Oakleaf: an S locus-linked mutation of Primula vulgaris that affects leaf and flower development

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Received: 19 September 2014
Accepted: 7 February 2015

New Phytologist (2015) 208: 149–161
doi: 10.1111/nph.13370

Key words: heterostyly, KNOX genes, Oakleaf, Primula vulgaris, S locus.

Summary

• In Primula vulgaris outcrossing is promoted through reciprocal herkogamy with insect-mediated cross-pollination between pin and thrum form flowers. Development of heteromorphic flowers is coordinated by genes at the S locus. To underpin construction of a genetic map facilitating isolation of these S locus genes, we have characterised Oakleaf, a novel S locus-linked mutant phenotype.
• We combine phenotypic observation of flower and leaf development, with classical genetic analysis and next-generation sequencing to address the molecular basis of Oakleaf.
• Oakleaf is a dominant mutation that affects both leaf and flower development; plants produce distinctive lobed leaves, with occasional ectopic meristems on the veins. This phenotype is reminiscent of overexpression of Class I KNOX-homeodomain transcription factors. We describe the structure and expression of all eight P. vulgaris PvKNOX genes in both wild-type and Oakleaf plants, and present comparative transcriptome analysis of leaves and flowers from Oakleaf and wild-type plants.
• Oakleaf provides a new phenotypic marker for genetic analysis of the Primula S locus. We show that none of the Class I PvKNOX genes are strongly upregulated in Oakleaf leaves and flowers, and identify cohorts of 507 upregulated and 314 downregulated genes in the Oakleaf mutant.

Introduction

Observations on different forms of Primula flowers date back nearly 400 yr (van Dijk, 1943; P.M. Gilmartin, unpublished). The development of two distinct floral forms, known as pin and thrum, attracted the attention of Darwin, who recognised and described their relevance and significance in his detailed studies of P. vulgaris and P. veris (Darwin, 1862). Primula produce either pin or thrum flowers, which exhibit reciprocal herkogamy and show different degrees of self-incompatibility (Darwin, 1862, 1877). Pin flowers have a long style with the stigma at the corolla mouth and anthers attached midway down the corolla tube; thrum flowers have anthers which are positioned at the corolla mouth and a short style which presents the stigma midway up the corolla tube (Darwin, 1862; Webster & Gilmartin, 2006). Elevation of the anthers in thrum flowers is caused by increased cell division in the corolla tube below their point of attachment, whilst in pin flowers the style is extended by increased cell elongation (Shivanna et al., 1981; Webster & Gilmartin, 2006). Differential floral architecture is orchestrated by different cellular mechanisms affecting anther elevation and style elongation (Webster & Gilmartin, 2006). Other morph-specific differences include pollen size, corolla opening diameter, stigma shape, stigmatic papillae length and style cross-section (Darwin, 1877; Haldane, 1933; Dowrick, 1956; Dulberger, 1975; Heslop-Harrison et al., 1981; Richards, 1997; Webster & Gilmartin, 2006). Darwin observed that within-morph pin-pin or thrum-thrum crosses were less fertile than intermorph pin-thrum or thrum-pin crosses (Darwin, 1877). This observation is underpinned by the presence of a sporophytic incompatibility system that in combination with the structural differences between the two forms of flower inhibits self-pollination and promotes outcrossing (Shivanna et al., 1981; Richards, 1997).

Floral heteromorphy in Primula is controlled by the S locus; pins are homozygous recessive (s/s), thrams heterozygous (S/s) (Bateson & Gregory, 1905; Haldane, 1933; Dowrick, 1956). Studies by Ernst (Ernst, 1928, 1933) and others (Pellow, 1928; Haldane, 1933; Dowrick, 1956; Lewis & Jones, 1993) suggested that the S locus comprises three dominant genetic functions: G, which suppresses style elongation; P, responsible for enlarged pollen; and A, which controls anther elevation. These genes represent a co-adapted linkage group. Other genes responsible for male and female sporophytic self-incompatibility functions are also linked (Lewis, 1949; Lewis & Jones, 1993; de Nettancourt,
Materials and Methods

Plant material and linkage analysis

Plants used in this study are wild-type Primula vulgaris Huds. and derived commercial cultivars. Primula vulgaris Oakleaf plants were originally obtained from Richards Brumpston (Woodborough Nurseries, Nottingham, UK) in 1999 and maintained by Margaret Webster as part of the National Collection of Primula, British Floral Variants. Plants were grown as described previously (Webster & Gilmartin, 2006). Hose in Hose, Jack in the Green and Jackanapes (Webster & Grant, 1990; Webster & Gilmartin, 2003) were crossed with Oakleaf, and controlled crosses between Oakleaf and wild-type were performed, in insect-free environments following emasculation of pollen recipients by removal of corolla and anthers. Seed was harvested from ripe seed capsules and stored at c. 4°C in air-tight containers.

Scanning electron microscopy (SEM)

Floral apical meristems and developing buds were dissected using scalpels and razor blades with a ¼ 20 hand lens. Samples were prepared for cryo-SEM, analysed and images recorded as described previously (Webster & Gilmartin, 2003).

Draft genome sequence acquisition

Paired-end and mate-pair genomic DNA sequence reads were generated by Illumina HiSeq2000 at The Genome Analysis Centre, Norwich Research Park, Norwich, UK. DNA was isolated from leaves of inbred self-fertile long homostyle P. vulgaris originating from Wyke Champflower, Somerset, UK (Crosby, 1940) for paired-end read sequencing. This genotype was chosen due to homozygosity compared with outbreeding pin and thrum plants. The assembly was scaffolded with mate-pair reads from a 9 kb thrum genomic DNA library. The paired-end reads provided ×60 genome coverage, and the mate-pair reads provided ×26 read coverage after filtering. A draft assembly was generated using ABySS v1.3.4 (Simpson et al., 2009) (kmer length = 81) to assemble paired-end reads, then SOAPdenovo v2.0.4 (Luo et al., 2012) to scaffold contigs using mate-pair reads (kmer length = 71). This process generated an assembly of 424 Mb comprising 102 442 sequences and a scaffold N50 of 47.8 kb. This draft assembly was used to identify the full complement of PvKNOX-like sequences and gene model assemblies for differential transcript analysis. Full details of the fully assembled and annotated P. vulgaris genome will be published elsewhere.

Gene model predictions for P. vulgaris KNOX (PvKNOX) genes

Arabidopsis thaliana KNOX proteins, KNAT1, KNAT2 (Lincoln et al., 1994), KNAT3, KNAT4, KNAT5 (Serikawa et al., 1996), KNAT6 (Belles-Boix et al., 2006), KNAT7 (Li et al., 2011) and STM1 (Long et al., 1996), were aligned to the draft P. vulgaris genome with Exonerate v2.2.0 (Slater & Birney, 2005).
(http://ccb.jhu.edu/software/tophat/index.shtml). *Primula vulgaris* KNOX loci were identified and gene models confirmed by transcript evidence from TopHat v2.0.8 and Cufflinks v2.1.1 (http://ccb.jhu.edu/software/tophat/index.shtml; http://cole-trapnell-lab.github.io/cufflinks/) (Trapnell et al., 2013) and by homology of the predicted proteins to KNOX proteins from the TAIR10 protein database (https://www.arabidopsis.org/). Parameters for protein sequence comparisons were ≥ 50% identity with ≥ 30% coverage of the KNOX query sequence. Gene models were curated manually where necessary with GenomeView (http://genomeview.org/). Sequences corresponding to *PvKNL1* were initially identified on two genomic contigs. The gene model was resolved as one locus by alignment to a Trinity (http://trinityrnaseq.github.io/) (Grabherr et al., 2011) assembly of the same Illumina RNA-Seq paired-end read data from pin and thrum flower RNA, as used for the Cufflinks analysis (Supporting Information Table S1). [Correction added after online publication 9 April 2015; in this section, URLs to TopHat and Trinity have been updated.]

**Results**

The *Oakleaf* mutant phenotype

The *Oakleaf* phenotype was identified in 1999 amongst commercial ornamental *Primula* plants. The pedigree and cultivar of *Oakleaf* are unknown. A division of the original mutant plant was obtained by Margaret Webster and an *Oakleaf* population established which was used in this study, alongside development of *Oakleaf* in polyanthus form as a commercial variety.

The *Oakleaf* phenotype is first visible in seedlings which sometimes produce normal and sometimes lobed cotyledons (Fig. 1a). However, the first true leaves consistently show the lobed appearance characteristic of *Quercus* species (Fig. 1a). The phenotype is variable but distinctive and easily recognisable. Mature leaves have an angular lobed appearance and contain wider and thicker leaf veins than wild-type (Fig. 1b). The lamina of the leaf is thicker and firmer than wild-type and the abaxial surface is pubescent. The effects of the mutation are not limited to the leaves; *Oakleaf* plants typically produce a distinctive floral phenotype. *Oakleaf* flowers are smaller than wild-type, typically 2 cm in diameter, and calyces are frequently split (Fig. 1c) with occasional yellow petaloid material in the sepals. The severity of the floral phenotype varies as seen in the F1 siblings from an *Oakleaf ×* wild-type cross (Fig. 1d–f). The most extreme floral phenotype presents five narrow straight-edged separate petals that look like the spokes of a wheel (Fig. 1d). Some plants produce an intermediate phenotype with attenuated rounded and separated petals (Fig. 1e), and in the least severe form, petals are similar to wild-type but sometimes with splits in the corolla to give partially separated petals (Fig. 1f). *Oakleaf* plants are fully fertile as both male and female parents.

We previously documented wild-type *Primula* flower development by cryo-SEM (Webster & Gilmartin, 2003). To investigate the timing of *Oakleaf* action and any impact on early flower development, we observed *Oakleaf* flowers from late stage 3 to late stage 4 (Fig. 2a). In both wild-type (Webster & Gilmartin, 2003) and *Oakleaf* flowers, sepals and anthers initiate at late stage 3 (Fig. 2a). Carpel development initiates at early stage 4 (Fig. 2a,d) and is accompanied by petal primordia bulges on the abaxial side of stamen primordia by mid stage 4 (Fig. 2a). *Oakleaf* does not therefore interfere with organ initiation or timing of development in early flower buds. However, at flower stage 6, the impact of *Oakleaf* on reduced petal and sepal development is visible. In *Oakleaf*, stage 6 petals are attenuated and the sepals have not expanded to engulf the developing stamens and carpels (Fig. 2b,c) as seen in wild-type flowers at this stage (Webster & Gilmartin, 2003).
2003). Comparison to wild-type flowers at mid stage 5 (Fig. 2d) reveals that by this earlier stage in wild-type, the sepals have already enclosed the flower. Standardisation of developmental stage comparisons between Oakleaf and wild-type were defined by equivalence of carpel development in Oakleaf and wild-type flowers; Oakleaf does not affect carpel development. There is no difference in the Oakleaf phenotype between pin and thrum plants.

Oakleaf plants occasionally produce ectopic meristems on the veins of leaves. These ectopic meristems can be vegetative, giving rise to leaves (Fig. 1g), or floral (Fig. 1h), leading to seed pods (Fig. 1i) but without viable seeds. Some aspects of the Oakleaf phenotype are reminiscent of the effects of ectopic expression of Class I KNOX homeodomain genes in Arabidopsis (Lincoln et al., 1994; Chuck et al., 1996; Hay & Tsiantis, 2010), and their role during normal development of lobed leaves in tomato and Cardamine hirsuta (Hareven et al., 1996; Bharathan et al., 2002; Hay & Tsiantis, 2006; Shani et al., 2009).

In order to explore the influence of Oakleaf on leaf and petal development and to examine whether the effects are organ-specific or whorl-specific, we combined Oakleaf with the following mutant phenotypes: Hose in Hose (Webster & Grant, 1990; Li et al., 2010), a dominant mutant phenotype in which sepals are converted to petals; Jack in the Green (Webster & Gilmartin, 2003), a dominant mutant phenotype in which sepals undergo a homeotic transformation to leaves; and Jackanapes (Webster & Gilmartin, 2003), a double mutant carrying both Jack in the Green and Hose in Hose dominant alleles, which produces hybrid petal/leaf structures in the first floral whorl.

Progeny from crosses of Oakleaf and Hose in Hose produce flowers with two whorls of Oakleaf type petals (Fig. 1j); progeny from crosses between Oakleaf and Jack in the Green produce flowers with characteristic Oakleaf petals surrounded by a calyx of miniature Oakleaf leaves (Fig. 1k); progeny from crosses between

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**Fig. 1** Developmental phenotypes of Primula vulgaris Oakleaf. (a) Seedlings from a wild-type × Oakleaf cross showing wild-type and mutant phenotypes, arrows indicate Oakleaf seedlings. (b) Leaf from Oakleaf plant. (c) Primula vulgaris Oakleaf mutant showing leaves and flowers. (d) Example of F1 plant from wild-type × Oak Leaf cross showing extreme attenuated petals phenotype. (e) Example of F1 plant from wild-type × Oakleaf cross showing partially attenuated petals. (f) Example of F1 plant from wild-type × Oakleaf cross showing near normal petals. (g) Leaves emerging from an ectopic meristem (indicated by arrow) on the main vein of an Oakleaf leaf. (h) Flower bud (arrow) emerging from an ectopic meristem on the tip of an Oakleaf leaf. (i) Seed capsule (arrow) arising from ectopic flower shown in (h) following pollination. (j) Flower on Hose in Hose – Oakleaf double mutant plant. (k) Flower on Jack in the Green – Oakleaf double mutant plant. (l) Flower on Hose in Hose – Jack in the Green – Oakleaf triple mutant plant. Bars, 1 cm.
Inheritance of Oakleaf

Preliminary analyses in horticultural crosses suggested that Oakleaf was dominant to wild-type (R. Brumpton, pers. comm.). Our crosses between Oakleaf and floral mutants reinforce this observation. To fully explore the inheritance of Oakleaf we undertook a series of controlled crosses. The first crosses (Fig. 3) used an Oakleaf thrum as both pollen recipient (Cross 1) and pollen donor (Cross 2) with a wild-type P. vulgaris pin plant. Seed from the Oakleaf thrum parent (Cross 1) yielded 44 progeny: 23 Oakleaf and 21 wild-type based on seedling phenotype (Fig. 3a); chi-squared analysis supports a 1 : 1 ratio (*P* > 0.70). Four Oakleaf and 14 wild-type plants were subsequently lost between seedling stage and flowering. The excess of pin Oakleaf and thrum wild-type progeny indicate linkage of Oakleaf to the S locus with coupling to the recessive s allele; three thrum Oakleaf progeny reveal recombination of Oakleaf from the recessive s allele to the dominant S allele. These small numbers suggest a map distance for Oakleaf to S of 11.5 cM.
Fig. 4 Confirmation of linkage between Primula vulgaris Oakleaf and the S locus. A recombinant P. vulgaris Oakleaf thrum plant was used in a reciprocal back cross with a wild-type pin plant. (a) Cross 3, Oakleaf as female parent. (b) Cross 4, Oakleaf as male parent. The phenotypes and genotypes, with respect to leaf shape (wild-type or Oakleaf), and the S locus (pin or thrum) of parent plants are indicated. The phenotypes, and predicted genotypes, of F1 progeny are shown along with numbers of pin- and thrum-type flowers found on each class of progeny lost before flowering is shown, as well as the number of pin- and thrum-type flowers found on Oakleaf and wild-type plants. Based on data from Fig. 4, the Oakleaf parent used in this cross carries the OKL locus in coupling to the dominant S allele of the S locus; genotypes of recombinant chromosomes in progeny and numbers of recombinant progeny are shaded grey. The map distance in cM between OKL and the S locus are indicated.

The reciprocal cross (Cross 2) with Oakleaf as pollen donor, confirmed linkage of Oakleaf to S and coupling to the recessive s allele (Fig. 3b). Of the 258 seeds planted, 45 germinated but died before producing secondary leaves and could not be scored. Of the remaining 112 Oakleaf and 54 wild-type plants, a further 18 Oakleaf and 29 wild-type plants died before flowering. These data (Fig. 3b) suggest a significant deviation from the anticipated 1:1 ratio (P<0.001) of Oakleaf to wild-type. Linkage of Oakleaf to the S locus is supported by the excess of Oakleaf pin and wild-type thrum plants. Four progeny, two Oakleaf thrums and two wild-type pins (Fig. 3b), are recombinants; these larger progeny numbers give a map distance between Oakleaf and the S locus of 3.3 cM.

In order to further confirm linkage to the S locus we backcrossed an Oakleaf thrum progeny plant to a wild-type pin plant. With the Oakleaf thrum as pollen acceptor (Cross 3), 28 progeny were obtained: 12 Oakleaf and 16 wild-type yielding the anticipated 1:1 ratio (P>0.3) (Fig. 4a). Three Oakleaf plants died before flowering and one Oakleaf plant produced pin flowers, revealing recombination between S and Oakleaf bringing Oakleaf back in coupling with s (Fig. 4a). The reciprocal cross (Cross 4) yielded 20 Oakleaf and 39 wild-type plants; eight plants were lost before flowering and no recombinants were found in the remainder (Fig. 4b). These data indicate distortion of the anticipated 1:1 ratio (P>0.01) of Oakleaf to wild-type, with Oakleaf progeny underrepresented. In this cross we did not observe any losses of plants at the seedling stage.

In order to investigate deviation from the anticipated 1:1 ratio of Oakleaf to wild-type plants in progeny from Cross 2 and Cross 4, we undertook further analyses. Reciprocal crosses between an Oakleaf pin and an Oakleaf thrum, with Oakleaf in coupling to the recessive s allele, are presented. (a) Cross 5, Oakleaf thrum as female parent. (b) Cross 6, Oakleaf pin as female parent. The phenotypes and genotypes, with respect to leaf shape (wild-type or Oakleaf), and the S locus (pin or thrum) of parent plants are indicated. The number of F1 progeny of each phenotype, classified with respect to leaf phenotype, and their predicted genotypes are shown; progeny were not scored with respect to flower morph.

Characterisation of the PvKNOX gene family

Aspects of the Oakleaf phenotype – namely lobed leaves, ectopic meristems and dominance – are reminiscent of the consequences of ectopic overexpression of Class I KNOX genes in Arabidopsis (Lincoln et al., 1994; Chuck et al., 1996; Hay & Tsiantis, 2010). We therefore set out to explore whether Oakleaf results from a constitutive overexpression mutation of a KNOX homeodomain gene. We considered and explored three possibilities: that the...
phenotype is caused by upregulation of a \( \text{PvKNOX} \) gene in mature leaves and flowers of \text{Oakleaf} \) plants; that the phenotype arises from a mutation in a \( \text{PvKNOX} \) gene that does not affect expression but confers a dominant gain of function in protein activity; that the dominant mutation is caused by upregulation of a gene unrelated to the \( \text{PvKNOX} \) gene family.

The \text{KNOX} \) homeodomain gene family in \text{Maize} \) (Vollbrecht \textit{et al.}, 1991), \textit{Arabidopsis} \) (Lincoln \textit{et al.}, 1994; Long \textit{et al.}, 1996; Serikawa \textit{et al.}, 1996; Belles-Boix \textit{et al.}, 2006; Li \textit{et al.}, 2011) and other species (Bharathan \textit{et al.}, 1999; Hay \& Tsiantis, 2010) have been characterised and classified as Class I or Class II based on phylogenetic relationships and expression dynamics (Kerstetter \textit{et al.}, 1994; Bharathan \textit{et al.}, 1999). We used this framework to define the full complement of Class I and Class II \( \text{PvKNOX} \) genes. Illumina RNA-Seq analysis of wild-type \( \text{P. vulgaris} \) leaf and flower transcriptomes, together with transcriptome analysis of \text{Oakleaf} \) mutant leaves and flowers, was used to generate a transcriptome dataset. We also included RNA-Seq datasets obtained from pin and thrum mixed stage flower samples to maximise the opportunity for \( \text{PvKNOX} \) related gene identification; these mixed pin and thrum flower RNA-Seq samples were not included in subsequent comparative expression analyses. A summary of read number, base coverage and transcript assemblies from these six RNA samples is presented in Table S1.

In parallel, we used Illumina sequencing to generate a draft \( \text{P. vulgaris} \) genome sequence. The full assembly and annotation of the genome will form the basis of a subsequent publication. We screened this draft genome assembly with \textit{A. thaliana} \text{KNOX} \) protein sequences using Exonerate c2.2.0 (Slater \& Birney, 2005) and identified nine genomic contig assemblies with \textit{KNOX} \) gene homology. Within these contigs we defined gene models using the RNA-Seq dataset with \textit{Tophat} v2.0.8 (Trapnell \textit{et al.}, 2012) and Cufflinks v2.1.1 (Trapnell \textit{et al.}, 2013). Seven of the genomic contigs were predicted to contain full-length \( \text{PvKNOX} \) gene models. Of the two remaining contigs, one contained three exons representing the 5’-end of a \( \text{PvKNOX} \) gene, the other contained two exons corresponding to the 3’-homeodomain region. It was not initially clear whether these models represented two partial loci or one locus split between two contigs due to an incomplete genome assembly. Both partial models were supported by RNA-Seq data. We therefore screened a \textit{de novo} \text{Trinity} \) (Grabherr \textit{et al.}, 2011) transcript assembly generated from RNA-Seq data of the \( \text{P. vulgaris} \) pin and thrum mixed stage flower bud RNA samples, and identified a single \text{Trinity} \) transcript assembly derived from a single locus (\( \text{PvKKNLI} \)) bridging the unjoined genomic contigs. This finding resolved that the \( \text{P. vulgaris} \) genome encodes eight \( \text{PvKNOX} \) genes; the predicted gene structures are shown in Fig. 6(a). Figure S1 presents the predicted amino acid sequence from each gene; a Clustal Omega sequence alignment of the eight proteins with conserved protein domains indicated is shown in Fig. S2.

Figure 6(b) shows a phylogenetic analysis of the eight predicted \( \text{PvKNOX} \) proteins (Fig. S1) in comparison to the \textit{A. thaliana} \text{KNOX} \) protein family, comprising seven KNAT proteins (Lincoln \textit{et al.}, 1994; Serikawa \textit{et al.}, 1996; Belles-Boix \textit{et al.}, 2006; Li \textit{et al.}, 2011) and STM1 (Long \textit{et al.}, 1996), together with \textit{KNOTTED-1} \) from \textit{Zea mays} (Vollbrecht \textit{et al.}, 1991). Following this analysis we named the \( \text{PvKNOX} \) genes and their encoded proteins \textit{KNOTTED-like} (\( \text{PvKNL} \)) and \textit{ShOOTmeristem-like} (\( \text{PvSTL} \)) based on encoded protein sequence similarity. \textit{Primula vulgaris} does not have a homologue of \textit{AtKNOT5}, but contains two \textit{STM-like} genes; it therefore has five Class I and three Class II \( \text{PvKNOX} \) genes.

Expression analysis and sequence comparison of \( \text{PvKNOX} \) genes in wild-type and \text{Oakleaf} \)

Identification of the full complement of Class I and Class II \( \text{PvKNOX} \) genes enabled us to compare the expression of each gene in leaves and flowers of wild-type and \text{Oakleaf} \) to determine whether constitutive upregulation of a \( \text{PvKNOX} \) gene was associated with the \text{Oakleaf} \) phenotype. Based on previous studies of overexpression of Class I \textit{KNOT} \) genes in other species (Smith \textit{et al.}, 1992; Lincoln \textit{et al.}, 1994; Chuck \textit{et al.}, 1996; Hareven \textit{et al.}, 1996; Bharathan \textit{et al.}, 2002; Hay \& Tsiantis, 2006; Shani \textit{et al.}, 2009) we explored whether the \text{Oakleaf} \) phenotype also resulted from constitutive upregulation of a \( \text{PvKNOX} \)-like gene. Our gene expression analyses, described earlier, used \textit{HTSeq} to create a data file of RNA-Seq reads aligned to each locus. We then used \textit{DESeq} to compare RNA-Seq read counts for each locus in \textit{Oakleaf} \) leaves, \textit{Oakleaf} \) flowers, wild-type leaves and wild-type flowers (Anders \& Huber, 2010). Graphical representation of the data is shown in Fig. 7. Normalised read counts for each gene in each tissue, and the \text{log} \text{2} \) fold-change between \textit{Oakleaf} \) and wild-type leaves, and \textit{Oakleaf} \) and wild-type flowers, are shown in Table S2.

The five Class I \( \text{PvKNOX} \) genes are expressed at very low levels in leaves of both wild-type and \textit{Oakleaf} \) plants (Fig. 7a). Only \( \text{PvKNL2} \) and \( \text{PvKNL6} \) produce measurable read counts from leaves (Table S2). Higher expression levels are observed for the Class II \( \text{PvKNOX} \) genes in wild-type and \textit{Oakleaf} \) flowers (Fig. 7b). When relative expression levels are compared between \textit{Oakleaf} \) and wild-type, all Class I \( \text{PvKNOX} \) genes show higher expression levels in \textit{Oakleaf} \) flowers than wild-type; only \( \text{PvKNL6} \) shows higher read counts in \textit{Oakleaf} \) leaves (Fig. 7c; Table S2), but the normalised read counts of only 15 and 7 reads, respectively, are only just above background. None of the Class I \( \text{PvKNOX} \) genes are strongly upregulated in \textit{Oakleaf} \) leaves (Fig. 7c; Table S2).

In contrast to the Class I \( \text{PvKNOX} \) genes, the three Class II \( \text{PvKNOX} \) genes – \( \text{PvKNL3}, \text{PvKNL4} \) and \( \text{PvKNL7} \) – show strong expression in both leaves and flowers of \textit{Oakleaf} \) and wild-type plants (Fig. 7a,b). Only one gene, \( \text{PvKNL3} \), is upregulated in both leaves and flowers of \textit{Oakleaf} \) (Fig. 7; Table S2). \text{PvKNL3} \) expression in \textit{Oakleaf} \) and wild-type leaves is represented by normalised read counts of 1571 and 1591 reads, respectively. Normalised read counts for \textit{Oakleaf} \) and wild-type flowers are 1704 and 1424, respectively (Table S2). These values give \text{Log}_2 \) fold upregulation in \textit{Oakleaf} \) of 0.31 for leaves and 0.26 in flower (Fig. 7c; Table S2).

It is possible that a dominant phenotype could arise through a splicing mutation that results in a protein lacking a critical regulatory domain. We therefore compared RNA-Seq read
abundance profiles across all predicted exons of all *PvKNOX* loci and saw no difference between *Oakleaf* and wild-type that would indicate alternate splicing profiles. We did, however, identify 18 polymorphisms between seven *PvKNOX* genes in *Oakleaf* and the wild-type *PvKNOX* sequences from the genome assembly that would cause amino acid substitutions (Table S3). The *Oakleaf* plant used was heterozygous for the *Oakleaf* locus in a pin genetic background. We therefore then compared the *Oakleaf* single nucleotide polymorphisms (SNPs) with *PvKNOX* genes expressed in the flowers and leaves of a wild-type pin plant to determine whether the SNP was *Oakleaf*-specific. Three SNPs in *PVKNL2* and *PvSTL1* were predicted to result in truncated proteins (Table S3). For the seven remaining SNPs in *PvSTL1*, *PvKNL3*, *PvKNL4* and *PvKNL7*, the potential impact of amino acid substitution was analysed using the SIFT prediction tool (Ng & Henikoff, 2003). Five SNPs were predicted to result in tolerated amino acid substitutions (Table S3) and could therefore affect protein function (Ng & Henikoff, 2003).

Differential gene expression between *Oakleaf* and wild-type plants

KNOX proteins are transcriptional regulators and we would therefore anticipate wider changes in patterns of gene expression of both direct and indirect target genes in response to any aberrant expression of a *PvKNOX* gene in *Oakleaf*. It is also possible that *Oakleaf* is caused by mutation of an unrelated gene that results in a similar phenotype to that predicted from overexpression of a *PvKNOX* gene. Either possibility would result in transcript profile changes between *Oakleaf* and wild-type plants. We therefore used *Oakleaf* and wild-type flower and leaf RNA-Seq data to explore global transcriptome changes between *Oakleaf* and wild-type plants.

Assembly of the RNA-Seq datasets through alignment to the draft *P. vulgaris* genome identified a total of 39 193 transcript models and created a data file of all RNA-Seq reads aligned to each of the corresponding loci. HTSeq and DESeq (Anders & Huber, 2010; Anders et al., 2014) were then used to generate normalised counts of RNA-Seq reads corresponding to each locus for each of the four RNA-Seq samples from leaves and flowers of the wild-type and *Oakleaf* plants. Analysis using a log₂ fold-change
threshold > 2 identified 1313 genes upregulated in Oakleaf leaves and 2854 genes upregulated in Oakleaf flowers. Of these genes, 507 were common to both tissues. Parallel analyses using the same threshold identified 2099 genes downregulated in Oakleaf leaves and 1285 downregulated in Oakleaf flowers, of which 314 were represented in both tissues. These data are summarised in Fig. 8. None of the P. vulgaris KNOX genes are included in these samples as the fold-change in expression for these genes is below the two-fold cut-off used. Summaries of genes which are upregulated, or downregulated, in both leaves and flowers of Oakleaf, including BlastX analysis of nonredundant protein and Arabidopsis TAIR databases, as well as Gene Ontology assignments, are presented in Table S4 and S5, respectively.

**Fig. 7** Differential expression of the PvKNOX gene family in *Primula vulgaris* Oakleaf and wild-type plants. Expression of the eight genes represented by normalized Illumina RNA-Seq read count from (a) RNA isolated from *P. vulgaris* Oakleaf leaves (closed bars) and wild-type leaves (open bars); (b) RNA isolated from Oakleaf flowers (closed bars) and wild-type flowers (open bars). (c) the Log2 fold increase or decrease in expression levels between Oakleaf leaf and wild-type leaves (closed bars) and Oakleaf and wild-type flowers (open bars). The wild-type was a pin plant. Class I and Class II PvKNOX genes are indicated.

**Fig. 8** Identification of up- and downregulated genes in *Primula vulgaris* Oakleaf compared with wild-type. Venn diagrams showing: (a) numbers of genes upregulated in Oakleaf leaves (light grey) and Oakleaf flowers (mid-grey) compared with pin wild-type leaves and flowers. The numbers of genes upregulated in both organs (dark grey) are shown. (b) Numbers of genes downregulated in Oakleaf leaves (light grey) and Oakleaf flowers (mid grey) compared with pin wild-type leaves and flowers. The numbers of genes downregulated in both organs (dark grey) are shown.

**Discussion**

Records of mutant phenotypes in *Primula* date back over 400 yr (Gerard, 1597; van de Passe, 1614; Parkinson, 1629) and predominantly affect floral phenotype. More recently identified mutants in *P. sinensis* include flower and leaf phenotypes (De Winton & Haldane, 1933, 1935), some of which are linked to the S locus. Contemporary studies in *P. vulgaris* (Webster, 2005) include two phenotypes linked to the S locus, *Hose in Hose* (Ernst, 1942; Webster & Grant, 1990; Li et al., 2010) and *sepaloid* (Webster, 2005; Li et al., 2008); others such as *double* are not linked to the S locus (Webster, 2005). Oakleaf is the third S locus-linked developmental phenotype in *P. vulgaris*. Oakleaf was identified as a spontaneous mutation; it is dominant and affects both flower and leaf morphology. A *P. sinensis* mutation described in 1911, and designated *o* caused oakt-shaped leaves and affected flower morphology, but was recessive and not linked to the S locus (Gregory, 1911). The shape and character of the lobed leaves in Oakleaf are variable but their presence is characteristic of the mutation. The attenuated petal phenotype is also variable as seen in the F1 siblings from an Oakleaf x wild-type cross (Fig. 1d–f). This observation may reflect differences in expressivity of the mutant locus in different organs in response to genetic background.

The mutation sometimes increases separation and size of sepals, but does not cause lobed sepals. Crosses of Oakleaf to other floral mutants reveal the organ-specificity of Oakleaf action (Fig. 1). In combination with *Hose in Hose*, both first and second whorls of petals show attenuation characteristic of Oakleaf petals.
In combination with *Jack in the Green*, the leaves that replace sepals have the lobed appearance of *Oakleaf* leaves (Fig. 1k). These two examples, and that of *Oakleaf* combined with *Jackanapes* (Fig. 1l), reveal that *Oakleaf* action is organ- and not whorl-specific. *Oakleaf* does not always affect cotyledons but is consistently present in the primary leaves. The developmental profile of *Oakleaf* suggests either organ-specific expression of the dominant locus, or restricted expression, or action, of downstream network components.

Genetic analyses with *Oakleaf* as the female parent, where *Oakleaf* is either in repulsion (Fig. 3a) or coupling (Fig. 4b) to the S locus, pollinated from a wild-type pin, show that *Oakleaf* is inherited as a single dominant locus (Figs 1–3). However, in the reciprocal crosses, with *Oakleaf* as the male parent (Figs 3b, 4b) we observed progeny numbers that deviated from the anticipated 1:1 ratio. In both cases, the missing progeny were consistent with reduced transmission of the dominant thrum S allele. Such distorted segregation ratios were not observed in all crosses (Fig. 5): we are unaware of other examples where the pin : thrum ratio distorts from the anticipated equal transmission of dominant and recessive S alleles (Darwin, 1862; Bateson & Gregory, 1905). It is therefore unlikely that the distorted ratios are due to poor transmission of the dominant S allele.

The data presented in Fig. 5(b) show a significant deviation from the anticipated 1:1 ratio (P<0.001) of *Oakleaf* to wild-type progeny. The reason for this is unclear, but in this cross 45 seedlings were lost before secondary leaf development. Intriguingly, chi-squared analysis of progeny numbers, including the 45 lost seedlings as wild-type, support a 1:1 ratio (P>0.30). *Primula* seedlings are susceptible to ‘damping off’ due to bacterial or fungal infection before secondary leaves emerge. Leaves of *Oakleaf* plants are thicker and firmer than wild-type. In three of four crosses (Figs 3, 4), progeny losses before flowering were higher for wild-type than *Oakleaf*. We speculate that if the *Oakleaf* mutation gives greater resilience to seedling loss under unfavourable conditions, or in response to pathogen exposure, this could account for the ratio distortion. Indeed, previous studies of asymmetric leaves 1 (*as1*) mutants in *Arabidopsis*, *Antirrhinum* and tobacco showed enhanced resistance to necrotrophic fungi (Nurberg et al., 2007). AS1 is involved in repression of KNOX gene expression, and as1 mutants have similar phenotypes to KNAT1 overexpression lines (Hay et al., 2002). This hypothesis for seedling resilience in *Oakleaf* needs to be tested. The reason for underrepresentation of *Oakleaf* progeny in Cross 4 (Fig. 4b) is unclear, and could reflect a statistical consequence of the small progeny numbers.

Based on data obtained from backcrosses (Figs 3a, 4a), and the reciprocal crosses between heterozygous *Oakleaf* plants (Fig. 5) which produce the predicted 1:1 and 3:1 progeny ratios, respectively, we conclude that *Oakleaf* is caused by a single dominant locus. Linkage of *Oakleaf* to the S locus is demonstrated by predominant cosegregation of *Oakleaf* with pin or thrum phenotypes in specific crosses, together with small numbers of recombinants. These crosses suggest a range of potential map distances, but the cross with the largest number of progeny (Fig. 3b) gives a map distance of 3.3 cM. This map distance is possibly an underestimate as the total progeny numbers do not include the 92 plants lost as seedling or before flowering.

By analogy to *Hose in Hose*, where upregulated expression of a transcription factor is responsible for the phenotype (Li et al., 2010), and based on similarities to the phenotype of Class I *KNOX* homeodomain gene overexpression in *A. thaliana* (Lincoln et al., 1994; Chuck et al., 1996; Hay & Tsiantis, 2010), we considered two possibilities as the basis for *Oakleaf*: dominant upregulation of a *PvKNOX* homeodomain gene; mutation in a *PvKNOX* gene that confers a dominant gain of function on the encoded protein, such as a point mutation that introduces an amino acid change, or through a splice site mutation that yields a truncated protein with dominant function; and dominant mutation of a gene unrelated to the *PvKNOX* homeodomain gene family.

We used a combination of *de novo* genome assembly and RNA-Seq to identify the full complement of eight *PvKNOX* genes (Figs 6b, S1, S2). A fully assembled and annotated *P. vulgaris* genome will form the basis of a future publication. Phylogenetic analysis (Fig. 6b) shows that *P. vulgaris* has five Class I and three Class II *PvKNOX* genes (Kerstetter et al., 1994; Bharathan et al., 1999). Alignment of RNA-Seq datasets from *Oakleaf* and wild-type leaves and flowers enabled us to investigate expression of each gene in *Oakleaf* and wild-type leaves and flowers (Fig. 7; Table S2). We also explored whether any of the *PvKNOX* genes showed constitutive upregulation in mature leaves and flowers of *Oakleaf*. In line with previous observations on the localised expression of Class I *KNOX* genes in *A. thaliana* (Bharathan et al., 1999; Hay & Tsiantis, 2010), we observed low expression of Class I *PvKNOX* genes in wild-type *Primula* leaves (Fig. 7; Table S2); none is strongly upregulated in *Oakleaf* leaves. Only *PvKNL6* has higher sequence read counts in both *Oakleaf* leaves and flowers (Table S2) but expression in leaves was low with only 15 and 7 reads in *Oakleaf* and wild-type, respectively. None of the Class I *PvKNOX* genes show strong upregulation in both flowers and leaves of *Oakleaf*.

Analysis of Class II *PvKNOX* gene expression (Fig. 7; Table S2) shows comparable expression levels in leaf and flower tissue and this is consistent with observations of broader expression profiles for Class II *PvKNOX* genes compared with Class I genes (Serikawa et al., 1997; Bharathan et al., 1999; Truernit et al., 2006). In *A. thaliana*, Class II *KNOX* genes have distinct functions from the Class I genes; KNAT3, KNAT4 and KNAT5 are implicated in root development (Truernit et al., 2006) and KNAT7 in secondary cell wall formation (Li et al., 2011, 2012). Of the three Class II *PvKNOX* genes, only *PvKNL3* is potentially upregulated in both leaves and flowers of *Oakleaf*, however, because *A. thaliana* Class II *KNOX* genes do not have roles in apical meristem identity, we do not consider *PvKNL3* as a strong candidate for *Oakleaf*. None of the *PvKNOX* genes is strongly upregulated in *Oakleaf* leaves and we conclude that dominant constitutive overexpression of a *PvKNOX* gene is not a basis of the *Oakleaf* phenotype.

In order to establish whether mutation within a *PvKNOX* gene is responsible for *Oakleaf*, we analysed RNA-Seq read profiles against *PvKNOX* gene models. We speculated that a change in amino acid sequence or expression of a truncated polypeptide
might lead to a dominant gain-of-function. Analysis of Oakleaf RNA-Seq read profiles for the eight P. vulgaris KNOX genes did not reveal differential splicing between Oakleaf and wild-type that might cause expression of a variant protein. However, several SNPs were identified between Oakleaf and the corresponding wild-type genome sequence. Those SNPs that were homozygous in Oakleaf, that were also found in RNA-Seq data from wild-type pin flowers, or that were predicted to lead to conservative amino acid substitutions, were discounted as the possible basis for Oakleaf (Table S3). Three SNPs in PvKNL2 and PvSTL1, all heterozygous in Oakleaf, would cause truncation of the encoded polypeptide, and two further heterozygous SNPs in PvKNL3 and PvKNL7 cause nonconservative amino acid substitutions. Although these five SNPs might affect KNOX protein function, those in PvKNL2 and PvSTL1 were observed only in Oakleaf flower but not leaf transcripts, and those in PvKNL3 and PvKNL7 were only observed in Oakleaf leaf but not flower transcripts; for PvKNL7 there were no RNA-Seq reads over this SNP in flower. It seems unlikely given the absence of the SNP in both flower and leaf samples that these are responsible for the dominant Oakleaf phenotype. However, the availability of a P. vulgaris genome sequence, and availability of SNPs for each gene will enable future segregation analyses to determine whether any of the P. vulgaris KNOX genes are linked to the S locus.

Transcriptome analysis of Oakleaf and wild-type identified cohorts of genes that are differentially up- and downregulated. These studies provide not only candidates for genes controlled by Oakleaf, but also potential candidates for Oakleaf if it proves not to be a P. vulgaris KNOX gene. The 507 genes which are upregulated and 314 genes downregulated (Log2 fold cut-off > 2) (Tables S4, S5) represent a broad spectrum of predicted function and we can only speculate which genes are the likely players in the regulatory networks operating downstream of Oakleaf. It has been shown that networks operating downstream of Class I KNOX genes in Arabidopsis thaliana involve upregulation of GA20 oxidase and downregulation of GA20 oxidase, alongside upregulation of IPT7, which alter gibberellin and cytokinin concentrations, respectively; genes involved in lignin synthesis such as COMT1, CCaOMT and AtIP12 are also downregulated by Class I KNOX genes (Hay & Tsiantis, 2010). Analysis of the differentially expressed genes in Oakleaf (Tables S4, S5) does not reveal the P. vulgaris homologues for these A. thaliana genes. It is possible that Oakleaf is not caused by overexpression of a Class I P. vulgaris gene, but is instead a phenocopy caused by a different pathway, as in the case of Waxy auricle in blade 1, a dominant mutant phenotype (Hay & Hake, 2004).

Here we have identified Oakleaf as a new S locus-linked phenotype that has enabled us to develop a genetic map of the S locus (Li et al., 2015). We have explored three possible explanations for the Oakleaf phenotype based on analysis of the complete P. vulgaris genome family and have identified potential candidate genes for Oakleaf, as well as candidate Oakleaf-regulated genes using RNA-Seq analysis. Future studies, facilitated by a Primula genome assembly and SNP analysis of candidate genes, will reveal potential candidates for Oakleaf on the basis of their linkage to the S locus.

Acknowledgements

We are grateful to Dr Richard Brumpton for the original Oakleaf plants and thank Martin Lappage, Mike Hughes and Pam Wells for horticultural support, and colleagues at The Genome Analysis Centre (Norwich, UK) for genome sequencing. This work was supported by BBSRC grant BB/H019278/2. We thank the University of Leeds, Durham University and Gatsby Foundation for support during early stages of this work. We thank the University of East Anglia for support and the John Innes Centre for hosting P.M.G.’s laboratory under the UEA-JIC Norwich Research Park collaboration.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Predicted amino acid sequences of PvKNOX proteins.

Fig. S2 Multiple sequence alignment of PvKNOX proteins.

Table S1 RNA-Seq read data from six paired-end read libraries

Table S2 Differential expression of PvKNOX genes

Table S3 Analysis of single nucleotide polymorphisms in PvKNL genes

Table S4 Genes upregulated in Primula vulgaris Oakleaf as compared with wild-type

Table S5 Genes downregulated in Primula vulgaris Oakleaf as compared with wild-type

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