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Typically, early in leaf development, SPCHpro::SPCH-YFP is expressed in many small cells, but fluorescence diminishes as cells become morphologically distinct meristoids (2) (Fig. 2K). Relative to equivalently staged SPCHpro::SPCH-YFP plants, SPCH variants with strong overproliferation phenotypes displayed increased numbers of YFP-positive cells early (Fig. 2L) and a trend toward increased protein persistence into meristemoid, GMC, and guard cell stages later (Fig. 2L and fig. S8). When expressed in a CA-YODA background (in which SPCH was predicted to be phosphorylated), full-length SPCH-GFP was not visible, nor could it promote stomatal development (figs. S2 and S9C). However, GFP-SPCHA49, which is missing phosphorylatable residues, was detectable and was able to drive asymmetric divisions (fig. S9D).

SPCH is closely related to two other bHLH transcription factors that control stomatal development. We have shown, however, that a novel domain of SPCH renders it uniquely subject to phospho-regulation by a group of kinases that have been demonstrated to transduce signals downstream of both cell-cell and plant-environment interactions (fig. S10). In general, the domain mediates repression of SPCH and does so in a quantitative manner; the more potential MAPK sites eliminated, the stronger the effect of the SPCH variant on stomatal development. However, one specific residue phosphorylated by MPK6, Ser389, is required positively for activity, which suggests that the MPKTD is the integration site for complex regulatory inputs. The MPKTD is of unknown origin; it is not present in Arabidopsis proteins other than SPCH but is found in SPCH homologs from a variety of plant species (fig. S11) (25), hence MAPK regulation of a stomatal bHLH is likely to be a widespread regulatory strategy.

SPCH solves a problem intrinsic to MAPK signaling—how is it a set of generally used MAPKs recruited to a specific biological event?—by providing the important effector in a spatially and temporally restricted domain. From the perspective of stomatal control, SPCH guards the entry into the stomatal lineage, including the production of self-renewing cells that contribute to later flexibility in epidermal development. This important decision point is likely the target of developmental, physiological, and environmental regulation (26, 27). Coupling the MPK3/6 signaling module to the activity of SPCH provides a unified, yet tunable, output for the complex set of inputs from these sources. Understanding the elements of the MAPK/SPCH regulatory system that coordinate stomatal production with the prevailing climate may allow the production of food or bioenergy crops with the ability to respond and adapt to changes in that climate.

References and Notes

See supporting material on Science Online.
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Figs. S1 to S11
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Regulatory Genes Control a Key Morphological and Ecological Trait Transferred Between Species

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Hybridization between species can lead to introgression of genes from one species to another, providing a potential mechanism for preserving and recombining key traits during evolution. To determine the molecular basis of such transfers, we analyzed a natural polymorphism for flower-head development in Senecio. We show that the polymorphism arose by introgression of a cluster of regulatory genes, the RAY locus, from the diploid species S. squalidus into the tetraploid S. vulgaris. The RAY genes are expressed in the peripheral regions of the inflorescence meristem, where they promote flower asymmetry and lead to an increase in the rate of outcrossing. Our results highlight how key morphological and ecological traits controlled by regulatory genes may be gained, lost, and regained during evolution.

Changes in regulatory genes have been implicated in a range of evolutionary transitions, operating from the micro- to macro-evolutionary scales (1–3). These changes have largely been considered as occurring independently within different species. However, it is also possible that interspecific hybridization plays an important role in evolution (4). One consequence of such exchanges is that they may allow traits that are lost because of short-term selective pressures to be regained at a later stage. For example, members of the sunflower family (Asteraceae) share a composite flower head, with each head comprising numerous small flowers (florets). In radiate species, the outer florets (ray florets) have large attractive petals, whereas the inner florets (disc florets) tend to be less conspicuous. Loss of the radiate condition has occurred multiple times within the Asteraceae, yielding nonradiate species with only disc florets (5). These events often correlate with shifts to higher levels of self-pollination (6), which should be favored when mates and/or pollinators occur at low densities (7). Partial or complete reversals from the nonradiate back to the radiate condition have been described (8), some of which appear to involve interspecific hybridization events (9). One explanation for such evolutionary gains and losses is that key regulatory genes control-

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ling the trait can be modified and exchanged between species. To test this idea, we analyzed a well-documented case of interspecific exchange in Senecio.

Senecio vulgaris (Groundsel) (Fig. 1A) is an allotetraploid nonradiate species, native to Europe and occurring throughout temperate zones. Radiate forms of S. vulgaris (Fig. 1B) arose in the United Kingdom after the introduction of S. squalidus (Fig. 1C), a diploid radiate species originating from Sicily. S. squalidus was brought to the Oxford Botanic Garden about 300 years ago (10), from where it spread. As S. squalidus became dispersed throughout the United Kingdom, it crossed with S. vulgaris, yielding triploid hybrids (11). Although such triploids have low fertility [seed set <0.02% (9)], some viable progeny occur as a result of backcrosses with S. vulgaris. Further rounds of backcrossing are thought to have led to introgression of the radiate trait into some populations of S. vulgaris (12, 13). The resulting polymorphism for the radiate condition in S. vulgaris is controlled by a single chromosome region or genetic locus, here termed RAY (14). Thus, the hypothesized introgression would have involved transfer of the RAY locus from S. squalidus into S. vulgaris.

The RAY locus affects floral symmetry. Disc florets have fivefold radial symmetry, whereas ray florets are bilaterally symmetrical (zygomorphic), having enlarged ventral (abaxial) and reduced dorsal (adaxial) petal lobes (Fig. 1D). CYCLOIDEA (CYC) is a primary gene controlling floral symmetry in Antirrhinum majus, a species with entirely zygomorphic flowers (15). CYC encodes a DNA-binding protein belonging to the TCP family (16, 17). Proteins from this family contain a conserved basic helix-loop-helix region that binds DNA (the TCP domain) and have a range of regulatory roles in plant development (16, 17). On the basis of Antirrhinum mutant phenotypes, it has been proposed that CYC-like genes might also control the development of ray florets in the Asteraceae (18). Supporting this theory, ectopic expression of a CYC-like gene from Gerbera hybridra, GhCYC2, has differential effects on ray and disc floret development in this horticultural species (19).

To determine whether CYC-like genes are involved in the RAY locus, homologs were isolated from S. vulgaris. RNA in situ hybridizations on radiate plants revealed that two of these genes, termed RAY1 and RAY2, were specifically expressed in ray floret primordia (Fig. 2, A and B). RAY1 and RAY2 were expressed in a similar pattern in radiate (R/R) and nonradiate (N/N) genotypes (Fig. 2, A to D). However, the signal appeared to be stronger in N/N compared with R/R. This difference was confirmed by the expression levels in RNA from young flower heads (Fig. 2E). Stronger expression of the N allele was also seen in RNA from N/R heterozygotes, suggesting that it reflects cis-regulatory changes (Fig. 2E). Phylogenetic analysis showed that RAY1 and RAY2 belong to a subfamily of TCP genes that include genes known to control flower asymmetry (clade in orange, Fig. 2F). RAY1 and RAY2 arose by a duplication event ~30 million years ago (20) that occurred early in the evolution of the Asteraceae, before divergence of Helianthus, Gerbera, and Senecio but after divergence of the Asteraceae from the Lamiidae (Fig. 2F). RAY2 appears to be orthologous to GhCYC2 from Gerbera, which is also expressed preferentially in ray florets (19).

Fig. 1. Flower head of nonradiate S. vulgaris (A), radiate S. vulgaris (B), and S. squalidus (C). Scale bars, 3 mm. (D) Section through a flower head and two individual florets taken by optical projection tomography. Disc floret petals are outlined in orange, whereas ray floret petals are outlined in red (dorsal) or yellow (ventral).

Fig. 2. (A) Expression pattern of RAY1 in a longitudinal section of a developing radiate (R/R) flower head. (B) Expression of RAY2 in radiate form. (C) Expression of RAY1 in nonradiate (N/N) form. (D) Expression of RAY2 in nonradiate form. In all cases, RAY1 and RAY2 are expressed in the outer floret primordia (marked by *). Scale bars, 100 μm. (E) Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) showing RAY1 and RAY2 expression in young flower heads of R/R, R/N and N/N genotypes. A common band for R and N is indicated with an arrow in the R/N genotype. 18S RNA control is also shown. (F) Phylogenetic relationships between RAY1, RAY2, and a sample of other genes from the TCP family on the basis of a maximum likelihood analysis of amino acid sequences. RAY1 and RAY2 belong to a clade with CYC and DICH, which control floral symmetry in Antirrhinum (15). Bootstraps of 500 replicates (where greater than 50%) are shown. Species abbreviations and GenBank accession numbers are given in (30).
Fig. 3. (A) RAY1: a 412-bp band that cosegregates with R and a 238-bp and a 174-bp band with N in an F2 population. (B) RAY2: a 540-bp and a 156-bp band that cosegregate with R and a 696-bp band with N. PCR products of RAY1 and RAY2 coding regions were digested with TaqI and EcoRI, respectively. (C) Variable sites at RAY1 and RAY2 in and around the coding regions for the four haplotypes N1, N1, R1, and N2. Polymorphisms that are diagnostic for N1/N1 versus R1/R1 haplotypes are shown surrounded by black and white, respectively. All other polymorphisms are highlighted in gray. Nucleotide polymorphisms that cause amino acid changes are indicated with asterisks. Positions of deletions of TAAGGAATTCGAAACCCA and ATAGAAA in the RAY2-R1 haplotype are marked with arrows.

Fig. 4. (A) Flower-head phenotypes of RR nontransgenic control plant. (B) Flower-head phenotype of RR nontransgenic control plant. (C) Flower-head with slightly short rays from a transgenic plant overexpressing an internal fragment of RAY1 N allele coding sequences in a radiate (R/R) S. vulgaris background. (D) Flower-head phenotype of RAY1 transgenic, as in (B), with very short ray florets. (E) Flower-head phenotype of RAY2 transgenic, as in (B), giving no ray florets. (F) Flower-head phenotype of RAY2 transgenic, giving tubular ray florets. (G) Section through a ventralized ray floret, color-coded as in Fig. 1D. All transgenics are T1 generation, obtained by self-pollinating the primary transformants. (G) RT-PCR showing expression levels of RAY1 and RAY2 in the transgenics, together with controls for 18S RNA and the kanamycin resistance gene (NPTII). R, normal radiate head; N, nonradiate or discoid head; SS, slightly short rays; S, short rays; VS, very short rays; T, tubular rays with ventralized petals. Scale bars, 2 mm [(A) to (E)] and 1 mm (F).

To determine whether RAY1 or RAY2 map to the RAY locus, their sequence was determined for parental radiate (R/R) and nonradiate (N/N) plants, allowing genotype-specific CAPS (cleaved amplified polymorphic sequences) to be designed (Fig. 3, A and B). Genotyping an F2 population derived from these parents revealed that both RAY1 and RAY2 segregated with flower-head phenotype, and we observed no recombinants in more than 700 plants. Linkage between RAY1 and the RAY locus was further confirmed by bulk segregant analysis on R/R and N/N genotypes, and no recombinants were observed out of 2800 chromosomes. CAPS were also used to genotype accessions of radiate and nonradiate forms from various locations in the United Kingdom (table S1). In all cases, the RAY1 and RAY2 genotypes matched the phenotype, confirming a tight association with each other and with the RAY locus. This was further confirmed by sequencing the RAY genes from several U.K. S. vulgaris accessions: All sequences from R/R genotypes were identical, while two minor variants were found among N/N genotypes, termed N and N1 (Fig. 3C).

Thus, both RAY1 and RAY2 are tightly linked and associated with RAY, and we were able to define three haplotypes: N1, N1, and R1. Because the radiate condition in S. vulgaris is thought to have originated from S. squalidus, RAY1 and RAY2 were also sequenced from various U.K. accessions of S. squalidus. This revealed two haplotypes, one identical to the R-haplotype of S. vulgaris and another that was a variant of R1 and was termed R1 (Fig. 3C and table S1). These results provide molecular proof that the radiate form of S. vulgaris arose through hybridization with S. squalidus plants and show that the R-haplotype was introgressed through this process.

Comparing the sequences of the haplotypes revealed several differences between N/N1 and R/R1 (Fig. 3C and fig. S1A). No diagnostic amino acid substitutions were found for RAY1, whereas two amino acid substitutions (S to F, D to E) were associated with the N/N1 alleles of RAY2. The substitutions found in the N and N1 alleles of RAY2 were also found in the radiate species S. vernalis and S. glaucus (fig. S1B), making it unlikely that they are responsible for the radiate condition. Several diagnostic differences were also found in the 5′ and 3′ noncoding regions. As these represent only a limited sample of flanking sequence, it is likely that further differences would also be found in regions extending further out from the genes. Thus, the N/N1 and R/R1 haplotypes have accumulated multiple nucleotide differences since they diverged from their common ancestor and it is likely that the functionally important changes lie outside the RAY1 and RAY2 coding regions.

The rapid spread of the radiate trait in S. vulgaris, despite the strong reproductive barrier between S. vulgaris and S. squalidus, suggests that the introgression of the R haplotype may have been driven by selection, presumably acting on differences outside the RAY1 and RAY2 coding regions. However, testing for selection by analyzing sequence variation at the RAY locus is not straightforward because most selection tests assume a single interbreeding population (21), whereas introgression of R involved exchange between two divergent species separated by a major reproductive barrier.

As a further test of whether RAY1 and RAY2 play a role in ray floret development, we transformed radiate S. vulgaris with two constructs, both of which are driven by the constitutive 35S promoter (the radiate background was chosen because N is semidominant and is thought to...
represent the derived condition). Expression of an internal fragment of the RAY1 coding region (N allele) that includes the conserved TCP and R domains, yielded 10 independent transformants. Five of these plants produced slightly shorter ray florets (Fig. 4, A and B), three produced very short ray florets (Fig. 4C), and two had only disc florets (Fig. 4D), resembling nonradiate plants. These results suggest that overexpression of RAY1 is repressing ray floret development, consistent with the higher levels of RAY1 expression observed in N/N genotypes. However, the level of transgene expression did not correlate in a simple manner with the severity of the phenotype; transgensics with slightly short ray florets had higher levels of expression than the discoid transgensics (Fig. 4G). There was also no correlation with the endogenous levels of RAY1 gene expression, because these levels were similar in transgensics with different phenotypes (fig. S1D). The variation in transgenic phenotype may reflect differences in the pattern of transgene expression, posttranscriptional interactions with the internal RAY1 fragment used in the transformations, or perhaps promotive as well as inhibitory effects of RAY1 on ray floret development. Whatever the explanation, the results indicate that RAY1 plays a critical part in controlling ray versus disc floret identity.

Expression of the entire RAY2 coding region (N allele) in the radiate background produced tubular ray florets in three independent transgenics (Fig. 4E). All petal lobes in these florets resembled the long ventral (abaxial) petal lobes of normal ray florets (Fig. 4, E and F), which suggests that RAY2 is involved in promoting ventral identity in ray florets. Unlike ectopic expression of GhCYC2 in Gerbera hybrida (19), disc floret development was not modified by expression of RAY2. This difference may reflect the fact that the innermost florets in Gerbera hybrida are not fully radially symmetrical (19) and may have some raylike character even in untransformed horticultural varieties.

We conclude that the RAY locus comprises a cluster of CYC-like genes that have played a key role in the evolution of the radiate condition.