IDENTIFICATION OF *HADELLA, HAGID1* AS WELL AS *HASLEEPY* AND *HASNEEZY* GENES INVOLVED IN GIBBERELLIN SIGNALING IN SUNFLOWER

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ABSTRACT

Mutations in the gibberellin (GA) biosynthesis or signaling pathway lead to significant changes in shoot growth (dwarfism/gigantism) and were responsible for the so-called 'green revolution' in cereals. Knowledge about the GA metabolism offers starting-points for targeted breeding in sunflower. However, so far we know little about the components of the GA signaling pathway in sunflower. Using the sunflower genome sequence database, kindly provided by the Loren Rieseberg group (www.heliagene.org), we screened for similar sequences to known DELLA, GID1 and SLEEPY proteins from other plant species. Next, identified sequences of the HA-412-HO scaffold database were used for primer design and the existence of the genes was verified in the line HA383 using DNA as well as cDNA. Here we describe the identification and characterization of four DELLA (HaDELLA1-2 and HaDELLA-like1-2), five HaGID1A-E and nine F-box genes (HaSLEEPY1A-F and HaSNEEZY1-3). For functional analysis, we studied sunflower SLEEPY and DELLA homologs in Arabidopsis thaliana. Our complementation studies of the Arabidopsis SLEEPY mutant sly1-10 demonstrated that HaSLY1A encodes a functional F-box protein, which is fully able to compensate the sly1-10 mutant phenotype. Overexpression of HaDELLA1 in wild-type Arabidopsis induced dwarfism and late-flowering.

Keywords: Gibberellins, *Helianthus annuus*, plant hormones, signal transduction

INTRODUCTION

Three major regulators are responsible for perception of GAs by the GA signaling pathway: the GA receptor GID1 (GA-insensitive dwarf1), the DELLA protein and SLY1/GID2 (SLEPPY1/GA-insensitive dwarf2) as part of the SCF E3 ubiquitin ligase. GID1 was characterized as soluble receptor for GA by studies of GA-insensitive dwarf rice mutants (Ueguchi-Tanaka et al., 2005). In Arabidopsis, three GID1 encoding genes (AtGID1A, AtGID1B and AtGID1C) were identified (Nakajima et al., 2006). Analyses of the gid1 double and triple mutants as well as interacting studies with DELLA proteins revealed that GID1 orthologs have redundant but also distinct roles in plant development (Iuchi et al., 2007). In contrast, there is very little knowledge available about GID1 encoding genes in sunflower. Recently, Blackman et al. (2011) has published one partial GID1-like sequence (about 37%, HaGID1B), which shows high similarities to GID1B (At3g63010) in Arabidopsis. However, transcriptome analysis with the focus on GA synthesis and signaling genes of Gerbera hybrida indicate that species of the compositae family contain various homologous GID1 genes (Kuang et al., 2013). In Gerbera, nine different GID1 transcripts were detected and in H. annuus three corresponding hits were found by local BLASTN indicating the existence of more than one GID1 gene in sunflower.

DELLA proteins are nuclear transcriptional regulators that repress the GA signaling pathway and belong to the GRAS gene family (Tian et al., 2004). Several plant species like rice (SLR1) and tomato (Pro) contain only a single gene encoding for DELLA (Ikeda et al., 2001; Jasinski et al., 2008). In contrast, other species like A. thaliana have multiple DELLA genes (Tyler et al., 2004). Ramos et al. (2013) have recently reported that the semidominant mutant sunflower allele, Rht1, which maps to linkage group 12 of the sunflower public consensus map, completely cosegregated with a haplotype of HaDELLA1. 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-pollinating conditions (Ramos et al., 2013). In addition to the known HaDELLA1 sequence similar EST sequences provide initial indications of a second HaDELLA gene (Blackman et al., 2011), but it remains to be determined whether that C-terminal sequence is part of a true DELLA protein or belongs to another member of the large GRAS gene family.

The F-box protein GID2/SLY1 interacts with DELLA proteins, especially in the presence of GA activated GID1, via the C-terminal GGF and LSL domains (Sun, 2008). The N-terminal F-box domain is necessary for the interaction with the SCF complex (Smalle & Vierstra, 2004). However, the molecular mechanisms behind GA signaling have been extensively studied in plants such as Arabidopsis and rice, which have only a single gene encoding SLY1/GID2. In sunflower, just one putative SLY1 sequence (HaSLY1) has been described so far (Blackman et al., 2011), but it seems to be very likely that sunflower, like Gerbera hybrida with six putative GID2/SLY1 transcripts (Kuang et al., 2013), will have more than one SLY1 homolog.

MATERIAL AND METHODS

Screening of the sunflower genome for GID1, DELLA and SLEEPY homologs

The genome database of the sunflower line HA-412-HO provided by Loren Rieseberg (www.heliagene.org) was screened for similar sequences to known GID1, DELLA1 and SLY1 sequences of Arabidopsis thaliana. Therefore the 3.1 GB scaffold fasta file (Celera_14libs_sspace2_ext.final.scaffolds) was converted into a local nucleotide database by using the stand alone blast program Blaststation-Local (www.blaststation.com). We also used published sequences of GA signaling genes of Lactuca sativa (Sawada et al., 2008) due to its close relationship to sunflower as well as previously published EST sequences of putative sunflower genes involved in GA signaling (Blackman et al., 2011).

In silico analyses of sunflower GA signaling homologs

To compare the hits of the scaffold database showing highest E-values with GID1, DELLA or SLY1 proteins we performed phylogenetic analyses. Therefore, we choose GID1, DELLA and SLY1 homologs of sequenced higher land plant genomes, which were described in the literature or detected by BLASTP and TBLASTN tools of the databases NCBI (National Center for Biotechnology Information) and Phytozome (http://www.phytozome.org). We also included amino acid sequences of Selaginella moellendorffii (lycophyte) as well as homologous protein sequences of Physcomitrella patens (moss). Several studies dealt with the subject of evolutionary origin of GA signaling. These studies indicate that the GA signaling evolved after the divergence of bryophytes from land plants (Wang et al., 2015). Furthermore, it was demonstrated that homologs of GID1, DELLA, and SLY1 work similar in S. moellendorffii and in flowering plants, whereas no evidences were found for functional conservation of genes in P. patens (Hirano et al., 2007). For this reason, sequences of P. patens were used as outgroup, whereas S.

moellendorffii sequences served as exclusion limits to eliminate similar but unrelated sequence, like other GRAS or F-box proteins. The "one click" mode of Phylogeny.fr (Dereeper et al., 2008) offered a quick and easy method for handling large volumes of sequences and was used to get a first impression how much of the hits really belonged to the GA signaling members. Next, Neighbor-joining trees were computed using the JTT matrix-based method and bootstrap test of 1000 replicates to confirm the evolutionary distance.

Verification of GA signaling scaffold sequences in HA383

Based on scaffold sequences forward and reverse primers were designed encompassing the respective coding sequence of GID1, DELLA and SLY1 homologs. Genomic DNA from leaves of HA383 was subjected to standard PCR (Taq DNA polymerase NEB). The obtained fragments were cloned in pGEM®-T Easy vector (Promega) and sequenced using SP6 and T7 primers designed for sequencing inserts cloned into this vector. Nucleotide polymorphisms were verified by using a proof reading Phusion DNA polymerase (Thermo Scientific). To get accurate information on the exon-intron structure of the genes, RNA from HA383 was isolated according to the protocol for RNA extraction from different tissues of grapevine and other woody plants (Gambino et al., 2008). All RNA samples have undergone a DNAse treatment (DNAse I; Fermentas) to remove DNA contaminations. Complementary DNA (cDNA) was obtained from total RNA by using RevertAid H minus cDNA synthesis kit (MBI Fermentas) and used for amplification of coding sequences.

Functional analyses of the sunflower SLEEPY and DELLA homologs HaSLY1A and HaDELLA1

PCR amplicons of HaSLY1A and HaDELLA1 coding sequences flanked by restriction sites were ligated into corresponding cloning sites of the 35S cassette. The entire 35S cassettes, now including cds of HaSLY1A or HaDELLA1, were excised and inserted into the vector pGREEN0029 via the EcoRV restriction site (http://www.pgreen.ac.uk). These vectors were used to transform Arabidopsis mutant sly1-10 or Landsberg erecta wild-type plants by using Agrobacterium tumefaciens strain GV3101psoup. After selection with BASTA (phosphinotricine, 0.1%), integration of the 35S:HaSLY1A or 35S:HaDELLA1 transgene was confirmed by PCR using a primer specific for the 35S promoter and reverse primers for the respective coding sequences (cds). In the screen for HaSLY1A complemented plants that were homozygous for sly1-10, we used the specific primer pairs sly1-10f/2-63r and sly1-10f/sly1-10r2 for PCR with genomic DNA to verify the absence of the wild-type Arabidopsis AtSLY1 gene and the presence of the sly1-10 allele, respectively (McGinnis et al., 2003). Primer for the gene At2g09990 encoding the 40S ribosomal protein S16 were used as positive control for DNA content. Arabidopsis plants were grown under a 10-h-light/14-h-dark (22°C/18°C) cycle at 100 to 150 µmol photons m^{-2} s^{-1} in controlled environment chambers, or a 12-h-light/12-h-dark cycle in the case of HaDELLA1-overexpression (OE) lines and the corresponding Arabidopsis wild-type.

RESULTS AND DISCUSSION

Identification of GID1 homologs in the sunflower genome

GID1, the soluble receptor for GA, was first characterized in rice (Ueguchi-Tanaka et al., 2005). A reason for this surely was that no other GID1 isoform was able to compensate phenotypic alterations based on mutations in this single gene. In contrast, in Arabidopsis three GID1 proteins

(AtGID1a, AtGID1b and AtGID1c) with redundant but also distinct roles in plant development were identified (Nakajima et al., 2006). In 2011, one sunflower EST sequence (BU028290) was published, which encodes the C-terminal part of an Arabidopsis AtGID1b similar protein (Blackman et al., 2011). However, our genome databases screenings revealed that the genomes of most eudicots contain at least two different genes encoding GID1 homologous proteins. In addition, Kuang et al. (2013) found three corresponding hits to GID1 homologs in Gerbera hybrida. In H. annuus, local BLASTN indicated several HaGID1 isoforms. By using the sunflower EST BU028290, which is similar to AtGID1b, for BLAST searches in the HA-412-HO scaffold database we detected an ORF of 1005 bp, which was identical to BU028290 in the 3'-end. The resulting amino acid sequence showed more than 90% identities when compared to Lactuca sativa LsGID1A and LsGID1B sequences (Sawada et al., 2012). Nevertheless the start ATG, as well as the next 13 amino acids could not be detected in that scaffold. By using the Lactuca GID1 sequences (LsGID1A and LsGID1B) we found a second scaffold, which was identical to the first scaffold in the 3'-end. In addition, this scaffold contained a start ATG followed by a 39-bp-sequence, which showed high similarities in the amino acid sequence to LsGID1A and LsGID1B, 890 bp upstream from the 1005-bp-ORF. To confirm the exon-inron structure we used RT-PCR with primers encompassing the full-length cds (binding in the 5'- and 3'-UTR region) and amplified the mature HaGID1B cds. We were thus able to guarantee that both scaffolds contained parts of HaGID1B, which consists of two exons and one intron of 890 bp. We utilized the HaGID1B sequence and found four other highly similar sequences (HaGID1A, C, D and E) within the HA-412-HO genome (Figure 1). Like HaGID1B, HaGID1A and HaGID1E consist of two exons separated by an intron of 577 bp and 885 bp, respectively. We anticipate that HaGID1C and HaGID1D have similar structures but the first exon and the following intron are missing due to truncated scaffold sequences. Phylogenetic analyses using the second exons, the main parts of HaGID1C and HaGID1D cds, have clearly shown that the resulting protein sequences belong to GID1 receptors (tree not shown). Consequently, our findings reveal that sunflower contains at least five HaGID1 isoforms and it seems to be very likely that HaGID1s, like Arabidopsis GID1 proteins, mediate a complex network with overlapping but also distinct functions.

Identification of DELLA homologs in the sunflower genome

DELLA proteins belong to a subfamily of the plant specific GRAS gene family. DELLAs contain a conserved C-terminal GRAS domain and a unique N-terminal DELLA domain, which is essential for GA-induced degradation (Sun, 2011). In rice and barley, one single gene codes for the only DELLA protein, called SLENDER1 due to the elongated stem phenotype (Ikeda et al., 2001; Chandler et al., 2002). In contrast, Arabidopsis contains five DELLA proteins with partly overlapping but also distinct functions (Daviere & Achard, 2013). There is some evidence that the genome of sunflower contains at least two different genes (HaDELLA1 and HaDELLA2) coding for DELLA proteins (Blackman et al., 2011). However, functional evidence for the HaDELLA proteins is missing, although Ramos et al. (2013) could show that the reduced height in some dwarf sunflower lines is based on a SNP in the DELLA motif of HaDELLA1, which leads to a single amino acid change from DELLA to DELPA (Ramos et al., 2013). At the beginning of our search for DELLA homologs in sunflower, we first aligned the EST sequences of HaDELLA1 published by Blackman et al. (2011). The ESTs coded for an N-terminal HaDELLA part as well as for a C-terminal GRAS domain, but did not overlap in the middle. For that reason, we tried to amplify the whole cds by using primers binding in the 5'- and 3'-UTRs and were able to fill the observed gap of 41 amino acids between the N- and C-terminal part. The HaDELLA1 amplicons differed in size by about 300 bp, when we used DNA or cDNA of the

line HA383. This is due to the fact that the HaDELLA1 cds is divided into two parts by a 325-bp-intron. In the next step, we wanted to find out whether the putative HaDELLA2 EST (CD850340), lacking the DELLA domain, is part of a true DELLA protein and searched for identical scaffold parts in the HA-412-HO genome. Indeed, CD850340 is part of an 1701-bp-ORF, not interrupted by introns, with a typical DELLA domain. In addition to HaDELLA1 and HaDELLA2, we detected two other coding sequences having putative DELLA domains. These sequences were named HaDELLA-Like1 and HaDELLA-Like2 due to the modified DELLE and DELLF motifs, respectively. Besides these DELLA sequences, we found many other hits of unrelated putative GRAS proteins, which were excluded by phylogenetic analyses. The HaDELLA-Like1 coding sequence consists of 1710 nucleotides. The HaDELLA-Like2 cds still remains fragmentary (1521 bp), lacking about 50-60 amino acid at the end. Despite many attempts to amplify transcripts, we could not detect expression of HaDELLA-like2 in different organs or developing stages of the line HA383, a prerequisite for RACE analyses. Like for HaDELLA-Like2, HaDELLA-Like1 transcripts could not be detected. However, it seems rather unlikely that HaDELLA-Like1 is just a pseudogene because HaDELLA-Like1 (also referred to as RGL2 by Mandel) was identified as a gene of likely agronomic importance in evolutionary analyses of crop-related traits in sunflower (Mandel et al., 2014). The authors speculated that HaDELLA-Like1, which co-localized with a QTL for seed dormancy, might perhaps have something to do with lesser or no dormancy of primitive and improved varieties compared to the strong seed dormancy observed in wild sunflowers. Investigations of Arabidopsis RGL2 revealed that RGL2 transcript levels rise rapidly following seed imbibition and then decline rapidly as germination proceeds (Lee et al., 2002). Further studies are needed to find out whether HaDELLA-Like1 is a functional equivalent of AtRGL2. Hower, it is conceivable that the expression level of HaDELLA-Like1 may be lower in improved varieties without dormancy.

Identification of SLEEPY and SNEEZY homologs in the sunflower genome

The two orthologous F-box genes GID2 and SLY1 of rice and Arabidopsis are needed for GA-stimulated DELLA degradation by the 26S proteasome (Wang & Deng, 2011). A second F-box protein SNE (SNEEZY) in Arabidopsis is only partially able to compensate the sly1 phenotype by overexpression. Extensive studies of AtSLY1 and AtSNE overexpression in the Arabidopsis sly1-10 mutant suggest that one reason, why SNE is unable to fully compensate the mutant phenotype, is that SLY1 regulates a broader spectrum of DELLA proteins than SNE (Ariizumi et al., 2011). However, rice and Arabidopsis have only a single gene for GID2/SLY1 and SNE, respectively, whereas other plant species like Gerbera hybrida have more SLY1 homologs (Kuang et al., 2013). In sunflower, one full-length cds of a putative SLEEPY protein (HaSLY1, now named HaSLY1A) was described so far (Blackman et al., 2011) but initial work of our group revealed the existence of a second HaSLY1 isoform (HaSLY1B).

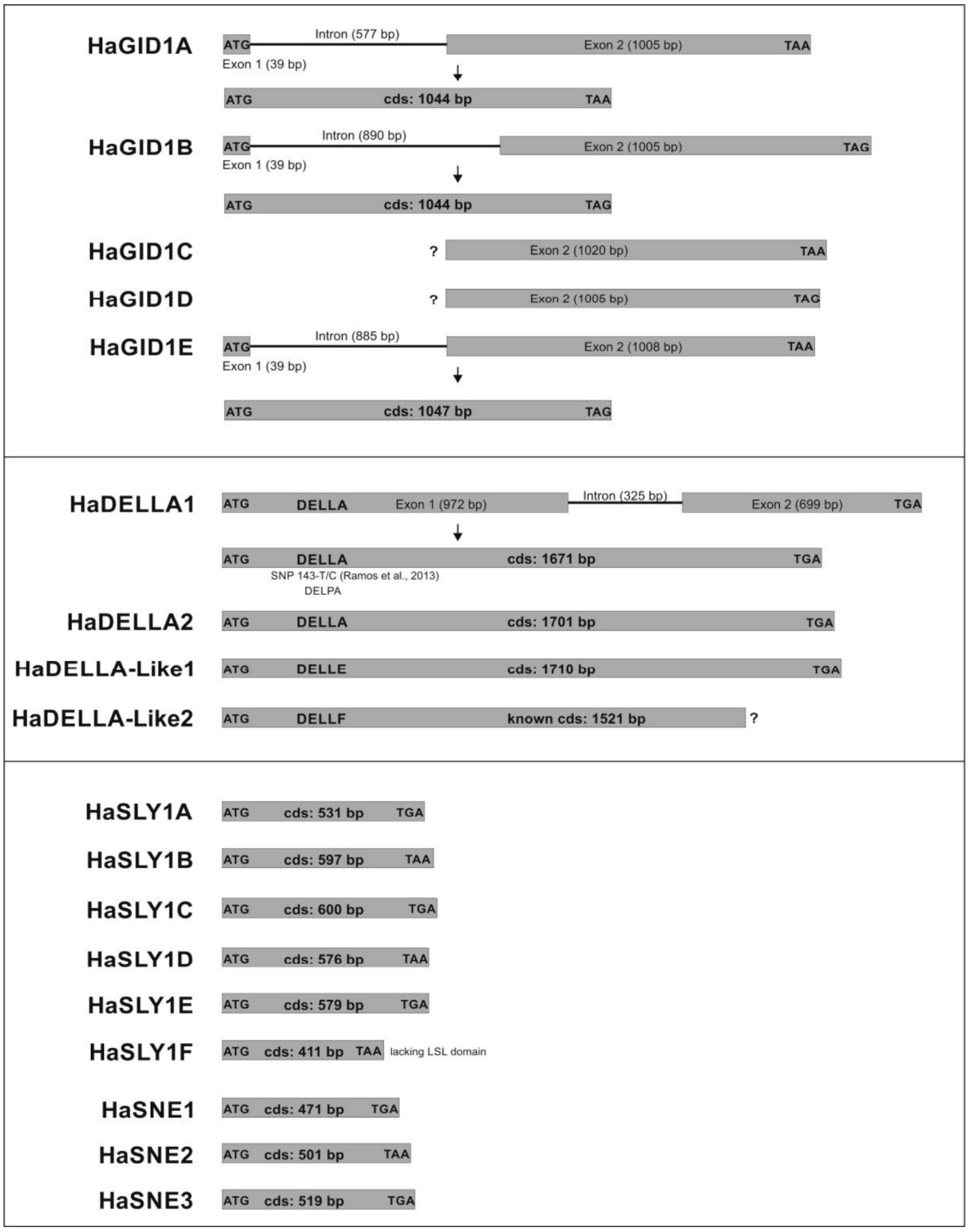

Fig. 16: Schematic overview of genes involved in GA signaling in the sunflower line HA383

Through the access to the genome database we were able to identify all in all nine coding sequences showing similarities to Arabidopsis SLY1, some of them being incomplete. Like HaSLY1A and HaSLY1B, four other coding sequences also showed high identity values to AtSLY1 and were named HaSLY1C, D, E and F. The last three related sequences, on the other hand, had a significantly lower correlation to AtSLY1 and HaSLY1s. We assumed that these

three proteins (now HaSNE1, 2 and 3) could be hitherto undetected sunflower SNEEZY homologs due to amino acid identities of about 50% to AtSNE. The coding sequence of HaSNE3 was incomplete in the scaffold database assembled by the program Celera but we detected the full-length cds in the Ha412Newbler20120907_gene database (www.heliagene.org). The complete cds of HaSLY1E, lacking the 5'-end in the scaffold databases, was obtained by RACE-PCR. Assuming that the HaSLY1F sequence was unusually short due to sequencing or assembly errors, we were surprised to find the same TAA stop codon in the sunflower line HA383 at position 409, which leads to a shortened F-box protein. Attempts to obtain RACE-PCR products as well as RT-PCR amplicons have failed. In contrast to all other HaSLY1s, HaSLY1F seemed to be not expressed. Complementation studies of truncated AtSLY1 forms provided evidence for malfunction of SLEEPY proteins without the LSL domain (Ariizumi et al., 2011). Together with the fact that no HaSLY1F expression was detectable the unusually short form suggests that HaSLY1F may represent a pseudogene.

In summary, our findings of nine F-box proteins in sunflower, as well as the detection of six putative SLY1 transcripts in the transcriptome of Gerbera hybrida ray florets (Kuang et al., 2013) suggest that in the Asteraceae family genomes may contain a large number of duplicated F-box proteins.

Functional analyses of sunflower SLEEPY and DELLA homologs

The very low regeneration rates in sunflower make it almost impossible to generate stable knockout or knockdown mutant lines. Therefore, we chose the model plant Arabidopsis for functional analyses of the sunflower GA signaling homologs. In this work, we describe our general approach for functional analyses using HaSLY1A and HaDELLA1 as examples. The sly1-10 mutant provides a good basis for complementation studies with SLY1 homologs. In Arabidopsis the overexpression of AtSLY1 under the control of the 35S-promoter fully complemented the dwarfism of the loss-of-function mutant sly1-10 (McGinnis et al., 2003). The full-length ORF of HASLY1A gene was cloned into plant transformation vector pGREEN0229 including the 35S-promoter and terminator (Fig. 17 A). The sly1-10 mutant was transformed with these constructs.

The genotyping analyses clearly showed that all lines contained the respective T-DNA and were homozygous for the sly1-10 mutation (Fig. 17 C). The growth of overexpression lines was compared with untransformed sly1-10 plants as well as wild-type (Ler) plants. HaSLY1A-overexpression lines grew significant faster than untransformed sly1-10 plants and resulted in a similar growth type as the wild-type plants (Fig. 17 E). These results show that the HaSLY1A protein was able to rescue the genetic defects of sly1-10.

Accumulation of DELLA proteins results in dwarfism and often in delayed flowering onset. Typical examples of this are GID1 and SLY1/GID2 knockout lines (Sasaki et al., 2003; Strader et al., 2004), as well as mutations in DELLA genes responsible for the so called 'green evolution' in cereals (Boss & Thomas, 2002). Therefore we tested the phenotypic changes of Arabidopsis wild-type plants (ecotype Ler) by overexpressing HaDELLA1 under the control of the 35S-promoter. The HaDELLA1 overexpression lines showed a delayed flowering onset, slightly decreased rosette diameter and shorter stems (Fig. 17 D). We have not yet made statistical evaluations (waiting for the T3-generation), but the phenotypic changes of HaDELLA1-OE line are in line with observation of plants showing DELLA accumulation.

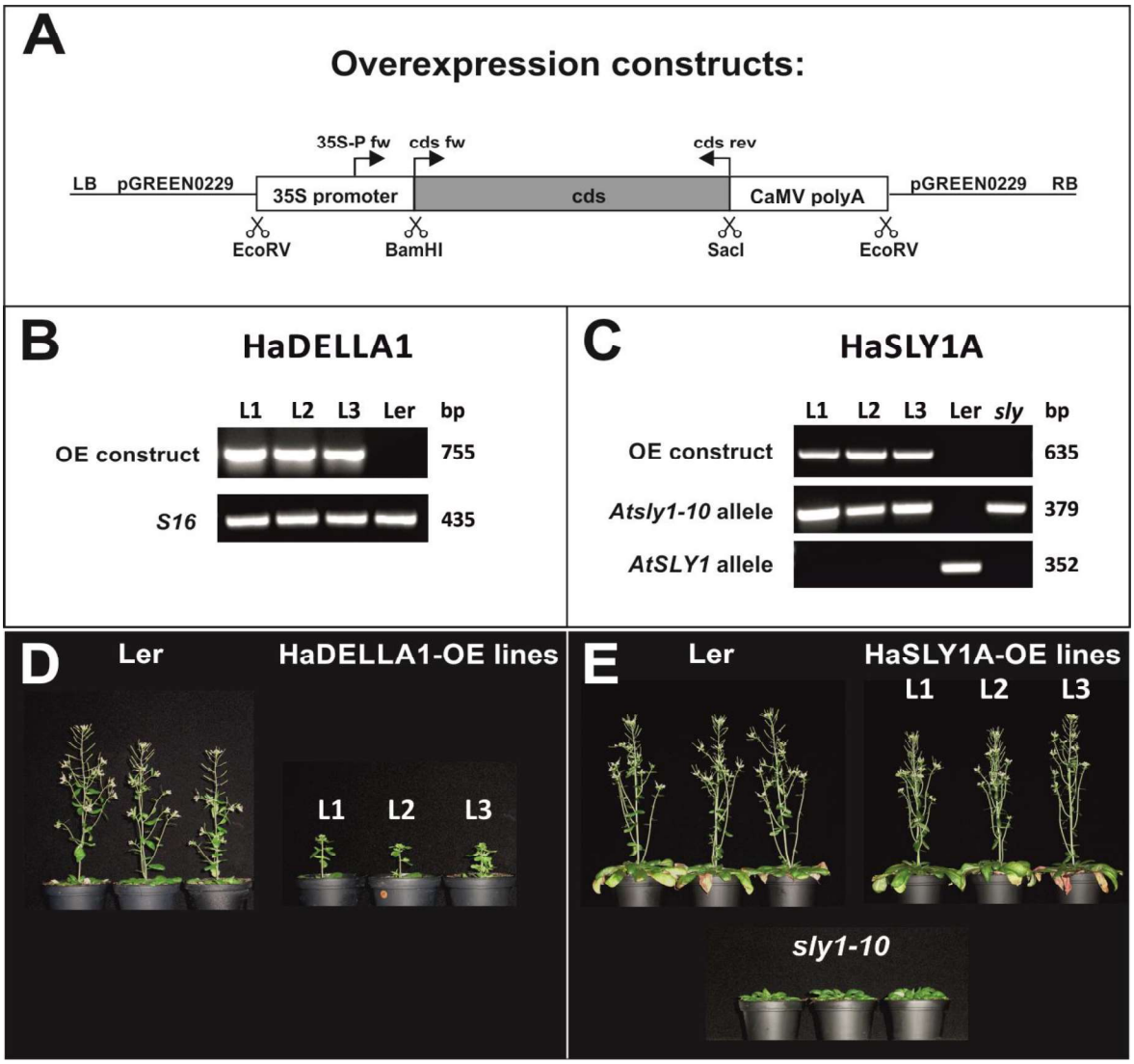

Fig. 17: Overexpression of HaDELLA1 in wild-type Arabidopsis induced dwarfism and late-flowering, whereas overexpression of HaSLY1A rescued sly1-10 dwarf phenotype. A: Schematic overview about cloning strategy of overexpression constructs (see methods for detailed information). B, C: Genotyping of HaDELLA1 (Ler background) and HaSLY1A (sly1-10 background) overexpression lines (L1-L3). D, E: Phenotyping of overexpression lines compared to corresponding Arabidopsis wild-type and sly1-10 mutant plants.

Currently, we analyze these overexpression lines, as well as overexpression lines of the other GA signaling genes detected in sunflower to get more detailed knowledge about their functions.

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