Early seedling growth and morphogenesis in the *xantha1* mutant of sunflower with alteration of chloroplast biogenesis

M. FAMBRINI[*], G. CIONINI[**], N. RASCIO[***], F. DALLA VECCHIA[***], and C. PUGLIESI[*,+]

Dipartimento di Biologia delle Piante Agrarie – Sezione di Genetica, Università di Pisa, Via Matteotti 1/B, 56124, Pisa, Italy[*]
Istituto di Biologia e Biotecnologia Agraria, CNR – Area della Ricerca di Pisa, Via Moruzzi 1, 56124, Pisa, Italy[**]
Dipartimento di Biologia, Università di Padova, Via Bassi 56/B, 35131, Padova, Italy[***]

Abstract

In the *xantha1* (*xan1*) mutant of sunflower (*Helianthus annuus* L.), the effects on organ anatomy and seedling growth did correlate to the alteration of chloroplast biogenesis. The *xan1* seedlings grown under 165 μmol(photon) m^{-2} s^{-1} revealed a severely altered chloroplast ultrastructure in cotyledons and leaves. Cross-sections or clarified tissues of the *xan1* cotyledons did not show evident alterations with respect to normal cotyledons suggesting that the impairment of chloroplast biogenesis has negligible consequences on embryonic leaves. By contrast, the analysis of *xan1* leaves showed that the defects in chloroplast biogenesis were correlated to a drastic reduction of organ size and to a clear enhancement of the trichome growth. The differentiation of palisade and spongy parenchyma in cotyledons and leaves of the *xan1* mutant was normal but both organs displayed a drastic reduction in the plastid number with respect to wild type. In addition, *xan1* hypocotyls showed a reduced development of the main vascular bundles in comparison with normal seedlings and an undersized central cylinder of the primary root. The exogenous supply of sucrose was not sufficient to revert *in vitro* the deficit of *xan1* growth and the constraints in morphogenetic processes.

Additional key words: chloroplast ultrastructure; *Helianthus annuus*; *in vitro* culture; pigment-deficient mutant; trichome development.

Introduction

Chloroplasts are lens shaped organelles with outer and inner envelopes localized especially in palisade parenchyma of photosynthetic leaves. Inside, a three dimensional apparatus of photosynthetic membranes arranged in stromal or grana thylakoids characterizes this plastid type (Mustárdy and Garab 2003). The biology of chloroplasts is a wide topic with primary importance to plant life. Green plastids are endosymbiotic remnants not only responsible for the positive carbon balance by photosynthetic process, but also required to biosynthesis of amino acids and fatty acids as well as hormones, vitamins, and many other metabolites (Neuhaus and Emes 2000). The complex functions of chloroplasts depend on the participation of two genomes physically separated into plastid and nucleus (Gray *et al.* 2002). The nucleus influences chloroplast gene expression, and chloroplast, in turn, can modulate the expression of nuclear genes encoding photosynthesis-related proteins (Gray *et al.* 2002).

The histological characterization of leaves in some mutants with defects of chloroplast biogenesis has suggested a model in which a putative plastid-to-nucleus signalling pathway operates early in leaf mesophyll to spread information about the developmental status of the chloroplast (Keddie *et al.* 1996). When the plastid ontogenesis is impaired, the anatomy of leaf mesophyll appears unusual with specific modification of palisade cell fate (reviewed in Rodermel 2001). The signal transduction pathway related to palisade cell development and the retrograde (chloroplast-to-nucleus) signalling that controls nuclear gene expression are probably not the same because in the *defective chloroplast and leaf-mutable* (*dcl-m*) mutant of tomato, the plastid regulation of leaf morphogenesis is specifically impaired (Bellaoui *et al.* 2003). Bellaoui and Gruissem (2004) suggest that essential compounds for organ development are produced by the early-developed plastids; however, the nature of this signal(s) is not known.

The palisade parenchyma cells are usually arranged in rows with columnar shape that facilitates radiation channelling to maximize photosynthesis. The cells in spongy parenchyma are more variable in shape and size and a conspicuous intercellular-space system is the peculiar

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+Corresponding author; fax: +39-50-576750, e-mail: cpuglies@agr.unipi.it

trait of these tissues. Leaf anatomy depends on several factors such as irradiance (Yano and Terashima 2004), hormone content (Barrero *et al.* 2005), growth habitat (Ramesar-Fortner *et al.* 1995), sensibility to phytochrome responses (Cookson *et al.* 2003), cell cycle activity (Dewitte and Murray 2003), or nutritional imbalance (Henriques *et al.* 2002). Moreover, aberrant mesophyll architecture has been described in genotypes characterized by increased sensitivity to ozone (González-Bayón *et al.* 2006).

Evidences about the relationship between the developmental status of chloroplasts and the morphogenesis of leaves are obtained studying two classes of mutants: leaf-variegated and albino plants. The first type is usually composed by viable genotypes characterized by leaves with the presence of green and white-yellow sectors (Sakamoto 2003, Aluru *et al.* 2006). Albino mutants are always lethal and in their cotyledons or true leaves the content of photosynthetic pigments is extremely reduced (Somerville 1986).

It is yet to be evaluated in depth how the metabolic status of the plastids influences the seedling development and growth in other types of genotypes with mutation in nuclear genes for plastid proteins. In this context, the *pale cress* mutant of *Arabidopsis thaliana* with light-green phenotype represents one of the few examples (Holding *et al.* 2000).

In the *xantha1* (*xan1*) mutant of sunflower the ultrastructural abnormalities such as preservation of few giant grana and the presence of fairly substantial amounts of plastid pigments are two traits very dissimilar if compared to the correspondent characteristics of white sectors in variegated plants or albino mutants (Fambrini *et al.* 2004). Evaluating different aspects of plant growth and organ histology we assessed if the abnormal biogenesis of chloroplasts observed in the *xan1* mutant is coupled to a significant alteration of seedling morphogenesis.

Materials and methods

Plant growth: Achenes from homozygous (*XAN1*/*XAN1*) and heterozygous (*XAN1*/*xan1*) self-pollinated plants were germinated in the dark at 25 °C in Petri dishes, on filter paper moistened with distilled water. After 3 d, germinated seeds were transferred to 8-cm diameter pots containing a mixture of soil and sands. Seedlings were grown for 12 d in a growth chamber at 25 °C with a schedule of 16 h of "white light". Irradiance was 165 μmol(photon) m^{-2} s^{-1} provided by cool-white fluorescent lamps (*Philips TLD 36W/33*, *Philips*, Eindhoven, The Netherlands). The growth of the root system was evaluated in seedlings developed from achenes placed between two filter paper sheets on a *Plexiglas* frame partially submerged in distilled water containing mineral nutrients (Lenzi *et al.* 1995). After 12 d of culture, each root system was collected, blotted between two sheets of folder paper, and weighed within 10 s using an electronic balance (±0.1 mg).

Clarification of tissues: Cotyledons and leaves excised from 12-d-old seedlings were cleared with NaOH and chloral hydrate using a method adapted from Ruzin (1999). Tissues were treated overnight in 5 % (m/v) NaOH and then bleached for 5–10 min in commercial bleach half strength. The clarification was completed by the treatment for 24 h with chloral hydrate at 24 °C. Plant material was rinsed several times in distilled water and then mounted for light microscopy (*Wild Makroskop M420*, Herbrugg, Switzerland).

Light and transmission electron microscopy: Cotyledons and leaf explants were excised in the middle portion of lamina from 12-d-old seedlings and immediately fixed overnight at 4 °C in 3 % (m/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9), post-fixed for 2 h in 1 % (m/v) osmium tetroxide in the same buffer and at the same temperature. The specimens were dehydrated in a graded series of ethyl alcohol and propylene oxide and embedded in araldite. Staining with uranyl acetate was carried out while dehydrating with 75 % (v/v) alcohol. Sections were cut with an ultramicrotome (*Ultracut*, *Reichert-Jung*, Vienna, Austria). For light microscopy thin sections (1 μm thick) were stained with basic toluidine blue [1 % (m/v) toluidine blue and 1 % (m/v) Na-tetraborate 1 : 1 by volume] and examined with a light microscope (*Ortholux*, *Leitz*, Wetzlar, Germany). For transmission electron microscopy, ultrathin sections (0.06 μm thick) were stained with lead citrate and observed with a transmission electron microscope (*TEM300, Hitachi*, Tokyo, Japan) operating at 75 kV. Hypocotyl and root explants were collected from 12-d-old seedlings. The sampling of hypocotyls was obtained in the median region while the root portions were excised 0.5–1.0 cm under the neck region. The materials were fixed for 24 h in FAA (formalin/glacial acetic acid/ethanol/distilled water, 10 : 5 : 50 : 35, v/v/v/v) at room temperature before being transferred into 70 % (v/v) ethanol (Fambrini *et al*. 2006). Paraffin-embedded tissues were sectioned using a rotary microtome (*Reichert*, Vienna, Austria). Transverse sections (10 μm thick) were stained in 1 % (m/v) safranin followed by 0.2 % (m/v) fast green and examined with a light microscope (*DMRB*, *Leitz*, Wetzlar, Germany).

Environmental scanning electron microscopy (ESEM) analysis: The observations were carried out on fresh leaves using an environmental scanning electron microscope (ESEM) *XL30 TMP Philips* (*Philips*, Eindhoven, The Netherlands) operating at 15 kV and 1.067×10^2 Pa.

***In vitro* culture**: Naked achenes of both wild type (WT) and *xan1* mutant were sterilized for 20 min in a 2.8 % (v/v) sodium hypochlorite solution [containing 0.01 % (v/v) *Triton X-100*] and rinsed several times in sterile distilled water. Achenes were germinated and grown for 30 d on solidified (8 kg m^{-3}) *Bactoagar*, *Oxoid Ltd.*, Basingstoke, UK) MS basal medium (Murashige and Skoog 1962) in presence of sucrose (30 kg m^{-3}) at 25 °C, under a 16-h photoperiod. Irradiance was 15 μmol(photon) m^{-2} s^{-1} provided by cool-white fluorescent lamps (*Philips TLD 36W/33*). To induce adventitious roots, single node cuttings from 10-d-old micro-propagated plants were excised and immediately sub-cultured in Erlenmeyer flasks on fresh MS basal medium in the presence of sucrose (30 kg m^{-3}) but without hormones (Fambrini *et al.* 2004). The induction of adventitious roots was evaluated after 30 d of cultivation. Hypocotyl segments, 1 cm long, were dissected from seedlings one-week-old. Explants were immediately weighed using an electronic balance (±0.1 mg) and immediately transferred in Petri dish on solidified MS basal medium supplemented with 1 g m^{-3} N^6-benzyl-adenine (BAP) and 1 g m^{-3} 1-naphtalenacetic acid (NAA) to induce callus proliferation. After 30 d of culture under a 16 h photoperiod [15 μmol(photon) m^{-2} s^{-1}], the explants were weighed. The mass increment was calculated as percentage with respect to initial mass of explants to evaluate the callus production.

Statistical analysis was carried out on all characters. Reported values are means from three independent experiments with 20 replicates each (seedlings and/or explants). Differences between mean values were tested by the Student's *t*-test. Either $p<0.05$ or 0.01 were considered significant. Mean values expressed as percentage were assessed statistically after arcsine transformation.

Results

The *xan1* mutant dies after depletion of cotyledonary reserves, and therefore the evaluation of plant growth and the morphological analysis were carried out *in vivo* during an early stage of growth (*i.e.* in 12-d-old seedlings) or *in vitro* (*i.e.* in plants grown for 30 d on MS medium supplemented with sucrose). The mutant seedlings are easily scored *in vivo* on the basis of a pigment-deficient phenotype (Fig. 1*A*). After a growth period of 12 d, under an irradiance of 165 μmol(photon) m^{-2} s^{-1}, the *xan1* mutant was characterized by smaller true leaves (Fig. 1*B*), shorter hypocotyl, and undersized cotyledons than in WT (Table 1). Moreover, in seedlings grown between two filter paper sheets on a *Plexiglas* frame partially submerged in distilled water, the dry mass of the *xan1* root system was significantly reduced in comparison to WT (Table 1).

Table 1. Phenotypic characters of wild type (WT) and *xantha1* seedlings of sunflower (*Helianthus annuus* L.) grown for 12 d in growth chamber under irradiance of 165 μmol(photon) $m^{-2}s^{-1}$. Comparisons between *xantha1* and WT followed by * or ** were different at 0.05 and 0.01 levels of significance according to Student's *t*-test, respectively.

Character	WT	*xan1*
Leaf area [cm^2]	1.71	0.44**
Cotyledon area [cm^2]	3.78	2.71*
Hypocotyl length [cm]	5.52	3.99**
Root dry mass [mg]	22.14	16.39**

Electron microscopic analysis was carried out on both cotyledons and leaves from seedlings grown under 165 μmol(photon) m^{-2} s^{-1}, in order to evaluate the effects of the *xan1* mutation on chloroplast ultrastructure and thylakoid organisation. In 12-d-old cotyledons of WT seedlings, the chloroplasts showed a well organized thylakoid system and a homogeneous stroma (Fig. 1*C*). By contrast, cotyledons of *xan1* seedlings grown under the same irradiance revealed a strong loss of the thylakoid membranes (Fig. 1*D*). The few and small thylakoid stacks were irregularly scattered in a stroma fragmented in a small globular mass (Fig. 1*D*). Well shaped and organized chloroplasts were present in leaves of 12-d-old WT seedlings grown under 165 μmol(photon) m^{-2} s^{-1} (Fig. 1*E*). Chloroplasts in leaves of *xan1* seedlings were irregularly shaped, with abnormal thylakoid stack chaotically distributed in a dishomogeneous stroma. They exhibited some thylakoid stacks irregularly distributed in such stroma and very electron-dense droplets possibly formed by aggregation of lipids and proteins from disassembled thylakoid membranes (Simidjiev *et al.* 1998) (Fig. 1*F*).

The effects of *xan1* mutation on seedling anatomy were analysed by cross sections of cotyledons, leaves, hypocotyls, and roots of seedlings grown under 165 μmol(photon) m^{-2} s^{-1}. In cotyledons and leaves of *xan1*, the organisation of both palisade and spongy parenchyma was not dissimilar with WT (compare Fig. 2*D* and *H* with Fig. 2*C* and *G*, respectively). However, *xan1* cells of both organs displayed a drastic reduction in plastid number with respect to WT (compare Fig. 3*B* and *D* with Fig. 3*A* and *C*, respectively). The extent and the pattern of vascularization (a reticulate venation pattern) were not abnormal in both cotyledons and leaves of the mutant (Fig. 2*A*, *B*, *E*, and *F*) but the falcate trichomes in the abaxial surface of *xan1* leaves were longer than in the WT (Fig. 4*A*–*D*). The *xan1* hypocotyl showed a reduced development of the main vascular bundles than in the WT (Fig. 5*A*, *B*). In addition, an undersized central cylinder characterized the primary roots of the mutant with respect to WT (Fig. 5*C*, *D*).

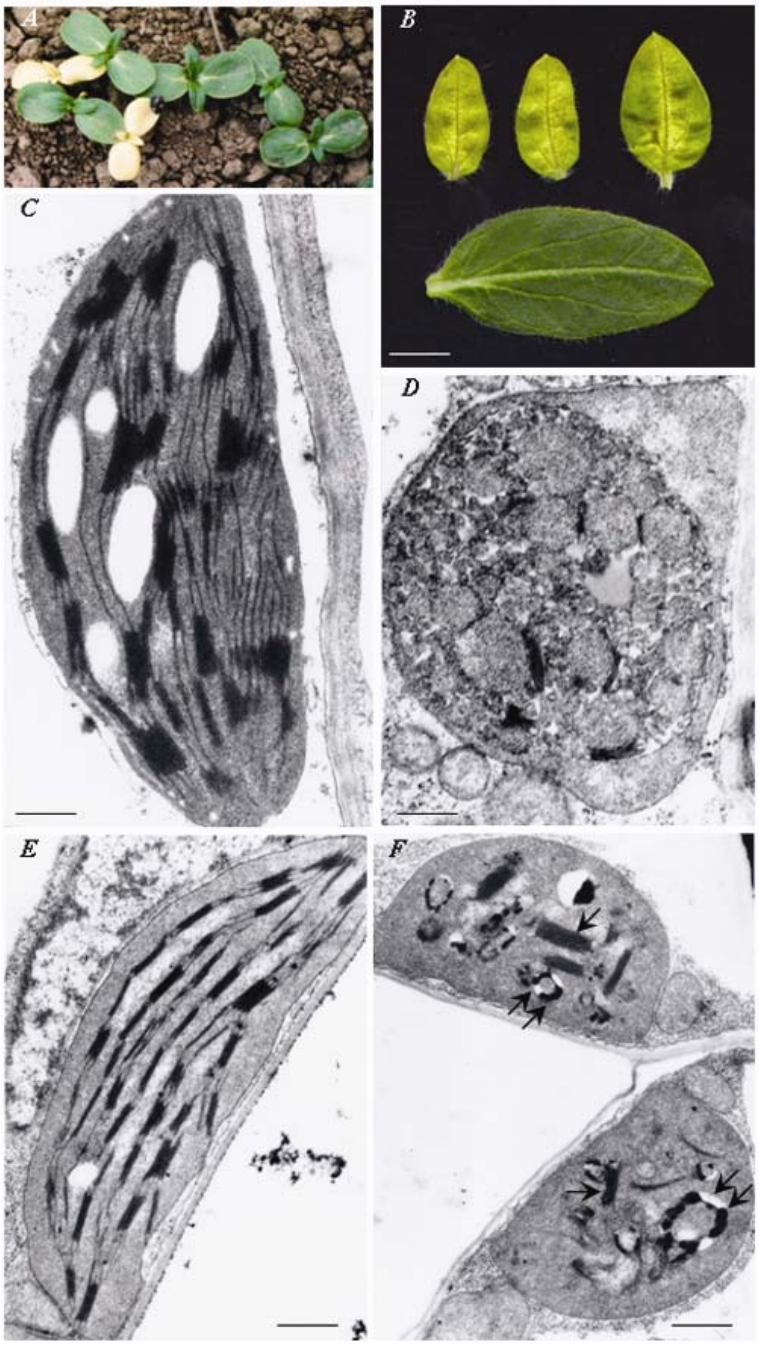

Fig. 1. *A*: Sunflower seedlings of a heterozygous progeny *XAN1*/*xan1*. The *xan1*/*xan1* seedlings are pigment-deficient. *B*: Leaves of the *xan1* mutant (*top*) and wild type (*bottom*) from 12-d-old seedlings grown under irradiance of 165 μmol(photon) m^{-2} s^{-1}. *C–F*: Chloroplast ultrastructure in wild type cotyledons (*C*) and leaves (*E*) and *xan1* cotyledons (*D*) and leaves (*F*) from 12-d-old seedlings grown under the same irradiance. *Bars* = *B*: 4.8 mm; *C*: 0.4 μm; *D*: 0.5 μm; *E*: 0.55 μm; *F*: 0.60 μm. *Arrows* = thylakoid stacks; *double arrows* = electron-dense droplets.

Net CO_2 assimilation rate in the *xan1* mutant is null and the seedlings die *in vivo* at the cotyledonary stage (Fambrini *et al.* 2004, Guidi *et al.* 2006); therefore, to study the plant growth at later stages, *in vitro* experiments were conducted. Despite the presence of exogenous sucrose the mutant seedlings showed a reduced growth in terms of both leaf number and total dry mass (Table 2). Notably, the capability to differentiate adventitious roots of *xan1* hypocotyls was reduced with respect to WT (Table 2). Instead, no differences between genotypes were detected in callus production from hypocotyl explants (Table 2).

Discussion

The processes that coordinate chloroplast differentiation with embryo and plant development have been poorly investigated and mutants characterized by alteration of plastid biogenesis are useful to obtain new insights on this topic (Rodermel 2001, Aluru *et al.* 2006). The developing chloroplast generates a cell-autonomous

signal, which is necessary for proper morphogenesis of palisade cells (Keddie *et al.* 1996). The characterization of albino and/or variegated genotypes in *A. thaliana* (Mandel *et al.* 1996, Aluru *et al.* 2001, Bisanz *et al.* 2003, Kuroda and Maliga 2003, Næsted *et al.* 2004, Hricová *et al.* 2006), tomato (Bonnema *et al.* 1995, Keddie *et al.* 1996), snap dragon (Chatterjee *et al.* 1996), and tobacco (Wang *et al.* 2000, Wycliffe *et al.* 2005), support this hypothesis. On the other hand, the alteration of mesophyll architecture is not necessarily linked to abnormal plastid development. In fact, in *reticulata* (*re*) mutant of *A. thaliana,* the marked change of mesophyll structure observed in interveinal regions was not dependent on plastid development (González-Bayón *et al.* 2006). To evaluate how in a *xantha* mutant the developmental processes are affected by chloroplast function, we analysed different aspects of seedling growth and organ histology of the sunflower *xan1* mutant that displayed specific defects in plastid ontogenesis and a yellow-pale green phenotype (Fambrini *et al.* 2004).

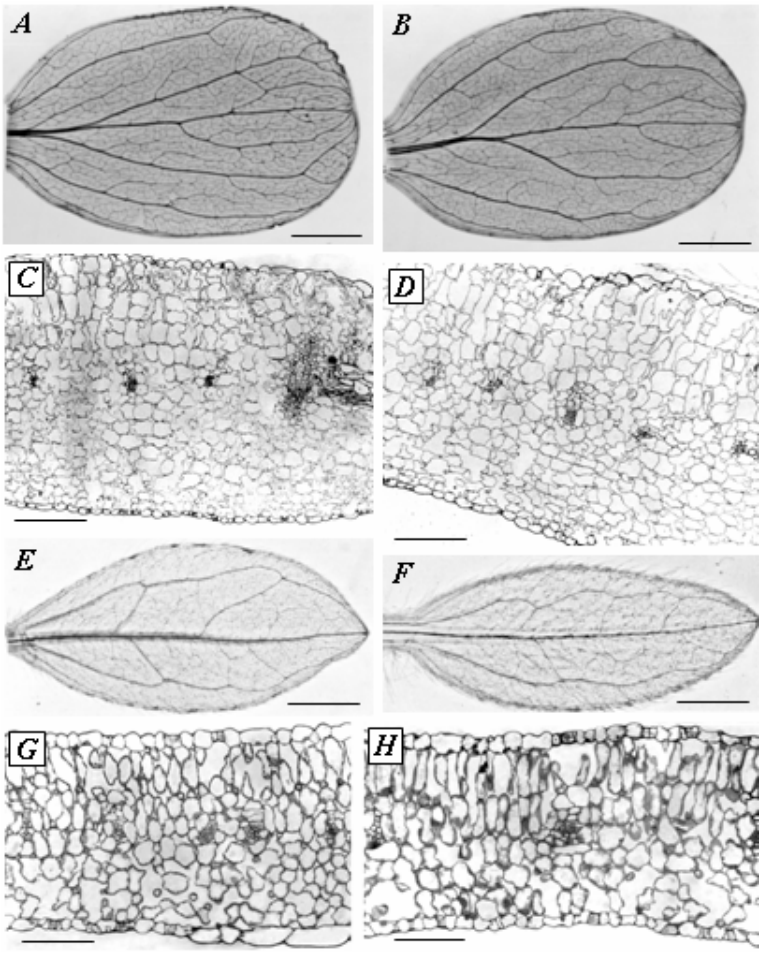

Fig. 2. *A, B, E, F*: Venation pattern in cleared cotyledons (*A, B*) and cleared leaves (*E, F*) of wild type, WT (*A, E*) and *xan1* (*B, F*) seedlings grown for 12 d under irradiance of 165 μmol(photon) m^{-2} s^{-1}. *C, D, G, H*: Cross sections of cotyledons (*C, D*) and leaves (*G, H*) of WT (*C, G*) and *xan1* (*D, H*) seedlings grown for 12 d under irradiance of 165 μmol(photon) m^{-2} s^{-1}. *Bars* = *A*: 4.35 mm; *B, E*: 4 mm; *C, D, G, H*: 100 μm; *F*: 3.1 mm.

Although very similar ultrastructure alterations affect the *xan1* plastids in cotyledons and leaves, amazing reduction of photosynthetic organ size, with respect to WT, was only observed in leaves. Moreover, the impairment of chloroplast biogenesis was compatible with the development of *xan1* cotyledons with a normal anatomic structure and a well-shaped network of main veins. Therefore, in the *xan1* cotyledons the main developmental aspects were not strictly dependent on the proper ultrastructure of chloroplasts. Analogously, the cotyledon histology of the *gun1-1* mutant of *A. thaliana* treated with norflurazon was preserved even in presence of a damaged thylakoid system (Susek *et al.* 1993).

The anatomy of the small *xan1* leaves was not disrupted because palisade and spongy parenchyma were distinguishable; moreover, the columnar shape of single palisade cell was maintained. On the contrary, the albino leaf sectors of the variegated *immutans* mutant of *A. thaliana* are characterized by severe impairment of palisade cell expansion (Aluru *et al.* 2001) while in tobacco, the palisade layer of the *Nonchromosomal Variegated* (*NCV)* mutant leaves showed cells organized in the manner of the spongy parenchyma (Bonnema *et al.* 1995). The contour of smaller *xan1* leaves was not altered and we never observed loss of blade portions, which was reported in other pigment-deficient mutants (Streatfield *et al.* 1999, Wang *et al.* 2000, Ahlert *et al.* 2003). Instead, the abaxial surface of *xan1* leaves was characterized

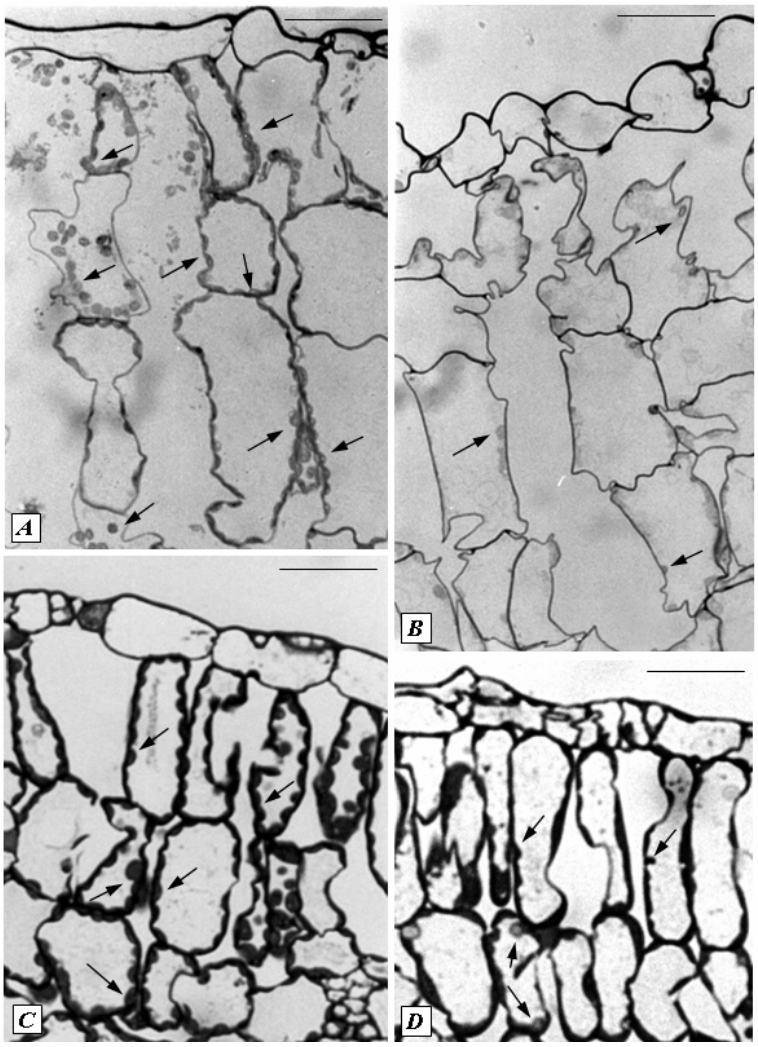

Fig. 3. Cross sections of the palisade region of wild type (*A, C*) and *xan1* (*B, D*) cotyledons (*A, B*) or leaves (*C, D*) from seedlings grown for 12 d under irradiance of 165 μmol(photon) m^{-2} s^{-1}. *The arrows* indicate the chloroplasts. *Bars* = *A*: 210 μm; *B*: 225 μm; *C*: 150 μm; *D*: 145 μm.

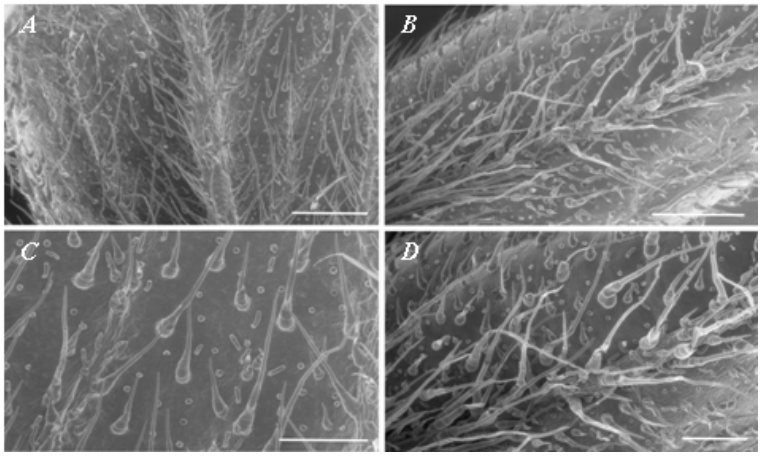

Fig. 4. Environmental scanning electron microscopy (ESEM) analysis of the abaxial surface of wild type (*A*) and *xan1* (*B*) leaves from seedlings grown for 12 d under irradiance of 165 μmol(photon) m^{-2} s^{-1}. *C*: Magnification of *A*. *D*: Magnification of *B*. *Bars* = *A* and *B:* 1 mm*; C* and *D*: 500 μm.

by packed development of elongated trichomes with analogy to the phenotype described for the albino leaves of the *plastid protein import 2* mutant of *A. thaliana* (Asano *et al.* 2004). The *xan1* mutant is very sensitive to irradiation stress (Fambrini *et al.* 2004, Guidi *et al.* 2006), and an ecological role of the increased pubescence observed could be correlated to the enhancement of reflectance properties in the leaf (Johnson 1975).

Taken together, our results obtained with the *xan1* mutant suggest that when the phenotype of seedlings is

yellow-green, the leaf morphogenesis is different with respect to albino leaves or white sectors of variegated plants. The dramatic consequences on mesophyll anatomy are correlated to the widespread and precocious loss of plastid ultrastructure. The thylakoid system of *xan1* chloroplasts in leaves grown for 12 d under 165 µmol m^{-2} s^{-1} is abnormal but some thylakoid membranes with unusual ultrastructure (few giant grana) are maintained also after the early stages of leaf development (see also Fambrini *et al.* 2004). By contrast, plastids in white tissues of albino mutants are usually characterized by only vesicular structures (Chatterjee *et al.* 1996, Wang *et al.* 2000, Aluru *et al.* 2001, Wycliffe *et al.* 2005).

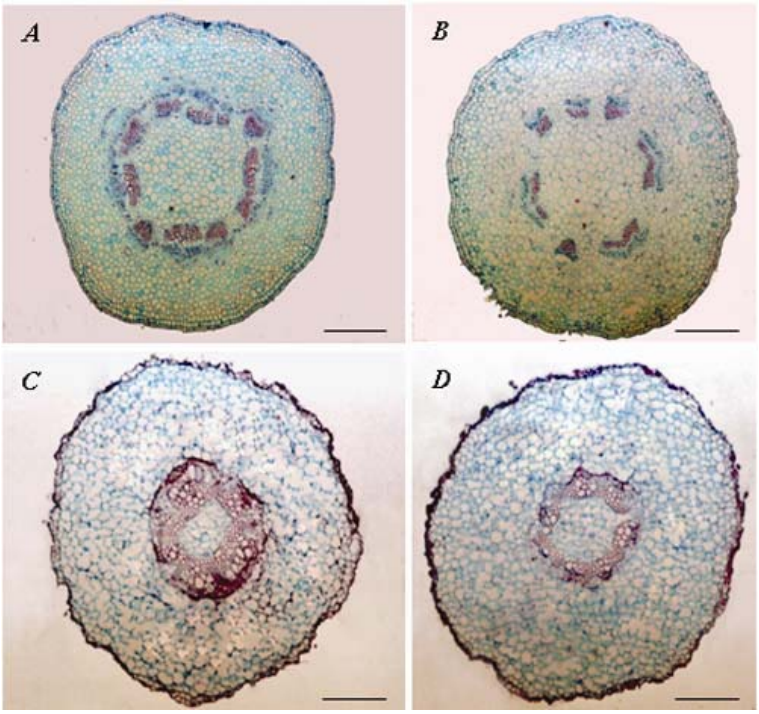

Fig. 5. Cross sections of wild type (*A, C*) and *xan1* (*B, D*) hypocotyls (*A, B*) or primary roots (*C, D*) from seedlings grown for 12 d under irradiance of 165 µmol(photon) m^{-2} s^{-1}. *Bars* = *A*: 5.2 mm; *B*: 4.5 mm; *C*: 2.6 mm; *D*: 2.5 mm.

Table 2. Phenotypic characters of wild type (WT) and *xantha1* plants of sunflower (*Helianthus annuus* L.) after 30 d of *in vitro* culture on MS. The root induction was evaluated on the basis of the percentage of rooted plants after one month of *in vitro* culture. Callus production was calculated as dry mass (DM) increment with respect to the initial DM of hypocotyl explants [%]. Comparisons between *xantha1* and WT followed by ** were different at 0.01 level of significance according to Student's *t*-test.

Character	WT	*xan1*
Number of leaves	12.1	7.6**
Plant DM [mg]	109.0	37.3**
Root induction [%]	100	30**
Callus production [%]	714	721

Photosynthetic organs are not necessarily the exclusive targets of the developmental signals arising from plastids. Abnormal flower morphology has been described in tobacco plants with recombination-induced knockout of chloroplast translation (Ahlert *et al.* 2003). Transgenic tobacco with constitutive expression of pea *Lhcb1-2* gene was characterized by tall size, more leaves, and delay of flowering (Labate *et al.* 2004). Moreover, the deregulation in plastid of the *4.5S* rRNA processing caused in *A. thaliana* several effects on vegetative as well as reproductive growth (Bellaoui and Gruissem 2004). On the basis of the *xan1* lethality, hypocotyl and primary root were the only two organs in which it was possible to analyse, at the histological level, the consequences of this mutation. Both organs of *xan1* plants showed differences in the development of vascular bundles with respect to WT. In particular, the main effect in *xan1* hypocotyl was the smaller size of the metaxylem whereas the central cylinder of the mutant primary root was globally undersized. A similar spectrum of changes has been described in a pigment-deficient mutant of *A. thaliana* deleted in the function of the gene *IRT1*, involved in the metal homeostasis (Henriques *et al.* 2002). On the contrary, tobacco *vdl* mutant deficient in a plastid DEAD box RNA helicase was characterized by the primary root with vascular cylinder completely disorganized (Wang *et al.* 2000).

In vivo growth of the mutant seedlings was altogether repressed because not only the photosynthetic organs of the mutant were smaller than in WT but also the root system was undersized. Moreover, despite the moderate growth irradiance [165 µmol(photon) m^{-2} s^{-1}], the length of *xan1* hypocotyl was remarkably reduced with respect to WT (3.99 *vs.* 5.52). On the contrary, when the seedlings were grown under dim irradiance

[3.5 μmol(photon) m^{-2} s^{-1}], no differences between genotypes were observed (data not shown).

The incompetence to photon energy utilisation displayed by *xan1* can be evocated to explain, almost in part, the reduced growth of the mutant (Fambrini *et al.* 2004, Guidi *et al.* 2006). However, in this early stage of development (the first two weeks after germination), the growth of sunflower seedlings is significantly supported by nutrients accumulated in cotyledons (Kutschera 1992). Therefore, in addition to photosynthetic incompetence other negative factors could be involved to deprive the *xan1* seedlings vigour. In line with this, the exogenous *in vitro* supply of sucrose was not sufficient to modify the scanty growth of *xan1* seedlings.

Notably, *xan1* hypocotyls displayed a lower ability for adventitious rooting than WT while no differences between genotypes were detected for callus proliferation. The root growth from stem internodes requires *de-novo* differentiation of tissues (Swingle 1940); on the contrary, callus is a muddled aggregate of cells with several types of fate (Vasil and Hildebrandt 1965). We hypothesize that the adventitious differentiation of new organs has metabolic requests (reviewed in Malamy 2005) not properly matched with chloroplast impairment. On the contrary, the proliferation of callus is probably not influenced by the plastid status but essentially dependent by the exogenous hormone treatments.

In conclusion, our experiments demonstrated that a lethal mutation, incompatible with the organization and maintenance of a stable and correct thylakoid system, is not linked to the disruption of the photosynthetic organ anatomy. Therefore, the range of developmental alterations in *high-chlorophyll-fluorescence* (*hcf*) mutants with yellow-green phenotype as *xan1* is different from the developmental changes described in albino or variegated mutants. At the same time, this genetic defect modifies the early seedling growth and morphogenesis in several aspects. To clarify the signal transduction pathway related to palisade cell development requires information how the plastid status is checked. Is the complete dismantling of thylakoid system sufficient to preclude the proper development of palisade layers? Does the pigment content play a distinctive role in this process? The thorough analysis of other *xantha* genotypes and/or *chlorina* mutants could be useful to solve some aspects of these questions.

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