Evolutionary Conservation and Transcriptome Analyses Attribute Perenniality and Flowering to Day-Length Responsive Genes in Bulbous Barley (*Hordeum bulbosum*)

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Abstract

Rapid population growth and dramatic climatic turnovers are challenging global crop production. These challenges are spurring plant breeders to enhance adaptation and sustainability of major crops. One intriguing approach is to turn annual systems into perennial ones, yet long-term classical breeding efforts to induce perenniality have achieved limited success. Here, we report the results of our investigation of the genetic basis of bulb formation in the nonmodel organism *Hordeum bulbosum*, a perennial species closely related to barley. To identify candidate genes that regulate bulb formation in *H. bulbosum*, we applied two complementary approaches. First, we explored the evolutionary conservation of expressed genes among annual Poaceae species. Next, we assembled a reference transcriptome for *H. bulbosum* and conducted a differential expression (DE) analysis before and after stimulating bulb initiation. Low conservation was identified in genes related to perenniality in *H. bulbosum* compared with other species, including bulb development and sugar accumulation genes. We also inspected these genes using a DE analysis, which enabled identification of additional genes responsible for bulb initiation and flowering regulation. We propose a molecular model for the regulation of bulb formation involving storage organ development and starch biosynthesis genes. The high conservation observed along a major part of the pathway between *H. bulbosum* and barley suggests a potential for the application of biotechnological techniques to accelerate breeding toward perenniality in barley.

Key words: transcriptome, perenniality, barley, *Hordeum bulbosum*, *Hordeum spontaneum*, crop wild relatives.

Significance

A major driver of modern agriculture is to increase food production while fostering sustainability. The transition of major annual crops into perennial life forms is gaining attention as a promising means to conserve ecosystem services while maintaining productivity. Here, we used evolutionary genomic and transcriptomic approaches to target candidate genes that are associated with perenniality in *Hordeum bulbosum*, a close wild relative to cultivated barley. A panel of candidate genes related to flowering and bulb formation was detected in response to a shift in day length, implying that the pathways are intertwined, yet the two processes can be distinguished. Moreover, the mechanism was largely conserved across *Hordeum* species, thus encouraging breeding toward a perennial barley crop.

Introduction

It is now recognized that agriculture should be made far more sustainable. One intriguing, yet challenging, approach is to transition major annual agricultural systems into perennial ones (Ryan et al. 2018). Perennials are year-round crops harvested multiple times over several seasons, a feature that

offers many environmental and economic benefits. Unlike annual crops, perennials are thriftier and improve soil structure and water-retention capacity, contribute to increased mitigation practices to cope with climate change, and promote biodiversity and ecosystem functions (Kantar et al. 2016; Lundgren and Des Marais 2020). Among annual crops that are cultivated across over 70% of global croplands, many staple crops are potential candidates for transition into perennials by hybridization and other genomic engineering techniques (Hübner and Kantar 2021). Broadly, there are two main approaches to developing a new perennial crop: de novo domestication of a wild perennial plant, and introgression of perennial traits into an annual crop through hybridization practices (Cui et al. 2018; Kantar et al. 2018). So far, attempts to develop a perennial cereal crop have had little commercial success. For example, efforts to develop perennial wheat varieties through hybridization with wild relatives were successful in generating stable amphiploids (Amstrong 1936; Larkin et al. 2014), yet grain yield declined quickly under field conditions. Introgression of perenniality into an annual crop is a long, challenging endeavor. Targeting crosses and directing selection based on a deep understanding of the molecular regulation of the perenniality traits can potentially accelerate this tedious process (Kantar et al. 2018).

Recent developments in biotechnology have prompted new methods for introducing a trait of interest into a crop through genome editing. This technology can be used for de novo domestication of wild species by targeting genes that regulate domestication syndrome traits, including seed shattering and awn length (Yu et al. 2021), fruit size and number, and their nutritional value (Gasparini et al. 2021). Another approach considers perenniality as a syndrome that includes a variety of interacting traits. Thus, understanding the underlying molecular mechanism is pivotal for an efficient breeding of perenniality traits (Lundgren and Des Marais 2020).

Barley (*Hordeum vulgare*) ranks fourth among cereal crops in cultivated area (www.fao.org/faostat) and is known for its enhanced adaptation to a wide range of environments (Haberer and Mayer 2015). However, only a few attempts to breed a perennial barley crop have been reported to date, although there are perennial species in the genus that are cross-compatible with cultivated barley.

Hordeum bulbosum is a nonmodel autopolyploid ($4n = 14$) wild cereal species that diverged from wild barley (*Hordeum spontaneum*) 4 M years ago and is abundant in the Mediterranean region (Blattner, 2009). Despite the morphological similarity between wild barley and *H. bulbosum*, the latter is a perennial species due to its bulb organ, enabling it to survive the dry summer season through dormancy. Its life cycle starts from a seed that germinates in the late fall, when the days shorten, temperatures decline, and the first rain of the season falls. The new *H. bulbosum* leaves sprout and the plant continues to grow and strengthen until the end of December, when the lengthening days signal the plant to shift from vegetative growth to flowering and to form a new bulb organ (Koller and Highkin 1960). The bulb organ is formed at the shoot's lowest internode by intensive cell division and elongation at the basis of the apical meristem, followed by accumulation of starch that leads to enlargement of this tissue (Leshem 1971). After the plant is fully mature, flowers, and produces new seeds, senescence begins as the temperatures increase and the plant enters a dormancy period during the dry warm summer season. With the coming of fall, new leaves sprout from regenerating buds that are located at the periphery of the bulb organ.

Previous studies in onion (*Allium cepa* L.) indicated that the transition from short to long days induces bulb formation and development through the regulation of flowering locus T (FT) homologs and other flowering regulating genes (Lee et al. 2013). However, little is known about the regulation of bulb formation in *H. bulbosum* and the underlying molecular mechanism remains largely unknown. To address this, advanced genomic infrastructure and proper molecular tools adjusted for *H. bulbosum* are required; yet, very few tools have been developed for this nonmodel species with its large and highly repetitive genome. Recently, a reference draft genome was developed for a double haploid *H. bulbosum* accession, but this resource is highly fragmented and remains un-annotated, thus the representation of the gene space is partial (Wendler et al. 2017). To test the collinearity between the *H. bulbosum* and barley, sparse genetic maps were developed from crosses between a doubled haploid *H. bulbosum* and cultivated barley (Wendler et al. 2017). Genetic maps generated from an interspecific cross and QTL mapping of perenniality can be highly valuable to target genetic regions that underlie the trait of interest if it segregates in the population and the mapping resolution is reasonable. Another efficient approach to identify genes of interest in a nonmodel organism is RNA sequencing, which enables efficient development of a reference transcriptome and testing of gene expression in response to a signal (Costa-Silva et al. 2017). Assembling a transcriptome for *H. bulbosum* has many benefits and can significantly improve the gene space representation and the identification of genes involved in bulb formation and development.

We analyzed the presence/absence variation (PAV) between annual and perennial species and assembled a reference transcriptome for *H. bulbosum* in order to identify candidate genes that are involved in the regulation of bulb formation. We then validated candidate genes using differential expression analysis in the congener (of the same genus) species *H. bulbosum* and *H. spontaneum*. We hypothesized that the development of a bulb organ is initiated in the shoot meristem during the vegetative stage in response to transition from short-day (SD) to long-day

(LD) regime. We further hypothesized that bulb formation is regulated by genes and pathways that are intertwined with the circadian clock and flowering, and the experimental design in this study enabled to partially deconvolute these two processes. Finally, we explored the set of candidate genes identified and, as a result, suggest a general molecular model for the regulation of bulb initiation in *H. bulbosum*.

Results

Developing a Reference Transcriptome for *H. bulbosum*

Seeds from a single spike of a tetraploid *H. bulbosum* plant were collected in the Galilee, Israel, and were used throughout the study. Plants were germinated and grown in a growth room controlled for temperature and day length, and different tissues were sampled for RNA extraction and high-throughput sequencing.

A total of 140 billion base pairs from 15 tissue samples were processed in order to assemble the *H. bulbosum* transcriptome after removing 2–4% of reads per sample due to unsatisfactory quality. The total length of the obtained transcriptome was 650 Mb comprising 707,824 transcripts (supplementary table S1, Supplementary Material online). Following the assembly, transcripts were annotated and filtered to include only genes that are present in the Spermatophyte super-division database. This filtering approach prevented potential contamination while maintaining information from species outside the Poaceae that may be relevant for the identification of bulb regulating genes (e.g., onion, garlic). A total of 138,696 transcripts remained after filtering, of which 42,045 were unigenes with a contig N50 of 2,758 bp.

To evaluate the completeness of the assembled transcriptome, BUSCO analysis was performed using the Poales genes database (4,896 BUSCOs) and the transcriptome mode. Altogether, 3,117 BUSCO genes were detected of which 92% were complete genes and 84% were single-copy genes. Next, the assembled transcriptome was compared with the available reference draft genome for *H. bulbosum* (Wendler et al. 2017) using the Poales genes database and genome mode. Expectedly, more genes were detected in the draft genome (4,119) than the transcriptome because the latter was targeted to specific tissues and timing. However, once unrepresented genes were excluded from both transcriptome and genome sequences, the number of complete genes was higher (92%) and the number of duplicated genes was lower (87%) in the transcriptome (fig. 1*A* and *B*; supplementary table S2, Supplementary Material online). These results emphasize the advantage of a transcriptome assembly for targeted studies in nonmodel organisms that harbor large and complex genomes.

To further explore the transcriptome composition and the expression profile in each tissue (leaf, root, flower organs, bulb, and embryo), a subset of 34,282 genes with a minimum coverage of 2× in at least one tissue was extracted. Genes that were expressed only in shoot meristems were excluded from this analysis due to over-representation among sequenced libraries (nine libraries). The number of genes detected in each tissue was quantified indicating that 43% of the expressed genes are shared among all six tissues, and 19% are tissue specific (fig. 1*C*). A high rate of tissue-specific genes was observed in the embryo (2,505), followed by roots (1,429), anthers (996), bulb (837), leaf (576), and stigma (392), after confirming that the number of genes detected in a tissue was not biased by the amount of RNA that was extracted ($r = 0.29$, $P = 0.57$). To further explore the underlying function of expressed genes across tissues or in a specific tissue, a gene ontology (GO) enrichment analysis was conducted (supplementary table S3, Supplementary Material online). Among GO categories, 19 were significantly enriched across all tissues and included response to stimulus, developmental process, and biological regulation. Within tissues, significantly enriched GO categories included biological processes that are associated with the corresponding tissue (fig. 1*D*). For example, gene expression in leaf tissue was enriched with photosynthesis processes including light harvest in photosystem-I (GO:0009768, $P = 1.70 \times 10^{-5}$) and chlorophyl biosynthesis (GO:0015995, $P = 8.17 \times 10^{-3}$), and embryo tissue gene expression was enriched with germination processes (GO:0009415, $P = 9.40 \times 10^{-8}$) and phyllotaxis (GO:0060772, $P = 2.30 \times 10^{-6}$), and so forth. A detailed list of GO terms identified in each tissue is provided in supplementary table S3, Supplementary Material online).

Evolutionary Conservation and PAV Analysis

The ability to generate a perennial organ is not abundant among Poaceae species. Within the *Hordeum* genus, *H. bulbosum* is special in its ability to form a bulb for use as a storage organ, enabling it to survive the dry Mediterranean summer. This trait strongly differentiates *H. bulbosum* and its closest congener species *H. spontaneum*; thus, the underlying genes are expected to be strongly diverged between *H. bulbosum*, *H. spontaneum*, and other annual grasses. To test this hypothesis and to identify candidate genes that potentially regulate bulb formation and development in *H. bulbosum*, the sequence conservation of transcribed genes was evaluated. To identify the least-conserved genes, we targeted sequences in *H. bulbosum* that had no hit (hereafter referred to as absent) in the genome assemblies of all other tested annual grass species in the Poaceae (*H. spontaneum*, *Trititcum urartu*, *Brachypodium distachyon*, and *Oryza sativa*). For

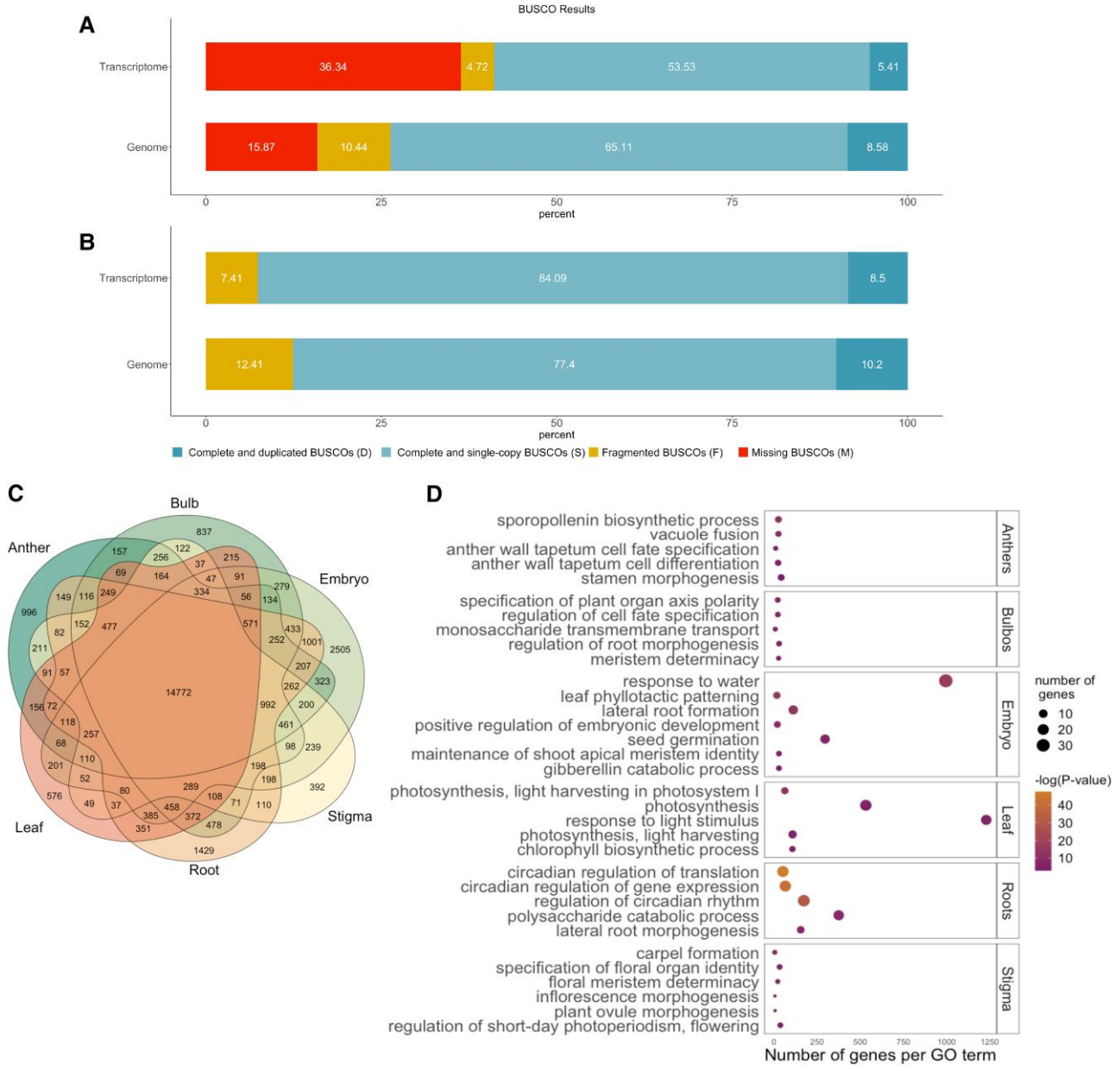

Fig. 1.—The *Hordeum bulbosum* transcriptome. (*A*) Proportions of all BUSCO gene counts in the assembled transcriptome and in the available draft genome for *H. bulbosum*. (*B*) Proportion of BUSCO gene counts after removing the missing BUSCO gene from the transcriptome and draft genome. (*C*) A Venn diagram of the shared and unique genes identified among tissues. (*D*) Significantly enriched biological process GO terms from each tissue that were included in the transcriptome. The circle size depicts the number of genes associated with each term.

each transcript, the longest isoform (42,045) was compared with the reference genome of all other species and the level of homology was quantified using BlastN. For each of the species, only absent genes were considered of poor conservation. This conservative approach enables targeting, with high confidence, genes in *H. bulbosum* that are absent or very poorly conserved in the other annual species, albeit with some level of false negatives (undetected genes).

A phylogenetic tree constructed from the PAV analysis across all genes supported the expected topology among the Poaceae (fig. 2*A*). The lowest rate of genes shared with *H. bulbosum* was observed for *O. sativa* (48% of *H. bulbosum* genes), which diverged from *H. bulbosum* 50 M years ago. The highest rate was observed for *H. spontaneum* (92%), which diverged from *H. bulbosum* 4 M years ago (fig. 2*A* and *B*). Among the 42,045 genes for which PAV was tested, 1,966 were present only among the

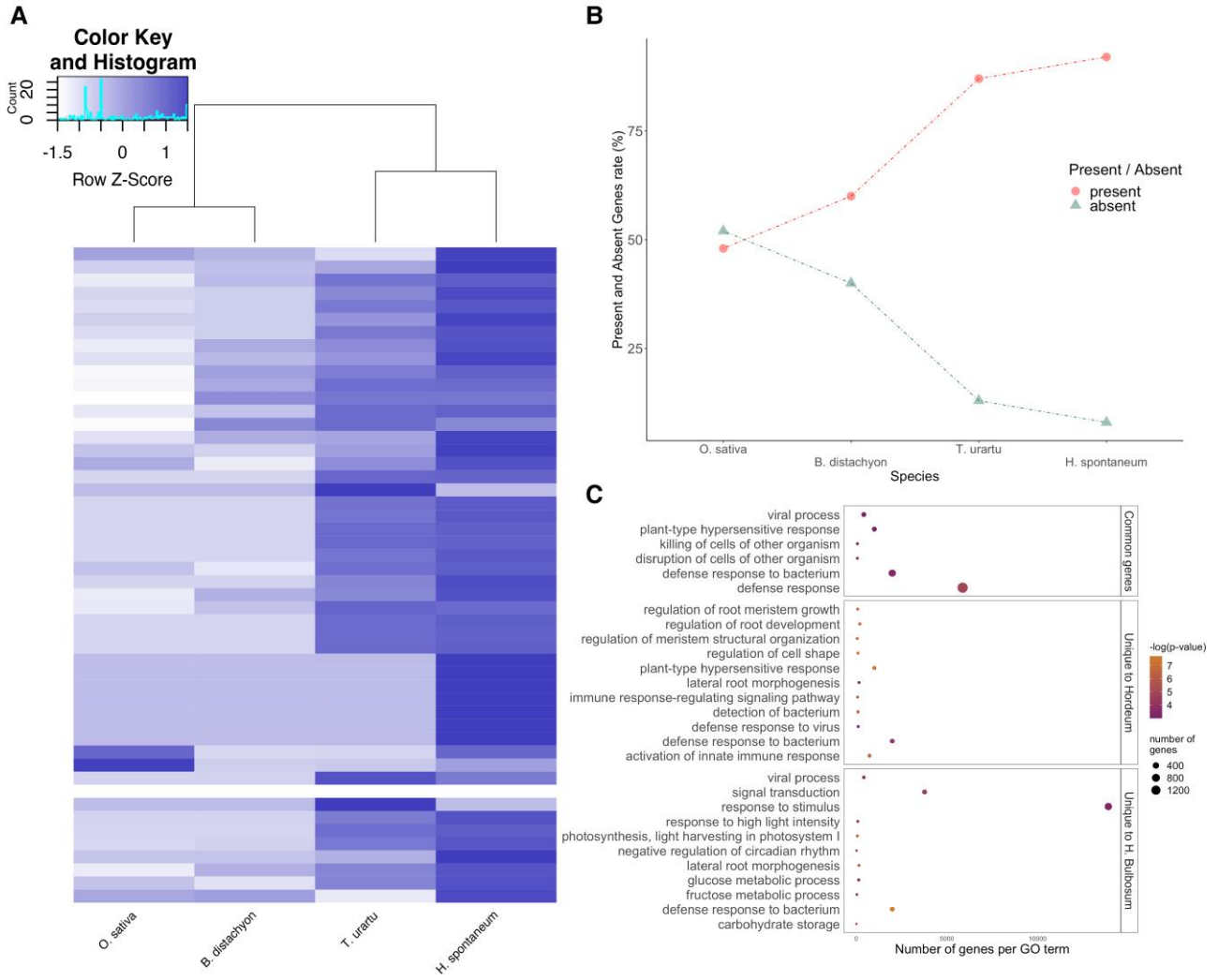

Fig. 2.—Sequence conservation analysis between *H. bulbosum* and annual species in the Poaceae. (*A*) Heatmap of 50 randomly chosen genes in the *H. bulbosum* transcriptome and their conservation in other species (*O. sativa*, *B. distachyon*, *H. spontaneum*, and *T. urartu*) based on *Z*-score normalized bit score values obtained from BLAST analysis. Absent genes are indicated in white and darker colors depict higher sequence conservation. (*B*) Sequence conservation along evolutionary distance indicated as the rate of presence or absence of genes between *H. bulbosum* and annual species. (*C*) Significantly enriched GO terms for genes that were conserved among all species (common genes), among *Hordeum* species (unique to *Hordeum*), and genes of lowest sequence conservation (no blast hit) between *H. bulbosum* and all other annual species (unique to *H. bulbosum*).

Hordeum species and are associated mainly with disease resistance (GO:0009626, $P = 6.7 \times 10^{-4}$; GO:0016045, $P = 2.5 \times 10^{-3}$), immune response (GO:0002218, $P = 1.6 \times 10^{-3}$; GO:0002764, $P = 2.9 \times 10^{-3}$), and defense from pathogens (GO:0042742, $P = 2.1 \times 10^{-2}$; GO:0051607, $P = 3.6 \times 10^{-2}$).

Interestingly, several significantly enriched categories were associated with root development and morphology (GO:0010082, $P = 1.8 \times 10^{-3}$; GO:0010102, $P = 3.1 \times 10^{-2}$), which may imply a special root structure that has evolved among *Hordeum* species. Among unique genes to *H. bulbosum* (1,906 genes), biotic stress and plant–pathogen categories were significantly enriched (GO:0042742, $P = 4.53 \times 10^{-4}$; GO:0016032, $P = 3.6 \times 10^{-2}$). Disease resistance genes tend to evolve faster due to a continuous arms race with the plant-specific pathogens; thus, low sequence conservation between species is therefore expected. However, lowest sequence conservation was also observed among genes associated with organ development, carbohydrate metabolism, and sugar accumulation categories (GO:0052576, $P = 2.1 \times 10^{-3}$; GO: 0006000, $P = 2.9 \times 10^{-2}$; GO: 0010102, $P = 2.8 \times 10^{-3}$; GO: 0048367, $P = 3 \times 10^{-2}$; fig. 2*C*, supplementary table S4, Supplementary Material online), supporting our hypothesis that genes associated with bulb formation have diverged strongly between the perennial and annual species. Among the detected candidate genes are the 14-3-3-like

gene which was previously identified as a tuberization promoting factor in potato (Teo et al. 2017), and hexokinase, which is involved in the development of a storage organ also in onion, casava, and potato (Zhang et al. 2016; Geng et al. 2017; Lehretz et al. 2021). A full list of the identified genes specific to *H. bulbosum* is provided in supplementary table S4, Supplementary Material online.

Expression Profiling of Candidate Genes

To further explore the functional context of the identified candidate genes, evaluate their potential contribution to bulb initiation, and suggest a model for the molecular pathway involved in perennial organ development in *H. bulbosum*, the expression profile of genes was quantified using RNA-seq. Previous studies have demonstrated that the signal for bulb initiation in *H. bulbosum* is the shift in day length and that the plant meristem response is intensified after 8–10 weeks in SD regime (Ofir and Koller 1974). Therefore, meristem tissues were sampled 9 weeks after germination and before transition to LD regime (T_0). Following the transition to LD, meristem tissues were sampled after 24 h (T_2) and 96 h (T_4) and RNA was extracted from each biological replicate and sequenced separately (fig. 3*A*). An average of 92 million reads were obtained for each sample and aligned to the assembled transcriptome. Among aligned reads, a high percentage (30%) were aligned to various contigs (transcripts). To test the source of these reads, a subset of 1,000 multi-mapped reads was randomly sampled and searched against the NCBI nonredundant database, indicating that the high rate of multi-mapped reads is attributed to the large number of duplicated genes in the assembled transcriptome of a polyploid species. To increase the robustness of the results, the expression analysis was restricted to genes with proper coverage (mean = 66%) and depth (mean = 38×), thus a total of 36,929 genes remained after filtering (supplementary tables S5 and S6, Supplementary Material online).

In *H. bulbosum*, the transition from SD to LD regime activates the formation of a bulb organ in addition to the transition to flowering. To decouple these two processes, the experiment was then conducted for the annual species *H. spontaneum* following the same experimental setup used with *H. bulbosum*. We hypothesized that flowering regulating genes would be expressed in both species, yet genes that contribute to bulb formation and development would be expressed only in the perennial species (*H. bulbosum*).

Following the transition from SD to LD (T_0/T_2), a total of 1,568 significantly differentially expressed genes (DEGs) were detected in *H. bulbosum* of which 837 were upregulated and 731 were downregulated (fig. 3*B*). Among the identified DEGs, six were also detected in the evolutionary conservation analysis including the hexokinase gene that contributes to storage organ development in perennial species (supplementary table S5, Supplementary Material online). After 4 days under LD (T_0/T_4), the effect of the shift in day length strongly declined, and only 102 genes were detected to be upregulated and 156 were downregulated. Among identified genes after 4 days under LD regime, three DEGs were also detected in the evolutionary conservation analysis (supplementary table S6, Supplementary Material online).

Unlike *H. bulbosum*, the expression profile observed in *H. spontaneum* was mild after 24 h (42 upregulated and 122 downregulated DEGs) but intensified after 4 days under LD regime (219 upregulated and 757 downregulated DEGs). These results suggest that the shift in day-length signal is indeed intensified in *H. bulbosum* due to the long period in SD (9 weeks), whereas in *H. spontaneum* the effect accumulates gradually after the transition to LD. Moreover, flowering-related genes were detected after 4 days (T_4) in LD in both species, implying that bulb initiation genes respond to the day-length signal before flowering regulating genes (fig. 3*C*).

To further study the functional context of the detected DEGs in *H. bulbosum*, a GO enrichment analysis was conducted. The number of significantly enriched GO terms was consistent with the declining trend observed in the expression analysis, thus 183 and 46 enriched GO terms were detected for T_0/T_2 and T_0/T_4, respectively. Among the enriched GO terms, 25 genes were associated with flowering (GO:0045595, $P = 0.0038$; GO:0009908, $P = 0.0036$), response to photoperiod (GO:0048577, $P = 0.0186$), and bulb formation (GO:0005986, $P = 0.0157$).

To further expand the list of candidate genes and propose a molecular model for perennial organ formation in *H. bulbosum*, we targeted DEGs that are associated with significantly enriched GO terms (supplementary table S7, Supplementary Material online). Bulb formation can be broadly divided into three phases: response to shift in day-length signal, morphogenesis and organ development, and accumulation of sugars in the bulb organ. Among DEGs that underlie enriched GO terms, several candidate genes were detected including CONSTANS (CO) (logFC = 1.48796, false discovery rate (FDR) = 4×10^{-4}), GIGANTEA (logFC = −1.61, FDR = 1.49×10^{-7}), and the pseudo-response regulator (PRR5) gene (logFC = −3.09, FDR = 1.71×10^{-17}), which interact in response to day-length and signal transduction. In addition, the morphogenesis and development regulating genes constitutive photomorphogenesis 9 (COP9; logFC = 0.6096, FDR = 0.001) and MADS-box16 (logFC = 4.33, FDR = 2×10^{-4}) were identified among DEGs, as was the fructose accumulation regulating gene SWEET17 (logFC = 2.11, FDR = 0.005). These genes were identified as key regulating factors in the formation of a storage organ (e.g., tuber, bulb) in other species (Chamovitz et al. 1996; Sawa et al. 2007; Chardon et al. 2013; Abelenda et al. 2016) and thus should

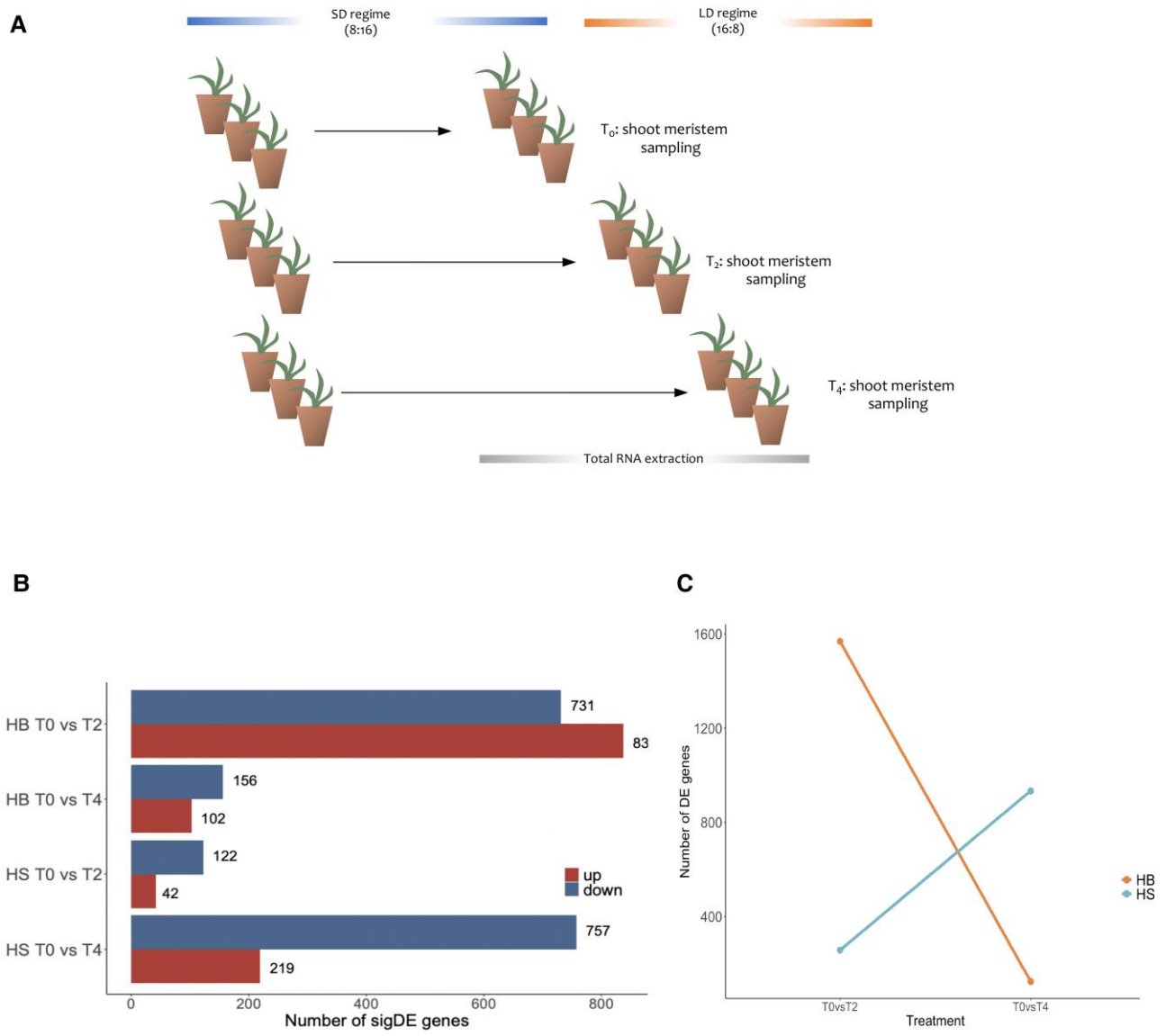

Fig. 3.—Expression profiling of meristem tissues before and after transition to LD regime. (*A*) A scheme of the experimental design. (*B*) Counts of up and down regulated DEGs identified along treatments (T_0, T_2, T_4) in *H. bulbosum* (HB) and *H. spontaneum* (HS). (*C*) Total number of DEGs in *H. bulbosum* and *H. spontaneum* along time points (treatments).

be further explored at the molecular level in order to illuminate their mode of action in *H. bulbosum*.

Discussion

Despite the many benefits of intensified modern agriculture, the consequential environmental impact must be reduced in accordance with current global efforts to mitigate harmful climate change. Agriculture must adopt a more sustainable strategy to face the 2-fold challenge of rising food demand and natural resource decline. One way to reduce agriculture's negative environmental impact is to shift crop plants from an annual to perennial system (Kantar et al. 2016, 2018; Mora et al. 2018; Lundgren and Des Marais 2020). Compared with annual species, perennials tend to be more tolerant to stress and require less irrigation and environmentally harmful inputs, including pesticides and fertilizers (Glover et al. 2010). However, previous attempts to develop a perennial crop through classical breeding methods have extended over long periods to little success. The main challenges are associated with crossing barriers between different species, a severe genetic drag, and declining yield under field conditions (Kantar et al. 2016; Lundgren and Des Marais 2020). Therefore, breeding toward perennial crops should become more targeted and incorporate biotechnological practices in the breeding process (Hübner and Kantar 2021).

To enable efficient and targeted breeding, perenniality has to be accurately defined. Conceptually, perenniality can be considered as a syndrome comprised of many interacting traits including growth rate, carbon fixation, root system development, and source-sink dynamics (Lundgren and Des Marais 2020) and involve different plant organs. Addressing multiplicity pragmatically, we defined perennially as the ability to form a bulb organ in *H. bulbosum* and explored the underlying molecular mechanism using two approaches: evolutionary conservation analysis and gene expression profiling.

Exploring Genes Associated with Perennial Life Habit in *H. bulbosum*

The annual/perennial life habit has shifted in both directions along the evolutionary history of many plant species (Heidel et al. 2016; Lundgren and Des Marais 2020). This shift is attributed to different genetic mechanisms including gain or loss of "perenniality genes," changes in the regulation of gene expression, or critical change in protein structure (Heidel et al. 2016). Along the evolution of the *Hordeum* genus, perennial and annual life habits have shifted a number of times independently (Blattner 2009). Among these shifts, the split between *H. bulbosum* and *H. spontaneum* is of particular applicative interest, yet little is known about the evolution of perennial/annual genes in these congener species. The split between *H. bulbosum* and *H. spontaneum* occurred 4 M years ago (Blattner 2009) and is likely associated with the evolution of life habit, thus genes that underlie this transition are expected to show poor sequence conservation among these species. Indeed, high sequence conservation was observed in *Hordeum* species (92%), except for disease responsive genes that tend to evolve quickly due to host–pathogen arms races (Stahl and Bishop 2000) and storage organ development genes, unique to *H. bulbosum* (fig. 2C). For example, the gene 14-3-3-like was detected as poorly conserved in *H. bulbosum* and all other annual species and was previously reported as an enhancer of storage organ formation in potato (Hannapel et al. 2017; Teo et al. 2017). Another interesting gene is hexokinase, which plays a central role in carbohydrate accumulation and was detected as a key factor in cassava, and as a contributing factor in potato and onion, for both carbohydrate accumulation and storage organ development (Geng et al. 2017).

Decoupling the Genetic Mechanism Involved in Flowering and Bulb Formation

To validate the results of the evolutionary conservation analysis and expand the list of potential candidate genes that are involved in the molecular regulation of perenniality in *H. bulbosum*, we quantified the expression profile of genes in response to shift in day length. In *H. bulbosum*, bulb formation is induced by a shift in day length, similar to onion and potato (Lee et al. 2013; Zhang et al. 2020), and develops at the bottom of the shoot meristem (Leshem 1971). Unlike onion and potato where transition to flowering inhibits the development of the storage organ (Lee et al. 2013; Hannapel et al. 2017), in *H. bulbosum*, flowering and bulb formation are coupled and occur simultaneously (Leshem 1971). Therefore, the molecular mechanism of both processes is expected to be regulated by the same genes at the top of the pathway cascade.

Comparison between the candidate genes identified in *H. bulbosum* and known regulation pathways of flowering and storage organ development in potato, onion, and Arabidopsis suggests that the mechanism is largely conserved (Putterill et al. 1995; Lee et al. 2013; Hannapel et al. 2017). Among the candidate genes identified in this study, CO was upregulated and GIGANTEA was downregulated (supplementary table S8, Supplementary Material online). This expression profile promotes flowering in *Arabidopsis* (Putterill et al. 1995), but suppresses tuberization in potato (Abelenda et al. 2016; Hannapel et al. 2017). Moreover, the morphogenesis regulating gene COP9 that induces organ development (Chamovitz et al. 1996; Wei and Deng 2003) was upregulated and so were several sugar transporters and starch biosynthesis genes. Among these, SWEET17 (Chardon et al. 2013), and hexokinase were detected, where the latter was also identified as a poorly conserved gene in *H. bulbosum* and annual species in the evolutionary conservation analysis (supplementary tables S4 and S5, Supplementary Material online).

This research is the first milestone toward understanding the molecular mechanism of perenniality in *H. bulbosum*. We cautiously suggest a general model for the molecular mechanism that underlies the development of a bulb organ based on the results of this study and available information from previous reports on other species that develop a storage organ. The activity of COP9 seems to trigger a rapid cell division followed by sugar biosynthesis and accumulation of starch through genes like hexokinase, SWEET17, and others. A major part of the pathway appears to be conserved among *H. bulbosum* and *H. spontaneum*, thus targeting of candidate genes can be further explored in an interspecific biparental mapping population where the bulb formation trait is segregating. To date, our efforts to generate a segregating population for perenniality traits from a cross between *H. bulbosum* and *H. spontaneum* have failed in the transition from hybrids to a population and further efforts are needed to overcome this limitation. Once QTLs are targeted, candidate genes can be further explored at the molecular level with higher confidence in order to pave the route for application in breeding programs of new perennial crop plants.

Materials and Methods

Plant Material

A random batch of seeds sampled at Alon HaGalil in northern Israel (32°45′35.2″N, 35°13′30.5″E) from single spikes of *H. bulbosum* and *H. spontaneum* plants was used in the experiments. Selected seeds were sown in planting trays, and placed in a cold (4 °C) dark room for 15 days to break dormancy. Germinated seedlings were transferred into a growth room under SD regime (8 h:16 h light:dark) and constant temperature of 16 °C. After an establishment period of 2 weeks, seedlings were transferred into 3 l pots containing a soil mixture ("GREEN 90," Ben-Ari Ltd., Israel) and slow release fertilizer (Osmocote, Everris International B.V., Heerlen, The Netherlands). Tissue samples from bulb, anthers, stigmas, leaf, root, and shoot meristems were dissected and immediately frozen in liquid nitrogen and stored in −80 °C until extraction of total RNA. For embryo tissue dissection, a single seed was soaked in water for 2 h to soften the seed and enable easy removal of the embryo.

RNA Extraction, Library Preparation and Sequencing

Total RNA was extracted from each sample using the RNeasy Plant Mini Kit (QIAGEN cat No./ID 74904) following the manufacturer protocol. For embryo RNA extraction, we used 500 µl RLT buffer and 10 µl β-ME, and for the leaf tissue 500 µl RLT buffer and 5 µl β-ME as recommended by the manufacturer. To confirm the integrity and quality, total RNA was inspected in both NanoDrop 1000 spectrophotometer and agarose gel electrophoresis. An optical desity (OD) 260/280 ratio of 2.0 and RNA concentration of 100 ng/µl were set as a minimum threshold for adequate quality and quantity. Sequencing libraries were constructed and sequenced at the Technion Genome Center (Haifa, Israel). Paired ends RNA-seq libraries were prepared using the TruSeq RNA Library Prep Kit v2 (Illumina Inc., USA) following the manufacturer instructions and sequenced on six lanes of a HiSeq-2500 Illumina machine. To avoid a lane bias in the expression profiling analysis, samples were pooled and the sixth portion of the pool was sequenced on each of the six lanes. The quality of the sequenced reads was inspected with the software FastQC v.0.11.5 (Andrews 2010) and low quality reads were trimmed and adapters were removed using the default parameters in Trimmomatic v.0.32 (Bolger et al. 2014). Finally, cleaned and high-quality reads were re-inspected and quality was confirmed using FastQC.

Transcriptome Assembly and Annotation

To develop a comprehensive and representative transcriptome for *H. bulbosum*, 15 RNA-seq libraries were obtained and sequenced for root, bulb, leaf, floral reproductive tissues (anthers and stigma), embryo, and shoot meristems. The *H. bulbosum* transcriptome was assembled de novo with the Trinity assembler v1.8 (Grabherr et al. 2011) using the reads from all tissues. Briefly, Trinity performs a full transcriptome assembly process in three main stages and includes partitioning the data, clustering and graphs construction, and tracing paths in the constructed graphs in parallel. At the end of this process, a linear sequence is obtained for each transcribed isoform.

To annotate the transcriptome, we used the full Trinotate pipeline v-3.1.1 (Bryant et al. 2017). Prediction of open reading frame in the assembled genes was conducted with TransDecoder v-5.5.0 (http://transdecoder.github.io). Homolog proteins detection was conducted with BlastX and BlastP against the SwissProt and UniProt (Boeckmann et al. 2005; The UniPort Consortium 2021) databases and an *e*-value cutoff of 10^{-5}. To identify conserved protein domains, trans-membranal regions and rRNA genes, the HMMER toolkit v-3.1b2 (Eddy 1998) was used to find hits against the PFam database (Finn et al. 2014). Signal peptides were predicted using SignalP (Petersen et al. 2011). To filter potential contamination in the transcriptome, transcripts that were annotated to species outside the Spermatophyta super division (seeded plants) were excluded from the data set. To further evaluate the quality of the assembly, cleaned reads from each sequenced library were aligned back to the filtered transcriptome using bowtie2 v2.3.5.1 (Langmead et al. 2009) and the alignment statistics were calculated with samtools v1.9-92 (Li et al. 2009). Finally, the transcriptome completeness was evaluated following the BUSCO analysis (Simão et al. 2015) using the Poales database and the transcriptome mode. Only the longest isoform per gene was inspected to minimize the false identification of duplicated genes.

Interspecific PAV Analysis

To identify genes that are highly diverged or conserved between *H. bulbosum* and other annual grasses, the assembled transcriptome was compared with available genome assemblies of *H. spontaneum* (Jayakodi et al. 2020), *T. urartu* (Ling et al. 2018), *O. sativa* (Wang et al. 2018), and *B. distachyon* (Vogel et al. 2010). All selected species are diploids to avoid a potential comparison bias, and they represent a continuous evolutionary distance from *H. bulbosum*. To identify poorly conserved gene sequences, the PAV was determined among species based on the sequence of each gene assembled in *H. bulbosum*. To avoid the complex comparison between a transcript and different gene models annotated in each species, only the longest isoform was used as a representative of each gene in the *H. bulbosum* transcriptome. The presence/absence of genes in each species was determined using BlastN analysis where no hit of a gene was considered

as the lowest conserved sequence and was used to identify potential candidate genes that are associated with bulb formation and perenniality in *H. bulbosum*.

DE Analysis

A DE analysis was performed using the RNA-seq data generated from shoot meristem tissues before and after transition from SD to LD photoperiod. The experiment included three treatment groups: T_0, T_2, and T_4, and three biological replicates were sampled for each group. A previous study on bulb physiology and development (Ofir and Koller 1974) has shown that the day-length signal intensifies 8–10 weeks after germination, and thus, plants were kept in the SD regime for 9 weeks before transition to LD. Shoot meristem tissues were sampled from the first group (T_0) before their transition to the LD regime and were immediately frozen in liquid nitrogen. After sampling the T_0 plants, the conditions in the growth room were shifted to the LD regime (16 h:8 h, light:dark) and shoot meristems were sampled from the T_2 plants after 24 h and from the T_4 group after an additional 96 h. This experiment was repeated with *H. spontaneum* seeds sampled at Alon HaGalil after two rounds of single seed descent and self-pollination procedure. Total RNA was extracted for library preparation and sequencing from each of the 18 samples (9 *H. bulbosum* and 9 *H. spontaneum*).

Obtained sequencing data were trimmed and cleaned with trimmomatic and only high-quality paired-end reads were used for the alignments. *Hordeum bulbosum* reads were aligned to the transcriptome with Bowtie2 v2.3.5.1 (Langmead et al. 2009), and *H. spontaneum* reads were aligned to the B1K-04-12 reference genome (Monat et al. 2019) with STAR v.2.7.3a (Dobin et al. 2013). To quantify the expression profile of genes in each sample, alignment files were processed with RSEM v.1.2.31 (Li and Dewey 2011), and genes with differentially expressed profiles between treatments were detected with the R package DESeq2 v1.22.2 (Love et al. 2014). To correct bias introduced by multiple testing, the Benjamini–Hochberg correction was implemented with a cutoff of FDR $\leq 5\%$. No other filtering threshold was set on the expression fold change ($\log_2$-FC) between treatments to avoid the exclusion of mild differences common among regulating genes and transcription factors (Shaar-Moshe et al. 2015).

GO Analysis

GO analyses were conducted to identify enriched biological processes among the different tissues that were used to assemble the transcriptome, among genes showing PAV, and among genes that were differentially expressed in response to the transition from the SD to the LD regime. GO terms were extracted from the annotation file generated for the transcriptome and used for enrichment analysis with the package topGO v3.11 (Alexa et al. 2006). The GO analysis was performed with default parameters and statistical significance was determined with Fisher's exact test. The *P*-values reported were corrected internally using the "sigGenes" command following the recommendation in the package manual. Heatmaps were generated with "gplot" v3.1.1 (Warnes et al., 2009) for the top significant GO terms.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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Author Contributions

D.F., B.S., and T.M. performed the experiments, D.F. performed the analyses, D.F. and S.H. wrote the manuscript, and S.H. conceived the study.

Data Availability

Sequencing data generated in this study will become available upon acceptance through the Sequence Read Archive PRJNA810915. Transcriptome and annotation files are available from github https://github.com/hubner-lab.

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