THE SUNFLOWER (*HELIANTHUS ANNUUS* L.) MUTANT *XAN1* IS IMPAIRED IN PHOTOSYNTHETIC ACTIVITY.

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Summary: A preliminary characterization of a lethal sunflower mutant (*xantha1; xan1*) defective in photosynthetic activity, has been performed. The nuclear mutation is spontaneous and influences the chloroplast pigments content, the Photosystem II activity, the hypocotyl elongation of de-etiolated seedlings and the capacity of abscisic acid biosynthesis of dehydrated leaves. The phenotype is sensitive to light stress and the accumulation of photoprotective xanthophylls is not able to prevent injury of the PS II reaction center. Further investigations are needed to detect the molecular nature of this mutation.

Introduction:

Sunlight is the primary source of energy for life on earth but its conversion in chemical energy, requires the transient formation of strong oxidants in chloroplast. Both photosynthesis and photoprotection are essential for biosphere, and the study of these processes has key significance for biology because many issues are unresolved.

The use of mutants as experimental tools in the analysis of complex phenomena as photosynthesis, is a well-established approach and large collections of mutants are available in *Zea mays*, *Hordeum vulgare*, *Arabidopsis thaliana*, *Pisum sativum*, *Nicotiana tabacum* and *Chlamydomonas reinhardii* (Somerville 1986; Henningsen *et al.* 1993; Meurer *et al.* 1996; Hippler *et al.* 1998). Nevertheless, few mutants specifically affected in photoprotection have been characterized in higher plants (Niyogi 1999; Shikanai *et al.* 1999). We are interested in studying in sunflower pivotal topics in photosynthesis or photoprotection, by genetic approach. For this purpose, several mutants of *Helianthus annuus* have been recently isolated and in the present work we report preliminary results on the pleiotropic effect of a nuclear mutation (*xantha1*) that precludes the passage from heterotrophyc to photoautrophyc stage.

Materials and Methods:

Plant material

The *xantha1* (*xan1*) analysed in this study is a spontaneous and monogenic mutant, found in a selfed progeny of the inbred line AC/2224 (Dipartimento di Biologia delle Piante Agrarie, Pisa, Italy). The mutant is lethal, and can be maintained by selfing heterozygous progenies only.

In vivo *growth conditions*

Achenes from segregating progenies were germinated in Petri dishes, on filter papers moistened with distilled water, in a growth chamber at 23 ± 1°C in the dark. After 2-3 days, germinated seeds were transferred to 8 cm diameter pots containing a mixture of soil and sand. Seedlings were grown in a growth chamber at 23 ± 1°C, under a 16-h photoperiod. Irradiance at the top of the seedling was provided by a mercury vapour lamp.

Pigment analysis and leaf gas exchange

Leaf samples of 50 mg (fresh weight) were collected from seedlings of the *xan1* mutant and wild type grown under different photosynthetically active photon flux density (PPFDs). Plant pigments were extracted in green light by homogenising leaves in 2 ml of 100% acetone in the presence of sodium ascorbate. The extract was filtered through a 0.2 μm Sartorius filter and immediately analysed by high-performance liquid chromatography (HPLC) using a Non-Endcapped Zorbax ODS column according to the method used by Ciompi *et al.* (1997). Leaf CO_2 assimilation rate (A) was measured in a temperature-controlled cuvette incorporated into an open gas exchange system as previously described (Guidi *et al.* 1997).

Chlorophyll fluorescence

In vivo chlorophyll *a* fluorescence was excited and detected with a pulse amplitude modulation fluorometer (PAM-2000, Heinz Waltz, Effeltrich, Germany) as described by Guidi *et al.* (1997). Measurements were carried out using leaves of seedlings grown at different light intensities, which were previously dark-adapted for 40 min.

Thylakoid isolation

Thylakoid membranes, from leaves grown under dim (3.5 μmol m^{-2} s^{-1}) or moderate light intensity (165 μmol m^{-2} s^{-1}), were isolated according to the method of Ciompi *et al.* (1997).

D1 protein immunoblotting

Thylakoid membranes were dissolved in 2% (w/v) SDS, 4.0 *M* urea, 0.611 *M* β-mercaptoethanol, 2.75 m*M* Tris HCl, pH 6.8, kept at 60°C for 30 min and then separated by SDS-PAGE in 14.6% (w/v) acrylamide, 0.4% (w/v) N,N'-methylene bisacrylamide, containing 6.0 *M* urea and the 3 *M* Tris buffer, as reported by Ciompi *et al.* (1997). Polyclonal antibodies raised against the D1 protein were used, the properties of which have been described by Barbato *et al.* (1992).

ABA measurements

For the estimation of endogenous ABA levels, *xan1* and wt seedlings were grown *in vivo* in the same conditions described above. The PPFD at the top of the seedlings was 3.5 or 635 μmol m^{-2} s^{-1}. The explants were instantly frozen in liquid nitrogen and stored at -80°C, or wilted by cutting off the aerial part from the roots and stored in glass vials when fresh weight (FW) was about 50% of the initial weight. ABA content was measured by immunoassay in crude aqueous extracts (Vernieri *et al.* 1989).

Results:

In vivo the *xan1* seedlings show a yellow-green phenotype and after two weeks, die at the cotyledonary stage. The nuclear mutation (Fambrini *et al.* in preparation) impairs the photosynthetic activity and the photochemical efficiency of Photosystem II (Table 1).

To evaluate the effects of light intensity to the mutant phenotype, seedlings were grown for 14 days in growth chamber in presence of dim (3.5 μmol m^{-2} s^{-1}) or high (635 μmol m^{-2} s^{-1}) light intensity. As reported in table 2, when the plants were grown under high light, the chlorophyll content in cotyledons or leaves of *xan1* is significantly lower with respect to wild type. On the contrary, under dim light condition, the characteristic depigmentated phenotype of mutant is scored only in cotyledons. Analogously, in presence of 3.5 μmol m^{-2} s^{-1} while the leaf carotenoid composition in the mutant is similar with respect that of wild type, in the cotyledons we detect unusual carotenoid complement with high level of VAZ (violaxanthin + antheraxanthin + zeaxanthin) and a low content of β-carotene. These differences with respect to wild type are accentuated when the seedlings were grown under high light. In particular, it is noteworthy the considerable content of zeaxanthin. The carotenoid composition of mutant leaves is strong altered respect to wild type only in high light condition. Furthermore the lutein level of *xan1* is never significant different when compared to that of wild type (Table 3). Under high light seedlings show short hypocotyl, marked dysfunction of PS II activity, loss of D1 protein and a strong reduction of ABA biosynthesis stress-induced (Table 4).

Discussion

The survival of the *xan1* mutant is restricted to the heterotrophic stage and the viability of seedlings can be improved *in vitro* by sucrose (data not shown). Leaf gas exchange analysis reveals that the *xan1* mutant is photosynthetically inactive while the chlorophyll *a* fluorescence shows a significative reduction of photochemical efficiency of Photosystem II. These results warrant the lethality observed *in vivo*. Several aspects of mutant phenotype depend on the light intensity utilised during the growth. Under high light the mutant leaves are chlorophyll deficient while xanthophylls involved in photoprotection are accumulated. The strong reduction of β-carotene content and the depletion of the D1 protein already at 165 μmol m^{-2} s^{-1}, suggest that in the mutant the reaction center of PS II is heavily damaged also in moderate light condition. Photosynthetic mutants as *xan1,* are frequently characterized by pleiotropic effects as recently demonstrated in the nuclear *hcf5* mutant of Arabidopsis (Dinkins *et al.* 1997), and their phenotype can be influenced by the light intensity of growth (Dauborn and Brüggemann 1998).

The impairment of photosynthesis makes the *xan1* mutant light-sensitive and this characteristic is confirmed by the data on table 4. If photosynthesis is not able to employ the light-energy adsorbed, photodamage can induce chlorosis and inactivation of PS II (Heck *et al.* 1999). Nevertheless, it is noteworthy that in *xan1* mutant the PS II activity and the pigment complement of cotyledons are altered with respect to wild type also in dim light condition. Therefore, at least for the last two characteristics of *xan1* mutant, is not possible to invoke photodamage. *xan1* cotyledons grown in dim light are not characterised by protochlorophyllide accumulation (data not shown). Consequently, the reduced content of chlorophyll in cotyledons is not probably associated to deficiency of biosynthesis. In sunflower during the etioplast-chloroplast transformation, thylakoids appear together with the chlorophyll synthesis (Rascio *et al.* 1979). For this reason, the depigmentated phenotype of *xan1* cotyledons can be associated to a defect of thylakoid biogenesis. To evaluate this hypothesis thylakoids analysis with electron microscopy will be necessary. An outstanding effect observed in *xan1* subjected to high light is the reduced increment of the ABA content in leaves or cotyledons (data not shown) after dehydration. In water-stress tissues the ABA biosynthesis depends on the cleavage of 9-cis-epoxycarotenoids and this reaction takes place in the thylakoids (Qin and Zeevaart 1999). In *xan1* seedlings the low ABA level after dehydration, can be a consequence of the premature senescence induced by light stress. During senescence, a sequential degradation of pigments, lipids and proteins is activated (Biswal 1995) and in detached leaves of *Citrus* the ABA increase after water stress, decreased in senescent tissues if compared to the accumulation of hormone detected in younger leaf (Norman *et al.* 1990). The biochemical nature of *xan1* mutation is actually unknown but the results here reported suggest that the product of XAN1 gene is essential to enable the conversion of light energy into reducing power.

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Table 1. Maximum CO_2 assimilation rate (A_{max}/gChl) and photochemical efficiency of Photosystem II (PS II) ratio (F_v/F_m) of wild type (wt) and *xantha1* (*xan1*) seedlings grown at 165 μmol m^{-2} s^{-1}.
F_m = maximal fluorescence; F_v = variable fluorescence.

PARAMETERS	wild type	*xantha1*
Amax	5.39 a	0.64 b
Fv/Fm	0.826 a	0.067 b

Values within the same line followed by different letters are significantly different (P = 0.001) according to Student's *t*-test.

Table 2. Effect of different light intensities (μmol m^{-2} s^{-1}) on chlorophyll (*a*+*b*) content (μg gFW^{-1}) in cotyledons and leaves of wild type (wt) and *xantha1* (*xan1*) mutant.

	3.5 μmol m-2 s-1		635 μmol m-2 s-1	
ORGAN	wild type	*xantha1*	wild type	*xantha1*
COTYLEDON	550.5 a	307 b	569.4 a	20.8 c
LEAF	668.2 b	579.8 b	1106.2 a	58.6 c

The data were treated using analysis of variance procedures and means were separated using Tukey's test. Values within the same line followed by different letters are significantly different (P = 0.01).

Table 3. Carotenoid composition (% of the total) of wild type (wt) and *xantha1* (*xan1*) grown in dim light (3.5 μmol m^{-2} s^{-1}) or high light (635 μmol m^{-2} s^{-1}).
VAZ = violaxanthin+antheraxanthin+zeaxanthin.

		3.5 μmol m-2 s-1		635 μmol m-2 s-1	
ORGAN	PIGMENTS	wild type	*xantha1*	wild type	*xantha1*
COTYLEDON	VAZ	11 a	17 b	20 b	41 c
	Lutein	52 ab	64 b	44 a	48 a
	Zeaxanthin	0 a	2 b	3 b	26 c
	β-carotene	28 c	13 b	28 c	6 a
LEAF	VAZ	28 a	21 a	27 a	41 b
	Lutein	45 a	48 a	48 a	51 a
	Zeaxanthin	0 a	0 a	0.3 a	29 b
	β-carotene	14 b	21 b	16 b	5 a

The data were treated using analysis of variance procedures and means were separated using Tukey's test after arcsin transformation of the percentage. Values within the same line followed by different letters are significantly different (P = 0.01).

Table 4. Pleiotropic effects of *xantha1* mutation on several characters of seedlings grown in different light intensities (μmol m^{-2} s^{-1}). $\Phi_{PS\ II}$ = actual quantum yield of PS II; D1 protein = *psbA* gene product (arbitrary units); Abscisic acid (ABA) increase (increase of ABA content after dehydration calculated as percentage respect to hydrated control; see Material and Methods).
(*****) The experiment of drought stress was conducted with seedlings grown under high light (635 μmol m^{-2} s^{-1}).

	3.5 μmol m-2 s-1		635 μmol m-2 s-1	
CHARACTER	wild type	*xantha1*	wild type	*xantha1*
Hypocotyl length (mm)	14.07a	13.57 a	5.52 a	3.99 b
Φ PS II	0.643 a	0.484 a	0.714 a	0.018 b
D1 protein content	15.9 a	16 a	58.5 a	5.1 b
ABA increase (*)	1113 b	1400 a	287 a	11 b

The statistical analysis was done separately for the two light intensities used. Values within the same line followed by different letters are significantly different (P = 0.01) according to Student's *t*-test.