mesophyll cell defective1, a mutation that disrupts leaf mesophyll differentiation in sunflower

M. FAMBRINI[*], E. DEGL'INNOCENTI[**], G. CIONINI[***], C. PUGLIESI[*,+], and L. GUIDI[**]

Dipartimento di Biologia delle Piante Agrarie, Sezione di Genetica, Via Matteotti 1B, I-56124 Pisa, Italy[*]
Dipartimento di Chimica e Biotecnologie Agrarie, Via del Borghetto 80, I-56124 Pisa, Italy[**]
Consiglio Nazionale delle Ricerche, Istituto di Biologia e Biotecnologia Agraria, Sezione di Pisa Via, Moruzzi 1, I-56124 Pisa, Italy[***]

Abstract

Mutants with altered leaf morphology are useful as markers for the study of genetic systems and for probing the leaf differentiation process. One such mutant with deficient greening and altered development of the leaf mesophyll appeared in an inbred line of sunflower (*Helianthus annuus* L.). The objectives of the present study were to determine the inheritance of the mutant leaf trait and its morphological characterisation. The mutation, named *mesophyll cell defective1* (*mcd1*), has pleiotropic effects and it is inherited as a monogenic recessive. The structure and tissue organization of *mcd1* leaves are disrupted. In *mcd1* leaves, the mesophyll has prominent intercellular spaces, and palisade and spongy tissues are not properly shaped. The mutant palisade cells also appear to be more vacuolated and with a reduced number of chloroplasts than the wild type leaves of equivalent developmental stage. The lamina thickness of *mcd1* leaves is greatly variable and in some areas no mesophyll cells are present between the adaxial and abaxial epidermis. The leaf area of the *mcd1* mutant is extremely reduced as well as the stem height. A deficient accumulation of photosynthetic pigments characterizes both cotyledons and leaves of the mutant. In *mcd1* leaves, chlorophyll (Chl) fluorescence imaging evidences a spatial heterogeneity of leaf photosynthetic performance. Little black points, which correspond to photosystem II (PSII) maximum efficiency (F_v/F_m) values close to zero, characterize the *mcd1* leaves. Similarly, the light-adapted quantum efficiency (Φ_{PSII}) values show a homogeneous distribution over wild type leaf lamina, while the damaged areas in *mcd1* leaves, represented by yellow zones, are prominent. In conclusion, the loss of function of the *MCD1* gene in *Helianthus annuus* is correlated with a variegated leaf phenotype characterized by a localized destruction of mesophyll morphogenesis and defeat of PSII activity.

Additional keywords: carotenoids; chlorophyll; chlorophyll fluorescence imaging; *Helianthus annuus* L.; leaf development.

Introduction

Leaves development initiates by recruitment of cells from the flanks of shoot meristems (Sinha 1999, Byrne 2005, Fleming 2005). Cells in the different meristematic layers undergo rounds of coordinated cell division to form a primordium. Cells on the flanks of this primordium in turn undergo rounds of cell division and expansion to form the lamina. The shape, size, and growth of leaves depend on the coordination of several processes, such as cell division, expansion, and differentiation (Tsukaya 2006). Leaves are not generated in a random fashion, but rather in a consistent pattern over space and time, producing the regular architecture of the plant (Fleming 2005, Reinhardt 2005, Smith *et al.* 2006).

In most angiosperms, a newly formed primordium undergoes lateral growth (*i.e.* becomes flatter) and growth along the proximal-distal axis (*i.e.* becomes longer). The phase of lateral growth results from dorsoventral (adaxial-abaxial) patterning events that occur during the earliest stages of leaf development (Waites and Hudson 1995, Eshed *et al.* 2004, Byrne 2005, Fleming 2005).

Received 15 June 2009, *accepted* 2 December 2009.
[+]Author for correspondence; fax: +39 (0) 502216661, email: cpuglies@agr.unipi.it
Abbreviations: Car – carotenoid; Chl – chlorophyll; Chl *a* chlorophyll *a*; Chl *b* – chlorophyll *b*; Chl *a/b* – chlorophyll *a/b* ratio; Chl (*a* + *b*)/Car – chlorophyll/carotenoid pool ratio; ETR – electron transport rate; F_0 – initial fluorescence; F_0' – light-adapted F_0; F_m – maximum fluorescence emission; F_m' – maximum fluorescence; F_t – current fluorescence yield; F_v – variable fluorescence; F_v/F_m – maximum photochemical efficiency of photosystem II; F_v'/F_m' – efficiency of excitation capture by open photosystem II reaction centers; *mcd1 – mesophyll cell defective1*; PPFD – photosynthetic photon flux density; PSII – photosystem II; q_{NP} – non-photochemical quenching of chlorophyll fluorescence; Φ_{PSII} – actual quantum yield of photosystem II electron transport.

The molecular mechanism underlying the spatially separated acquisition of adaxial and abaxial fate is still being explored, but a process of intercellular communication coupled to transcription factor patterning has been elucidated (Waites and Hudson 1995, McConnell and Barton 1998, McConnell *et al.* 2001). Small RNAs have been found also to play a crucial role in this process and specify mutually antagonistic fates (reviewed in Chitwood *et al.* 2007). Subsequent to events that direct leaf polarity, the control of final leaf shape and size continues by coordinated regulation of cell division and expansion along the length and width of the leaf. Cessation of cell division and differentiation proceeds along the proximodistal plane from leaf tip to base. In the dorsoventral plane, continued divisions in the adaxial mesophyll differentiate a thicker cuticle and a densely packed layer of palisade cells, from the abaxial spongy mesophyll (Tsukaya 2006).

Mutations that disrupt the cellular environments of mesophyll cells may provide useful tools for probing either the leaf development or/and the photosynthetic processes (Roth *et al.* 1996, Brutnell *et al.* 1999, Asano *et al.* 2004, Janošević *et al.* 2007, Covshoff *et al.* 2008).

Chl fluorescence imaging represents a non-invasive and quantitative tool by which it is possible to determine localized changes in the photosynthetic process (Baker 2008). The light energy absorbed by antennae systems has three possible fates: it can be utilized to drive the photosynthetic process after transfer to reaction centres, it can be dissipated by non-photochemical pathways and, in particular, as heat in the light-harvesting antennae, or re-emitted as fluorescence characterized by a longer wavelength than the incident light. The analysis of Chl fluorescence during the photosynthetic induction and under different illumination permits to determine numerous parameters as F_v/F_m, the proportion of absorbed light which is utilized for photosynthetic electron transport Φ_{PSII} or the coefficient q_{NP}, which estimates the amount of energy dissipated nonphotochemically as heat (Maxwell and Johnson 2000).

In this study we investigated the inheritance of a new spontaneous mutant of sunflower characterized by a disruption of the leaf mesophyll and for this reason named *mesophyll cell defective1*(*mcd1*). A histological analysis of leaves is presented. The pleiotropic effect of the *mcd1* mutation on morphological characters and pigment accumulation is also presented. Finally, we examined the physiological effect of this mutation on photosynthesis efficiency by monitoring Chl fluorescence imaging.

Materials and methods

Plant material and inheritance study: Fifty-three stunted plants characterized by altered leaf morphology, and a chlorotic aspect were identified in a progeny of 210 plants of the sunflower (*Helianthus annuus* L.) inbred line AC53 field-grown (Department of Crop Plant Biology of the University of Pisa, Pisa, Italy). It is likely that this segregating progeny was originated by a selfed plant heterozygous for a spontaneous recessive mutation. This mutant *mcd1* differentiated a small inflorescence and produced few seeds. No segregation was observed within progenies obtained from seeds produced by mutant plants. For the genetic analysis, flowers of mutants were hand-emasculated and pollinated with wild type pollen to obtain the F_1 generation. F_1 plants were self-fertilized and the F_2 generation was obtained. Plants were classified in all population into mutant or normal types. Intermediate type was never observed. Chi-square test was used for testing the goodness-of-fit of observed and expected frequencies of different phenotypic classes in the F_2 generation. F_2 progenies were pooled after the chi-square test for heterogeneity (Snadecor 1956).

Plant growth conditions: Plants used to carry out the morphological and pigment analyses were cultivated under field conditions as described in Fambrini *et al.* (2006). Briefly, mutant and wild type plants were grown in 50-cm inter-row spacing with 25–30 cm between plants. Cotyledons and leaves collected for histological analysis were obtained in growth chamber from seedlings and plants, respectively. The temperature was 25 ± 1°C while the daily photoperiod was 16 h of light and 8 h of dark. Irradiation was set at 165 μmol m^{-2} s^{-1} (photosynthetic photon flux density, PPFD) with a mixture of cool-white fluorescent (*TLD 30W/33*, *Philips*, Eindhoven, The Netherlands) and mercury-vapour (*HPI-T 400W*, *Philips*, Eindhoven, The Netherlands) lamps.

Morphological analysis: Phenotypic observations were made on both mutant and wild type of randomly selected plants (n = 10) of progenies, this in three replicate. Most of morphological parameters were recorded at the onset of anthesis. By contrast, the inflorescence diameter was evaluated at the end of flowering. Anthesis of the first tubular flower was recorded to determine the blooming date. Length and width of leaves from 4th to 6th node were measured. Stem height was measured from the insertion of cotyledons to the inflorescence receptacle.

Pigment analysis: Pigments were extracted by tissue homogenization in 100% acetone from cotyledons of 7-day-old plants and from leaves (4th and 5th nodes) of 30-day-old plants of wild type and mutant as previously described (Fambrini *et al.* 2004). Spectrophotometric analysis was performed using an *UV-VIS Scanning Spectrophotometer* (*UV-2101PC*, *Shimadzu Italia*, Milan, Italy). The concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll [Chl (*a* + *b*)] and

of total carotenoids (Car) were calculated according to the equations (Lichtenthaler, 1987):

$$\text{Chl } a = (11.24 \times A_{661.6}) - (2.04 \times A_{644.8}) \quad (1)$$
$$\text{Chl } b = (20.13 \times A_{644.8}) - (4.19 \times A_{661.6}) \quad (2)$$
$$\text{Chl } (a + b) = (7.05 \times A_{661.6}) + (18.09 \times A_{644.8}) \quad (3)$$
$$\text{Car} = (1000\ A_{470} - 1.90\ \text{Chl } a - 63.14\ \text{Chl } b)/214 \quad (4)$$

where A is the absorbance measurement determined at 661.6, 644.8 and 470 nm. Eight extracts were made for each genotype, and two measurements were made per extract.

Histological analysis: Cotyledons from 7-day-old wild type and mutant seedlings grown in the dark or under a 16-h photoperiod were collected. Samples were fixed for 24 h in formaldehyde/glacial acetic acid/ethanol/distilled water (10:5:50:35 v/v) at room temperature before being transferred into 70% ethanol (Fambrini *et al.* 2006). Water was removed by graded ethanol series while the dehydrated material was cleared in xylene with 5 steps according to Ruzin (1999). Paraffin-embedded tissues were sectioned using a rotary microtome (*Reichert*, Vienna, Austria). The serial transverse sections obtained (8 μm thick) were stained with a solution containing alcian blue 8GX, bismarck brown Y and safranin O according to Graham and Trentham (1998). Results were observed with a light microscope (*DMRB*, *Leica,* Wetzlar, Germany) and a selection of digital images was recorded with a camera unit (*PowerShot A590 IS*, *Canon,* Tokyo, Japan).

Chl fluorescence imaging: Leaves from 4th and 5th nodes of 30-day-old wild type and *mcd1* mutant plants grown under a 16-h photoperiod in a growth chamber at 25 ± 1°C were used for Chl fluorescence analysis. Irradiation (165 μmol m^{-2} s^{-1} PPFD) was provided as above described. The imaging technique was performed by a Chl fluorescence imaging system (*IMAGING-PAM*, *Walz*, Effeltrich, Germany). Details of the capture of Chl fluorescence imaging are reported by Guidi *et al.* (2007). The current fluorescence yield (F_t) was continuously measured and the F_0 images were recorded in a quasi-dark state. The maximum fluorescence yield (F_m) was determined with a saturating pulse of 8,000 μmol $m^{-2}s^{-1}$ PPFD for 1–2 s. The images of F_0 and F_m were subtracted and divided $[(F_m - F_0)/F_m]$ to generate the image of F_v/F_m. The F_t and the maximum light adapted fluorescence (F_m') were determined in the presence of an actinic illumination of 400 μmol m^{-2} s^{-1} PPFD, then Φ_{PSII} was computed as the quotient $(F_m' - F_t)/F_m'$ (Genty *et al.* 1989). Nonphotochemical quenching $[q_{NP} = (F_m - F_m')/(F_m - F_0')]$ was calculated according to Schreiber *et al.* (1994). Correct F_0' determinations require the application of a far red light, which would disturb the fluorescence imaging. Therefore, instead of measuring, F_0' was estimated using the approximation of Oxborough and Baker (1997) $[F_0' = F_0/(F_v/F_m + F_0/F_m)]$. Images of the fluorescence parameters were displayed by means of a false colour code ranging from 0.00 (black) to 1.00 (purple). Single leaflets in triplicate for each genotype were used for the analyses. In every leaflet analysed, an internal square portion of the lamina was chosen and, inside it, the value of each single parameter was measured in different points and an average value was calculated. The area, the position and the number of points were the same in all the leaflets analysed. The apparent rates of photosynthetic electron transport (ETR) were estimated as: ETR = $(\Phi_{PSII}) \times 0.5 \times PFD \times 0.8$, where 0.5 accounts for the excitation of both PSII and PSI and 0.8 represents the average value for leaf absorptance. To determine the response of ETR, Φ_{PSII} and q_{NP} to increasing light intensity, leaves were adapted for the specific light level for 10 min.

Statistical analysis: Differences between means were tested using the Student *t*-test (p = 0.05 or 0.01). In addition, regression analysis was carried out for experiments shown in Fig. 5.

Results and discussion

The most obvious alterations in growth and development of the *mcd1* mutant were the stunted growth, the deficient greening of leaves, and the aberrant development of both cotyledons and leaves (Figs. 1–3). Most of *mcd1* seeds germinated normally but the expansion of the cotyledons, which showed ample necrotic areas, was slower as compared to the wild type (Fig. 1*A*,*B*). In cotyledons, the incidence of cell disruption was related to light. In fact, histological examination of seedlings grown in the dark for two weeks revealed no differences between *mcd1* and wild-type cotyledons (data not shown). By contrast, in light-grown seedlings, the cotyledon adaxial surface (compare Fig. 2*A* with Fig. 2*C*) and the cotyledon tips (compare Fig. 2*B* with Fig. 2*D*) of *mcd1* mutant displayed an abnormal enlargement of mesophyll cells (Fig. 2*C*) and a collapse of both epidermis and mesophyll cells (Fig. 2*C,D*). Necrotic areas were also observed at the leaf tip of the first true leaves of *mcd1* (Fig. 1*B*), whereas in leaves of other nodes several areas characterized by deficient mesophyll development and very light green color were spotted on the whole lamina (Fig. 1*F*,*G*). Despite this phenotype, as observed in the *reticulate* mutants of *Arabidopsis thaliana* (González-Bayón *et al.* 2006), the overall leaf shape was maintained in the *mcd1* mutant (Fig. 1*F*,*G*). In contrast to wild-type plants, a severe reduction in plant size was observed for *mcd1* plants during every developmental stage (Fig. 1*A–D*).

Histological analysis revealed that the internal

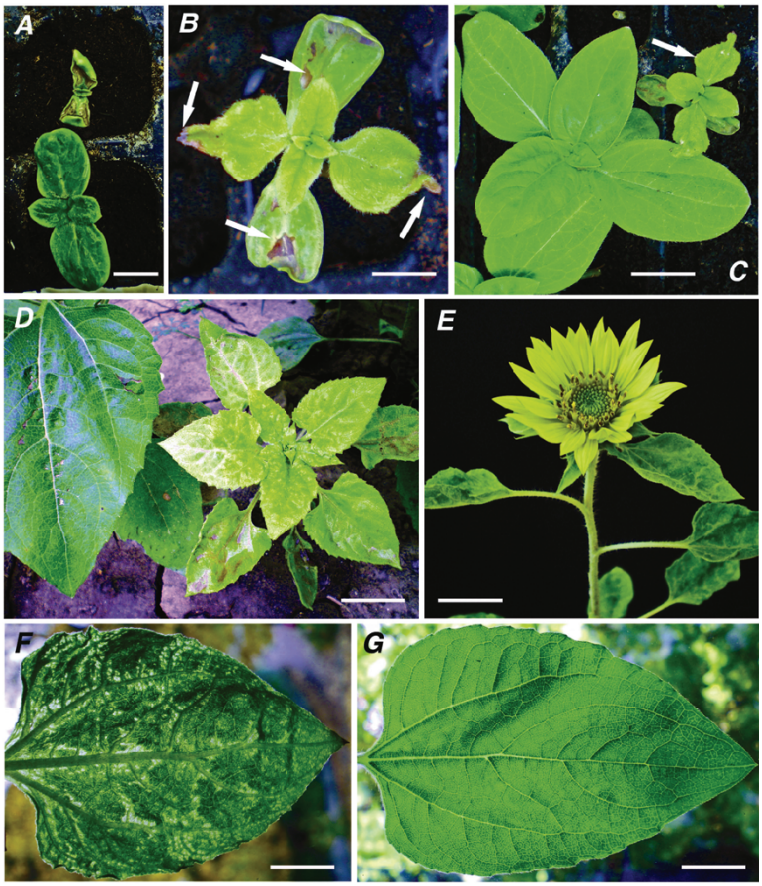

Fig. 1. Developmental phenotype of the *mesophyll cell defective1*(*mcd1*) mutant of sunflower (*Helianthus annuus* L.). *A*: 7-day-old seedlings of wild type (*bottom*) and *mcd1* mutant; note the reduced dimension as well as the necrotic areas on cotyledons of *mcd1* mutant. *B*: *mcd1* mutant showing necrotic areas (*arrows*) on cotyledons and leaf tips. *C*: 20-day-old plants of wild type and *mcd1* mutant (*arrow*). *D*: A 30-day-old *mcd1* plant in the field. *E*: Inflorescence of the *mcd1* mutant at the onset of anthesis. *F–G*: Expanded leaves of *mcd1* (*F*) and wild type (*G*) plants; in the mutant, areas with disrupted mesophyll are evident as transparent sectors. Bars – *A*: 16 mm; *B*, *G*: 14 mm; *C*: 27 mm; *D, E*: 26 mm; *E*: 20 mm; *F*: 10 mm.

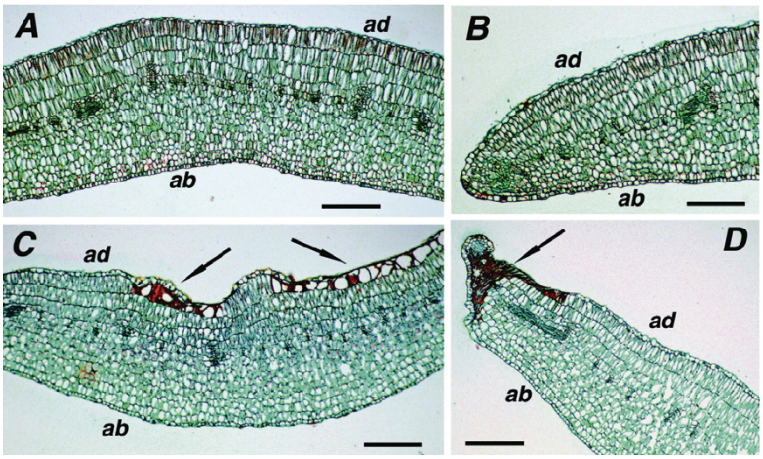

Fig. 2. Developmental phenotype of the *mesophyll cell defective1*(*mcd1*) mutant of sunflower (*Helianthus annuus* L.). Transverse sections of wild type (*A*,*B*) and *mcd1* (*C*,*D*) cotyledons of 7-day-old seedlings. Sections were stained with a solution containing alcian blue 8GX, bismarck brown Y and safranin O. *Arrows* indicate cell disruption on the adaxial surface. *ad* = adaxial; *ab* = abaxial. Bars – *A*, *C*: 360 μm; *B*, *D*: 300 μm.

structure and tissue organization of *mcd1* leaves was dramatically affected (Fig. 3*A–F*). The lamina thickness was variable (Fig. 3*C*), and although regions of *mcd1* leaves displayed a near normal phenotype (Fig. 3*D*), most areas of *mcd1* leaves had prominent intercellular spaces lacking in palisade and spongy tissue differentiation (Fig. 3*C*,*E*,*F*). Notably, palisade cells in wild type (Fig. 3*B*) and near normal leaf sectors of *mcd1* (Fig. 3*D*) leaves were dorsoventrally elongated, whereas those in highly modified *mcd1* leaves showed a spherical rather than a columellar shape (Fig. 3*E*,*F*). It is likely that normal elongation of palisade mesophyll cells during leaf development was also disrupted. A reduced number of chloroplasts characterized the palisade and spongy cells of more altered regions of *mcd1* leaves (Fig. 3*E*,*F*). The mutant palisade cells also appeared more vacuolated that in the wild-type sections of equivalent developmental stage (compare Fig. 3*B* with Fig. 3*E–F*). In some areas of *mcd1* leaves no mesophyll cells were present between the adaxial and abaxial epidermis (Fig. 3*E*), suggesting that in *mcd1* leaves the proliferation of mesophyll cells was partially suppressed, or that an unusual cell death was

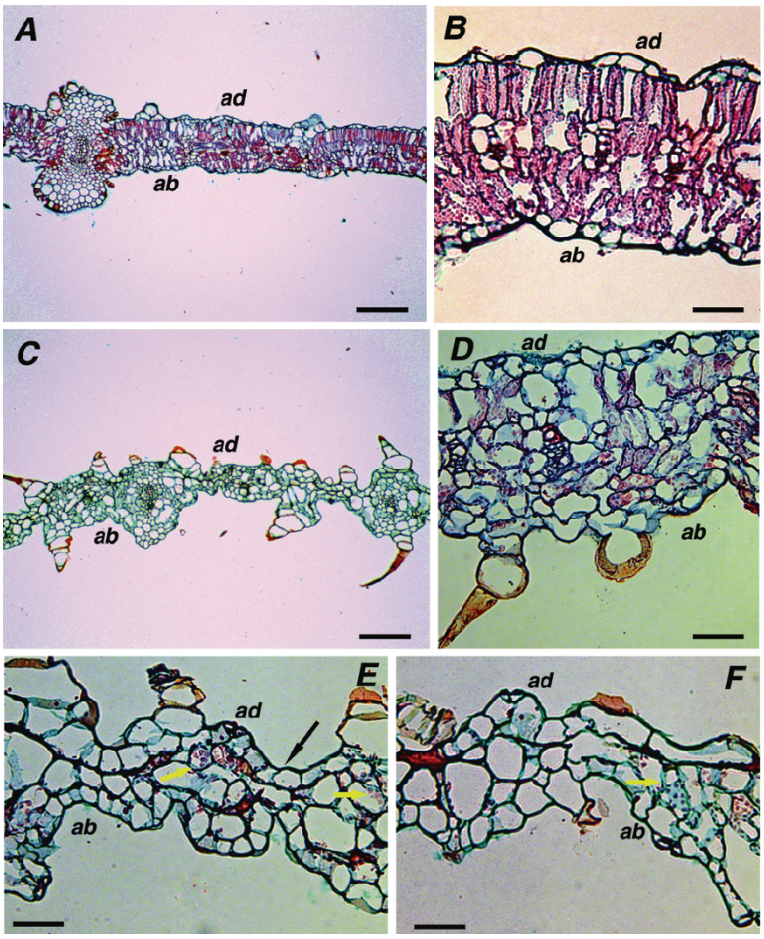

Fig. 3. Developmental phenotype of the *mesophyll cell defective1* (*mcd1*) mutant of sunflower (*Helianthus annuus* L.). Transverse sections of wild type (*A*,*B*) and *mcd1* (*C*–*F*) leaves of 30-day-old plants. Sections were stained with a solution containing alcian blue 8GX, bismarck brown Y and safranin O. *ad* = adaxial; *ab* = abaxial. *The black arrow* in *E* indicates a region without mesophyll. *The yellow arrows* in *E* and *F* indicate mesophyll cells with few chloroplasts. Bars – *A*: 215 μm; *B*,*E*,*F*: 60 μm; *C*: 175 μm; *D*: 45 μm.

Table 1. Effects of the *mesophyll cell-defective* (*mcd1*) mutation on some morphological characters of sunflower (*Helianthus annuus* L.) plants grown in field conditions. Leaf width and length were evaluated in expanded leaves from 4th to 6th node. The inflorescence diameter was evaluated at the end of flowering. Values are means (± SD) from three progenies, with ten replicates each (plants). $^{**}p<0.01$; n.s. – not significant (Student's *t*-test).

Parameter	wild type	*mcd1*
Number of leaves	25.42 ± 2.89	22.70 ± 2.67 $^{n.s.}$
Leaf width [mm]	185.2 ± 31.2	45.3 ± 9.1**
Leaf length [mm]	193.7 ± 24.8	63.2 ± 11.5**
Total leaf area [m^2]	0.534 ± 0.026	0.026 ± 0.011**
Stem height [mm]	840.7 ± 149.9	312.9 ± 124.6**
Stem diameter [mm]	19.1 ± 3.4	5.2 ± 1.3**
Inflorescence diameter [mm]	136.8 ± 30.2	34.2 ± 9.7**
Onset of flowering [d]	64.09 ± 2.91	74.11 ± 1.90**
Number of ray flowers	51.65 ± 4.07	30.67 ± 3.54**

active in the mutant. The *mcd1* mutation also affected the layered arrangement of mesophyll tissue because a clear distinction between palisade and spongy layer was misplaced in the thinner sectors (compare Fig. 3*A,B* with Fig. 3*E,F*).

Sometimes the destruction of genes (*i.e. DAG*, *PLASTID PROTEIN IMPORT2*, *HANDSHAKE*) involved in leaf developmental anatomy shows also pleiotropic effects (Chatterjee *et al.* 1996, Asano *et al.* 2004, Janošević *et al.* 2007). The leaf area of the *mcd1* mutant was extremely reduced as well as height and stem diameter. Also *mcd1* plants showed a delay in the onset of flowering as compared to the wild type (Table 1). By contrast, the leaf number in the *mcd1* mutant (Table 1) as well as the phyllotactic pattern showed no defect with respect to normal plants (data not shown). The small *mcd1* inflorescences differentiated fewer ray and disk flowers (Fig. 1*E*, Table 1), but ovules and pollen were fertile and seeds were produced.

In contrast to wild-type leaves that were dark green in color, the *mcd1* leaves showed a much lighter color (Fig. *1B–E*). The chlorotic phenotype of *mcd1* plants was due to a reduction of total Chl *a*, Chl *b* and Car in both cotyledons and leaves (Table 2); *mcd1* leaves also displayed a reduced Chl *a*/Chl *b* ratio with respect to the wild type.

The F_1 plants obtained from the cross of the *mcd1* mutant with its wild type were normal indicating that the mutant character is recessive to normal (Table 3). Four progenies of the F_2 generation were used, and a total

Table 2. Effects of the *mesophyll cell-defective* (*mcd1*) mutation of sunflower (*Helianthus annuus* L.) on cotyledon and leaf pigment content. Values are means (± SD) from two independent experiments, with eight replicates each (cotyledon or leaf). $^{*}p < 0.05$; $^{**}p < 0.01$; n.s. – not significant (Student's *t*-test). The statistical analysis in cotyledons and leaves was done separately. Car – carotenoid; Chl – chlorophyll.

Pigment [μg mm^{-2}]	Cotyledon wild type	*mcd1*	Leaf wild type	*mcd1*
Chl *a*	2939.7 ± 359.7	1781.6 ± 356.3**	3823.8 ± 276.9	1309.1 ± 43.4**
Chl *b*	945.9 ± 93.4	658.8 ± 81.4**	1275.1 ± 88.4	656.1 ± 17.6**
Total Chl (*a* + *b*)	4068.1 ± 463.6	2574.7 ± 429.1**	5099 ± 364.6	1965.2 ± 40.8**
Total Car	804.8 ± 101.6	600.6 ± 66.7*	1108 ± 76.4	450.6 ± 33.8 **
Chl (*a* + *b*)/Car	5.128 ± 0.114	4.604 ± 0.341*	4.604 ± 0.196	4.455 ± 0.316$^{n.s.}$
Chl *a*/*b*	3.127 ± 0.126	2.731 ± 0.381$^{n.s.}$	2.998 ± 0.037	1.997 ± 0.098 **

Table 3. Inheritance of the *mesophyll cell-defective1* (*mcd1*) mutation in sunflower (*Helianthus annuus*). Summary of chi-squares for the F_2 generation (3: 1 segregation) and chi-square test of heterogeneity among F_2 progenies.

Cross	Generation	No. of progenies	No. of plants Normal	Mutant	χ^2 (3:1)	*P*	Heterogeneity χ^2 (3:1)	*P*
Mutant × Normal	F_1	2	26	–	–	–	–	–
Mutant × Normal	F_2	4	701	237	0.0355	0.80–0.90	0.9758	0.80–0.90

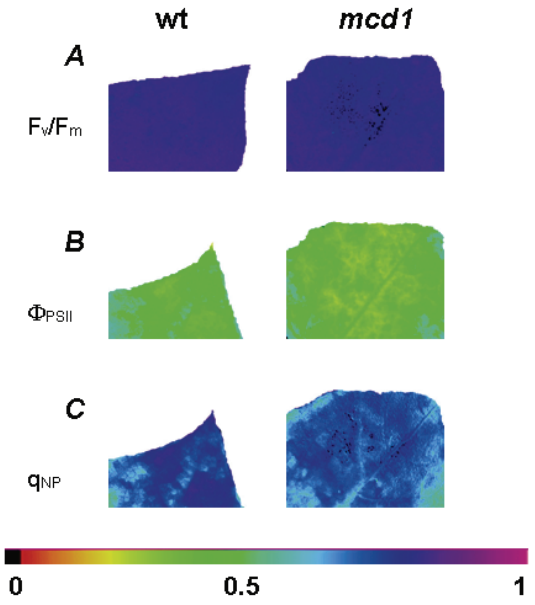

Fig. 4. Analysis of chlorophyll fluorescence parameters in wild type (wt) and *mesophyll cell defective1* (*mcd1*) mutant plants of sunflower (*Helianthus annuus* L.). *A–C*: Fluorescence images of the maximum efficiency of PSII (F_v/F_m; *A*), the proportion of absorbed light, which is utilised for photosynthetic electron transport (Φ_{PSII}; *B*), and the nonphotochemical quenching coefficient (q_{NP}; *C*), in representative leaves from wild type (*left column*) and *mcd1* mutant (*right column*), are shown.

of 938 plants were observed. The pooled F_2 population fit a monogenic ratio of 3 normal: 1 mutant plant (χ^2 = 0.0355, *P*>0.80). The test for heterogeneity showed no significant variation in the segregation of characters among the individual F_2 populations (χ^2 = 0.9758, *P*>0.80). The *mcd1* defect therefore results from a recessive mutation in a single nuclear gene locus.

In addition to an aberrant leaf anatomy, mesophyll cell defective mutants are severely compromised in establishing photoautotrophic growth (Covshoff *et al.* 2008). For instance, *bundle sheath defective2* (*bsd2*) seedlings do not accumulate Rubisco (Roth *et al.* 1996, Brutnell *et al.* 1999) and lack a functional Calvin cycle (Smith *et al.* 1998). Mesophyll cell defective mutants that lack PSII are unable to generate electron flow and likely result in overly oxidized linear photosynthetic electron transport chains. Imaging Chl fluorescence is a noninvasive and quantitative tool by which it is possible to determine localized changes in the photosynthetic process (Buschmann *et al.* 2000, Oxborough 2004, Baker 2008). This is particularly the case when leaves are characterised by a significant surface heterogeneity of Chl fluorescence emission. In Fig. 4*A*, images of F_v/F_m in wild type and *mcd1* leaves after dark adaptation are shown. In wild type, F_v/F_m values were homogeneous. By contrast, in Chl fluorescence images of *mcd1* leaves, little black points, which correspond to values of F_v/F_m close to zero, were evidenced. More obvious differences between wild type and *mcd1* leaves were displayed in illuminated leaves. The values of Φ_{PSII} showed a considerable homogeneous distribution over wild-type leaf lamina, while the damaged areas in *mcd1* leaves, represented by yellow zones in Chl fluorescence images, were significantly prominent (Fig. 4*B*). By contrast, no significant differences were detected for the q_{NP} coefficient (Fig. 4*C*).

In Fig. 5, the changes in fluorescence parameters in the last saturating pulse of the kinetic fluorescence induction with respect to different intensities of actinic

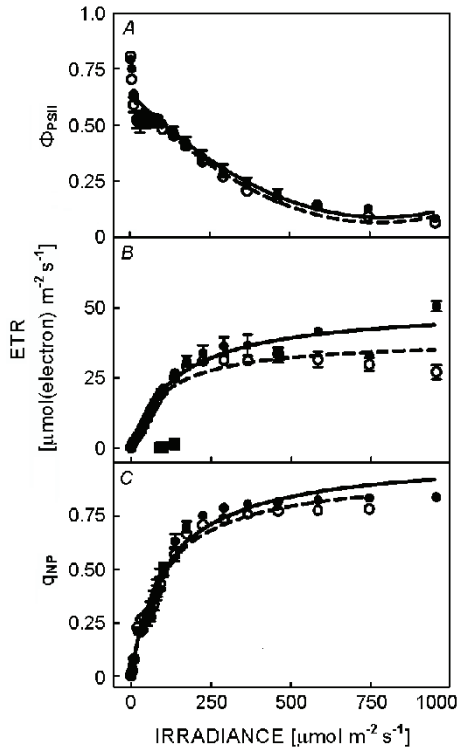

Fig. 5. Effect of light intensity on the relationship among Φ_{PSII} (*A*), ETR (*B*) and q_{NP} (*C*) in wild type (wt) leaves (*closed circle*) and *mesophyll cell defective1* (*mcd1*) mutant leaves (*open circle*) of sunflower (*Helianthus annuus* L.). Bars indicate SD (n = 6). The equations of the regression lines for the wt (*solid line*) and *mcd1* mutant (*dashed line*) are the following: (*A*) y = 0.64 – 0.001 x + 0.0000008 x^2 (r^2 = 0.890) for wt, y = 0.64 – 0.001 x + 0.0000009 x^2 (r^2 = 0.876) for *mcd1*; (*B*) y = 50 x/(142 + x) (r^2 = 0.913) for wt; y = 39 x/(100 + x), (r^2 = 0.912) for *mcd1*; (*C*) y = x/(111 + x) (r^2 = 0.946) for wt, y = 0.96 x/(101 + x) (r^2 = 0.954) for *mcd1*.

light are shown. The actual photochemical efficiency (Φ_{PSII}) experienced a gradual decrease with respect to different intensities of actinic light. This parameter showed a slight reduction in *mcd1* leaves as compared to wild type (Fig. 5*A*). The ETR parameter increased with increasing light intensity; even ETR showed lower values in *mcd1* leaves as compared to the wild type (Fig. 5*B*). The q_{NP} coefficient is considered a good estimate of the amount of energy dissipated non-radiatively by plants (Baker 2008). It increased markedly with larger light intensities of actinic light. The values of q_{NP} were similar in wild type and mutant leaves (Fig. 5*C*).

Heterogeneity of images in the mutant suggested that pigment composition and concentration as well as stomatal function undoubtedly differed in cells from different regions of the leaf, contributing to leaf spatial differences in photochemical activity (Chaerle *et al.* 2003). However, in the mutant, areas that were not chlorotic showed higher values of Φ_{PSII} indicating that a higher percentage of the absorbed quanta was converted into chemically fixed energy by photochemical charge separation at PSII reaction centres. In conclusion, damaged areas of *mcd1* leaves were not able to carry out electron transport rate while no alterations were observed in the other portions of the leaf lamina.

The *mcd1* mutant characterized in this study is apparently different from other leaf mutants described in sunflower elsewhere (Luczkiewicz 1975, Pugliesi *et al.* 1995, Jambhulkar and Joshua 1999). The spotted distribution of leaf chlorotic areas is a common trait of variegation mutants (Yu *et. al.* 2007) and some mutants of this type have been isolated and characterized in sunflower (Usatov *et al.* 2004); however, these mutations affect plastidial genes and no details about mesophyll morphogenesis have been reported. The *mcd1* mutation causes severe defects in cotyledon and leaf anatomy, a pale green phenotype and a stunted growth. In particular, the mesophyll of *mcd1* leaves contains fewer (if any) cells and more intercellular spaces as compared to the wild type. The chlorotic phenotype of the *mcd1* mutant seems to be mainly due to reduced mesophyll cell number with few chloroplasts and increased air space. *mcd1* plants also exhibit a spatial heterogeneity of leaf photosynthetic performance. The *variegated 3* mutant of *Arabidopsis thaliana* displays a phenotype with remarkable analogies compared to *mcd1*. The nuclear gene *VARIEGATED 3* (*VAR3*) encodes a novel zinc-finger protein required for both chloroplast development and palisade cell morphogenesis (Næsted *et al*. 2004). At the moment, the molecular nature of the *mcd1* mutation is unknown but the functional classes of *VAR3* and *MCD1* genes are probably closely related.

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