Sunflower ‘Sunspot’ is Hyposensitive to GA₃ and has a Missense Mutation in the DELLA Motif of *HaDella1*

Norman B. Best¹, Xingang Wang, Scottie Brittsan, Eric Dean, Seth J. Helfers, Ryan Homburg, Mariah L. Mobley, Tiffeny L. Spindler, Bofan Xie, Menglu Zhang, Paul M. Hasegawa, Robert J. Joly, and David Rhodes

Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907

Brian P. Dilkes¹

Department of Biochemistry, Purdue University, West Lafayette, IN 47907

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ABSTRACT. Gibberellins (GAs) are phytohormones that regulate plant height and flowering time in plants. Plants with reduced GA or disrupted in GA signaling exhibit a dwarf phenotype. DELLA proteins are transcriptional repressors that attenuate GA-mediated promotion of plant growth. Alleles in which the eponymous DELLA motif in these proteins is disrupted result in constitutive repression of GA signaling and a dominantly inherited dwarf phenotype. We found that the dwarf *Helianthus annuus* (sunflower) cultivar Sunspot is hyposensitive to GA₃ as compared with the tall cultivar Mammoth Grey. Sequencing of the *HaDella1* gene indicates that ‘Sunspot’ has a single nucleotide polymorphism resulting in a missense mutation in the DELLA motif as compared with ‘Mammoth Grey’ and the reference sequence. *Helianthus annuus* has five genes encoding DELLA proteins, including *HaDella1*. We propose that the DELLA motif alteration in the *HaDella1* gene results in a dominant mutation in ‘Sunspot’ and is the cause of its dwarf phenotype.

Sunflower is an important crop for production of vegetable oil and forage. It is also a significant ornamental plant due to its attractive foliage and large inflorescence. Sunflower is extensively cultivated as landscape plants and for cut flowers. Introduction of ornamental plants is essential to diversify an urban landscape (Suleiman et al., 2009). Dwarf cultivars which permit layering by height across the landscape are commercially significant in a wide variety of landscape plant species, including sunflower. Molecular advances permit the identification of single gene effects responsible for shared phenotypic traits across cultivated plants. We have explored the causes of dwarfism in the commercially important dwarf cultivar Sunspot.

GAs are essential phytohormones and determinants of plant height (Hooley, 1994). Mutants altered in GA biosynthesis or signaling can exhibit a dwarf phenotype (Helliwell et al., 1998; Peng et al., 1997). GA has been shown to control multiple aspects of sunflower growth and development, including plant height and flowering time (Blackman et al., 2011; Ramos et al., 2013). The “Green Revolution” used semidwarfed cereal cultivars to resist plant lodging and increase yield (Hedden, 2003). The semidominant mutants in *Triticum aestivum* (wheat),

exploited during the “Green Revolution,” are altered-function alleles of *Rht-1* encoding a DELLA transcription factor (Peng et al., 1999). DELLA transcription factors are repressors of GA signaling. They typically comprise an amino-terminal domain including the DELLA motif and a plant-specific carboxyl-terminal GRAS domain (Hussain and Peng, 2003). Genetic alterations in the DELLA motif of these genes in *Arabidopsis thaliana*, *Oryza sativa* (rice), and *Zea mays* (maize) have been shown to result in constitutive repression of GA signaling (Asano et al., 2009; Lawit et al., 2010; Willige et al., 2007).

GA stimulates growth by overcoming the DELLA-mediated repression of growth promoting gene expression. In *A. thaliana*, for example, bio-active GAs bind to the soluble GA receptor, *GID1*. The *GID1* protein interacts with the DELLA proteins in a GA-dependent manner and promotes the degradation of the DELLA proteins via the 26S proteasome (Murase et al., 2008). A functional DELLA motif is required for the interaction with *GID1* and relief of transcriptional repression by turnover of the DELLA-domain proteins (Dill et al., 2001).

In this study, we investigated the genetic and physiological basis of the dwarfism exhibited by the sunflower cultivar Sunspot. Comparisons of responses to GA₃ and uniconazole (UCZ), a GA biosynthetic inhibitor, application with the tall cultivar of sunflower Mammoth Grey indicated that ‘Sunspot’ is GA insensitive. Recently, Ramos et al. (2013) mapped dwarfism in a dwarf cultivar to the *HaDella1* locus. Miller and Hammond (1991) previously described the progenitor of this dwarf cultivar, DDR, as a reduced-height genotype obtained from Zentralinstitut für Genetik und Kulturpflanzenforschung, Gatersleben, Germany. The DNA sequence of the *HaDella1* allele in ‘Sunspot’ identified a missense mutation within the DELLA motif predicted to result in constitutive repression. Sequence comparisons and phylogenetic

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¹Corresponding authors. E-mail: nbbest@purdue.edu; bdilkes@purdue.edu.

analyses of the gene family identified five DELLA proteins in sunflower. Four of the five DELLA genes from sunflower were used to perform phylogenetic analysis of the DELLA genes from 33 fully sequenced plant species. All four sunflower genes are grouped in the same clade as the *A. thaliana* DELLA genes *gibberellic acid insensitive* (*gai*), *repressor of ga* (*rga*), *rga-like1* (*rgl1*), *rga-like2* (*rgl2*), and *rga-like3* (*rgl3*).

Materials and Methods

PLANT MATERIAL AND CHEMICAL TREATMENTS. Seeds of ‘Sunspot’ and ‘Mammoth Grey’ were purchased from Bennett’s Greenhouses (Lafayette, IN), and were planted in 20.3-cm pots and filled with a 2:1 (v/v) mixture of peat germination mix (Conrad Fafard, Agawam, MA) to calcined clay (Turface MVP; Profile Products, Buffalo Grove, IL). Seedlings were grown under greenhouse conditions with supplemental high-pressure sodium lighting and were allowed to establish for 14 d before chemical application. Seedlings were treated with 100 μ M GA₃ (Gold Biotechnology, St. Louis, MO) + 0.2% ethanol or 100 μ M UCZ (95% pure; Jiangsu Sevencontinent Green Chemical Co., Zhangjiagang, China) + 0.2% ethanol or with just 0.2% ethanol for control conditions as a soil drench 14 d after germination and were allowed to grow for an additional 3 weeks. Plant height and stem diameter were measured every 7 d starting at the time of treatment. Plant height was measured from soil surface to apex of plant and stem diameter was measured at the first leaf node. Data were collected from two independent replications of n = four or five plants for each cultivar and treatment.

DNA EXTRACTION AND GENOMIC SEQUENCING. Plants used for polymerase chain reaction (PCR) and sequencing of ‘Mammoth Grey’ and ‘Sunspot’ were grown under conditions as previously described. An F₁ cross of ‘Mammoth Grey’ and ‘Sunspot’ was obtained from open-pollinated ‘Mammoth Grey’ and ‘Sunspot’ plants planted in close proximity under field conditions. Seeds from the open-pollinated ‘Mammoth Grey’ were planted in flats and screened for individuals with a dwarf phenotype. One dwarf plant was selected for DNA extraction and sequencing. Plants were allowed to grow for 6 weeks to confirm phenotype. Tissue from the first leaf of two ‘Mammoth Grey’ plants, two ‘Sunspot’ plants, and one dwarf F₁ plant from the open-pollinated cross of ‘Mammoth Grey’ x ‘Sunspot’ was collected and frozen at –80 °C. Tissue was homogenized in liquid nitrogen and DNA was isolated using a small-scale cetyltrimethylammonium bromide method (Allen et al., 2006).

The *HaDella1* gene sequence was amplified by PCR using primers from Ramos et al. (2013) and DyNAzyme II DNA polymerase (Thermo Fisher Scientific, Waltham, MA). PCR was conducted on a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) with a temperature cycling protocol as follows: 94 °C for 2 min followed by 30 cycles of 94 °C for 28 s, 55 °C for 28 s, and 72 °C for 40 s and 72 °C for 5 min final extension. Amplified DNA fragments were separated by electrophoresis and visualized in a 1% agarose gel. PCR products were submitted to Macrogen USA (Rockville, MD) for Sanger sequencing (Sanger et al., 1977). Comparison of the nucleotide and amino acid sequences were performed using ClustalW version 2.1 (Larkin et al., 2007), and the output was observed using Mesquite version 3.03 (Maddison and Maddison, 2015).

DATABASE SEARCHES AND PHYLOGENETIC ANALYSIS. To identify the DELLA domain protein family, sequences similar to

AT1G14920 were obtained using the Inparanoid method as employed by the Phytozome version 10.2.2 database (Goodstein et al., 2012). Parameters for the dual affine Smith–Waterman alignment score were set to greater than 1400 and a sequence similarity of greater than 50%. All sequences were downloaded using BioMart (Smedley et al., 2015) combined with our sunflower sequences in Mesquite version 3.03 (Maddison and Maddison, 2015), and aligned using ClustalW version 2.1 (Larkin et al., 2007). An approximate maximum-likelihood (ML) tree was created from the aligned sequences using FastTree version 2.1.7 (Price et al., 2010). The ML tree was used as a starting tree for MrBayes version 3.2.5 (Ronquist et al., 2012) using a GTR+I+r model with a variable partition rate. The analysis was allowed to run for 10 million generations.

Results and Discussion

‘SUNSPOT’ IS HYPOSENSITIVE TO GA₃. Dwarfism was only observed for plant height and there was no significant difference in stem diameter between ‘Sunspot’ and ‘Mammoth Grey’ (Fig. 1). To determine whether ‘Sunspot’ dwarfism was caused by deficiencies in GA biosynthesis and/or signaling, chemical applications to both ‘Sunspot’ and ‘Mammoth Grey’ were conducted. GA₃ application to ‘Sunspot’ resulted in no significant increase in plant height 21 d after application (Fig. 1A). In contrast, ‘Mammoth Grey’ plant height was increased 14 d after treatment in response to the application of GA₃ (Fig. 1B). Plant height for both genotypes was reduced in UCZ treatments. ‘Sunspot’ treated with UCZ resulted in a significant reduction in plant height after just 14 d and ‘Mammoth Grey’ treated with UCZ showed significant reduction in plant height after 21 d (Fig. 1A and B). No significant effect of GA₃ or UCZ on stem diameter of ‘Sunspot’ or ‘Mammoth Grey’ was observed (Fig. 1C and D). These results demonstrate that ‘Sunspot’ is insensitive to GA signaling compared with ‘Mammoth Grey’. Triazole compounds, including UCZ, have been shown to inhibit multiple cytochrome P450 enzymes (Rademacher, 1991). UCZ has been shown to inhibit GA biosynthesis by blocking activity of *ent*-kaurene oxidase, brassinosteroid biosynthesis in *Lemna minor*, cytokinin biosynthesis in *A. thaliana*, and abscisic acid catabolism in *A. thaliana* (Asami et al., 2000; Saito et al., 2006; Sasaki et al., 2013; Todoroki et al., 2012). The response to UCZ application may be due to nonspecific inhibition of any of these pathways, and cannot be unambiguously interpreted as evidence that additional reduction in GA biosynthesis can cause further dwarfing in ‘Sunspot’.

ANALYSIS OF *HADELLA1* ALLELE SEQUENCES. Previous work identified a polymorphism in *HaDella1* (GenBank accession no. DQ503809.1) as the causative dwarfing locus in the reduced height of DDR-derived inbred line RHA358 (Ramos et al., 2013). A similarity search between GenBank accession no. DQ503809.1 and the sunflower transcriptome (HeliaGene, 2016) using Basic Local Alignment Search Tool (BLAST) (Tatusova and Madden, 1999) identified the most similar annotated transcript as HaT131002279. The conceptually translated annotated protein HaT131002279 was the most similar to DQ503809.1 with 100% identity match at the amino acid level and 99% identity match at the nucleotide level. When we sequenced this locus in ‘Sunspot’ and ‘Mammoth Grey’ to determine if ‘Sunspot’ has a mutation in the *HaDella1* gene,

Fig. 1. Plant height and stem diameter of sunflower cultivars Sunspot and Mammoth Grey treated with gibberellin A₃ (GA₃) or uniconazole (UCZ). Seedlings were allowed to grow for 14 d, and were then treated with a soil drench application of 100 μ M GA₃, 100 μ M UCZ, or control treatment. Data are presented as means from two independent experiments of n = 4 or 5 for each experiment. Plant height was measured on (A) 'Sunspot' (n = 8 or 9) and (B) 'Mammoth Grey' (n = 9). Stem diameter was also measured on (C) 'Sunspot' (n = 8 or 9) and (D) 'Mammoth Grey' (n = 9). Error bars represent sd and asterisks indicate Student's *t* test for significance difference (*P* < 0.01) between treatment and control at a given time point.

Fig. 2. Nucleotide and protein sequence alignments of *HaDella1* alleles. (A) Nucleotide and (B) protein sequence alignment of sunflower cultivars Mammoth Grey and Sunspot to HaT131002279. (A and B) Only the first 260 nucleotides and amino acid residues are shown. Black arrow indicates the polymorphism that distinguished 'Sunspot' from 'Mammoth Grey' and the reference sequence.

we found a single T to C transition at nucleotide position 143 after the start site in 'Sunspot' but not in 'Mammoth Grey', nor in the reference sequence (Fig. 2A). This single nucleotide polymorphism was also identified in the DDR-derived lines described by Ramos et al. (2013) and results in a missense mutation that converts the second leucine (Leu⁴⁸) residue to proline in the DELLA motif of *HaDella1* (Fig. 2B). The

progeny from the F₁ cross of 'Mammoth Grey' × 'Sunspot' also exhibited a dwarf phenotype, demonstrating that the mutation distinguishing the two cultivars is dominant. DNA from this F₁ plant was extracted and the *HaDella1* locus was subjected to Sanger sequencing. The Sanger sequencing chromatogram from DNA amplified from the genomic DNA of this individual had a mixture of T and C signals from the polymorphism at position 143 demonstrating that this plant was heterozygous at this position (data not shown). In the previous study, a second T to C transition at 174 nucleotides after the start site distinguished the DDR-derived dwarf sequence from the DQ503809.1 reference sequence accession (Ramos et al., 2013). We found that only the sequence accession DQ503809.1 used in the previous study has this T allele.

GA-induced degradation of *rga* in *A. thaliana* is dependent upon the amino acid sequence of the DELLA motif (Dill et al., 2001; Silverstone et al., 2001). Disruption of this motif can result in constitutive accumulation and action of the protein even

with the presence of GA. The T to C transition at position 143 of the coding sequence of HaT131002279 results in a missense mutation that converts the DELLA motif into a DELPA, with the dissimilar and more structurally unhindered proline residue replacing leucine. Previous mutations in the DELLA motif of this protein family have resulted in dominant loss of function mutations that render these as constitutive transcriptional repressors that no longer bind the GA-receptor, *gid1* (Hussain and Peng, 2003; Willige et al., 2007).

PHYLOGENETIC ANALYSIS OF DELLA GENES IN SUNFLOWER. To investigate the phylogenetic relationships between DELLAs in sunflower and similar genes in other plant species, we used a Bayesian Markov chain Monte Carlo approach implemented by MrBayes to construct the consensus tree shown in Fig. 3. The annotated gene HaT131002279 was used as a starting sequence to BLAST against the sunflower transcriptome (HeliaGene, 2016) to identify additional potential annotated protein sequences in sunflower. There were four highly similar predicted protein sequences (blast cutoff e-value < 1e-100) to HaT131002279 in the sunflower transcriptome. A strict BLAST value cutoff was used due to the high homology and family number of the carboxyl-terminus GRAS domain (Hirsch and Oldroyd, 2009). The loci HaT131001200, HaT131002552, and HaT131008690 had greater than 50% sequence identity with HaT131002279. The locus HaT131004515 had less than 50% sequence identity with the other loci and initial phylogenetic analysis showed long branch length compared with all species analyzed. Therefore, it was removed from further phylogenetic analysis. In addition, the protein sequence of HaT131002279 was used as a query in a sequence similarity search using the BLASTp algorithm against the *A. thaliana* proteome (TAIR10).

Fig. 3. Phylogenetic tree of the DELLA family of transcription factors from 33 taxa. Maximum likelihood, Bayesian Markov chain Monte Carlo, phylogenetic consensus tree of nucleotide-coding sequences derived from the genes encoding presumed DELLA transcription factors identified within plant genome assemblies in Phytozome (Goodstein et al., 2012) and the sunflower transcriptome (HeliaGene, 2016). Scale bar indicates the number of codon changes per orthologous site. Posterior probabilities for some nodes are indicated. The sunflower *Della1* gene and *Arabidopsis thaliana* genes, *gai*, *rga*, *rgl1*, *rgl2*, and *rgl3*, are indicated by bold text.

The closest hit was AT1G14920 (*gai*) with 63% sequence similarity. The *A. thaliana* gene *gai* was used as a query to identify similar genes in other species. Upon further examination of alignments, several annotated genes were missing the amino-terminal end of the encoding sequence and were therefore left out of the phylogenetic analysis. These included *Selaginella*

*moellendorffii*_139506, *Cucumis sativus*_Cucsa.031210, *Prunus persica*_ppa005944m.g, *Malus domestica*_MDP0000950387, and *Medicago truncatula*_Medtr3g065980. This resulted in a total of 92 sequences from 33 species. The grass species [rice, maize, *Panicum virgatum* (switchgrass), *Sorghum bicolor*, *Setaria italica*, and *Brachypodium distachyon*] were used as an outgroup for phylogenetic analysis.

After the split between dicot and monocot, dicot DELLA genes were duplicated, often retained, and diversified. Only one orthologous DELLA gene was observed in monocot species except switchgrass and maize, each of which had two. Of the 27 dicot species analyzed, all had two or more DELLAs except for the two species in the family Solanaceae (*Solanum tuberosum* and *Solanum lycopersicum*). All four sunflower genes used in the phylogenetic analysis are grouped within the dicot clade but neither within the *gai/rga* nor *rgl1-rgl3* subclades which formed divisions specific to the Brassicaceae (Fig. 3). The sunflower paralogs did not form a single group. The gene *HaDella1* is grouped in the same subclade as HaT131002552 and HaT131001200, with HaT131008690 forming its own subclade (Fig. 3).

In conclusion, the sunflower cultivar Sunspot is hyposensitive to GA₃ treatment as compared with the cultivar Mammoth Grey. Sunflower has four completely sequenced DELLA genes. These all fall within the grouping of the dicots, but do not separate together with the diverged Brassicaceae subclades of *gai/rga* and the *rgl1-rgl3* genes. ‘Sunspot’ exhibits dominant dwarfism, consistent with the expected effects of a missense mutation in the DELLA motif. The cultivar encodes a missense mutation in *HaDella1* altering the DELLA domain to DELPA. This mutation was also found previously and cosegregated with dwarfism in the DDR line of sunflower (Ramos et al., 2013). These results identify the likely molecular cause of dwarfism in ‘Sunspot’, and explored the relationships of the DELLA genes in sunflower. This identifies the DELLA domain proteins as future targets for genetic modification for plant height in sunflower and other species used for landscape plantings.

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