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DIVERGENCE IN GENE EXPRESSION IS UNCOUPLED FROM DIVERGENCE IN CODING SEQUENCE IN A SECONDARILY WOODY SUNFLOWER

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Premise of research. The transition from herbaceous annual to woody perennial has occurred numerous times during angiosperm evolution, but the suite of genetic changes that accompanies this life history shift remains poorly understood. Here we analyze genetic sequence and gene expression divergence between the annual *Helianthus annuus* and a recently diverged woody perennial ecotype from California.

Methodology. We grew plants from populations of typical *H. annuus* and the woody perennial ecotype in a common garden and measured days to flower, mature height, and basal stem density. We looked for evidence of genome size evolution in the woody ecotype using flow cytometry and conducted anatomical observations of stem development in seedlings. We sequenced the aboveground seedling transcriptomes of six individuals of each ecotype and identified genes with high fixation index (F_{ST}) and significant divergence in gene expression. Finally, we examined the gene ontology annotation of differentiated genes and assessed the extent of overlap between genes with divergent sequence and those with divergent expression.

Pivotal results. Plants from the novel woody ecotype flowered later and were taller at maturity than typical *H. annuus* plants. Despite differences in the initiation and extent of secondary growth, the two sunflowers were similar in basal stem density at 6 mo. The ecotypes do not differ in genome size. We discovered 575 genes (3.5%) with significantly different expression between the ecotypes and identified seven biological processes associated with divergent gene expression. Surprisingly, only five of the differentially expressed genes were also present among genes with the most coding sequence divergence (top 3.5% of F_{ST} distribution).

Conclusions. The recent transition in *H. annuus* from annual to woody perennial has been accompanied by numerous genomic changes, including divergence in gene expression and in coding sequence. These two mechanisms may represent alternative pathways toward the same adaptive optimum, or genes may be constrained to evolve via one mechanism or the other over short timescales.

Keywords: adaptation, secondary woodiness, sequence divergence, gene expression, RNA-Seq, *Helianthus*.

Online enhancements: appendix figures.

Introduction

Throughout evolutionary history, flowering plants have repeatedly transitioned from an annual herbaceous habit to a perennial woody life history (Dulin and Kirchoff 2010). Also termed "secondary woodiness," this shift has long fascinated botanists and other natural historians (Van Steenis 1973; Carlquist 1975). Darwin (1859) remarked that this transition often occurred on islands, citing the advantages of stature in competition against other herbs. Later researchers posited a number of adaptive hypotheses to explain the evolution of secondary woodiness, including mechanical support, architecture, hydraulics, resistance to frost and drought stress, and defense against herbivores and pathogens (Poorter et al. 2009; Spicer and Groover 2010; Lens et al. 2013*a*, 2013*b*). Given the widespread nature of this phenomenon, which has been described in at least eight eudicot orders (Carlquist 1969, 1992, 1995; Böhle et al. 1996; Baldwin 1997; Kim et al. 1999; Ballard and Sytsma 2000; Lens et al. 2005), each of these hypotheses may hold true in at least one instance. Among these cases, anatomical variation in vascular development has been well documented (Spicer and Groover 2010). Many secondarily woody species share juvenile, or paedomorphic, characteristics of wood anatomy, which may result from differences in the timing of developmental events (Carlquist 2009; Olson et al. 2009).

Comparatively little is known about the genomic changes that accompany the evolution of secondary woodiness, with the exception of some excellent functional work in *Arabidopsis* and other model systems (Groover 2005; Lens et al. 2011). These studies demonstrate that the evolution of secondary woodiness is a complex developmental process involving changes in transcription, cell wall development, and phytohormone signaling (Groover and Robischon 2006). A woody perennial-like phenotype can be induced in *Arabidopsis thaliana* via mutations in two flowering-time regulatory genes (Melzer et al. 2008), but studies of transcriptional regulation

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of secondary growth often report complex genomic changes (Oh et al. 2003; Ko et al. 2004; Schrader et al. 2004). It appears that many of the networks involved in regulating transcription during secondary growth also play important roles during primary growth in the shoot apical meristem (Schrader et al. 2004) and that gene function is often conserved across herbaceous and woody taxa (Groover and Robischon 2006; Yang et al. 2008; Lens et al. 2011). Further work is needed to clarify the dynamics of genomic evolution during the transition to secondary woodiness in wild populations.

In *Helianthus*, secondary growth and life history seem to be particularly labile (Heiser and Smith 1969). Within the annual section, which likely derived from a perennial taprooted ancestor, there is repeated gain of wood development and, to a lesser extent, facultative bienniality and perenniality (Heiser and Smith 1969). Like many plant taxa, *Helianthus* also exhibits rapid chromosomal evolution, with several well-documented large-scale rearrangements and duplications (Burke et al. 2004). The study of adaptive variation in the wild benefits greatly from access to genomic tools, and in *Helianthus* there is access to an ever-growing set of genomic resources (Heesacker et al. 2008; Bowers et al. 2012; Kane et al. 2013).

RNA-Seq, or Illumina-platform sequencing of transcriptomes, is a promising approach for identifying genomic changes that accompany key evolutionary transitions such as the shift to secondary woodiness. Leveraging recent advances in next-generation sequencing technology, this method yields more data more accurately and at a lower cost than traditional Sanger sequencing or gene expression microarrays (Wang et al. 2009). In addition to the quantification of relative gene expression, RNA-Seq data can be used to identify other genomic polymorphisms, including splicing isoforms and sequence variants (Wilhelm and Landry 2009). RNA-Seq has the further advantage of requiring no a priori information about transcribed genes and so can easily be used in nonmodel systems (Stapley et al. 2010). Researchers can now directly examine the relationship between gene expression divergence and gene sequence divergence and ask how evolution in these genomic arenas may coincide or differ.

We know that gene expression, like nucleotide sequence, can evolve under natural selection (Jordan et al. 2005; Rifkin et al. 2005), and like nucleotide sequence, gene expression can also diverge neutrally (Khaitovich et al. 2005). During the early stages of adaptation, we might hypothesize that the evolution of gene expression and nucleotide sequence could be correlated at the allelic or functional level. That is, those genes involved in the same pathways or processes might show similar patterns of divergence when evolving under similar selective pressures, even using different mechanisms. A few studies have demonstrated this kind of correlation between gene expression and sequence divergence (Nuzhdin et al. 2004; Hunt et al. 2013). On the other hand, it is possible that sequence and expression variation could act as alternative routes toward the same adaptive optimum (so any particular gene or pathway would use only one mechanism) or that particular genes or pathways might be constrained to evolve via one mechanism rather than the other.

In this study, we focus on *Helianthus annuus*, the common sunflower, and a putatively recently derived woody perennial ecotype from the Sierra Nevada foothills in California. We present several phenotypic and genomic comparisons between *H. annuus* and the new ecotype, including observations of seedling stem anatomy; comparisons of the flowering time, size, and stem density of mature plants; and measurements of relative genome size for each ecotype. Using RNA-Seq data, we ask: (1) How has this new ecotype diverged from *H. annuus* in gene expression and genetic sequence? (2) What biological processes are associated with the genes most highly differentially expressed or highly divergent in sequence? (3) Are the genes diverging in sequence the same as those being differentially expressed?

Material and Methods

Study System and Collections

Helianthus annuus, the common sunflower, is a widely distributed annual plant native to much of North America, where it is often found in open country on moist, mesic soils (Heiser 1954). Heiser and others have hypothesized that the ancestral range of *H. annuus* was originally restricted to the Great Plains and expanded in conjunction with human disturbance (Heiser and Smith 1969; Dorado et al. 1992), inferring this on the basis of the plant's increased affinity for marginal or disturbed habitats and frequent hybridizations with congeners outside of the region. If this hypothesis is correct, it is likely that *H. annuus* arrived in California via the Sierra Nevada Mountains sometime in the past few thousand years, likely with the aid of indigenous traders or European settlers. Regardless of its mode of introduction, *H. annuus* today is found throughout the state, with well-documented collections in almost every county (Baldwin et al. 2012). Wild populations of *H. annuus* typically exhibit some degree of secondary growth at the base of the primary stem, although this varies among individuals and populations.

A set of populations of *Helianthus* recently discovered in California (J. C. Stebbins, personal communication) closely resemble *H. annuus* but exhibit several key character differences: they are perennial, flower almost continuously throughout the year, and while maturing acquire dense secondary growth throughout the primary stem (fig. 1). These populations are found exclusively on ungrazed hillsides with southern exposure, in relatively poor, xeric, and shallow soils, directly east of the San Jose Valley in Fresno and Tulare Counties (fig. 2). The local climate can be characterized as Mediterranean, with hot, dry summers and cool, wet winters. Hereafter, we will refer to this ecotype as "winteri," because a species description of *Helianthus winteri* is in progress (J. C. Stebbins, personal communication). We made seed collections from populations of this ecotype and several California populations of typical *H. annuus* in 2009, 2010, and 2011 (table 1; fig. 2). Since then, four additional populations have been discovered in similar habitat in the hills between the Boyd and Squaw and Valley populations (J. C. Stebbins, personal communication).

We took care to preserve maternal families (half-sib seed lots) while collecting and to maximize distance between maternal plants to minimize sampling of close relatives. Because this ecotype is perennial, we also made sure to use only collections from a single year for each experiment to avoid accidentally incorporating the same maternal family twice. All studies described below were conducted using wild collected

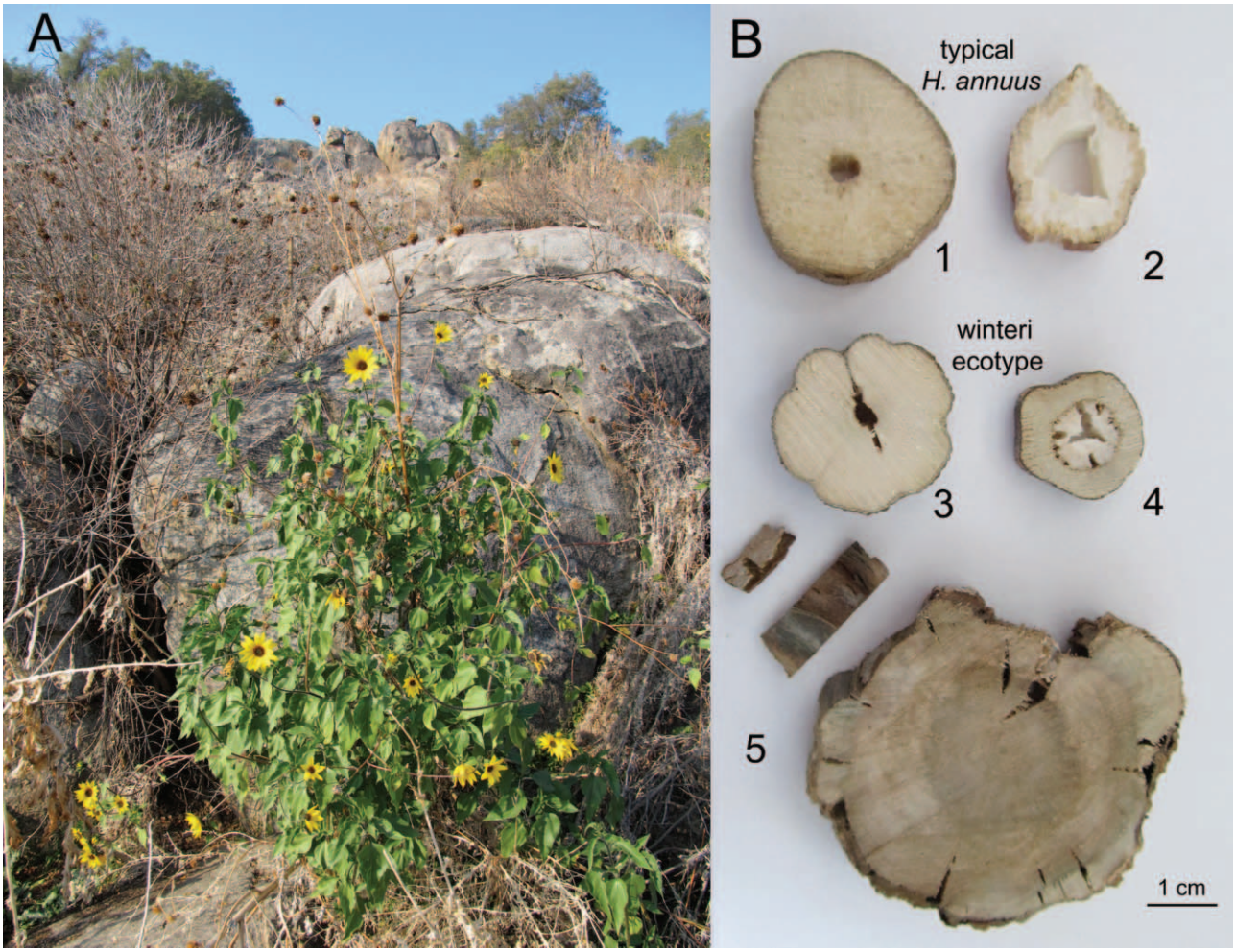

Fig. 1 A recently diverged woody perennial ecotype of *Helianthus annuus* from California. *A*, A mature winteri individual in typical habitat (shallow, rocky soil on an ungrazed, southern-exposed slope). *B*, Stem sections from primary stems show variation within individuals and between ecotypes: 1 and 3 are basal sections taken from typical *H. annuus* (1) and winteri ecotype plants (3) grown in the same environment and harvested at 6 mo postgermination, while 2 and 4 are sections taken at 1 m height from the same plants (2 = *H. annuus*, 4 = winteri); *5* is a basal section taken from a wild winteri individual of undetermined (2+ yr) age, including pieces of putative bark.

seeds, so it is important to keep in mind that maternal effects may play a role.

Anatomical Observations of Stem Development

We germinated seeds from five maternal families from each of three populations of the two ecotypes (*H. annuus*: Manteca, Academy, and Canal; winteri: Squaw, Valley, and Boyd; table 1). We scarified seeds by cutting and imbibed them with water on filter paper in petri dishes. Once the plants had produced cotyledons and a primary root, we transplanted them into sterile media mixed with bonemeal in 2-in-diameter Conetainers. These plants were grown in a climate-controlled greenhouse (25°/18°C day/night) with supplementary lighting set to 16-h days. Every 3 d, starting at 12 d postgermination, we made anatomical observations on the stem development of one individual from each maternal family (15 plants/ecotype/sampling day). Immediately after harvesting each seedling, we made 8–12 cross sections by freehand sectioning with a double-edged razor blade at approximately halfway between the cotyledons and the first true leaves. We transferred these sections to a slide and stained them with 0.05% toluidine blue O in a 0.1 M phosphate buffer at pH 6.8 (O'Brian et al. 1964) for ~90 s. We then washed the sections with water, examined them under a Nikon Eclipse 80i light microscope, and photographed the clearest one or two sections at ×4 and ×10 magnification using a Nikon DS-U2/L2-Ri1 camera integrated into the microscope (Nikon Instruments, Melville, NY). We scored initiation of vascular cambium development when any differentiation of tissue was observable at ×4 magnification between the primary phloem and xylem.

At 24 d postgermination, we harvested two plants per family (30 plants/ecotype). We made cross-section preparations and photographed them as described above. We then made measurements of various anatomical features on the digital images using ImageJ v1.42q (National Institutes of Health, http://

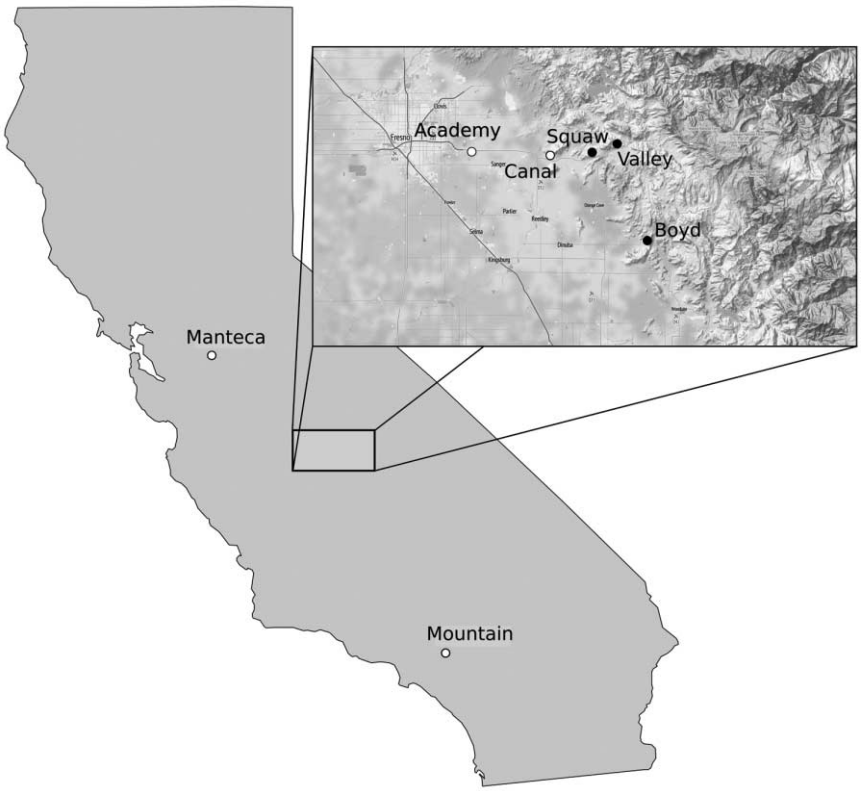

Fig. 2 Collection localities for populations used in analyses (open circles = typical *Helianthus annuus*, filled circles = the winteri ecotype). *Helianthus annuus* is widespread across North America and a relative newcomer to California via human-assisted migration, while the perennial woody winteri ecotype is restricted to southern-exposed, ungrazed slopes in the lower foothills east of the San Jose Valley.

rsb.info.nih.gov/ij). These features included the area of the stem (from a mean of three measurements of diameter) and the area of fascicular cambium (the sum of height by width of secondary tissue between each primary xylem and phloem bundle). We tested for differences in these traits between the ecotypes using two-sample *t*-tests within a linear mixed-effects model (nlme package in R; Pinheiro et al. 2013), with ecotype as a fixed effect and population as a random effect. We counted the number of vascular bundles separated by parenchymatous tissue and tested for a difference with a *z*-test in a generalized linear model (glm) with a Poisson error distribution implemented in R (R Development Core Team 2011). We also calculated the relative area of fascicular cambium as the ratio of fascicular cambium to total stem area and tested for a difference using a glm as described above but with a binomial error distribution (link = "logit").

Common Garden

In 2012, we grew two individuals from 9–19 half-sib families collected from five populations of typical *H. annuus* and three of the winteri ecotype (100 and 64 plants, respectively; table 1) at the University of British Columbia (UBC) Farm in Vancouver, British Columbia, Canada. We germinated seeds by scarifying in early April and transplanted seedlings into 1-in-diameter Cone-tainers once they had produced cotyledons and a primary root. We grew seedlings in a climate-controlled greenhouse for 3 wk and then moved them outdoors to harden for 3 d before transplanting them into a complete randomized design in the field in early May. We censused plants twice weekly for flowering, scoring the number of days from germination to the day the first reproductively active flower (pollen or stigma) was observed. In early October, when all of the *H. annuus* individuals had passed peak flowering and started to senesce, we measured each plant's height and harvested a 20-cm section from the base of every primary stem. We calculated stem density as the ratio of dry stem mass to fresh volume. We tested for differences in phenotype between typical *H. annuus* and the winteri ecotype using two-sample *t*-tests within a linear mixed-effects model (nlme package in R; Pinheiro et al. 2013), with ecotype as a fixed effect and population and family as nested random effects.

Genome Size Estimation with Flow Cytometry

We harvested fresh, fully expanded leaves from 4-wk-old seedlings of typical *H. annuus* and the winteri ecotype, sampling two individuals from different maternal families from each population included in the common garden (table 1). Immediately after harvesting, we finely chopped each sample in a chilled, slightly modified de Laat's nuclear isolation buffer with an equivalent mass of *Zea mays* leaf (de Laat 1984). *Zea mays* makes an excellent standard for flow cytometry measurements of diploid *Helianthus* samples, as the maize diploid

Table 1

Population Collection Information for Typical California *Helianthus annuus* and the Woody Perennial Winteri Ecotype

Population	Ecotype	Latitude (°N)	Longitude (°W)	Year collected	Data[a]	Maternal families[b]
Squaw	Winteri	36.719	−119.335	2009, 2010	CG, A, FC, RNA-Seq	10
Valley	Winteri	36.722	−119.312	2010, 2011	CG, A, FC, RNA-Seq	10
Boyd	Winteri	36.556	−119.194	2011	CG, A, FC	11
Canal	*H. annuus*	36.720	−199.398	2010	CG, A, FC, RNA-Seq	9
Academy	*H. annuus*	36.736	−119.560	2010	CG, A, FC, RNA-Seq	12
Manteca	*H. annuus*	37.781	−121.235	2010	CG, A, FC, RNA-Seq	9
Mountain	*H. annuus*	34.162	−117.675	2011	CG, FC	19

[a] The analyses in which each population was included (CG = common garden, A = anatomical observations, FC = flow cytometry, RNA-Seq = transcriptome sequencing).

[b] The number of half-sib families grown in the common garden in 2012.

genome size (2C = 5.67 pg DNA; Doležel et al. 1998) is sufficiently distinct yet does not differ more than twofold from the size of a typical *H. annuus* genome (~7 pg). After homogenization, we strained the samples through two layers of Miracloth and centrifuged them for 5 min at 1200 rpm. We resuspended the pellet in nuclear isolation buffer and treated each sample with RNAse before staining with propidium iodide for 10–20 min. We analyzed samples on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) at the "slow" delivery rate and counted a minimum of 5000 particles for each sample. We estimated sample genome size from a minimum of 1000 particles per gated peak (peak CV < 5%), scaled to the *Z. mays* standard, using the software FlowJo (v9, Tree Star, Ashland, OR). We tested for a difference in genome size between *H. annuus* and the winteri ecotype using a two-sample *t*-test.

In addition, four samples of the winteri ecotype were paired with *H. annuus* samples as standards. These data are not reported here; all pairings produced a single, uniform peak, indicating no major difference in genomic content between the samples.

Transcriptome Sequencing and Alignment

Our transcriptome analysis includes one sample from each of two families in three California *H. annuus* populations (Canal, Academy, and Manteca) and three families in two winteri populations (Squaw and Valley), for a total of six individuals from each ecotype (table 1). We started seeds as described for the anatomical observations above and grew them in 2-in pots in 12-h days at 22°C in a UBC growth chamber, watering regularly with a fertilizer solution. At 3 wk postgermination, when differences in stem vascular development may already be observed, we harvested all aboveground tissue and flash froze it in liquid nitrogen. We extracted RNA from each tissue sample with a modified TRIzol Reagent protocol (Invitrogen, Carlsbad, CA) and eluted the final product into nanopure water. We then checked RNA quality and quantity on agarose gels and with a NanoDrop (Thermo Fisher Scientific, Waltham, MA) and retrotranscribed each RNA sample into cDNA. These cDNA samples were sequenced on a GAII sequencing platform (Illumina, San Diego, CA) at the Genome Sciences Centre in Vancouver, British Columbia, Canada. We used paired-end sequencing (2 × 100 bp reads) and multiplexed two samples per lane.

We used Trimmomatic v0.20 to clean the raw sequence data by discarding reads containing one or more positions in either paired end with a Phred quality score of 0 (Illumina 1.3+ fastq format) and removing vector sequences (Lohse et al. 2012). We next used BWA v0.6.1 to align each cleaned sequence file to a *H. annuus* reference transcriptome with the "aln" and "sampe" commands (read trimming parameter set to –q 20 and all other parameters as default; Li and Durbin 2009). Our reference transcriptome includes 16,311 nuclear contigs and the complete chloroplast and ribosomal sequences assembled from expressed sequence tag libraries representing multiple genotypes (Renaut et al. 2012).

Single Nucleotide Polymorphism Calling and Fixation Index Calculations

After aligning to the reference, we used the RealignerTargetCreator and IndelRealigner tools from GATK v1.4-37 to correct for alignment errors near indel regions (McKenna et al. 2010) and SAMtools' v0.1.18 "pileup" to extract per-site base pair data (Li et al. 2009). We treated any site with fewer than three aligned reads as missing data. We then called the genotype at each site heterozygous only if the minor allele frequency was at least 10% and the minor allele was represented by at least three reads. These quality thresholds are meant to minimize sequencing errors being called allelic variants. For our final filtering step, we concatenated all genotype calls for all individuals at all sites aligned to the *H. annuus* reference transcriptome. We kept only polymorphic sites with no missing data, with an observed heterozygosity within the limits of 0.1–0.9, and without proximity of 10 bp or less to a site with poor sequence coverage or extremely low/high heterozygosity. These filters are relatively conservative and allow us to be confident that our polymorphic sites primarily represent single nucleotide polymorphisms (SNPs) rather than paralogues or sequencing errors. Finally, we calculated pairwise fixation index (F_{ST}) between the *H. annuus* and winteri ecotype samples at each polymorphic locus using the "basic.stats" function of the R package hierfstat (Goudet 2005), which is equivalent to Weir and Cockerham's (1984) F_{ST}. We use F_{ST} as an estimate of sequence divergence in this system.

Gene Expression and Gene Ontology Analyses

To allow for gene expression comparisons among individuals and genes, we normalized each sample by scaling the raw number of reads aligned per gene to the median expression of that sample across all genes and then normalized all genes in all individuals by gene length. Using these normalized values and the Bioconductor package DESeq (Anders and Huber 2010), we calculated the mean expression difference between the typical *H. annuus* and winteri samples as the $\log_2$ absolute value of mean expression for one group divided by mean expression for the second group. With the same DESeq package, we tested for statistically significant differences in expression using a Poisson-distributed error model and the Benjamini-Hochberg procedure to account for multiple testing ($\alpha = 0.1$).

We conducted three gene ontology analyses of biological process terms using an approach that takes into account relative ranking on an ordered list (receiver operator characteristic scoring) rather than the relative values of a score with an arbitrary threshold, as implemented in the software package ErmineJ (Lee et al. 2005; Gillis et al. 2010). First, we created a gene ontology annotation database by blasting the *H. annuus* reference transcriptome against the *Arabidopsis* TAIR 10 transcript database (Berardini et al. 2004; Lamesch et al. 2011), keeping only the best hit with a bit score greater than 60. We were able to find annotations for slightly less than half (7296, or 44.7%) of the *H. annuus* reference genes. ErmineJ uses this database alongside an input file of ranked genes to score for significantly enriched annotation terms and controls for multiple testing using the Benjamini-Hochberg correction ($\alpha = 0.1$; Gillis et al. 2010). We used three input files for three independent analyses: (1) ranking of genes by the absolute value of $\log_2$-fold change in gene expression, calculated above; (2) ranking by mean F_{ST} per gene, calculated by averaging SNP F_{ST} values across each gene; and (3) ranking by the SNP with the maximum F_{ST} value per gene.

Finally, we compared the list of genes that were significantly differentially expressed between *H. annuus* and winteri to lists generated by taking the same proportion of those genes with highest mean and maximum F_{ST}. We tested whether the overlap between these lists differed from that expected by chance using a two-sided Fisher's exact test, calculating the *P* value as the sum of all probabilities less than or equal to that of the observed contingency table.

Results

Typical *Helianthus annuus* and the woody perennial winteri ecotype exhibit differences in stem anatomy early in development (fig. 3). By 21 d postgermination, all winteri seedlings we surveyed had initiated development of a vascular cambium, and the youngest winteri individual with a distinguishable vascular cambium was 15 d old. By contrast, the first typical *H. annuus* seedling to show vascular cambial initiation was at 18 d postgermination, and all *H. annuus* seedlings we surveyed did not exhibit a vascular cambium until 24 d postgermination. At 24 d, winteri seedlings had smaller stems than typical *H. annuus* seedlings ($t = 4.495$, df = 4, $P = 0.011$; table 2) but had developed a similar area of fascicular cambium ($t =$

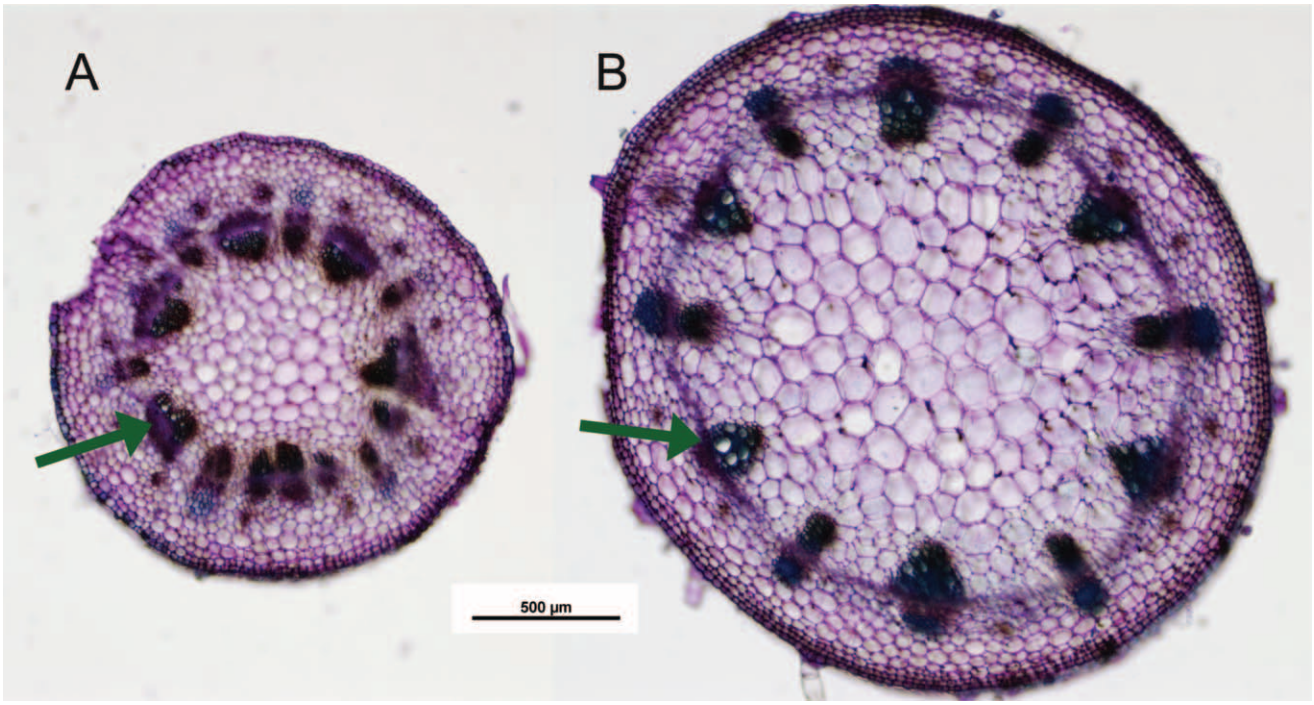

Fig. 3 Stem cross sections of typical winteri ecotype (*A*) and *Helianthus annuus* (*B*) seedlings at 24 d postgermination. Sections were prepared fresh by freehand sectioning at approximately halfway between the cotyledons and first true leaves and then stained in 0.5% toluidine blue O before photographing. Note the differences in relative size and development of fascicular cambium (green arrows; see also table 2).

Table 2

Phenotypic Comparisons of Seedling Stem Anatomy and Mature Plant Traits between Typical *Helianthus annuus* and the Woody Perennial Winteri Ecotype

	H. annuus		Winteri ecotype			
	Mean	SE	Mean	SE	Test statistic[a]	*P* value
Stem anatomical observations:[b]						
Stem area (mm^2)	3.241	.291	2.543	.170	$t = 4.495$	.011*
Fascicular cambium area (mm^2)	.115	.018	.160	.014	$t = 2.001$	.116
Number of vascular bundles	12	0	13.3	.6	$z = .817$	.414
Relative area of fascicular cambium[c]	.0391	.0042	.0630	.0036	$z = 1.962$	.049*
Common garden experiment:[d]						
Flowering initiation (d)	89.59	.63	95.26	.57	$t = 4.292$	.005**
Mature height (cm)	142.46	3.21	167.30	4.55	$t = 3.424$	.014*
Basal stem density (g/mL)	.307	.007	.344	.008	$t = 1.675$	.145

[a] All *t* values and accompanying *P* values are from two-sample *t*-tests on linear mixed-effects models with ecotype as a fixed effect and population as a random effect (stem anatomy, df = 4) or population and family as nested random effects (common garden, df = 6). For the number of vascular bundles and the relative area of fascicular cambium, differences were assessed with *z*-tests on generalized linear models with Poisson/binomial error distributions, respectively.

[b] Measurements were made on digital photographs of cross sections at ×4 magnification, made from the stem between the cotyledons and first true leaves of seedlings at 24 d postgermination. Sections were stained in 0.05% toluidine blue O in a phosphate buffer (O'Brian et al. 1964).

[c] The proportion of stem area consisting of fascicular cambial tissue in cross section (fascicular cambium area/stem area).

[d] Plants were grown at the University of British Columbia Farm in 2012. Flowering was scored as the first day we observed a reproductively active flower postgermination, height was measured along the primary stem at first flowering, and basal stem density is the ratio of dry mass to fresh volume of a 20-cm basal section of the primary stem harvested at ~6 mo postgermination.

* $P < 0.05$.

** $P < 0.01$.

2.001, df = 4, $P = 0.116$; table 2), thereby devoting a larger proportion of their stems to secondary tissue (relative area of fascicular cambium; $z = 1.962$, $P = 0.050$; table 2). All typical *H. annuus* seedlings had exactly 12 primary vascular bundles, while some winteri seedlings had as many as 18; however, this difference is not statistically significant ($z = 0.817$, $P = 0.414$; table 2). In addition, the overall development of the vascular cambium in the winteri ecotype appears less well ordered, with the interfascicular cambium often difficult to distinguish and the primary bundles varying in size (fig. 3).

In a common garden, the winteri ecotype plants flowered almost 6 d later than typical *H. annuus* from the same geographic area ($t = 4.292$, df = 6, $P = 0.005$; table 2) and were on average ~25 cm taller ($t = 3.424$, df = 6, $P = 0.014$; table 2). Surprisingly, *H. annuus* plants and the winteri plants did not have significantly different basal stem densities when harvested at 6 mo postgermination ($t = 1.675$, df = 6, $P = 0.145$; table 2).

There is no evidence for whole-genome duplication or any major segmental genome duplication or loss in the winteri ecotype relative to typical *H. annuus*. We measured six winteri and eight *H. annuus* individuals, representing two families from each of seven populations, and found no significant differences between the ecotypes ($t = -0.577$, df = 10.904, $P = 0.576$). The mean genome size across all samples is 3.47 Gb.

After aligning our 12 RNA-Seq samples to a *H. annuus* reference transcriptome, we identified 73,996 well-supported SNPs in 8961 genes. Across all SNPs, *H. annuus* and the winteri ecotype have a mean F_{ST} of 0.034, with a range of 0–0.8487. Of the 13 genes with mean F_{ST} greater than 0.34 (i.e., an order of magnitude above the global mean), five have sequence homology with annotated *Arabidopsis thaliana* genes, including FLOWERING BHLH, a regulator of flowering time via *CONSTANS* transcription; GALACTURONOSYL TRANSFERASE-LIKE 8, involved in cell wall development through xylan biosynthesis in the Golgi apparatus; MAP3Kε protein kinase 1, involved in pollen and embryo development; SAR DEFICIENT 1, a key regulator of isochorismate synthase induction and salicylic acid synthesis involved in the MAPK cascade; and an unnamed protein kinase superfamily protein located in the plasma membrane, AT1G11390.

We identified 575 (3.5%) of the 16,311 genes as having significant differential expression between the two ecotypes in 3-wk-old seedlings (fig. A1, available online). The list of genes ranked by expression pattern was significantly enriched for seven biological processes, including response to gibberellin stimulus and karrikin, cell death, DNA packaging and nucleosome organization, chemical homeostasis, and phenylpropanoid metabolism (table 3). We also analyzed two lists of genes ranked by either mean F_{ST} or highest F_{ST}; these analyses contain no significantly enriched biological processes after controlling for multiple testing. However, we report the seven highest-ranked biological processes for both lists (table 3). These top gene ontology terms have no overlap with those from the gene expression analysis, although the regulation of phenylpropanoid metabolism and flavonoid biosynthesis are biologically related to phenylpropanoid metabolism (table 3).

To better compare the analyses, we identified the 315 genes (top 3.5% of genes with sequence divergence) that were highest ranked for either mean F_{ST} or highest F_{ST} of genes. This is the same proportion of genes that we had identified as having sig-

Table 3

Seven Highest-Ranked Biological Processes for Gene Expression Divergence and F_{ST} between Typical *Helianthus annuus* and the Woody Winteri Ecotype

Biological process	GO ID[a]	ROC rank[b]	*P* value[c]
Gene expression analysis:[d]			
Response to gibberellin stimulus	GO:0009739	1	.033**
Cell death	GO:0008219	2	.069*
Response to karrikin	GO:0080167	3	.076*
DNA packaging	GO:0006323	4	.069*
Chemical homeostasis	GO:0048878	5	.085*
Nucleosome organization	GO:0034728	6	.076*
Phenylpropanoid metabolic process	GO:0009698	7	.099*
Mean and highest F_{ST} analyses:[e]			
Energy derivation by oxidation of organic compounds	GO:0015980	1/3	ns
Cellular respiration	GO:0045333	2/4	ns
Root hair elongation	GO:0048767	3/5	ns
Coenzyme catabolic process	GO:0009109	4/6	ns
Regulation of phenylpropanoid metabolic process	GO:2000762	5/1	ns
Regulation of flavonoid biosynthetic process	GO:0009962	6/2	ns
Protein targeting	GO:0006605	7/59	ns
Regulation of secondary metabolic process	GO:0043455	11/7	ns

Note. ns = not significant.

[a] The unique identifier associated with a gene ontology (GO) term (Berardini et al. 2004).

[b] Annotation terms were evaluated using the receiver operator characteristic (ROC) method implemented by ErmineJ (Lee et al. 2005), which takes into account the relative ordering of a list and ranks associated gene ontology terms.

[c] Corrected *P* value, accounting for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.1$).

[d] Genes in this analysis were ordered by the absolute value of $\log_2$-fold change of gene expression.

[e] Separate analyses were conducted on genes ordered by mean and by highest F_{ST} per gene. ROC rank is represented as mean/highest F_{ST}.

* Corrected $P < 0.1$.

** Corrected $P < 0.05$.

nificant differential expression. These gene lists also have very little overlap in identity: only two are shared among all analyses and an additional three between the differentially expressed genes and the genes with highest mean F_{ST} (fig. 4). An overlap of five genes is not statistically different from the overlap expected by chance (two-sided Fisher's exact test, $P = 1.00$). Of these five genes, four have no homology to *Arabidopsis* genes, and the last is homologous to a structural constituent of the ribosome involved in protein targeting to the chloroplast and embryonic development, EMBRYO DEFECTIVE 1473.

Discussion

If *Helianthus annuus* arrived in California within only the past few thousand years as hypothesized by Heiser and others (Heiser 1954; Heiser and Smith 1969; Dorado et al. 1992), then this woody perennial ecotype is very recently evolved. Indeed, although it can be genetically distinguished with sufficient sampling of its genome, the ecotype is closely related to nearby *H. annuus* populations on the valley floor ($F_{ST} = 0.034$) and shows reduced heterozygosity consistent with a possible founder event or population bottleneck (B. T. Moyers and L. H. Rieseberg, unpublished data). Such an abrupt shift in life history and development seems very likely to have been driven by strong selective pressures. We have observed that the habitat where the winteri ecotype grows is relatively harsh, with poor, extremely shallow (10–70 cm) granite decomposition soils on steep hillsides. In combination with seasonal drought and locally patchy rainfall, these populations likely experience water stress regularly. We might hypothesize that increased secondary growth affords the winteri ecotype greater resistance to embolisms inside vessels under negative water potentials (Choat et al. 2012; Lens et al. 2013*a*, 2013*b*) and/or that perenniality allows individuals who establish a foothold in this harsh environment to capitalize on their success.

Indeed, seedlings of the winteri ecotype exhibit differences in secondary growth when compared to typical *H. annuus* seedlings as early as 3 wk postgermination. These include differences in both developmental timing (i.e., heterochrony; Carlquist 2009) and possibly also morphology, although further anatomical work is needed to confirm this observation. Unexpectedly, in a common garden environment, first-year winteri plants did not have increased basal stem density relative to *H. annuus* plants. Perhaps this is because winteri plants do not differ in basal secondary growth relative to *H. annuus* until after the first year, or perhaps we failed to observe an increase in stem density at the UBC Farm due to a plastic response to the nutrient-rich mesic soils and abundant water. Had we also measured stem density at a higher point on the primary stem, it is likely we would have seen a difference (see fig. 1*B*). Regardless, the winteri plants were characterized by later flowering and increased height and were still vigorously

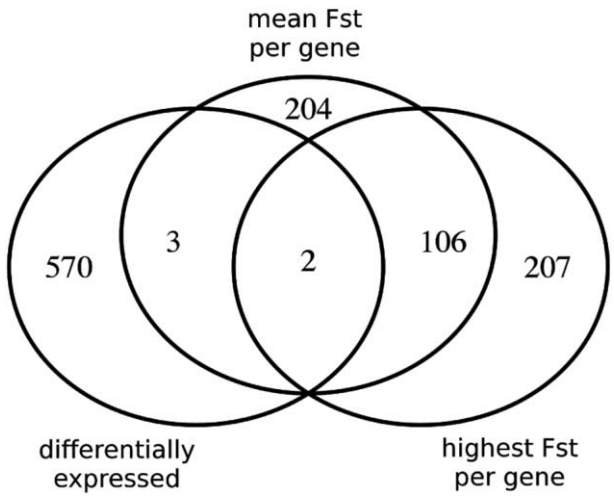

Fig. 4 Venn diagram representing the overlap in gene identity among the 3.5% of genes that are differentially expressed between typical *Helianthus annuus* and the woody ecotype winteri (*left*), the top 3.5% of genes with the highest mean F_{ST} per gene (*center*), and the top 3.5% of genes with the highest maximum single nucleotide polymorphism F_{ST} (*right*).

flowering in early October, when almost all *H. annuus* plants had begun to senesce.

Notably, the gene with the second-highest mean F_{ST} in this analysis (at 0.498) is homologous to *Arabidopsis thaliana*'s FLOWERING BHLH, a transcription factor that activates expression of *CONSTANS*, the photoperiodic flowering-time regulator, and thereby affects flowering time (Ito et al. 2012). We know that transcription during primary growth in the shoot apical meristem, which controls the transition from vegetative to reproductive growth, shares regulatory elements with transcription during secondary growth (Schrader et al. 2004; Groover and Robischon 2006). Many studies in model systems on secondary woodiness also report altered flowering phenology (Ko et al. 2004; Melzer et al 2008). This gene is thereby a strong candidate for involvement in the transition to secondary woodiness in *H. annuus*, and a case could also be made for another high-F_{ST} gene (homologous to GALACTURONOSYL TRANSFERASE-LIKE 8) because of its potential role in cell wall development via xylan synthesis (Rennie et al. 2012).

We chose the population differentiation estimator F_{ST} as a proxy for coding sequence divergence for several reasons. First, F_{ST} is both widely used and widely understood (Holsinger and Weir 2009). Second, in our transcriptome sequence analysis, typical *H. annuus* and the winteri ecotype have no fixed genetic differences, which makes some measures of sequence divergence (e.g., any involving substitution rates) inappropriate for our data. Third, the overall mean F_{ST} between typical *H. annuus* and the winteri ecotype is quite low (0.034; see also fig. A2, available online). Together with the geographic proximity of the ecotypes and the putative recent origin of the winteri ecotype, this suggests either that gene flow between the populations is ongoing and/or that evolution in the winteri ecotype may have primarily been from standing genetic variation. In either case, large allelic frequency differences between the ecotypes (i.e., high F_{ST} values) provide a most sensitive means for detecting loci that may have diverged due to selection. Finally, F_{ST} is robust to many demographic effects (Beaumont 2005) and to relatively small sample sizes at the individual level (Willing et al. 2010).

One early hypothesis we considered for the rapid evolution of perenniality in *Helianthus* was whole or partial genome duplication, which is correlated with the perennial habit in angiosperms (Otto and Whitton 2000). Our data allow us to conclusively reject that hypothesis: although *Helianthus* species may in general be prone to rapid chromosomal evolution (Burke et al. 2004), wild *H. annuus* and the winteri ecotype have virtually identical genome sizes.

Among the seven biological processes we identified as significantly enriched in our gene expression analysis, most are difficult to interpret in specific reference to the evolution of the winteri ecotype. The highest-ranked biological process, response to gibberellin stimulus, may relate to lignin biosynthesis, although gibberellins are broadly involved in many aspects of plant development. However, it has been demonstrated in both poplar (Israelsson et al. 2005) and tobacco (Biemelt et al. 2004) that overexpression of the gibberellin biosynthetic enzyme GA-20 oxidase results in an increase of lignin biosynthesis and stem lignification. Also of potential interest is phenylpropanoid metabolism. The phenylpropanoid pathway sits upstream from a number of important biosynthetic pathways and ultimately generates a vast array of secondary metabolites in plants, among them flavonoids, sporopollenin, and lignin (Vogt 2010). Phenylpropanoid metabolism is involved in response to nutrient, temperature, and water stress; herbivore resistance; pathogen response; signaling; and even UV protection (Dixon and Paiva 1995). We might easily speculate that any number of these associations could play a role in differentiating the winteri ecotype from typical *H. annuus*. Additionally, the regulation of phenylpropanoid metabolism and flavonoid biosynthesis are two of the top seven highest-ranked biological processes associated with genes with high sequence divergence, which is suggestive of a connection with the gene expression pattern, although these terms are not statistically significant.

Perhaps surprisingly, none of the other top-ranked biological process terms share any apparent connection between the two analyses. Similarly, only five genes are shared between those that are significantly differentially expressed and those with the highest mean or highest single SNP F_{ST}. It is difficult to speculate on the identity of those shared genes, as only one has a known homologue in *Arabidopsis* and the homologous gene is unremarkable. Furthermore, the co-occurrence of five genes on both lists is not different from what we would expect by chance. It appears that divergence in gene expression in the winteri ecotype is uncoupled from coding sequence divergence.

Sequence divergence in this system almost certainly extends to untranslated and noncoding regions of the genome (Bird et al. 2006). This kind of polymorphism is undetectable in RNA-Seq data, so our analysis is limited to only the subset of nucleotide sequences that is expressed. It is probable that expanding our data set to include sequence polymorphism across the entire genome would increase the similarity of patterns of divergence, because much of the variation in gene expression ultimately must stem from changes in genomic sequence (Cheung and Spielman 2002). However, our data do allow us

to compare more proximate changes and to conclude that the genes that have diverged in expression pattern are different from those that have diverged in coding sequence.

The apparent independence of gene expression and coding sequence divergence between typical *H. annuus* and the winteri ecotype may be the result of the short timescale of this transition. Even if gene expression and gene sequence might eventually show similar patterns of divergence, it is possible that not enough time has passed for the right mutations to accumulate or other processes to act. At least in this case, the genomic architecture of the transition to secondary woodiness seems quite complex. Many genes, divergent in sequence and expression, are involved in the adaptation of the *Helianthus* winteri ecotype to the foothills of California.

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