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Characterization of a sunflower (*Helianthus annuus* L.) mutant, deficient in carotenoid synthesis and abscisic-acid content, induced by in-vitro tissue culture

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Abstract. Genetic variation induced by tissue culture has been characterized in many species. The present study was conducted to genetically and phenotypically characterize an albino mutant in sunflower induced by in-vitro culture. A single recessive gene defective in carotenoid biosynthesis eventually leads to a chlorophyll loss due to photobleaching, absence of seed dormancy, and a low level of endogenous abscisic acid (ABA) in cotyledons and leaves. Further characterization has shown that the endogenous level of the hormone does not increase after drought stress and that the mutation prevents anthocyanin synthesis.

Key words: Abscisic acid – Carotenoid-deficient mutant – *Helianthus annuus*

Introduction

Changes in both quantitative and qualitative traits have been described amongst regenerated plants. An analysis of the selfed progenies of regenerants has demonstrated that many of the observed changes are heritable due to genetic differences. These include numerical and structural chromosome variation, point mutations, transposition of DNA sequences, and changes in the mitochondrial and chloroplast genome (Karp 1991).

In sunflower, we have previously described (Pugliesi et al. 1991) the genetic variation induced by in-vitro tissue culture by scoring the chimerism of regenerants which derived from plants heterozygous for two monogenic characters expressed in the fruit epidermis and in the ray flowers of the capitulum. Furthermore, the analysis of the selfed progeny of regenerated plants showed the presence of albino seedlings. In this report, we describe an albino mutant of *Helianthus annuus*, hereafter called *non-dormant* (*nd-1*), induced by in-vitro tissue culture, in which the genetic lesion seems to impair one of the early steps in carotenoid biosynthesis resulting in both photobleaching and abscisic acid (ABA) deficiency. The features of this mutant support the idea that in *Helianthus*, as previously demonstrated with corn (Moore and Smith, 1985) and *Arabidopsis* (Duckham et al. 1991; Rock and Zeevaart 1991), ABA is synthesized from carotenoid precursors.

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Materials and methods

Plant material

The *nd*-1 mutant analyzed in this study was found in the selfed progeny of an in-vitro regenerated plant. Regeneration was obtained from cotyledonary explants of an inbred isogenic (nine generations of selfing) sunflower line (Pugliesi et al. 1991). The *nd-1* mutant is not capable of independent existence. Under normal light conditions, the seedlings lack chlorophyll and form only one pair of true leaves. Under low intensity illumination, chlorophyll is formed, but in both cases seedlings live only for 2–3 weeks, after which they die. The origin of the genetic material used in this study is summarized in Fig. 1.

To determine chlorophyll and carotenoid levels, seedlings were grown for 7 days after germination in a growth chamber at 25 ± 1 °C. For anthocyanin measurements, 1-week-old seedlings grown at two different temperatures (5 °C and 25 °C) were used. In all experiments, seedlings were maintained at a 16 h photoperiod. White light (WL) was obtained from Philips (Eindhoven, The Netherlands) TLM 40W/33 RS fluorescent tubes.

In some experiments, seedlings were grown for 1 week in darkness.

Chlorophyll determination

Seedlings of the *nd*-1 mutant and of the green sibs (i.e., collected from the same head) were grown in the dark and under WL and

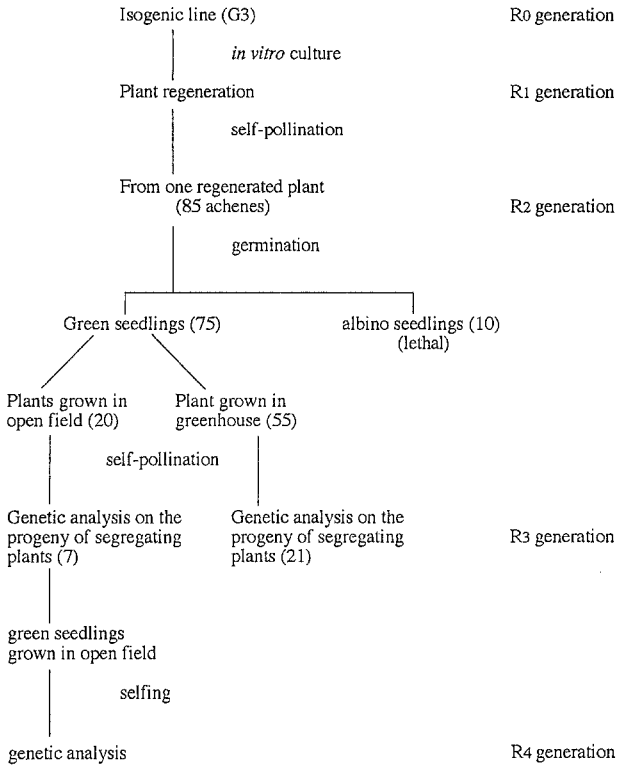

Fig. 1. Origin of the albino mutation and of the genetic materials used in this study. The *numbers* in brackets represent the number of seedlings or plants

the standard photon fluence rates were 12 and 180 μmol m^{-2} s^{-1}. Twenty to thirty 1.13 cm^2 leaf disks of each genotype and light treatment were removed and placed in N-N dimethylformamide (DMF) (Moran 1982). Samples were incubated in the dark at 4 °C for 24 h. Absorption readings were made on a UV-visible light spectrophotometer Shimazu at 625, 647 and 664 nm. All values presented are the means of experiments replicated at least three times (9–12 replicates).

Anthocyanin measurement

Hypocotyls of the *nd*-1 mutant and of green sib-seedlings gorwn in the dark at two different temperatures and under WL (standard photon fluence rate of 180 μml m^{-2} s^{-1}) were extracted in 10 cm^3 of acidified (1% HCl, w/v) methanol for 2 days at 4 °C, with shaking at 12-h intervals. Extracts were clarified by filtration and absorbance values were measured at 530 and 657 nm. According to Mancinelli and Rabino (1985), the formula $A_{530}-A_{657}$ was used to compensate for the absorption of chlorophyll derivates at 530 nm. All values presented are the means of experiments repeated at least three times.

Carotenoid determination

From 40 seedlings grown under WL at the standard photon fluence rate of 180 μmol^{-2} s^{-1}, samples of the two genotypes were extracted in the dark with 3 ml/gm tissues of acetone. After the addition of 3 ml/gm tissue of petroleum ether, the extract was clarified by centrifugation. The upper phase was then dried under a stream of nitrogen and resuspended in petroleum ether (b.p. 35–65 °C). Absorption spectra were obtained with a Beckman (DU-70 spectrophotometer. When necessary, the petroleum ether extract was further fractionated by thin layer chromatography on silica gel plates (Giuliano et al. 1986).

Table 1. Summary of genetics results for the R_3 generation of the albino phenotype

Growing conditions	Number of independent R_3 progenies	Number of selfed progeny plants		
		Green	Albino	χ^2(3:1)
Field	7	935	148(13.7)	84.2***
Greenhouse	21	2140	660(23.6)	5.8

In brackets: the percentage of albino seedlings
*** $P > 0.001$

ABA measurements

For the estimation of endogenous ABA levels, *nd*-1 and green sib-seedlings were grown for 2 weeks in a growth chamber (temperature 25 °C at 70% relative humidity, 16-h fluorescent light at 12 μmol m^{-2} s^{-1}, 12-h dark). The tissues collected (cotyledons and leaves) were either instantly frozen in liquid nitrogen and stored at −20 °C, or wilted by cutting off the aerial part from the roots and stored in glass vials. Fresh weight was determined every half-hour. When the fresh weight was about 50% of the initial, cotyledons and leaves were frozen as above. The last weighting was used to correct for weight loss.

ABA was measured by immunoassay in crude aqueous extracts of tissue (50–100 mg FW). Samples frozen in liquid nitrogen were extracted with distilled water for 16 h at 4 °C in the dark. Aliquots (50 μl) of extract were then analyzed using a solid-phase RIA based on monoclonal antibody raised against free s(+)-ABA, as previously described (Vernieri et al. 1989).

Results

Mode of inheritance of the albino phenotype and absence of dormancy in the nd-1 mutant

Twenty plants from seventy-five of the R_2 seeds were grown in an open field and, after self-pollination, seeds from seven of them produced albino seedlings on germination. In Table 1 the total number of germinating achenes and the number of albino seedlings per head are reported. The percentage of albino seeds in heterozygous heads was 13.67 which is not consistent with a single recessive ratio as shown by a chi-square (χ^2) test.

On the other hand, the segregation data for seeds from 55 self-pollinated R_2 plants grown in a greenhouse under controlled environmental conditions (Table 1) gave an almost perfect single recessive ratio (23.57%).

The R_3 green seedlings derived from plants grown in the field were self-pollinated and the number of heads segregating for the albino character was 600 in a total of 904, showing a ratio between heterozygous and homozygous plants of 1.97 and confirming that the albino phenotype is controlled by a single recessive gene.

Further observations on self-pollinated, field-grown heterozygous plants showed that before harvest, in presence of a high relative humidity, premature germination of the albino seeds occurred in the head. These seedlings

Table 2. Effect of white fluence rates on the chlorophyll content of the *nd-1* mutant and an isogenic line

Fluence rate (μmol m^{-2} s^{-1})	*nd-1* mutant				wt-sibs			
	Proto-	a	b	Total	Proto-	a	b	Total
Total darkness	12.9	4.4	6.2	23.5	13.7	3.7	3.8	21.2
	(54.89)	(18.72)	(26.38)		(64.62)	(17.53)	(17.92)	
SE	1.1	0.6	0.7		1.2	0.3	0.5	
12	15	100	20	135	21	154	28	203
	(11.11)	(74.07)	(14.81)		(10.34)	(75.86)	(13.79)	
SE	4.1	9.01	4.03		3.2	15.1	10.9	
180	0	0	0	0	116	891	353	1,360
					(8.52)	(65.51)	(25.95)	
SE					20.03	45.1	15.3	

Chlorophyll content: mg/kg fresh weight. In brackets: percentage of pigment

survived for 2–3 days, until death was caused by drying of the head.

We have observed a similar lack of dormancy in sunflower seeds homozygous for the albino character in germination experiments involving seeds freshly harvested from self-pollinated heterozygous plants. The homozygous recessive *nd-1* seeds germinated uniformly and quickly, whereas no germination was observed for green sib-seeds. Their germination increased after storage for 1 or 2 months from 0 to almost 100%. In the precocious germination of albino seeds no differences were observed between decoated and complete fruits.

Since the *nd-1* mutant must be maintained as a heterozygote, the lack of dormancy can be used to identify the segregating heads of heterozygous plants.

Analysis of pigments

In Table 2 quantitative determinations of the chlorophyll concentrations in the *nd-1* and green sib-seedlings grown in total darkness and at two light conditions are given.

The values of protochlorophyll, chlorophyll a and chlorophyll b in total darkness agree quite closely between the mutant and its green sibs. From data of chlorophyll production and accumulation after prolonged exposure to weak light the chlorophyll content in the albino mutant is 2/3 that of green sibs with little or no difference between them in respect to of a/b ratios observed. With a fluence rate of 180 μmol m^{-2} s^{-1} the mutant seedlings are completely white because they totally lack chlorophyll. It is particularly interesting to note that white light and temperature (Table 3) have no measureable effect on anthocyanin synthesis, while a high fluence rate resulted in a stimulation of anthocyanin synthesis in the green sibs which breed true for this character.

Figure 2 shows the absorption spectra of petroleum ether extracts of the *nd-1* mutant and the wild-type when grown under high photon fluence rate. In the green line the observed maxima indicate the presence of chloro-

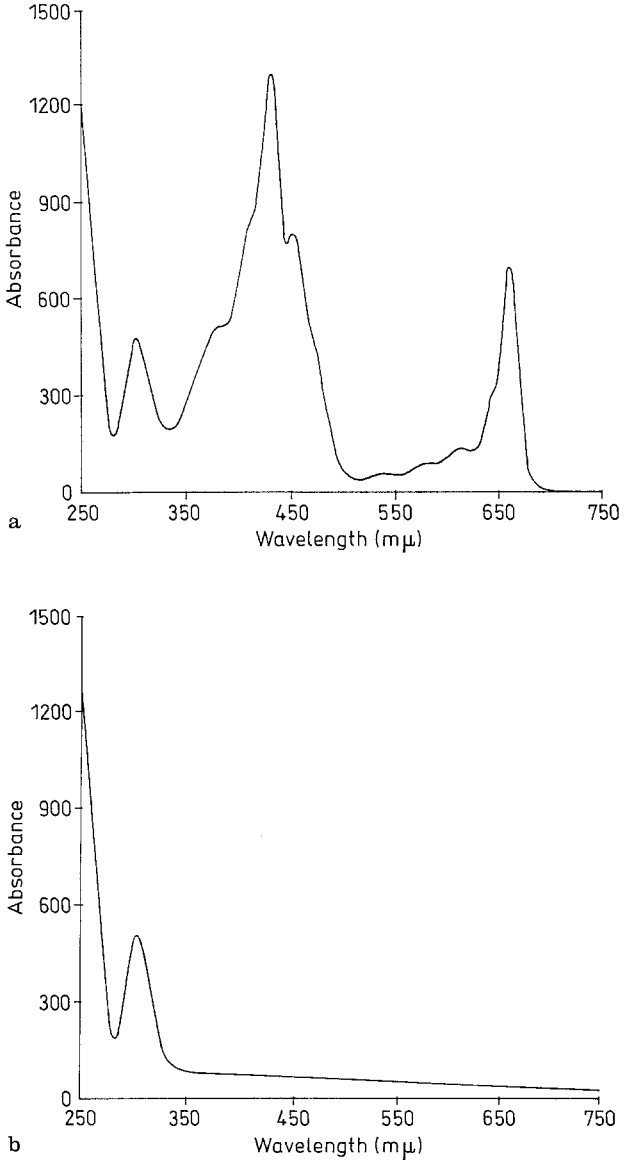

Fig. 2. A, B. Absorption spectra of green-sib (**A**) and *nd-1* mutant (**B**) seedlings

Table 3. Effect of white light (180 $\mu mol^{-2}\ s^{-1}$) and temperature on the anthocyanin content of the *nd-1* mutant and an isogenic line. Anthocyanin content: absorbance for 100 g of fresh material ± standard error

Treatment	Temperature (°C)	Genotype	
		nd-1	Isogenic line
Dark	5	0	0
	25	0	0
WL	5	0	0.631 ± 0.02
	25	0	0.568 ± 0.03

Table 4. Levels of ABA (ng/g fresh weight) in non-stressed (NS) and stressed (S) cotyledons and leaves of the *nd-1* mutant and its green sibs

Genotype	Tissue	Treatment	
		NS	S
nd-1 mutant	Cotyledons	5.1 ± 0.5	4.3 ± 0.5
	Leaves	2.2 ± 0.6	0.5 ± 0.2
Green sibs	Cotyledons	10.3 ± 1.6	29.3 ± 1.4
	Leaves	13.5 ± 0.9	30.8 ± 1.5

phyll and carotenoids while in the *nd-1* mutant no evidence for the presence of chlorophyll or the coloured carotenoids was found.

No evident peaks diagnostic of UV-absorbing carotenoid precursors (phytoene, phytoluence or ζ-carotene) were observed (Guiliano et al. 1986; Scolnik et al. 1987). Nevertheless, since a peak with an absorption maximum around 280 nm, which could mask tiny amounts of accumulated phytoene, is observed both in the wild-type and in the mutant, the extract was further fractionated by thin layer chromatography using as a standard phytoene derived from seedlings of the tomato *ghost* mutant (Scolnik et al. 1987). No compound comigrating with phytoene was observed in this system.

Taken together, these data indicate that carotenoid biosynthesis is presumably arrested in the mutant before the formation of phytoene.

ABA levels in albino mutant and wild-type

Quantitative estimation of levels of ABA in extracts of cotyledons and leaves of the *nd-1* mutant and green sibs (Table 4) show that the albino mutation is associated with a significant reduction in endogenous ABA level ($P<0.01$). Statistical analysis of the results was performed using a paired sample t-test. Detached cotyledons and leaves of the wild-type responded to a rapid stress by producing ABA. After about 2 h the levels had increased from an average of 12 to 30 nmol g^{-1} (150% increase). Detached leaves of *nd-1* failed to respond to the stress by synthesizing ABA.

Discussion

Mutants with a low endogenous content of abscisic acid (ABA) and a reduced capacity to accumulate the hormone in response to water stress have been isolated from a number of species (Koornneef 1986). All ABA-deficient mutants in dicot species have normal levels of chlorophyll and many show a wilty phenotype due to their abnormal stomatal behaviour.

The best characterized wilty, ABA-deficient, mutants are the *notabilis* (*not*), *flacca* (*flc*) and *sitiens* (*sit*) mutants of tomato (Tal 1966; Tal and Nevo 1973; Neil and Horgan 1985). Wilty ABA-deficient mutants of potato (*droopy;* Quarrie 1982), pea (*wilty*; Wang et al. 1984), *Arabidopsis thaliana* (*aba*; Koornneef et al. 1982) and *Nicotiana plumbaginifolia* (*CRK1*; Blonstein et al. 1991) have also been reported.

In some mutants in monocost-species, low ABA concentrations are accompanied by a large reduction in chlorophyll content and often they show precocious germination. Some of the viviparous mutants of maize fall in this category. In these mutants, the genetic lesions involved specific steps in the carotenoid pathway (Moore and Smith 1985).

The *nd-1* mutant of sunflower, as is the case for all ABA-deficient mutants, is monogenic recessive and is carotenoid deficient like the maize *viviparous* mutants. The primary lesion leads to the following multiple metabolic defects: chlorophyll is destroyed under high light intensity, the endogenous ABA content is lower than in the isogenic line, the capacity to accumulate the hormone in response to water stress is lacking, and seeds show a lack dormancy. Le Page-Degivry et al. (1990) showed in sunflower, that the endogenous ABA level increased sharply during embryo development and an application of fluridone, an inhibitor of carotenoid biosynthesis, prevented both ABA synthesis and the development of embryo dormancy. The results with the *nd-1* mutant confirm that the lack of dormancy may be correlated with the lack of ABA synthesis in developing embryos. Our observation that the seed coat does not control the dormancy indicates that its induction occurred because the embryos were able to synthesize ABA and was independent of the presence of maternal ABA.

The relationship between the blocks in the carotenoid pathway and the effects on ABA provide strong arguments for the indirect C_{40} (apo-carotenoid) route (Li and Walton 1990; Duckham et al. 1991; Rock and Zeevaart 1991; Parry and Horgan 1992).

In sunflower, a non-dormant, carotenoid-free, white mutant which has not been characterized with respect to ABA was described by Wallace and Habermann (1959).

The mutant was obtained from the progeny of a single ultrasonically-treated seedling and is no longer available. However, Walles (1965) when describing the structural organization of the plastids in leaves of the white mutant reported that white seedlings could develop anthocyanin colour at low temperature. The *nd-1* mutant is unable to accumulate anthocyanin under light and low temperature treatments suggesting that the metabolic block is probably located in a different step of the pathway. Many stimuli can initiate anthocyanin synthesis. These can be environmental signals, such as cold, hormone levels and sensitivity, quality of light, or developmental age. The arrest in anthocyanin synthesis in the *nd-1* mutant is not completely understood. Carotenoid-deficient mutants of tomato (*ghost*) (Scolnik et al. 1987) and maize (*Vp7/ps-1*) (Neill et al. 1986) are able to synthesize anthocyanins in response to white light. The only mutant in which an interaction of ABA and anthocyanin biosynthesis has been reported is the *Vp-1* mutant of maize which is a regulatory mutant insensitive to ABA and deficient in anthocyanin synthesis (Robichaud and Sussex 1986; Mc-Carty et al. 1989). Hattori et al. (1992) suggest that *Vp1* activates C1 specifically during seed maturation by interacting with one or more ABA-regulated transcription factors. The failure to achieve this interaction in *Vp-1* would be consistent with the hormone-insensitive phenotype of this maize mutant. Our results in sunflower indicate that the mutation which affects the level of ABA in the seedlings also inactivates the anthocyanin pathway, thus supporting the regulatory pathway proposed by the previous authors (Hattori et al. 1992).

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