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ORIGINAL PAPER

Phenotypic characterization, genetic mapping and candidate gene analysis of a source conferring reduced plant height in sunflower

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Abstract Reduced height germplasm has the potential to increase stem strength, standability, and also yields potential of the sunflower crop (*Helianthus annuus* L. var. *macrocarpus* Ckll.). In this study, we report on the inheritance, mapping, phenotypic and molecular characterization of a reduced plant height trait in inbred lines derived from the source DDR. This trait is controlled by a semi-dominant allele, *Rht1*, which maps on linkage group 12 of the sunflower public consensus map. Phenotypic effects of this allele include shorter height and internode length, insensibility to exogenous gibberellin application, normal skotomorphogenetic response, and reduced seed set under self-pollination conditions. This later effect presumably is related to the reduced pollen viability observed in all DDR-derived lines studied. *Rht1* completely cosegregated with a haplotype of the *HaDella1* gene sequence. This haplotype consists of a point mutation converting a leucine residue in a proline within the conserved DELLA domain. Taken together, the phenotypic, genetic, and molecular results reported here indicate that *Rht1* in sunflower likely encodes an altered DELLA protein. If the DELPA motif of the *HaDELLA1* sequence in the *Rht1*-encoded protein determines by itself the observed reduction in height is a matter that remains to be investigated.

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Introduction

The huge increase in cereal yields during the years of the ''Green Revolution'' led by Norman Borlaug, Monkombu Swaminathan and Gurudev Khush was enabled by the application of large amounts of agrochemicals in combination with the introduction of semidominant dwarfing mutations into plants causing height reductions associated with yield increases in several different crop species (Hedden 2003). The varieties used today, in particular for crops such as wheat, rice and sorghum, are shorter, more resistant to lodging and have increased grain yields compared to earlier ones (Evans 1998). There are various factors responsible for dwarfism in plants, but gibberellins (GA) and brassinosteroids (BR) are the most intensely studied (Fujioka and Yokota 2003; Yamaguchi 2008).

GA are essential phytohormones that regulate many aspects of plant growth and development, including seed germination, leaf expansion, stem and root extension, flower induction and development, seed development, and fruit expansion (for review, see Fleet and Sun 2005; Olszewski et al. 2002; Swain and Singh 2005; Yamaguchi 2008). The dwarf genes used in the Green Revolution, *sd1* in rice and *Rht-B1b* and *Rht-D1b* in wheat, are involved in the GA biosynthesis and signaling pathways, respectively (for review, see Maluszynski and Szarejko 2005). The rice semi-dwarf gene encodes a defective GA biosynthetic enzyme, GA20 oxidase, which causes a deficiency of bioactive GA in the *sd1* mutant. The application of exogenous GA to *sd1* plants was sufficient to restore normal plant height (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). In contrast, wheat mutants containing dwarf genes *Rht-B1b* and *Rht-D1b* could not be rescued with exogenous GA treatment. The wild-type *Rht* alleles, *Rht-B1a* and *Rht-D1a*, encode DELLA proteins that serve as GA signaling repressors.

GA-induced degradation of DELLA proteins is required for normal GA signaling. *Rht-B1b* and *Rht-D1b* encode mutant DELLA proteins that are resistant to GA-induced degradation and GA signaling is constitutively blocked (Peng et al. 1999; Silverstone et al. 2001).

The major bioactivities associated with BR application are the stimulation of cell elongation and cell division (Mandava 1988). BR gained attention as a major class of plant hormones after the analysis of *Arabidopsis* mutants deficient in BR biosynthesis. These mutants showed major developmental defects including dwarfism, dark-green leaves, male sterility, and delays in flowering and senescence (Bishop 2003). The dwarfism, which was mainly due to shorter organs such as leaves and stems, could be partially reversed by supplementing mutant plants with end products of BR biosynthesis (Szekeres et al. 1996). The most salient features of BR biosynthesis and signaling mutants are that they are dark green dwarf that often exhibit a de-etiolated phenotype when grown in the dark, i.e., they undergo photomorphogenetic development in the absence of light (Bishop 2003).

Sunflower (*Helianthus annuus* L. var. *macrocarpus* Ckll.) is grown all over the world with three main purposes: beauty (ornamental sunflower), direct consumption of the seeds (confectionary sunflower) and oil production (oilseed sunflower). By far, the last of them is the most important objective in terms of acreage and production (Miller and Fick 1997). Sunflower oil has been traditionally viewed as a healthful vegetable oil and it is considered a premium oil for salad, cooking, and margarine production and also is being evaluated as a source of biodiesel (Sánchez Muñiz and Cuesta 2003). Stem lodging, defined as the permanent displacement of the stem from its vertical position, causes important yield losses in sunflower. The prostrate head of lodged plants is not retrieved during mechanical harvesting causing significant losses. Although yield is known to increase up to densities higher than those currently used, lodging also tends to increase. For this reason, lodging may contribute to fix the upper limit to commercially viable crop population density in sunflower (López Pereira et al. 2004; Hall et al. 2010). Progress in improving the standability of standard-height sunflower has been slow (Miller and Fick 1997). Therefore, reduced height germplasm has the potential to increase stem strength and also yields potential of the sunflower crop. However, the potential of reduced height germplasm as a strategy to increase yield potential and reducing stem lodging still deserves to be fully explored in sunflower (Sala et al. 2012).

Dwarfism in sunflower controlled by recessive genes in lines with a reduced number of leaves has been reported by Vranceanu (1974), Fick (1978), Beretta de Berger and Miller (1984, 1985), Cecconi et al. (2002), Jambhulkar (2002), Jagadeesan et al. (2008) and Fambrini et al. (2011). In particular, *dwarf1* (*dw1*, Cecconi et al. 2002) and *dwarf2* (*dw2*, Fambrini et al. 2011) are severe dwarf mutants affecting vegetative and reproductive growth which can be restored to the wild type phenotype by exogenous GA applications. In fact, *dw2* is the only dwarf mutant that has been characterized at the molecular and physiological levels up to the present (Fambrini et al. 2011). Nevertheless, none of them has been used to improve yield as yet because of the excessively severe phenotypes of these mutants. On the other hand, three sources of reduced height—'DDR', 'Donsky' and 'Donskoi 47'—with an equal or similar number of leaves as standard-height sunflowers were reported (Tolmachov 1991; Miller and Hammond 1991). DDR and Donsky were used to develop several restorer and maintainer lines (Miller and Gulya 1989; Miller 1993; Velasco et al. 2003b). The inheritance of reduced plant height in the sunflower line Dw89, which traces back to Donsky, was reported to be controlled by two recessive alleles, designated *dw1* and *dw2* (Velasco et al. 2003a). Reduced height in Donskoi 47, on the other hand, is controlled by a single dominant gene, *Dw* (Tolmachov 1991). The inheritance of dwarfism in the source DDR has not been reported yet.

In this paper, we report on the phenotypic characterization, inheritance, and mapping of a semidominant gene for reduced plant height in inbred lines derived from DDR. We also determined the effect of the application of exogenous GA on plant height in these lines. Using a candidate gene approach and an analysis of cosegregation, we also confirmed that this semidominant gene encoded an altered DELLA protein.

Materials and methods

Plant materials

Standard-height public inbred lines HA89, RHA274 and HA821, and DDR-derived reduced height inbred lines RHA356, RHA357, RHA358, RHA359, HA378 and HA379 were used. DDR is a reduced height genetic material originally obtained from the Zentralinstitut für Genetik und Kulturpflanzenforschung (Gatersleben, Germany) and used to develop the reduced-height lines mentioned above (Miller and Gulya 1989; Miller 1993). Restorer lines RHA356, RHA357 and RHA358 are BC_2F_4-derived BC_2F_7 lines from the cross RHA274*3/DDR. RHA359 is an F_5-derived F_7 restorer line selected from the cross RHA274/DDR, utilizing the pedigree breeding method. RHA274 is a standard-height restorer line released by the USDA in 1973 (Miller and Gulya 1989). Maintainer lines HA378 and HA379 are F_6-derived F_7 lines selected from the cross HA821/DDR. HA821 is a standard-height maintainer line released by the USDA-ARS and the North Dakota Agricultural Experiment Station in 1983 (Miller 1993).

Phenotypic characterization

Standard and reduced-height lines were sown under field conditions in Nidera Experimental Station at Venado Tuerto, Santa Fe, Argentina (32°40′S, 61°58′W; 110 m.a.s.l.), on October 15th 2009. Each line was sown by hand in three rows 0.7 m apart and 0.25 m among plants. At R5.1 stage of development (Schneiter and Miller 1981), measurements were recorded on ten plants in full competence for the following characters: days to flowering, plant height, stem diameter, number of leaves, pollen stainability and relative autogamy. Days to flowering are the number of days from emergence (VE stage) to R5.1; plant height was taken as the distance between the cotyledon node and the point where the stem is attached to the capitulum. Stem diameter was measured between the 3rd and 4th true leaves. The number of leaves of each plant was recorded in the main stem and not in the apical branches of the plants. Pollen stainability was assessed microscopically by buffalo black staining (Jackson 1988; Mendoza Villarreal et al. 2006). Relative autogamy was determined from the mean weight of seed produced by ten self-pollinated plants and expressed as percentage of the mean weight of seed produced by ten plants in open-pollinated conditions (Miller and Hammond 1991). Self-pollination was achieved by covering each head with a bag previous to flowering.

Response to exogenous GA treatments

Response to exogenous GA treatments was assessed under greenhouse conditions. Seeds of the reduced-height line RHA358 and the standard-height line RHA274 were sown in Petri dishes and, after germination, seedlings were transplanted into potting media consisting of equal parts of vermiculite, soil and sand in 20-by-20-by-30 cm pots. Plants were grown in a greenhouse under natural light conditions supplemented with 400 W sodium halide lamps to provide a 16-h photoperiod. Day/night temperatures were 25 and 20 °C, respectively. After the first pair of leaves was fully expanded (V2 stage), a drop of 0, 1.4×10^{-2}, 1.4×10^{-1}, 1.4 or 14 mM GA_3 (Sigma) in ethanol:water solution was applied to the shoot tips of ten seedlings for each treatment once a week till the R1 stage. Plants were arranged as a completely randomized design with four treatments per genotype and ten replications. Height of each plant was recorded as described above.

Growth response under dark conditions during germination

Morphology of the seedlings of the inbred lines RHA358 and RHA274 was assessed under dark conditions to determine if the reduced-height line has abnormal skotomorphogenesis. To do this, four replications consisting in 25 seeds of each line were used. These seeds were sown into sand with 13 % (w/w) moisture content in 20-by-20-by-5 cm pots. One pot of each line was placed in a 50-by-70-by-30 cm dark chamber containing 1,000 cm^3 of water and maintained closed until the end of the experiment. Dark chambers were kept in a greenhouse at a day/night temperatures of 25 and 20 °C, respectively. The experiment was arranged as a completely randomized block design, with three replications (dark chambers). Five days after sowing, one dark chamber was opened and the plantlets of both genotypes were observed to determine the presence of "hook" and open or close cotyledons. Twelve days after sowing, hypocotyl length was recorded for each seedling in the remaining dark chambers.

Analysis of variance was performed on these experimental data using the statistical programming package R (R Development Core Team 2011).

Inheritance of reduced height in RHA358

The standard-height inbred line HA89 was crossed with the reduced height inbred line RHA358 and one of the resulting F_1 plants was backcrossed to HA89 to obtain BC_1F_1 seeds. In addition, HA89 was crossed with RHA274 and the resulting F_1 seeds were used as standard-height check in the experiments described below. Plants of the BC_1F_1 population were sown in 20-by-20-by-30 cm pots in a greenhouse under natural light conditions supplemented with 400 W halide lamps to provide a 14-h daylength. Day/night temperatures were 25 and 20 °C, respectively. During flowering, height of each BC_1F_1 plant was measured as described above and its head was covered with a pollination bag to obtain BC_1F_2 seeds. Each BC_1F_2 family was sown under field conditions in a row of 6 m long and 0.7 m apart and visually scored at R1 for their segregation for height. To do this, each BC_1F_2 family was visually scored as standard-height or segregant for height in comparison with the parental and F_1 plants grown at the same environment and used as checks. Chi-square test was used for testing the goodness-of-fit of observed and expected frequencies of different phenotypic classes.

Mapping the reduced height phenotype from RHA358

DNA was extracted from each individual BC_1F_1 plant by the method of Dellaporta (Dellaporta et al. 1983). Bulk segregant analysis (Michelmore et al. 1991) was used to detect polymorphic markers linked to the reduced height trait. To do this, the two parental lines and two bulks of ten BC_1F_1 individuals each composed of plants contrasting by their height were screened by 180 microsatellites markers. Polymorphic markers which also discriminate parents and bulks

were scored in each of the 94 BC_1F_1 individuals. PCRs were performed in 20 μL of reaction mixture containing 2 μL 10× PCR buffer, 2.5 mM Mg^{2+}, 0.2 mM each of dNTPs, 7.5 pmol of each primer, 0.7 units of Platinum *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 20 ng of genomic DNA. PCRs were then performed in a PTC200 thermocycler (MJ Research, Waltham, MA, USA). The PCR products were separated by electrophoresis in Metaphor agarose gels [4.0 % (w/v) in TBE pH 8.3] or denaturing polyacrylamide gels [6 % (w/v) acrylamide/bis-acrylamide, 20:1, 8 M urea in TBE, pH 8.3]. Agarose gels were stained with a SYBR Safe nucleic acid gel stain (Molecular Probes, Eugene, OR, USA). Polyacrylamide gels were analyzed using a LI-COR 4300 DNA Analyzer system (LI-COR Biosciences, Lincoln, NE, USA).

Map distances were determined by maximum likelihood estimations with the computer program MAPMAKER 3.0 (Lander et al. 1987), using default parameters of LOD = 3 and a maximum Kosambi distance of 50 cM and the default algorithm.

PCR amplification and direct sequencing of *HaDELLA1* gene sequence from genomic DNA

The *HaDELLA1* gene sequence was PCR amplified from DNA isolated from RHA358. PCR amplification was accomplished with Platinum *Taq* DNA polymerase and associated reagents using standard methods. The forward primer named G-D1-F (5′-ATGAAACGTGACTACCCAA ATC-3′) corresponds to base pairs 68-89 of GenBank Accession No. DQ503809.1 (Liu and Burke 2006). Reverse primer coded as G-D1-R (5′-GGTTGCTACTTTCCGCAT CGC-3′) corresponds to base pairs 742-722 of the same accesion. PCR was conducted on PTC200 thermocycler as follows: heat denaturation at 95 °C for 2 min followed by 45 cycles of 94 °C 30 s, 55 °C 20 s, 72 °C 1 min and 72 °C 5 min final extension. The PCR products were analyzed in 1.5 % agarose gels and visualized as described above.

PCR product from RHA358 was submitted for direct sequencing. Sequence trace files were imported into ContigExpress (Invitrogen, Carlsbad, CA, USA), trimmed, aligned, and evaluated for nucleotide polymorphisms by comparing the corresponding control Accession No. DQ503809.1 and the sequencing trace contigs. Nucleotide and amino acid multiple sequences alignments were generated using ClustalW (http://www.ebi.ac.uk/clustalw), and the output was edited and annotated using GeneDoc software (http://www.psc.edu/biomed/genedoc). Gene sequence from RHA358 was deposited in GenBank under Accession No. JQ042689.

Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/, Ng and Henikoff 2003) is a sequence-based amino acid substitution prediction method that predicts whether an amino acid substitution affects protein function. Its prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through Position Specific Iterated-BLAST. SIFT assigns a severity score to each mutation from 0 to 1, where 0 is damaging and 1 is neutral. Scores lower than 0.05 are predicted to be deleterious, although there is a 20 % false-positive rate (Ng and Henikoff 2006).

Diversity of *HaDELLA1* gene sequence in *Helianthus*

To determine if the SNP found in *HaDELLA1* gene sequence from RHA358 is present in other genotypes of *H. annuus* or other species of *Helianthus*, a nucleotide BLAST (Basic Local Alignment Searching Tool, Altschul et al. 1990) was performed using the corresponding accession number JQ042689 in the *H. annuus* database.

On the other hand, *HaDELLA1* gene sequence was PCR amplified from *H. annuus* open pollinated populations Havasupai (PI 369358) and Hopi (PI 432504), and other wild diploid *Helianthus* species, *H. ciliaris* (PI 435647), *H. floridanus* (PI 468715), and *H. debilis* subsp. *debilis* (AMES 10829). These sequences were deposited in GenBank under accession numbers: JQ342829, JQ342828, JQ319320, JQ319321, JQ319322, respectively.

Developing an allele-specific PCR marker for the *HaDELLA1* gene sequence in RHA358

Allele-specific PCR-based markers were generated to detect single nucleotide polymorphisms (SNP) of *HaDELLA1* gene sequence from RHA358. Allele-specific primers were designed to both alleles in the forward orientation; G-delp-F sequence (5′-CGGAGATGACGAGCTGCC-3′) is identical to G-dell-F (5′-CGGAGATGACGAGCTGCT-3′) with the exception of the single base pair responsible for the mutation in DELLA1 motif in *HaDELLA1* of RHA358. Allele-specific primer combinations for the DELLA motif with G-D1-R were expected to give a 549 bp PCR fragment for each genotype. Furthermore, each set of allele-specific primers were used in combination with G-D1-F to produce a PCR fragment of 675 bp as internal control. PCR conditions were the same as described in the previous step. PCR analysis was performed in 2.5 % Synergel (Andwin Scientific, NC, USA).

Cosegregation analysis

Analysis of cosegregation of reduced height and the allele-specific markers developed above was carried out on the 94 plants of the BC_1F_1 population described. Likewise, these markers were tested in the DDR-derived reduced height inbred lines RHA356, RHA357, RHA358, RHA359, HA378 and HA379, and in their standard-height parents

(RHA274 and HA821). Previously described DNA extraction and PCR procedures were used. DNA fragments were separated in 1.5 % agarose gels.

Results

Phenotypic characterization of the reduced-height lines derived from DDR

All the tested lines derived from bi-parental or backcross populations obtained by crossing DDR with standard-height sunflower lines showed significant reduced height ($p < 0.001$) with respect to their standard-height parents (Table 1). This reduction in height with respect to the standard-height parental lines ranged from 44.5 % for HA379 to 61.8 % for RHA358. Reduced height lines showed a significant greater (e.g.: RHA356), equal (RHA357) or lesser (RHA359) number of leaves than their standard-height parental lines. Mean internode length of the reduced height lines ranged from 2.1 to 2.6 cm and was significant smaller ($p < 0.001$) than the internode length of the standard-height parental lines (6.2 and 3.8 for RHA274 and HA821, respectively). Stem diameter ranged from 20.9 ± 4.5 to 27.7 ± 3.8 cm, but it did not differ between reduced height lines and their standard-height parents. With respect to the phenology, reduced height lines derived from RHA274 were significantly later than this last line. Reduced height lines tracing back to HA821 also showed differences in their phenology. In fact, HA379 showed the same number of days to flowering than HA821 but HA378 was 8 days later (Table 1).

Relative autogamy of the standard-height height lines ranged from 78.3 ± 6.7 to 93.4 ± 7.5 %. Reduced height lines, however, showed a drastic reduction of their seed set under self-pollination conditions, with relative autogamy ranging from 9.1 ± 5.4 % for RHA359 to 24.8 ± 3.6 % for HA378 (Table 1). This significant reduction can be explained by the presence of a pollen-pistil incompatibility mechanism that would prevent self-fertilization to occur or by a reduction in pollen viability of the reduced-height lines in comparison with their standard-height parental lines. In this sense, pollen stainability—as a measure of pollen viability—of the reduced height lines ranged from 45.8 ± 2.6 to 68.8 ± 2.7 % and it was significantly lower ($p < 0.001$) than that observed in the standard-height lines (Table 1). Apart from the presence of non-stained or aborted pollen grains—which is reflected by pollen stainability—micropollen, microspores with rudimentary exine without the characteristic spines of viable sunflower pollen grains, and oily yellow granules surrounding the pollen grains were observed in the pollen samples from the reduced height lines (Fig. 1).

Response to exogenous GA treatments

Genotype and GA doses significantly ($p < 0.001$) contributed to plant height after exogenous GA treatments of RHA358 and RHA274 inbred lines. However, a significant line × GA doses interaction was not detected, indicating that the increase in height in response to increasing concentrations of exogenous GA showed similar trends in both lines. Moreover, application of GA did not restore the reduced height phenotype of the line RHA358 to the height of its standard parental line RHA274 (Fig. 2).

Growth response under dark conditions during germination

Reduced height line RHA358 and its standard-height recurrent parent RHA274 showed a normal skotomorphogenic response when germinated under dark conditions, i.e., presence of the characteristic "hook" determined by the acute angle formed by the cotyledons and the hypocotyl, and "closed cotyledons" since both cotyledons were closely linked together. Mean hypocotyl length after 12 days of germination in the dark was significantly greater ($p < 0.0015$) in RHA274 (17.45 ± 1.83) than in RHA358 (10.57 ± 1.77 cm). These results indicate that RHA358 seedlings grown in the dark were morphologically similar to its standard-height recurrent parent. This response under dark conditions suggests that the BR pathway is probably not involved in the reduced height phenotype of RHA358.

Inheritance of reduced height in RHA358

The phenotypic expression of the BC_1F_1 generation, obtained by backcrossing one F_1 plant from the cross RHA358/HA89 to the standard-height parent, was investigated under greenhouse conditions. Heights of the BC_1F_1 plants showed a bimodal distribution from 56 to 209 cm, with two peaks separated by approximately 110 cm (Fig. 3). F_1 plants from the cross HA89/RHA358 showed a height of 112 ± 5.8 cm, whereas the plants of its near isogenic hybrid HA89/RHA274 showed a height of 219 ± 7.1 cm under the same environmental conditions. Height of the parental lines permitted to roughly classify BC_1F_1 plants into two groups of reduced and standard-height phenotypes. BC_1F_2 families obtained by self-pollination of each of these plants were analyzed under field conditions to confirm this classification. All the families obtained from the BC_1F_1 plants previously classified in the group of standard-height phenotypes did not show any plant shorter than the standard-height parent. On the other hand, the BC_1F_2 families tracing back to the group of plants classified before in the group of reduced height phenotypes showed a clear segregation of reduced and standard-height plants. This observation confirms that the

Table 1 Phenotypic characterization of reduced-height (RH) inbred lines derived from the source DDR and their standard-height (SH) parental lines

Genotype	Type	Plant height (cm)	Stem diameter (mm)	Number of leaves	Days to flowering	Pollen stainability (%)	Relative autogamy (%)
RHA274	SH	112.0 ± 12.1 a*	23.2 ± 4.6 ab	18 ± 2.4 d	54.2 ± 0.8 e	91.5 ± 4.8 a	78.3 ± 6.7 b
RHA356	RH	62.6 ± 8.7 bc	27.7 ± 3.8 a	27.8 ± 3.9 ab	69.2 ± 1.3 b	61.8 ± 7.1 bc	10.4 ± 4.1 de
RHA357	RH	54.8 ± 5.8 cd	25.0 ± 2.4 ab	20.8 ± 2.6 cd	68.8 ± 0.8 b	50.4 ± 4.8 d	15.8 ± 5.7 d
RHA358	RH	69.2 ± 5.8 b	24.7 ± 0.9 ab	28 ± 3.6 ab	69.6 ± 1.3 b	45.8 ± 2.6 d	16.1 ± 5.1 de
RHA359	RH	59.4 ± 3.4 c	20.9 ± 4.5 b	24.4 ± 1.9 bc	69.2 ± 0.8 b	68.8 ± 2.7 b	9.1 ± 5.4 e
HA821	SH	107.2 ± 3.6 a	23.1 ± 2.0 ab	28.2 ± 3.0 a	63 ± 1.0 c	95.3 ± 3.8 a	93.4 ± 7.5 a
HA378	RH	55.4 ± 1.1 cd	26.9 ± 4.8 a	26.2 ± 1.6 ab	71.2 ± 1.1 a	64.5 ± 2.4 bc	24.8 ± 3.6 c
HA379	RH	47.4 ± 10.0 d	25.7 ± 3.9 a	22 ± 2.5 c	61.4 ± 1.1 d	59.1 ± 3.1 c	15.7 ± 4.9 d
LSD		9.2	4.6	3.6	1.3	7.5	6.2

* Mean values with the same letter do not differ among inbred lines

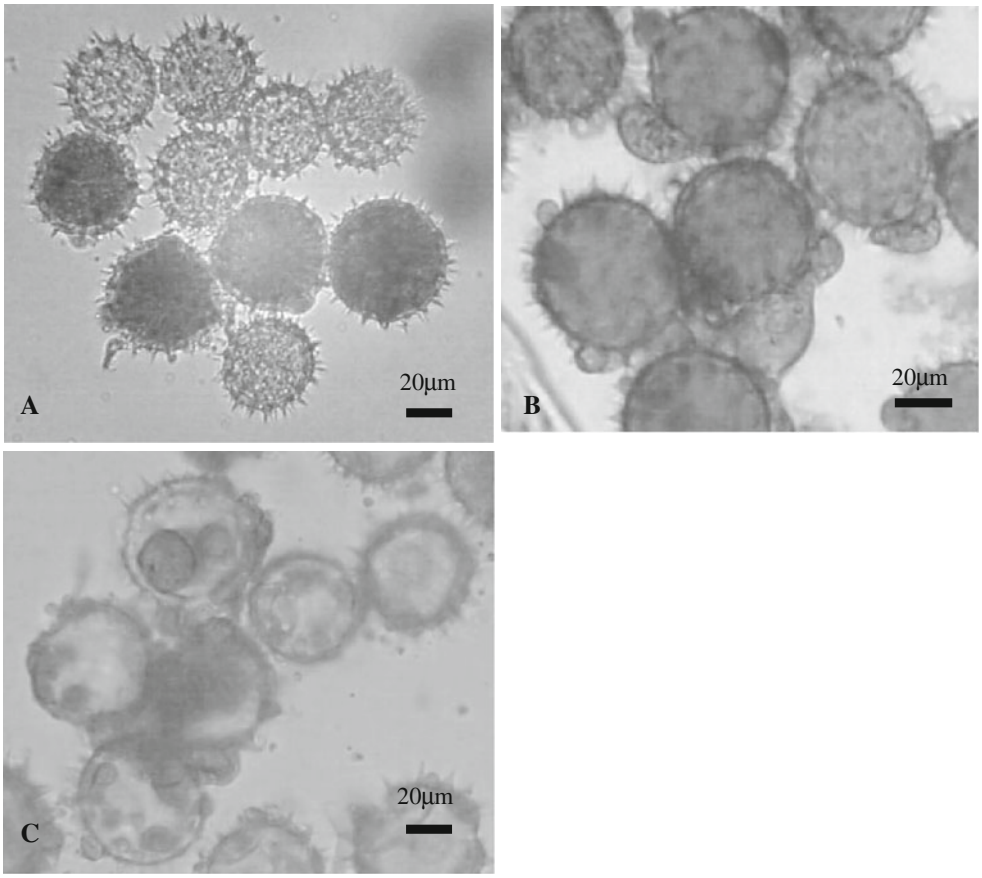

Fig. 1 Pollen grains morphology of reduced-height lines derived from DDR. **a** Pollen grains from RHA358 showing aborted grains with reduced size in comparison with normal non-aborted pollen grains. **b** Pollen from inbred line HA378 showing oily yellow deposits surrounding the pollen grains. **c** Aborted pollen grains of inbred line RHA359 without well-developed exine spines and surrounded with oily granules

phenotypic classes distinguished in BC_1F_1 corresponded to two genotypic classes based on segregation analysis in BC_1F_2. Observed segregation ratio of reduced and standard-height phenotypes in BC_1F_1 fitted the expected 1:1 ratio ($p = 0.84$), indicating that the reduced height phenotype is controlled by one dominant allele, which was named *Rht1*.

Mapping reduced height in RHA358

Bulk segregant analysis identified two markers that were associated with the reduced height phenotype: CRT24 and ORS609. Both markers map to linkage group (LG) 12 of the sunflower public map (Tang et al. 2002) and for this

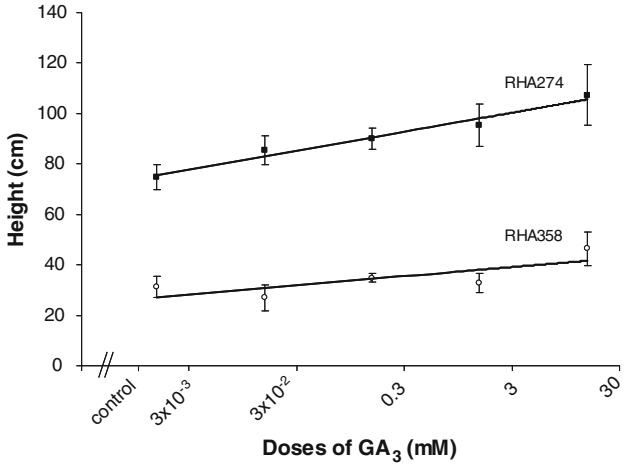

Fig. 2 Height response of standard-height line RHA274 and reduced-height line RHA358 to increasing doses of GA_3

reason, polymorphism for additional SSR markers in this LG was screened. By this way, another set of three markers was found that demonstrated association with the reduced height phenotype. All the individuals of the BC_1F_1 population were genotyped with these five markers and the information was used to construct the map of the genomic region around *Rht1* (Fig. 4). The closest markers to this gene were CRT24 at 14.7 cM and HA3396 at 8.8 cM.

Search of candidate genes for *Rht1* indicated that *HaDella1*, a member of the GA pathway, maps to this region on this LG (Blackman et al. 2011). As DELLA mutations govern reduced height phenotypes in several crops (Yamaguchi 2008), this sequence was thoroughly investigated in RHA358 and its standard-height isoline, RHA274.

HaDELLA1 sequencing and diversity analysis

The *HaDELLA1* partial gene sequence was PCR amplified from RHA358 as only one expected fragment of 675 bp. The resulting PCR product was sequenced. An alignment of this nucleotide sequence versus nucleotide sequences of GenBank Accessions No. DQ503809.1, EU112602.1 (RHA274) and *Lactuca sativa DELLA1* gene sequence (GenBank Accession No. AB370238.1, Sawada et al., 2008) is shown (Fig. 5). The alignment revealed that *HaDELLA1* gene sequence from RHA358 had two point mutations relative to the *HaDELLA1* from RHA274. Both mutations are T-to-C transition corresponding to nucleotide positions 143 and 177 in *HaDELLA1* from RHA358 (Fig. 5). An alignment of the predicted amino acid sequence of *HaDELLA1* nucleotide sequences from RHA274, RHA358, and *L. sativa* (GenBank Accession No. BAG71200.1, Sawada et al. 2008) is shown (Fig. 6). Relative to *HaDELLA1* amino acid sequence of RHA274, the SNP at position 143 converts a leucine residue in a proline within the conserved DELLA—thus, leading to DELPA motif—(Fig. 6). This amino acid position corresponds to amino acid position 57 in the full-length amino acid sequence encoded by the *L. sativa DELLA1* nucleotide sequence of GenBank Accession No. BAG71200.1 (Sawada et al. 2008). The other point mutation in RHA358 gene sequence, at position 177, does not introduce any change in the amino acid sequence.

In silico prediction of amino acid substitution severity was performed. SIFT selected 57 out of 188 protein sequences closely related to the deduced amino acid sequence of *HaDELLA1* from RHA358. In fact, no other sequence showing the DELPA motif was found (Table S1). The point mutation resulting in the amino acid substitution leucine-to-proline in *HaDELLA1* of RHA358 was predicted to affect the protein function with the score of 0.00, suggesting that this change should have an impact in the DELLA motif structure.

All sequences reported for cultivated sunflower and wild *Helianthus* species display a conserved DELLA domain, which indicate that the leucine-to-proline change in the DELLA motif is restricted to reduced-height lines that trace back to DDR. Moreover, this change is absent in all the reported *HaDELLA1* sequences of standard-height sunflower genotypes, annual or perennial species of *Helianthus*

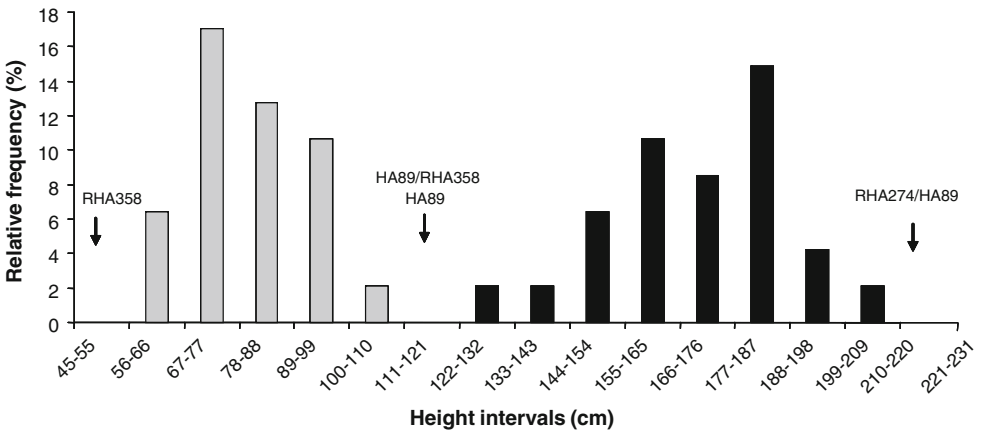

Fig. 3 Frequency distribution of height for BC_1F_1 plants from the cross HA89//HA89/RHA358. Parental lines, F_1 among them and RHA274/HA89, were also included for comparison. *Gray* and *black bars* correspond to the reduced-height and standard-height plants, respectively, based on their individual height confirmed later by progeny test

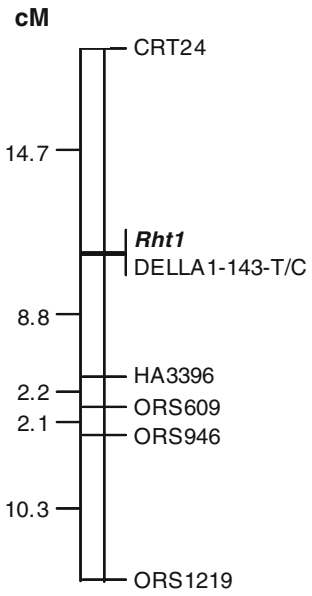

Fig. 4 Map location of the *Rht1* locus and DELLA1-143C/T markers on linkage group 12 of the public map of the sunflower genome

(Table S2). Interestingly, another remarkable feature of the *HaDELLA1* sequences is the VHYNP motif. Although, this domain is highly conserved among all lineages of Angiosperms (Table S1), this motif is changed uniformly to VH[Q]NP across all the reported sequences of *Helianthus* (60 sequences belonging to eight species, Table S2).

Validation of PCR-based markers

Allele-specific markers based on the above-described SNP were developed. Marker DELLA1-143C gave the expected 549 bp PCR fragment in the inbred line RHA358 and no PCR amplification was observed in RHA274 and HA89 (Fig. 7a). On the other hand, a PCR fragment of the expected size was observed in RHA274 and HA89, when the marker DELLA1-143T was amplified. No-corresponding PCR product to this marker was observed in RHA358 (Fig. 7b). Furthermore, any primer cross reactions were observed.

Cosegregation analysis

The cosegregation of reduced or standard height phenotypes and DELLA1-143 markers was assessed on the 94 BC_1F_1 plants segregating for height. Forty-six plants with reduced height from the BC_1F_1 population produced PCR fragments of 549 bp when amplified with both DELLA1-143C and DELLA1-143T markers. On the other hand, the 48 plants with standard-height phenotype from this population amplified one PCR fragment of the expected size when the DELLA1-143T marker was utilized and they only amplified the internal control fragment when the DELLA1-143C marker was used (Table 2). Reduced height progeny was heterozygous for the HA89 and RHA358 haplotypes (T/C), whereas standard height progenies were homozygous for the HA89 haplotype (T/T). The *HaDELLA1* haplotypes completely cosegregated with phenotypes for reduced and standard height, 46 C/T: 48 T/T.

Cosegregation of reduced or standard-height and DELLA1-143 markers was further assessed over standard-height public inbred lines RHA274 and HA821 and their DDR-derived reduced-height inbred lines RHA356, RHA357, RHA359, HA378 and HA379. The analysis revealed that all the DDR-derived lines were homozygous

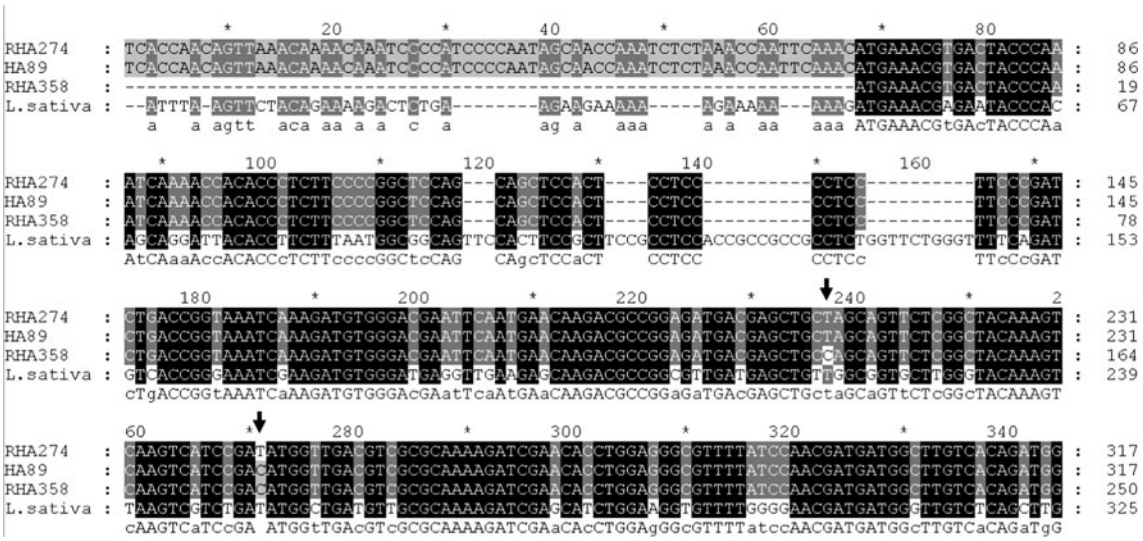

Fig. 5 Nucleotide sequences alignment of *HaDELLA1* from HA89, RHA274, RHA358, and *DELLA1* from *L. sativa*. Point mutations between RHA358 and RHA274 *HaDELLA1* sequences are indicated by *arrowheads*

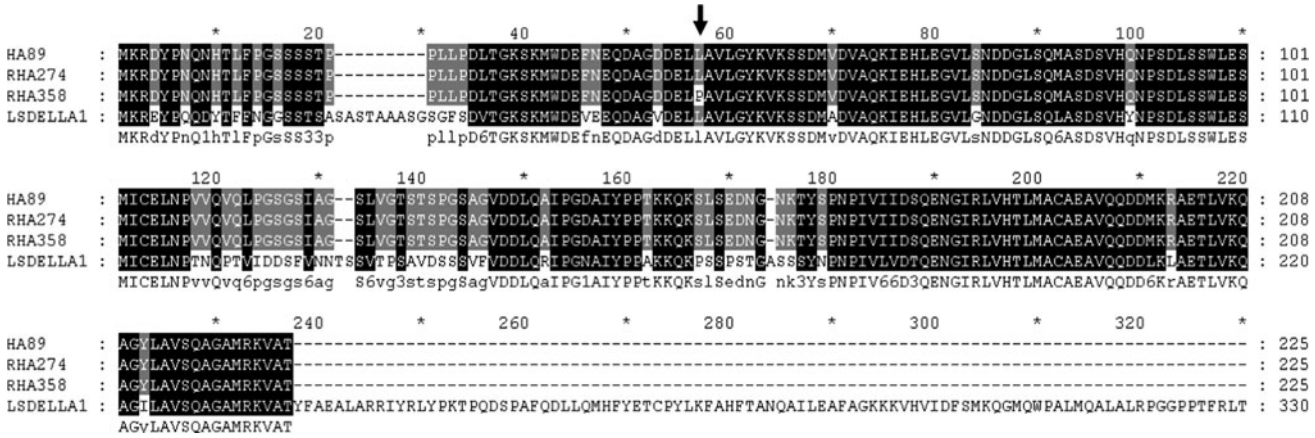

Fig. 6 Predicted HaDELLA1 proteins of HA89 and RHA274 are compared to the mutant HaDELLA1 from RHA358 and LsDELLA1. The T/C point mutation at position 143 in *HaDELLA1* of RHA358 results in a leucine to proline substitution within the DELLA domain (*arrowhead*) creating a DELPA motif

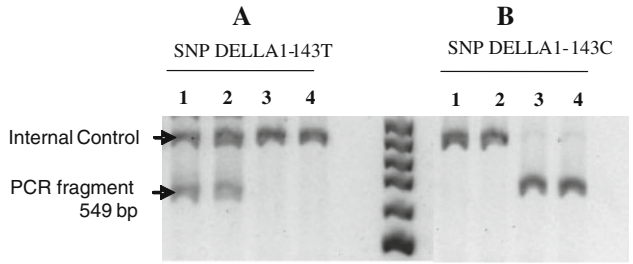

Fig. 7 Allele-specific markers DELLA1-143 for *HaDELLA1*. **a** Allele-specific marker to detect the SNP DELLA1-143T **b** Allele-specific marker to detect the SNP DELLA1-143C. *Lanes 1* HA89, *2* RHA274, *3* and *4* RHA358

for the RHA358 haplotype, whereas the standard-height lines were homozygous for the HA89 haplotype (Fig. 8).

Discussion

Nomenclature of genes conferring reduced height or a drastic dwarf phenotype in sunflower has become confusing. Chronologically, *Dw* was used to designate a dominant allele conferring reduced height and tracing back to the source Donskoi 47 (Tolmachov 1991), *dw1* designates a severe dwarf mutant sensitive to exogenous GA application (Cecconi et al. 2002), *dw1* and *dw2* were used to designate two recessive alleles postulated to control reduced height in the lines tracing back to the source Donsky (Velasco et al. 2003a). Finally, *dw2* was used again to designate another severe dwarf mutant which can be rescued by GA (Fambrini et al. 2011). To avoid this misleading nomenclature, we propose to reserve the gene designation "dw_x" for severe dwarf sunflower mutants, as those described by Cecconi et al. (2002) and Fambrini et al. (2011), and to use "Rht_x" for the genes conferring reduced height in sunflower.

The results of this research indicated that reduced plant height in RHA358 is controlled by a single allele which is designated *Rht1*. This allele exerts dominance over the wild type allele conferring standard height, *rht1*, in such a way that heterozygous *Rht1/rht1* plants showed almost half of the height than the homozygotes *rht1/rht1* of the same genetic background. In addition, the BC_1F_1 population obtained by crossing heterozygotes *Rht1/rht1* to the standard height parental line showed a bimodal distribution for height. This distribution is the result of the combined effect of the locus *Rht1/rht1* plus the epistasy effect of several minor segregating genes controlling height in both parents. The effect of these minor genes would explain the heterosis observed when both standard-height lines were crossed.

Previous genetic studies on the reduced-height source DDR concluded that the trait was quantitatively inherited since no distinct classes were observed in the F_2 generation (Miller and Hammond 1991). Our results, however, indicate that the trait is controlled by a single semidominant gene. One possible explanation for this apparent discrepancy is that we used a BC_1F_1 generation to study the inheritance of the trait instead of a F_2 population, so that the number of phenotypic classes was reduced and, hence, the possible overlap between them was also decreased. Second, in the present work the phenotypic score was taken over plants grown under greenhouse conditions with a photoperiod of 14 h that, in our experience, tends to maximize differences in plant height among standard height lines with respect to their reduced-height derived lines. In the previous study, on the other hand, plants were grown under field conditions where photoperiod and competence effects tend to obscure the differences attributable solely to genetic causes. Third, in the present study the heterosis for height in the absence of *Rht1* (i.e., the height of the hybrid HA89/RHA274, see Fig. 3) was taken into account to interpret the results. This last fact was not

Table 2 Co-segregation of reduced height and DELLA1-143C/T haplotype markers (T = HA89 haplotype, C = RHA358 haplotype) in a BC_1F_1 population derived from the cross RHA358/HA89//HA89

Phenotype of BC1F1 plants	No of plants	DELLA1-143 Haplotype segregation	
		T/T	T/C
Reduced height	46	0	46
Standard height	48	48	0
Segregation ratio tested	1:1		
χ^2 *p* value	0.84		

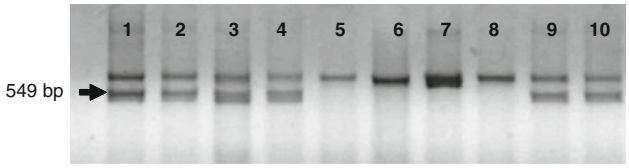

Fig. 8 Polymorphism for the marker DELLA1-143C among standard- and reduced-height lines: *1* RHA356; *2* RHA357; *3* RHA358; *4* RHA359; *9* HA378; *10* HA379 and standard-height lines: *5* HA821; *6* RHA274; *7* HA89; *8* RHA271

considered in previous works addressing the same issue which can lead to misleading results. For example, in studying the inheritance of reduced height in the line Dw89 (an isoline of HA89 with reduced height tracing back to the source Donsky, Velasco et al. 2003a) the trait was considered recessive since the height of the F_1 hybrid Dw89/RHA271 was almost the same as the standard height line RHA271 (Velasco et al. 2003b). However, this "semidwarf hybrid" (using the terminology of Velasco et al. 2003a) was reported to be 27 % shorter than its standard height isohybrid HA89/RHA271 (Velasco et al. 2003a), which indicates that the reduced-height trait is dominant.

Dwarf mutants deficient in endogenous GA have been described for several plant species (for review, see Sakamoto et al. 2004; Yamaguchi 2008). In sunflower, three dwarf mutants have been reported (*dw1*: Cecconi et al. 2002; Jambhulkar 2002, *dw2*: Fambrini et al. 2011). These mutants displayed abnormal development of flower organs without seed development. GA treatments were able to revert *dw1* and *dw2* to wild type or near wild type phenotype, although severe aberrations of reproductive organs were retained (i.e., precocious abort of pollen; Cecconi et al. 2002). The most obvious alterations of *dw2* plants were the lack of stem growth, reduced size of leaves, petioles and flower organs, and retarded flower development. Pollen and ovules were produced but the filaments failed to extrude the anthers from the corolla (Fambrini et al. 2011). A deletion in the ent-kaurenoic acid oxidase gene sequence generated aberrant mRNA-splicing, causing a premature stop codon in the aminoacid sequence. This mutation severely reduced the flux through the biosynthetic pathway leading to bioactive GA by hampering the conversion of ent-kaurenoic acid to GA_{12}. The aberrant phenotype in plants carrying the *dw2* mutation is due to the lack of enough GA production, so application of exogenous GA can restore the wild type phenotype (Fambrini et al. 2011). In contrast to these mutants, exogenous GA applications did not revert the height of the inbred line RHA358. This indicates that *Rht1* is insensitive to exogenous GA application and, therefore, it is not a GA biosynthesis mutant.

Previous studies have established that the mutants which were defective in the biosynthesis of BR or in the BR response pathway showed similarity to the mutants with severe GA-deficiency, i.e., short stature, dark-green leaves, reduced fertility, and robust stems when grown in the light (Fujioka and Yokota 2003). Even though that these attributes correspond to those displayed by RHA358, studies carried out on *Arabidopsis* showed that the BR mutants also displayed a de-etiolated growth habit with short hypocotyls, open cotyledon and no hook (Fridborg et al. 1999).The absence of this skotomorphogenic response in RHA358 indicates that *Rht1* probably is not involved in the BR pathway.

The described results suggest that *Rht1* is defective in the GA response pathway. This pathway is controlled by the DELLA repressors, which are characterized by their N-terminal DELLA domain (Pysh et al. 1999). Characteristically, this type of mutants encodes an altered form of the DELLA protein that are resistant to GA-induced degradation and constitutively blocks GA signaling (Peng et al. 1999). DELLA proteins encoded by *GAI* (Peng et al. 1997) and *RGA* (Silverstone et al. 1998) in *Arabidopsis*; *d8* in maize (*Zea mays*, Winkler and Freeling 1994); *VvGAI* in grape (*Vitis vinifera*, Boss and Thomas 2002); *SLN1* in barley (*Hordeum vulgare*, Chandler et al. 2002), *SLR1* in rice (*Oryza sativa*, Ikeda et al. 2001, Itoh et al. 2002), *BnRGA* in rapessed (*Brassica napus*, Liu et al. 2010), *BrRGA1* in *B. rapa* (Muangprom et al. 2005) and *LeGAI* in tomato (*Solanum lycopersicum*, Bassel et al. 2004) have been isolated and have conserved functions as GA signaling repressors.

In addition to their reduced height, the results obtained indicate that all the analyzed DDR-derived lines showed several abnormalities in the later stages of pollen development, rendering them partially sterile. This observation suggests a pleiotropic effect of *Rht1* over pollen development. GA is not only involved in plant growth but also in reproductive development (Pharis and King 1985; King and Evans 2003). For example, analysis of *A. thaliana* GA-deficient and GA-insensitive mutants has demonstrated that GA synthesis and signaling are important for flower induction and the development of flower organs, such as

petals and stamens (Wilson et al. 1992; Goto and Pharis 1999; Dill and Sun 2001; Cheng et al. 2004). In fact, it has been demonstrated in several species that mutants showing defects in GA synthesis and GA signaling block anther and pollen development (Koornneef and Veen 1980; Tyler et al. 2004; Kaneko et al. 2003; Chhun et al. 2007; Plackett et al. 2011). Moreover, GA exogenous application during early reproductive stages of sunflower cause failures in the microsporogenesis leading to pollen abortion and subsequent male sterility (Schuster 1961).

Genetic studies of *gai* in *Arabidopsis*, *D8* in maize, and *Rht-B1b/Rht-D1b* in wheat mutants (Koornneef et al. 1985; Harberd and Freeling 1989; Peng and Harberd 1993; Peng et al. 1997, 1999) identified common properties of these various mutant alleles that resembles the phenotypic effect of *Rht1* described here: they act in a genetically dominant fashion and encode active (altered function) mutant products that decrease GA response and, hence, confer reduced height. Since the phenotype of *Rht1* resembles that of several DELLA mutations governing reduced height phenotypes reported in the above-mentioned crops (Yamaguchi 2008), this sequence was thoroughly investigated in RHA358 and its standard-height isoline, RHA274. Linkage analysis indicates that *Rht1* maps on sunflower LG12, where the putative candidate gene, *HaDELLA1*, was previously mapped (Blackman et al. 2011). A SNP was found in the sequence of *HaDELLA1* of RHA358 respect to the sequence of RHA274 which determines an aminoacid residue change in the DELLA motif of the DELLA protein. This polymorphism completely cosegregated with the reduced height phenotypes in the mapping population. Furthermore, the presence of the haplotype of RHA358 in all the analyzed DDR-derived lines with reduced height and its absence from the standard-height parental inbred lines, other cultivated sunflower genotypes, annual or perennial *Helianthus* species indicates that *Rht1* probably is a modified *HaDELLA1* sequence.

Discovery of the molecular identity of the endogenous plant GA-opposable growth inhibitory factor resulted from the molecular cloning of genes encoding DELLA proteins (see Harberd et al. 2009 and Sun 2010 for reviews). Several DELLA domain mutations have been described that result in GA-insensitive growth in different plant species. A 17 aminoacid deletion in the conserved DELLA domain, which is the mutation present in the dominant *Arabidopsis gai-1* mutant, renders mutant *gai* and *rga* proteins insensitive to GA induced proteolysis, and plants expressing these mutant DELLA repressors are GA-insensitive, dark-green, late-flowering dwarfs (Peng et al. 1997; Dill and Sun 2001; Silverstone et al. 2001; Fleck and Harberd 2002; Itoh et al. 2002; Dill et al. 2004). Mutations in the DELLA domain were also identified in dwarfing alleles of the DELLA repressors *Rht1* from wheat, *d8* from maize, and *Sln1* from barley, and these mutations were hypothesized to be the molecular cause for the GA insensitivity of the respective alleles (Gale and Marshall 1976; Peng et al. 1999; Chandler et al. 2002). In fact, DELLA proteins have a conserved structure which consists of a DELLA domain at the N-terminus and a conserved GRAS domain at the C-terminus. DELLA domain is unique to all GA-response members of the GRAS family of plant regulatory proteins. The DELLA domain is constituted by a DELLA motif, a VHYNP motif and a poly S/T region. DELLA domain plays an important roll in the degradation of DELLA proteins (Thomas and Sun 2004). Deletions or truncations (*Arabidopsis gai-1*; wheat *Rht-B1b* and *Rht-D1b*; maize *D8-1* and *D8-Mp1*), or point mutation in the DELLA motif (grape *Vvgai1*, Boss and Thomas 2002) or very close to it (barley *Sln1d*, Fu et al. 2002) rendered in dwarf phenotypes resistant to GA-induced degradation. In consequence, the modifications in DELLA domain block the interaction with a GA signal, DELLA proteins is not recruited for degradation and remains as a growth repressing form, resulting in a dwarf phenotype (Schwechheimer and Willige 2009; Sun 2010). The obtained results indicate that the point mutation in the *HaDELLA1* gene sequence of RHA358 converts a leucine residue in a proline within the conserved DELLA motif, rendering a DELPA sequence for this important motif. To the best of our knowledge, this sequence has not been described yet. In silico prediction of aminoacid substitution severity performed suggests that this change could have an impact in the DELLA motif structure and hence, in the functionality of the altered DELLA protein. Therefore, it is likely that the point mutation observed in *Rht1* and the resulting DELPA motif would be a new example of an altered DELLA protein conferring reduced height.

In summary, reduced height in the lines derived from DDR is controlled by a semidominant allele, *Rht1*, which maps on LG12 of the sunflower public consensus map. Phenotypic effects of this allele included shorter height and internode length, insensibility to exogenous GA application, normal skotomorphogenetic response, and reduced seed set under self-pollination conditions, an effect presumably related with the observed reduced pollen viability. Finally, this phenotype completely cosegregated with the *Rht1* sequence of the *HaDELLA1* gene. Taken together, all the phenotypic, genetic and molecular results reported here suggest that sunflower *Rht1* locus likely encodes an altered DELLA protein. If the DELPA motif of the *HaDELLA1* sequence in the *Rht1*-encoded protein determines by itself the observed reduction in height is a matter that remains to be investigated.

Acknowledgments We wish to dedicate this work to the memory of Aurelio Luciano (1922–2012), who pioneered scientific research on genetics and breeding of sunflower. Additionally, we wish to acknowledge the expertise and continuous effort of our lab and greenhouse staff.

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