoms of leaf aging are usually first expressed in chloroplasts (Butler and Simon 1971), chloroplasts of different leaf ages were examined using transmission electron microscopy (TEM). TEM revealed that 20-d-old leaf chloroplasts were approximately 6.8 μm long (Table 1). The chloroplasts conserved its normal disc shape and membrane integrity as evidenced by the differentiation of the continuous thylakoid membrane network into regular domains of closely appressed membranes, the grana stacks which were interconnected by non-appressed single lamellae, the stroma thylakoids; both realms of the membrane organization were oriented parallel to the long axis of the organelle cross section (Fig. 2*A*). In the young leaves, the plastoglobuli, the lipid-protein globules (Guiamét *et al.* 1999, Kutík *et al.* 2001), were few and small (Table 1) and confined to the stroma (Fig. 2*A*). Apart from possessing a panoramic network of membrane system, the young chloroplasts of cotton exhibited proficiency in accommodating high contents of thylakoid and stroma proteins (Pettigrew and Vaughn 1998). These ultrastructural features as displayed by the young chloroplasts delineate the ideal membrane architecture required to carry out and maximize photosynthesis; any aberration to such membrane assembly is most likely to decrease photosynthetic activity (Šesták *et al.* 1985, Bondada *et al.* 1994).

Table 1. Chloroplast and plastoglobuli characteristics in aging cotton leaves. Values within a column followed by the same letter are not significantly different at $p \leq 0.05$.

Leaf age [d]	Chloroplast length [μm]	Chloroplast area [μm^2]	Plastoglobulus area [μm^2]	Area covered by plastoglobuli
20	6.8 a	9.7 b	1.85 b	20.4 b
40	7.5 a	12.8 a	5.68 a	45.2 a
60	7.2 a	12.3 a	5.79 a	46.7 a

With the progression of leaf senescence, there was a loss of membrane integrity manifested by disintegration of grana stacks and stroma lamellae as observed in the chloroplasts of 40- and 60-d-old leaves. The most dramatic change in the chloroplast ultrastructure with leaf age was the proliferation and the size of the plastoglobuli as indicated by its increased cross-sectional area (Table 1). This was concomitant with the disruption of the membrane system (Fig. 2*B*). For instance, plastoglobuli from chloroplasts of 40-d-old leaves exhibited an average area of 5.68 μm^2 (45.2 % of the chloroplast area) per chloroplast profile (Table 1) contributing to reductions in the volume of the stroma (Fig. 2*B,C*). Some of the grana stacks were pushed by the plastoglobuli towards the periphery of the organelle pressing the thylakoids unusually close together (Fig. 2*B*). Furthermore, large plastoglobuli distended the chloroplasts and distorted the normal disc shape of the organelle (Fig. 2*B*), conspicuous in the 60-d-old chloroplasts (Fig. 2*C*) that resulted in an increase only in chloroplast area, the chloroplast length was not affected (Table 1). These senescent symptoms signify the loss of photosynthetic capacity (Wittenbach *et al.* 1980) and hence, the credibility of membrane dam-

age, acknowledged by the conception of plastoglobuli as a major contributing factor to the frequently observed photosynthetic deterioration in old cotton leaves, is considerably high.

Numerous large plastoglobuli accumulate in the stroma of chloroplasts whenever thylakoid assembly breaks down suggesting that these globuli secreted by a chloroplast serve as the repository for the degraded photosynthetic components such as lipids, chlorophyll, carotenoids, and proteins (Lichtenthaler 1968, Tevini and Steinmüller 1985). One of the leaf factors predominantly associated with such phenomenon of the chloroplasts includes leaf age or senescence during which the membrane loss is accompanied by the formation of plastoglobuli which ordinarily remain in the stroma of the chloroplast (Butler and Simon 1971). In our study, however, a very unique phenomenon was observed in the chloroplasts of old cotton leaves wherein the plastoglobuli, perhaps serving as the pool of photosynthetic components, protruded through the chloroplast envelope and emerged into the cytoplasm (Fig. 2*B*). The genesis of protrusions appeared to be a consequence of plastoglobuli pressing against and squeezing through the envelope membranes of the chloroplast (Fig. 2*C*) providing cues that globule emergence is a secretory process. What prompted the efflux of plastoglobuli into the cytoplasm remains unclear. Nonetheless, it has been suggested that the plastoglobuli are discharged into the cytoplasm for degradation by lipases and proteases to release nutrients, thus the efflux of plastoglobuli into cytoplasm constitutes an important pathway in the breakdown of senescing chloroplasts for redeeming nutrients which are used elsewhere (Guiamét *et al.* 1999).

At first glance, the degradation of the thylakoid membrane system in the chloroplasts of old leaves seemed to be a wasteful process, but in a morphologically complex crop such as cotton it most plausibly serves a constructive role by providing nutrients and growth promoting substances to the developing bolls. Unlike other crops, the morphological complexity of the cotton crop hinders the synchronization of the highest photosynthetic rate of the 20-d-old cotton leaf with the commencement of assimilate utilization by the young subtended boll, thereby suggesting that the yield productivity of bolls is supported by a declining photosynthetic system (Wullschleger and Oosterhuis 1990). Our study provided considerable insight into how the older leaves (Fig. 2*B,C*) with depreciated photosynthetic systems might have accomplished such a feat using cellular secretory mechanisms. Upon senescing, the membrane system of chloroplast degenerates and the products of membrane degeneration are garnered as plastoglobuli for secretion into the cytoplasm where they are enzymatically degraded to unleash nutrients and growth promoting substances for utilization by the acquisitive bolls. If such a feeding mechanism exists, then, this may contribute to concurrent declines in N content and photosynthesis in senescing leaves as observed frequently in cotton plants (Bondada and Oosterhuis 1998) since leaves sequester a large portion of N into their chloroplasts to optimize photosynthesis (Evans 1989).

In conclusion, striations of the epicuticular wax were sparsely distributed on the 10- and 20-d-old leaf surface, and thereafter, the wax spread increased with leaf age. The chloroplast of a 20-d-old photosynthetically active leaf was healthy and disc shaped with infallible membrane integrity evidenced by well developed grana and stroma lamellae aligned parallel to the long axis of the organelle cross section. The internal membrane organization of chloroplasts of leaves older than 20 d was disrupted as manifested by disintegration of grana and stroma lamellae paralleled with the presence of numerous large spherical plastoglobuli which contributed to the distortion of the shape of the organelle. These ultrastructural alterations may have induced inhibitions in photosynthesis during leaf senescence in cotton.

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