Comparative Genomics of Flowering Time Pathways Using *Brachypodium distachyon* as a Model for the Temperate Grasses

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Abstract

*Brachypodium distachyon* (Brachypodium) is a model for the temperate grasses which include important cereals such as barley, wheat and oats. Comparison of the Brachypodium genome (accession Bd21) with those of the model dicot *Arabidopsis thaliana* and the tropical cereal rice (*Oryza sativa*) provides an opportunity to compare and contrast genetic pathways controlling important traits. We analysed the homologies of genes controlling the induction of flowering using pathways curated in Arabidopsis Reactome as a starting point. Pathways include those detecting and responding to the environmental cues of day length (photoperiod) and extended periods of low temperature (vernalization). Variation in these responses has been selected during cereal domestication, providing an interesting comparison with the wild genome of Brachypodium. Brachypodium Bd21 has well conserved homologues of circadian clock, photoperiod pathway and autonomous pathway genes defined in Arabidopsis and homologues of vernalization pathway genes defined in cereals with the exception of VRN2 which was absent. Bd21 also lacked a member of the CO family (CO3). In both cases flanking genes were conserved showing that these genes are deleted in at least this accession. Segmental duplication explains the presence of two CO-like genes in temperate cereals, of which one (*Hd1*) is retained in rice, and explains many differences in gene family structure between grasses and Arabidopsis. The conserved fine structure of duplications shows that they largely evolved to their present structure before the divergence of the rice and Brachypodium. Of four flowering-time genes found in rice but absent in Arabidopsis, two were found in Bd21 (*Id1, OsMADS51*) and two were absent (*Ghd7, Ehd1*). Overall, results suggest that an ancient core photoperiod pathway promoting flowering via the induction of *FT* has been modified by the recruitment of additional lineage specific pathways that promote or repress *FT* expression.

Introduction

The switch from vegetative growth (the production of stems and leaves) to reproductive growth (the production of flowers) is an important developmental step in the life cycle of plants. Flowering needs to occur when conditions for pollination and seed development are optimal and consequently most plants restrict flowering to a specific time of year. They commonly achieve this by using reliable environmental cues such as day length (photoperiod) and temperature. In addition, nutrient and water availability and plant size can be important.

The genes and molecular mechanisms controlling flowering have been extensively studied in the model dicot *Arabidopsis thaliana*, subsequently Arabidopsis (reviewed by [1,2,3,4,5,6,7,8,9,10,11,12,13,14]). As part of this study the Arabidopsis flowering pathways were curated in Arabidopsis Reactome (http://www.arabidopsisreactome.org [15]) to provide an electronic knowledge resource allowing for further developments such as integration with protein-protein interaction datasets, overlaying with micro-array data and electronic projection into all newly sequenced plant genomes. Using this we compiled a list of genes and gene families with a known role in flowering time in Arabidopsis.

Flowering time has also been extensively studied in crop species (reviewed by [5,6,9,11,12,13,16,17,18,19]). Flowering time is important for adaptation to specific environments and the world's major crop species provide a particularly interesting opportunity for study because they are grown in areas outside the ecogeographical limits of their wild ancestors. In addition, they are adapted to different farming practices such as fall (autumn) sowing or spring sowing in temperate regions. Adaptation to different environments and practices has been achieved by manipulation of flowering time responses and this makes flowering pathways an excellent system for comparison between and within domestic and wild species.

Comparative studies between Arabidopsis and the tropical cereal rice (*Oryza sativa*) have shown that rice has homologues of many flowering-time genes and that aspects of the photoperiod and autonomous pathway are well conserved (reviewed by [6,9,11,12,13,16,17,19]). Experimental studies have also shown that a gene may retain a role in flowering but with important
changes of action. For example the CONSTANS (CO) gene of Arabidopsis promotes flowering in long days while the equivalent gene in rice (HiII [20]) promotes flowering in short days but represses flowering in long days [9]. In addition, novel flowering-time genes have been found in rice, showing that different plant lineages have evolved new flowering controls. Examples are the rice GhB7, OsID1, Eh1 and OsMADS51 genes that are discussed individually in the results section.

Vernalization pathways are significantly different between Arabidopsis and the grasses as the key flowering repressors (FLC in Arabidopsis and VRT2 in cereals) are not conserved (reviewed by [5,11,12,13,16,17,18,19]). Understanding the control of flowering time in a range of plant species therefore gives us insights into the ancestral control of flowering time and the evolution of alternative mechanisms in different plant lineages.

The wild grass Brachypodium distachyon (subsequently Brachypodium) has emerged as an important model for temperate species which include important grain crops such as wheat (Triticum monococcum, T. durum and T. aestivum), barley (Hordeum vulgare) and oats (Avena sativa) and forage grasses such as Lolium and Festuca species. The availability of a complete genome sequence for Brachypodium enables the evolutionary relationships of chromosomes to be revealed [21,22] and is a powerful method for identifying candidates for QTLs identified in individual crop species.

In this paper we used the complete genome sequences of Arabidopsis, rice and Brachypodium to find homologues of genes known to have a role in flowering time in Arabidopsis or other species. For our purposes the genes of interest were those known to affect flowering time measured as leaf number or days to bolting or flowering in Arabidopsis, or as days to panicle emergence, ear emergence or anthesis in cereals. Genes may have been identified from mutation screens or from studies of natural variation. Our aim was to analyse a range of genes involved in known pathways rather than to complete an exhaustive study of all possible flowering-time genes. From this basis phylogenetic analyses of gene families were used to investigate the evolutionary relationships of genes and the impact of segmental duplications on the number of genes in families. Segmental duplications are collinear regions containing paralogous genes that derive from likely whole genome duplication events that occurred in the ancestors of modern species (recently reviewed by [23]). Tandem duplications are instances in which paralogous genes reside side by side along a chromosome and these are likely to result from evolutionarily recent amplification events. The results give us an insight into the evolution of flowering-time genes at the monocot/dicot divide and the relationship between temperate cereals (long-day plants with a vernalization requirement) and tropical cereals (short-day plants with no vernalization requirement).

Results

The genes and pathways selected are shown in Figure 1 and are listed with names and genomic identifiers in Tables 1 and S1. For clarity, we prefix the gene name with two letters showing the species when more than one species is being discussed: At for Arabidopsis thaliana, Bd for Brachypodium distachyon, Os for Oryza sativa (rice), Hv for Hordeum vulgare (barley), Ta for Triticum aestivum (bread wheat); Tm for T. monococcum (einkorn wheat); Sb for Sorghum bicolor (sorghum) and Zm for Zea mays (maize). Following Arabidopsis nomenclature, we use italics when referring to genes and non-italic upercase to refer to proteins in the text.

Brachypodium homologues were identified by BLASTP and TBLASTN searches using Arabidopsis, rice, barley or cereal predicted proteins. The approaches and stringencies are described in full in the materials and methods section. Putative orthologues in Brachypodium were confirmed by reciprocal BLAST searches (Table S1). In cases where flowering-time genes and their close homologues belonged to large gene families, searches were performed using profile Hidden Markov Models (HMMs) [24]. Homologous proteins were aligned using the Probabilistic Alignment Kit (PRANK) [25] which recognises insertions and deletions as individual evolutionary events and takes into account the evolutionary distances between sequences. Phylogenetic trees for each family were estimated from these alignments using distance matrix methods.

It is well known that the Arabidopsis, rice and Brachypodium genomes contain segmental duplications as a result of whole genome duplication events in relatively recent ancestors [26]. The genes residing in these regions may exhibit some functional redundancy which is important to recognise in the present study. We used information on flowering-time genes from the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication) and the segmental duplications function of Rice annotation 6.1 (http://rice.plantbiology.msu.edu/). We also carried out additional TBLASTN searches to compare genomic sequences. Paralogues arising from segmental duplications are shown in the phylogenetic tree diagrams. Paralogues that are tandem duplications on individual chromosomes are shown separately on the phylogenetic trees because they are a result of more recent and localised duplication events and the genes are more likely to be functionally redundant.

As an additional check against rice or, where available, other cereals, flanking genes around the gene of interest were also identified and compared. This provided a genomic context that helped support the orthology of individual genes. Where genes were not found in Brachypodium, the presence of conserved flanking genes and intervening continuous sequence provided evidence that the gene was deleted.

1. Light signalling pathways

1.1 Photoreceptors. Light perception by the plant is primarily by the PHOTOTROPIN (PHOT) and CRYPTOCHROME (CRY) blue light receptors and the PHYTOCHROME (PHY) red/far red light receptors. PHOT genes do not have a known flowering role and were not analysed. Brachypodium CRY genes were similar to genes previously described in rice, with two CRY1 like genes and one CRY2 like gene (Table 1). The ZEITLUPE (ZTL) protein is also a blue light receptor (see section 1.2). Three phytochrome genes (PHYA, PHB and PHC) were found in Brachypodium, as in rice. Three additional PHYA loci are known in barley from RFLP mapping but these may be pseudogenes [27]. This is supported by the absence of the additional copies in Brachypodium. Comparison of flanking genes showed the PHYA gene in Brachypodium to be colinear to the rice PHYA gene and the likely functional copy on the barley and wheat group 4 chromosomes.

1.2 Circadian clock components. Light and temperature inputs entrain the circadian clock, allowing plants to coordinate metabolic pathways that need to be attuned to the day. The circadian clock also provides the time piece against which day length is measured, allowing flowering to be induced by specific inductive day length conditions (reviewed by [3,4,7,14]). In Arabidopsis, two genes encoding related mth transcription factors (CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTIL (LHY)) are expressed in the morning and PSEUDO-RESPONSE REGULATOR 1 (PRR1, also called TOC1) is expressed in the evening, forming a feedback loop. These
Figure 1. Genetic pathways controlling flowering in Arabidopsis, rice and temperate grasses. Arrows show promoting effects, T-bars show repressing effects. Environmental cues are shown as lower case letters in square boxes; v is extended cold (vernalization); ld is long days; sd is short days; c is continuous light (non-vernalizing) temperatures and p in non-photoperiodic conditions. Proteins are shown as rectangles and arrows indicate activation at all stages but we make this distinction only in cases where separate controls are known to exist for transcription and protein function or stability. # indicates inhibition of protein function. The pathways were compiled from the following reviews [1,2,3,4,5,6,7,8,9,11,12,13,14,16,17,18,19] plus individual articles referenced in the text. Table A. In Arabidopsis genes assigned to pathways are shown in colour (photoperiod pathway in green, vernalization pathway in blue, autonomous pathway in light blue). Flowering pathway integrators are shown in red. Triple headed arrows indicate activation by red or blue light. Box B. Rice genes identified as homologues of those in Arabidopsis are shown in black. Ambiguous homology is indicated with a question mark. Genes with published roles in photoperiod regulated flowering are shown in green and orthologues of pathway integrators in red. Box C. In the temperate grasses, box Brachypodium genes identified as homologues of those of Arabidopsis or rice are shown in black. The VRN2 and CO3 genes from barley and wheat are also included. Genes with published roles in photoperiod or vernalization regulated flowering in barley or wheat are shown in green or blue, respectively, and orthologues of pathway integrators in red. In B and C likely orthologues of Arabidopsis genes are shown in equivalent positions within the figure (e.g. Hdl is the rice orthologue of CO and Hd3a and RFT1 are rice orthologues of FT). Tables and S1 gives information on gene names and synonyms. Square brackets in box B show three maize genes with known flowering time effects. Notes: *1 TOC1 represses PRR9, PRR7 and PRRS [130]. *2 SUF4 interacts with FRI and FRL1. LD blocks SUF4 action to reduce FLC expression [110]. *3 Cerdan and Chory [131] identified PFT1 and proposed that it promotes flowering by a photoperiod independent pathway activating FT. The model here follows Wollenberg et al. [132] who propose an alternative in which PFT1 acts by repressing Pfr phytochrome signalling. *4 SWR1 is a protein complex including SWC6, ARP6 and PIE1. *5 CO expression from phloem-specific promoters complements the co mutation, but expression from mesistem-specific promoters does not. CO triggers flowering from the phloem by the cell-autonomous activation of (HD-ZIP) protein. The FWA protein interacts with FT protein and so ectopic expression of monococcum model here follows Wollenberg et al. [132] who propose an alternative in which phenotype of dominant replacement of histone H2A.Z with H2A in nucleosomes. Mutation of ARP6 (part of the SWR1 complex) blocks this process. The late-flowering effects. Notes: *1 TOC1 represses PRR9, PRR7 and PRRS [130]. *2 SUF4 interacts with FRI and FRL1. LD blocks SUF4 action to reduce FLC expression [110]. *3 Cerdan and Chory [131] identified PFT1 and proposed that it promotes flowering by a photoperiod independent pathway activating FT. The model here follows Wollenberg et al. [132] who propose an alternative in which PFT1 acts by repressing Pfr phytochrome signalling. *4 SWR1 is a protein complex including SWC6, ARP6 and PIE1. *5 CO expression from phloem-specific promoters complements the co mutation, but expression from mesistem-specific promoters does not. CO triggers flowering from the phloem by the cell-autonomous activation of (HD-ZIP) protein. The FWA protein interacts with FT protein and so ectopic expression of FWA delays flowering by impairing FT function [134]. FWA is not expressed during the vegetative phase in wild type plants and normally has no role in flowering so homologues are not considered in this paper. *7 TWIN SISTER OF FT (TSF) is closely related to FT, CO has a similar role in flowering and is repressed by SVP [135]; reviewed by [4,102]. *8 Activation of AP1 and LFY requires two paralogous homeobox genes (PRR9/WRI1 (PWY), aka BELLRINGER and REPLUSLESS) and POUND-FOOLISH (PFN)). Double null mutants express SOC1 and FUL but not AP1 or LFY. The double mutants can receive floral inductive signals but do not properly restruct the shoot apical meristem to make flowers [136,137]. *9 Gl promotion of Hd1/CO is shown directly but could act through the FKF1/CDF route as in Arabidopsis. *10 Mayfield et al. [80] found that 14-3-3 proteins physically interact with CO in Arabidopsis. *11 It is unclear if there is a direct connection between OsMADS99 and OsMADS14/15 as well as the connection via Edh1. *12 Ppd-H1 promotion of CO is shown directly but could act through the FKF1/CDF route as in Arabidopsis. *13 Downregulation of VR2 by vernalization and a role in repressing VRN1 is proposed by Kane et al. [107,108] but is not supported by Trevaskis et al. [109] so VR2 is shown in two positions. *14 Shitsukawa et al. [89] found that WSOC1 was expressed preferentially in leaves. Expression was not affected by photoperiod or vernalization but was upregulated in seedlings by a GA treatment. *15 X indicates an unknown target gene [97]. Key to figures of phylogenetic trees A PRANK alignment of protein sequences (shown in the supplementary datasets) was used to construct phylogenetic trees using the neighbor-joining algorithm (subsequently NJ trees) with bootstrap support from 1000 iterations (values of >50% shown). Gene identifiers show the species as follows: At for Arabidopsis thaliana, Bradi for Brachypodium distachyon, Os for Orzya sativa (rice), Hv for Hordeum vulgare (barley), Ta for Triticum aestivum (bread wheat), Tm for Triticum monococcum (einkorn wheat), Sb for Sorghum bicolor (sorghum) and Zm for Zea mays (maize). barley and wheat proteins are highly homologous and usually only one is shown. Gene names follow the identifiers where relevant to the text and genes with known flowering time functions are shown in colour following Figure 1. In several instances we identified pairs of genes in grasses (paralogues) in segmental duplications (SegDup) which usually result from a grass whole genome duplication that predate the divergence of the rice, maize, sorghum, Brachypodium, barley and wheat lineages. These duplication events are shown as solid circles. Similarly, solid diamonds show duplication events in Arabidopsis; these are independent from the duplication event in the grasses. Boxed T shows tandem or closely arranged paralogues resulting from duplications within individual species. Where the protein belongs to a large family we show small clades containing genes of interest selected from analyses of whole families. The clades were identified either by using an online interrogatory tree of the transcription factor family using known flowering-time genes as query sequences [98] or from the family trees shown in the supplementary material. Key to figures of segmental duplications in these figures the size and spacing of genes in the respective genomic sequences is shown to the physical scale indicated in each figure. The components of each half of the duplication are grouped under the bars at the top of the figure. For example, Figure 3 shows part of rice chromosome 3 which is colinear with part of Brachypodium chromosome 1 and is related to part of rice chromosome 7 by segmental duplication. This section of rice 7 is colinear with a separate section of Brachypodium 1. Identifiers and names of flowering-time genes and their homologues are shown, with colours following Figure 1. Gene identifiers are also shown at the top and bottom of each segment to allow orientation to the respective genome browsers. The segmental duplications are recognized by conserved genes (black boxes connected by black lines). Some figures have grey boxes which show genes that are not conserved across the duplication in all species. Within these regions, other genes are conserved between species but not across the duplication (red boxes connected by red lines). These are deletion/insertion events that occurred after the duplication arose but before the divergence of the respective species. Genes in yellow are not conserved across species and represent candidates for deletion/insertion events that occurred after species divergence. Hypothetical genes and sequences annotated as transposons are excluded. In most cases the segmental duplications extend further than shown as we only use those portions that are relevant to the relationships of the flowering-time genes discussed in the text. doi:10.1371/journal.pone.0010065.s001

three genes form the central oscillator of the circadian clock (Figure 1). The Arabidopsis CCA1 and LHY genes have a single counterpart in rice (OsCCA1 [29]) and Brachypodium (called BdCCA1 here). A single homologue of PRR1/TOC1 is present in Brachypodium, rice and barley. The PRR1-like genes could clearly be recognized as an outgroup of the PRR gene family. All PRR homologues had a conserved 6 exon structure and were the only PRR genes lacking an intron in the CCT1 domain.

In rice, the Heading date6 (Hd6) gene was identified as the α subunit of CASEIN KINASE 2 (CK2) which is thought to have a role in CCA1 protein regulation by phosphorylation [29]. The Brachypodium orthologue of Hd6 was identified as Bradi1g07810. Paralogous genes exist in rice and Brachypodium as a result of segmental duplication (Figure 2A).

CCA1 HIKING EXPEDITION (CHE) interacts with TOC1 to repress CCA1 [30]. CHE is a TCP family protein. There are 11 high homology matches in Brachypodium to this protein but the amino acid sequence outside the TCP domain was conserved sufficiently between CHE and the Bradi3g60350 protein to suggest that the latter protein was a Brachypodium orthologue (Figure S1).

In Arabidopsis, the circadian clock has additional feedback loops. A morning loop contains members of the PRR family and an
Table 1. Flowering-time genes in Arabidopsis and rice and their Brachypodium homologues.

| Gene | Arabidopsis | Rice | Brachypodium | Cereal | At vs Os | At vs Bd | Os vs Bd | Bd vs cereal | Tree |
|-----------------|--------------|------|---------------|--------|----------|----------|----------|--------------|------|
| PHYA            | At1g09570    | Os03g51030 | Bradi1g10520  | HvPHYA | 63       | 63       | 88       | 88           |      |
| PHYB            | At2g18790    | Os03g19590 | Bradi1g64360  |        | 71       | 70       | 93       |              |      |
| PHYC            | At5g35840    | Os03g54084 | Bradi1g98400  |        | 59       | 59       | 88       |              |      |
| CRY1            | At4g08920    | Os02g36380 | Bradi3g46590  |        | 64       | 63       | 85       |              |      |
| CRY2            | At1g04400    | Os02g14550 | Bradi3g9420   |        | 52       | 56       | 80       |              |      |
| CKAI            | At5g67380    | Os03g55389 | Bradi1g07810  |        | 93       | 80       | 95       |              | Fig. 2A|
| ZTL             | At5g57360    | Os06g47890 | Bradi1g33610  |        | 78       | 76       | 92       |              | Fig. 2B|
| ELF3            | At2g25930    | Os01g38530 | Bradi2g14290  | TaELF3 | 25       | 27       | 56       | 78           | Fig. 2C|
| ELF4            | At2g40080    | Os11g40610 | Bradi4g12330  |        | 33       | 37       | 81       |              | Fig. 2D|
| GI              | At1g22770    | Os01g08700 | Bradi2g05230  | HvGl   | 68       | 66       | 90       | 93           |      |
| LHY             | At1g01060    | Os08g06110 | Bradi3g16515  |        | 40       | 38       | 73       |              |      |
| CHE             | At5g08330    | Os03g60350 | Bradi3g60350  |        | 50       |          |          |              | Fig. S1|
| TOC1            | At5g61380    | Os02g40510 | Bradi3g48880  |        | 41       | 39       | 79       |              | Fig. 3A|
| PRR3            | At2g60100    | Os07g49460 | Bradi1g16490  | HvPpd-H1| 36       | 37       | 68       | 71           | Fig. 3A|
| COP1            | At2g12950    | Os02g53140 | Bradi3g57670  |        | 69       | 68       | 88       |              |      |
| CDF1            | At5g62430    | Os03g07360 | Bradi1g73710  | HvCDF  | 40       | 40       | 75       | 82           | Fig. 4|
| CO              | At5g15840    | Os06g16370 | Bradi1g3670   | HvCO1  | 43       | 36       | 70       | 73           | Fig. 5A|
| SPA             | At2g46340    | Os01g52640 | Bradi2g48660  |        | 31       | 41       | 65       |              |      |
| TOE1            | At2g28550    | Os05g03040 | Bradi2g37800  | ZmRAP2.7| 45       | 48       | 69       | 65           | Fig. 6A|
| TEM1            | At1g25560    | Os01g49830 | Bradi2g47220  |        | 49       | 51       | 73       |              | Fig. 7A|
| Ehd1            | Os10g32600   | Os10g28330 | Bradi3g26910  | ZmID1  | 57       | 56       | 56       |              | Fig. 7C|
| CB1             | At4g34530    | Os10g40150 | Bradi3g60350  |        |          |          |          |              |      |
| FT              | At1g65480    | Os06g06320 | Bradi1g4830   | HvFT1  | 70       | 69       | 86       | 95           | Fig. 8A|
| HAP3A           | At2g38880    | Os05g38820 | Bradi2g2940   |        | 73       | 73       | 89       |              | Fig. 8B|
| HAP5A           | At3g48590    | Os03g14669 | Bradi1g67980  |        | 57       | 55       | 81       |              | Fig. 8C|
| GF14            | At5g16050    | Os08g33370 | Bradi3g36480  |        | 83       | 82       | 94       |              | Fig. 9A|
| SOC1            | At2g45660    | Os03g03070 | Bradi1g77020  | TaSOC1 | 54       | 50       | 61       | 62           | Fig. 9B|
| MADS51          | Os01g69850   | Bradi2g5190 | TaAGL41      |        | 66       | 85       |          |              | Fig. 9B|
| LFL1            | Os01g51610   | Bradi2g48060 |          |        | 62       |          |          |              |      |
| LFY             | At5g61850    | Os04g51000 | Bradi5g20340  |        | 51       | 51       | 80       |              |      |
| AP1             | At1g69120    | Os03g54160 | Bradi1g08340  | TaVRN1 | 52       | 51       | 86       | 86           | Fig. 10A|
| Ghd7            | Os07g15570   | Os01g59760 | Bradi2g1820  | TaFDF2 | 21       | 17       | 62       | 84           | Fig. 12|
| SPL             | At2g33810    | Os01g59760 | Bradi2g1820  | TaFDF2 | 21       | 17       | 62       | 84           | Fig. 12|
| FLC             | At5g10140    | Os03g63440 | Bradi1g0520   |        | 22       | 19       | 47       |              | Fig. 13C|
| SVP             | At2g22540    | Os03g08754 | Bradi1g72150  | HvBM1  | 51       | 52       | 72       | 89           | Fig. 13B|
| FRI             | At4g06550    | Os03g63440 | Bradi1g0520   |        | 22       | 19       | 47       |              | Fig. 13C|
| SUF4            | At1g30970    | Os09g38790 | Bradi4g38000  |        | 51       | 57       | 76       |              |      |
| FCA             | At4g16280    | Os09g03610 | Bradi4g08730  | HvFCA  | 35       | 35       | 69       | 69           |      |
| FY              | At5g13480    | Os01g72220 | Bradi2g0820   |        | 56       | 57       | 89       |              |      |
| FLD             | At3g10390    | Os04g47270 | Bradi5g18210  |        | 69       | 69       | 89       |              |      |
| FVE             | At2g19520    | Os01g51300 | Bradi2g47940  |        | 75       | 76       | 90       |              |      |
| FIE1            | At3g02740    | Os08g04270 | Bradi3g14520  |        | 68       | 69       | 87       |              |      |
| MSI1            | At5g58230    | Os03g43890 | Bradi1g13930  |        | 85       | 86       | 94       |              |      |
| VRN5            | At3g24440    | Os12g34850 | Bradi4g05950  | TmVIL1 | 40       | 41       | 75       | 82           | Fig. 13D|
| ARP6            | At3g33520    | Os01g16414 | Bradi2g10130  |        | 64       | 63       | 86       |              |      |
evening loop contains EARLY FLOWERING 3 (ELF3), EARLY FLOWERING 4 (ELF4), LUX ARRHYTHMO (LUX) and GIGANTEA (GI) (Figure 1 [3,7,14,31,32]). CYCLING DOF FACTOR (CDF), CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), ZTL and LOV KELCH PROTEIN 2 (LKP2) proteins all interact with these feedback loops. ZTL, highly homologous LKP2 and closely related FLAVIN-BINDING, KELCH REPEAT, F BOX 1 (FKF1) gene encode ubiquitin-ligase proteins. The COP1 and ELF3 proteins interact and regulate GI by promoting the degradation of GI protein in the dark [33]. Interactions between GI and other proteins allow different functions to be performed. In the circadian clock, the ZTL gene is constitutively expressed but blue light enhanced interaction between clock controlled GI and ZTL is required to stabilize the latter. This provides circadian oscillation of ZTL that directs the degradation of TOC1 [34]. In the photoperiod pathway, blue light enhanced interaction of GI and FKF1 are involved in the induction of CONSTANS (CO) expression (see section 1.3). Arabidopsis ZTL, LKP2 and FKF1 also act to regulate CDF transcription factors (section 1.3; Figure 1 [35]).

GI is a highly conserved single copy gene in Arabidopsis, rice and Brachypodium (where it has a proven role in flowering time [36,37] and in barley (Table 1). Rice and Brachypodium have two paralogous ZTL-like genes with similar homologies to AtZTL and gene a Arabidopsis Rice Brachypodium Cereal

| Gene a | Arabidopsis | Rice | Brachypodium | Cereal | At vs Os b | At vs Bd b | Os vs Bd b | Bd vs cereal b | Tree c |
|--------|-------------|------|--------------|--------|------------|------------|------------|--------------|--------|
| ELF2   | At5g51230   | Os09g13630 | Bradi3g03110   |         | 50         | 47         | 80         |              |        |

a An expanded list of genes including complete details of gene names, synonyms and accession numbers is provided in Table S1. 
b ClustalW pairwise alignment scores comparing representative genes for gene families in Arabidopsis (At) rice (Os) Brachypodium (Bd) or a cereal: Barley (Hv), Wheat (Ta) or Maize (Zm). c Please refer to phylogenetic trees in figure listed.

d UniProt sequences used for the following loci: Os06g16370 (Q30DN4_ORYSI), Os03g03070/100 (MAD5Q_ORYSJ), At4g35900 (FD_ARATH), At4g00650 (Q52596_ARATH), doi:10.1371/journal.pone.0010065.t001

Figure 2. Phylogenetic relationships within gene families containing circadian clock associated proteins. A) NJ tree of Heading date6 (Hd6) and related CASEIN KINASE II proteins (whole protein alignment; Dataset S2). B) NJ tree of ZEITLUPE and related proteins (whole protein alignment; Dataset S3). C) NJ tree of EARLY FLOWERING 3 and related proteins (whole protein alignment; Dataset S4). D) NJ tree of EARLY FLOWERING 4 and related proteins (whole protein alignment; Dataset S5). doi:10.1371/journal.pone.0010065.g002
the closely related \textit{AtLKP2}. This suggests independent gene duplication in the monocot and dicot lineages after divergence. One additional gene in rice and Brachypodium was more similar to \textit{AtFKF1} (Figure 2B). \textit{Arabidopsis COP1} had a single orthologue in rice and Brachypodium (Table 1). \textit{Arabidopsis ELF3} and \textit{SIMILAR TO ELF3} (At3g21320) identified two rice genes both of which were most closely related to \textit{AtELF3} (Figure 2C). At least one rice gene has a role in flowering as an \textit{Os01g38530} loss of function mutation causes late flowering in short or long days [30].

Two genes were also found in sorghum, one of which was in the same sub-group as the two rice genes. One Brachypodium homologue was found, but flanking genes surrounding the \textit{OsELF3}-like genes did not identify the same Brachypodium location. This suggests that two genes were originally present in grasses, one of which was lost from the temperate grasses. The other gene was lost from rice and the remaining rice gene was subsequently duplicated.

Overall, the results suggest that \textit{GI} and its various interactors are well conserved but that independent duplications affect the \textit{\textit{ZTL/LKP2}} homologues giving the same numbers of genes in \textit{Arabidopsis} and grasses by different routes.

Two other \textit{Arabidopsis} genes (\textit{ELF4} and \textit{LUX}) that are involved in the regulation of \textit{CCA1} and \textit{LHY} were also investigated. A single orthologue of the \textit{Arabidopsis LUX} gene was identified in rice and Brachypodium (Table 1). Four rice and three Brachypodium genes were found with homology to \textit{Arabidopsis ELF4} gene, but homology was closest to related members of the \textit{Arabidopsis} gene family and no convincing orthologue of \textit{ELF4} was identified (Figure 2D).

The \textit{Pseudo-Response Regulator} (\textit{PRR}) family consists of five genes in \textit{Arabidopsis} (\textit{PRR1} (\textit{TOC1}), \textit{PRR3}, \textit{PRR5}, \textit{PRR7} and \textit{PRR9}) and five \textit{PRR} genes in rice (\textit{OsPRR1}, \textit{OsPRR37}, \textit{OsPRR73}, \textit{OsPRR59}, \textit{OsPRR95}) [28]. Five genes were found in Brachypodium and these were closely related to the respective rice genes (Figure 3A). The \textit{PRR1} (\textit{TOC1}) genes were clearly recognizable across all species but the relationships of the remaining grass and \textit{Arabidopsis} genes was less clear, as reflected in the rice nomenclature.

\textit{PRR37} and \textit{PRR73} are paralogues arising from a segmental duplication (Figure 3B). The gene content of the two segments is

\textbf{Figure 3. Phylogenetic relationship of PRR proteins.} A) NJ tree of PSEUDO-RESPONSE REGULATOR proteins based on alignment of the PSEUDO-RECEIVER (PF00072) and CCT (PF06203) domains; Dataset 56; Full length Barley sequences; Dataset S1). B) Structure of a segmental duplication originally defined in rice using RFLP markers [138] that contains the \textit{PRR37} (\textit{Ppd-H1}, \textit{Hd2}) and \textit{PRR73} genes.

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consistent with a model in which duplication occurred and experienced a period of gene loss so that the majority of the genes remaining were present in one copy. The derived chromosomes were largely stable and retained a well conserved gene order during the divergence of the major grass lineages. All the duplicated regions we examined were consistent with this model, but the example in Figure 3B was unusual in that the rice 7 region and its Brachypodium equivalent contained many fewer genes, suggesting preferential gene loss from one of the duplicated segments.

PRR37 and PRR73 are most similar to AtPRR7. PRR37 is also known as PHOTOPERIOD 1 (Ppd1) as mutations in this gene provide important flowering time variation in barley (Ppd-H1 [39]) and wheat (Ppd-A1, Ppd-B1, Ppd-D1 [40,41]). PRR37 in rice has been identified as the Heading date 2 (Hd2) locus [42]. The parologue (PRR73) is not known to provide flowering time variation in any of these species. This may reflect subfunctionalization, and the genes are known to have different expression profiles in rice [43]. AtPRR7 is thought to have roles in clock function and photoperiod response (Figure 1A), but the cereals genes may have partly separated these functions, allowing photoperiod response to be manipulated by mutation of the PRR37 gene without compromising clock function.

The two other PRR genes in grasses are more similar to AtPRR5 and AtPRR9 (Figure 3A). The relationship between these genes is not clear and a further ambiguity is that AtPRR5 has six exons and AtPRR9 has seven while the cereal PRR59 and PRR95 genes have the same eight exon structure as OsPRR37, O-PRR73 and AtPRR7. Therefore, while the PRR family has the same number of genes in Arabidopsis and grasses, they may have achieved this number by different routes and the genes in grasses do not relate to the Arabidopsis genes in a simple one to one manner.

1.3 Photoperiod pathway genes. In Arabidopsis the CONSTANS (CO) gene plays a key role in photoperiod response (reviewed by [9]), but other members of the family are also likely to be involved in flowering (e.g. COL5 [44]). CO is characterized by two B-boxes (a class of zinc-finger domain) near the amino-terminus and a CCT domain at the COOH-terminus. Its transcription is regulated by outputs from the circadian clock and peaks late in the day. CO transcription at the start of the day is directly repressed by binding of the Dof transcription factor CDF1 [45]. CDF1 transcription is repressed by PRR genes [46] and the CDF1 protein is removed from the CO gene by a blue light activated complex of GI and FKF1 proteins [47]. In this way GI acts as a positive regulator of CO expression. CO expression is also repressed by CDF2, CDF3 and CDF5 [35]. CDF2 is known to be regulated by FKF1 and the related ZTL and LKP2 genes [35]. The activity of the CO protein is also regulated. In short days the expression peak occurs in the dark where CO protein is rapidly degraded. PHYB and SUPPRESSOR OF PHYA-105 (SPA) proteins remove CO protein at the start of the day. If the expression peak coincides with light, which occurs in long days, the CO protein is stabilized by a mechanism involving PHYA and CRY proteins and can then activate FLOWERING LOCUS T (FT) (section 2.1 [48,49]).

Four Brachypodium and four rice genes were most closely related to AtCDF1, 2 and 3 but the grass and Arabidopsis genes formed separate subgroups (Figure 4) suggesting that the CDF family has increased in size after monocot/dicot divergence. Conserved domains were highly conserved between all these proteins while other regions were diverged. Therefore, the relationships between them were ambiguous and a role for individual CDF genes in the photoperiod pathway in grasses would need to be established by experimental approaches.
having a single B-box that is likely to have arisen from a two B-box ancestor by internal deletion [51]. No homologue of HzCO3 was found in Brachypodium. Genes flanking OsCO3 had clear orthologues on Brachypodium chromosome 4 which were separated by three additional genes with different rice locations, suggesting BcCO3 may have been lost by a complex rearrangement (Figure 3D). Other CO family genes were well conserved between Brachypodium, barley and rice. The CO4 and CO5 genes are paralogues arising from segmental duplication as are the CO6 and CO7 genes (Figure 5A).

In Arabidopsis, SUPPRESSOR OF PHYA-105 (SPA) proteins interact with the CO protein to reduce its stability, inhibiting flowering in short days [Figure 1]. Mutations in SPA1 cause early flowering under short-day (SD) but not long-day (LD) conditions and this is independent of PHYA. Mutations in SPA1, SPA3 and SPA4 in combination have an enhanced phenotype and flower at the same time in LD and SD [48]. The spa1 mutation is also reported to have an effect on the circadian clock, slightly shortening the circadian period of CCA1, TOC1 and SPA1 transcript accumulation under constant light conditions [54].

Rice and Brachypodium each had three genes with homology to AtSPA. One was the COPII orthologue which has a similar WD repeat domain to SPA in the C-terminal region of the protein and which also has a role in degrading CO protein [55]. The related Os05g09590 and Bradi2g15900 genes lacked a protein kinase domain found in AtSPA1,3 and 4 so that only one rice and one Brachypodium gene (Os01g52640 and Bradi2g48660; Table 1) were identified as likely orthologues of AtSPA.

In addition to activation by CO, FT is regulated by GI through an independent pathway in Arabidopsis where GI upregulates microRNA172 which acts as a repressor of the APETALA 2 (AP2) domain gene TARGET OF EAT1 (TOE1) which in turn encodes a repressor of FT [56]. TOE1 is a member of a gene family characterized by two AP2 domains. Other orthologues include APETALA 2 itself, involved in inflorescence development. The TOE1 subfamily members have a smaller first AP2 domain due to an internal deletion of 15 amino acids and all contain a miR172 target site. Genes of this subfamily are present in grasses (Figure 6A) and include the Q gene of wheat and the INDETERMINATE SPIKELETT 1 (IDS1) gene of maize, both affecting inflorescence structure [57]. However, TOE1 is unusual in having a deletion of 15 amino acids in the second AP2 domain. No gene with this deletion was found in Brachypodium or rice but the maize VEGETATIVE 1 (RAP2.7_Vgt1) gene, which affects flowering time [58], has the 15 amino-acid deletion (Figure 6A). It is unlikely that identical deletions would occur independently in Arabidopsis and maize so it is possible that this type of gene has been lost from the rice/Brachypodium lineage. Genes flanking Vgt1 in maize were found on rice chromosome 1 and Brachypodium chromosome 2 (Figure 6B). These regions contained a distantly related AP2 domain gene related to Arabidopsis BABYBOOM that is too dissimilar to Vgt1 to appear in the clade of AP2 domain proteins shown in Figure 6A.

TOE2 and SCHLAFMUTZE (SMZ) also have diverged second AP2 domains. SMZ is an additional factor repressing FT, and this repression requires the MADS-box transcription factor MADS AFFECTING FLOWERING 1 (MAF1) gene [59]. No homologue of SMZ was identified in rice or Brachypodium (Figure 6A) nor was a homologue of MAF1 found (see section 5).

In Arabidopsis, TEMPRANILLO 1 and 2 (TEM1 and TEM2) have been identified as additional repressors of FT [60]. TEM proteins have one AP2 domain and a B3 domain. TEM-like genes were found in rice and Brachypodium but no clear orthologues of TEM1 or TEM2 were found (Figure 7A). This suggests that TEM1 and TEM2, with two RAV genes, may have originated by gene duplication after the monocot/dicot divergence.

Early heading date (Ehd1) is a B-type response regulator identified in rice which has no counterpart in Arabidopsis [61]. Its role is to upregulate Hv3a (FT) in inductive conditions (short days) and it acts independently of Hd1/CO (Figure 1B). No orthologue was identified in Brachypodium, although flanking genes were well conserved (Figure 7B), or in temperate grass ESTs suggesting that this is likely to be an additional flowering component that evolved within the rice lineage or was deleted from the temperate grasses.

The rice MADS-box transcription factor OsMADS51 is regulated by OsGI and upregulates Ehd1 in short days, providing an alternative route for the upregulation of Hv3a (FT) in inductive conditions (Figure 1B). OsMADS51 has no clear Arabidopsis orthologue but in contrast to Ehd1 there were Brachypodium and wheat (AGL41) orthologues. The relationship of the OsMADS51 group to other MADS-box proteins is shown in Figures 7 and 9B. The lack of an Ehd1 gene in temperate grasses suggests either that the OsMADS51 orthologue has a different function or that there are additional targets linking it to flowering control.

INDETERMINATE 1 (ID1) was first identified in maize (Zea mays) where loss of function mutation causes extremely late flowering [63]. ID1 encodes a C2H2 zinc-finger transcription factor which has no clear Arabidopsis orthologue. In rice, loss of function mutations of the ID1 orthologue (RID1; Os10g28330) also called Early heading date2 (Ehd2) show extremely late flowering under short- or long-day conditions [64,65,66]. The expression of ID1 and RID1 is independent of photoperiod and is necessary for the expression of genes such as Ehd1 and GhD7 involved in regulating the switch from vegetative to floral development (Figure 1B). A phylogenetic tree was constructed for ID1-like proteins in rice and Brachypodium which form a distinct grass specific clade of the C2H2 proteins (Figure 7C). In Brachypodium Bradi3g260910 was identified as the closest equivalent by sequence and the presence of flanking genes. The BcID1 genomic sequence had a 1 bp insertion giving a frame shift mutation. This could be a feature of the Bd21 accession or a sequencing error.

In Arabidopsis, FT expression is also promoted independently of CO by CRYPTOCHROME-INTERACTING BASIC-Helix-LOOF-Helix 1 (CIB1) in a blue light dependent interaction with CRY2 [67]. Weak homologues to CIB1 were found in rice and Brachypodium in the bHLH domain but no convincing orthologue could be identified (Figure S4).

2. Flowering pathway integrators

2.1 The FLOWERING LOCUS T (FT) family. The induction of FT expression is a key step in flowering. Long-day
Figure 6. Phylogenetic relationship of TOE1 and related AP2 family genes a physical maps of the maize Vgt1 region. A) NJ tree of TARGET OF EAT1 and related proteins (whole protein alignment; dataset S9; clade selected from Figure S2 using outgroup root At1g16060). Sequence alignment shows a 15 amino acid deletion in TOE1 and Vgt1 (red bar) that was not found in any Brachypodium or rice gene. The position of the conserved miRNA172 target site is also shown. B) Position of Vgt1 on maize chromosome 8 and colinear regions of rice chromosome 1 and Brachypodium chromosome 2 showing the absence of Vgt1 homologues from rice and Brachypodium.

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and short-day plants differ in how FT expression is regulated but in both cases flowering is associated with increased FT expression, suggesting that FT is a conserved activator of flowering [9]. In Arabidopsis, FT protein is a mobile signal that moves from leaf to apex where it interacts with the FD protein to activate the APETALA 1 (AP1) gene and initiate the transition to reproductive growth [9,68,69]. FT and its close homologue TWIN SISTER OF FT (TSF) are members of a phosphatidylethanolamine binding domain (PEBP) gene family that is more extensive in grasses [70,71]. The clade of PEBP proteins most similar to FT and TSF is encoded by two subgroups of genes in the grasses (Figure 8A). The first comprised OsFTL1 and its orthologues BdFTL1 and HvFT2. The second comprised two rice genes (OsFTL2 and OsFTL3) that are arranged in tandem and are likely to be a recent duplication so that both can be regarded as orthologues of HvFT1 and BdFTL2. The rice numbering follows Chardon and Damerval [70] and we have also used this for orthologous Brachypodium genes. OsFTL2 (Hd3a) and OsFTL3 (RFT1) promote flowering [72,73,74]. HvFT1 and HvFT2 are also likely to be flowering promoters because they can act as such in rice [75] but HvFT1 was thought to be the most likely orthologue of FT because it is upregulated at the floral transition [71].

Generally, other rice and Brachypodium genes grouped as recognizable orthologues. However, rice OsFTL8 and 11 had no counterpart in Brachypodium. HvFT5 had no detectable expression in barley and may be a pseudogene [71]. It had no counterpart in Brachypodium. The HvFT3 gene, which is a candidate for the Ppd-H2 flowering-time locus [71,75] was present in Brachypodium. The OsFTL7 orthologue in Brachypodium was present as a tandem triplication (Figure 8A).

In Arabidopsis the TERMINAL FLOWER 1 (TFL1) gene is important in regulating meristem determinacy. Three TFL1-like genes were found in Brachypodium. Two had rice orthologues while the third was orthologous to two rice genes known to be part of a recent segmental duplication involving rice chromosomes 11 and 12 [76]. The rice MOTHER OF FT 1 (OsMFT1) gene has two counterparts in Brachypodium (Figure 8A). The Brachypodium FT/TFL family was therefore similar to barley and rice with differences attributable to the fate of genes in segmental duplications and to different tandem duplications.

Figure 7. Phylogenetic relationships of TEM, ID1 and Ehd1 proteins. A) NJ tree of TEMPRANILLO and related proteins (whole protein alignment; Dataset S10). The TEM subgroup was identified with an online interrogatory tree using known Arabidopsis flowering-time genes as query sequences [98]. B) Colinear segments of rice chromosome 10 and Brachypodium chromosome 3 showing the absence of Ehd1 from Brachypodium. C) NJ tree of INDETERMINATE1 proteins (using an alignment of the ID domain; Dataset S11). Two related IDD proteins are included to show that ID proteins are a distinct subgroup of the C2H2 zinc-finger transcription factor family (Figure S3).

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Figure 8. Phylogenetic relationship of FT and HAP family proteins. A) NJ tree of FLOWERING LOCUS T and related proteins (PEBP (PF01161) domain alignment; Dataset S12). B) NJ tree of HAP3 and related proteins (whole protein alignment; Dataset S13). C) NJ tree of HAP5 and related proteins (whole protein alignment; Dataset S14). In B and C only the HAP3 and HAP5 clades selected from Figure S5 are shown.

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2.2 The HEME ACTIVATOR PROTEIN (YEAST) HOMOLOG (HAP) family. The CO protein is thought to act as a transcription factor but has no direct DNA binding activity. Recently it has been shown to form a complex with proteins similar to yeast HAP proteins and it is this complex that activates FT expression in Arabidopsis [77]. HAP proteins are present as large families in cereals (Figure S5 [18]) and there were several possible counterparts for the HAP5 and HAP3 proteins involved in CO interaction in Arabidopsis (Figures 3B and 6C, respectively). This suggests diversification of the HAP family following monocot/dicot divergence and further clarification of the relationships will require experimental approaches. The HAP family is of interest in grasses because key photoperiod (Hd1, Ppd-H1) and vernalization (VRN2) proteins belong to the CCT family (sections 1.3 and 3.2) and are likely to act in complexes with HAP proteins. Variation in HAP proteins or complexes could therefore provide functional variation.

FT is also regulated at the protein level by interaction with 14-3-3 proteins (PF00244) that typically modulate the activity of phosphorylation-dependent protein–protein interactions [78]. In rice, the GF14c Os0g04305000 protein (Os0g033370) acts as an inhibitor of flowering by binding to the Hd3a protein and thereby preventing activation of its targets [79]. GF14c falls in a clade containing six closely related rice proteins (Figure 9A); putative orthologues are present in Brachypodium suggesting that this type of control also exists in the temperate grasses. In Arabidopsis, 14-3-3 proteins are also implicated in the regulation of CO activity [80].

2.3 SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1). In Arabidopsis, FT upregulates the MADS-box transcription factor SOC1 (AGL20) which is also controlled by the gibberellic acid pathway (not investigated in this paper) and repressed by FLOWERING LOCUS C (FLC) (Figure 1A). SOC1 is also controlled post-transcriptionally by EARLY FLOWERING 9 (ELF9) (Figure 1 [81]). SOC1-like genes have been reported from rice and wheat but although the wheat gene can partially complement an Arabidopsis soc1 mutation, its expression was not found to be regulated by photoperiod or vernalization [82]. However, two paralogous rice genes (OsMADS50 and 56) have been shown to be involved in flowering in long days by regulating Ehd1 via antagonistic regulation of LEC2 and FUSCA3 like 1 (LFIL1) (Figure 1 [83,84]). Brachypodium orthologues of OsMADS50 and 56 were found from the phylogenetic analysis but their relationship to Arabidopsis is complicated by the observation that the grass genes also resemble other Arabidopsis genes (AGL14, 19, 42, 71 and 72; Figure 9B).

2.4 LEAFY (LFY). RFL (Os0g510000), the rice homologue of Arabidopsis LFY, has been shown to have a dual role. RFL acts as a regulator of plant architecture through its effects on apical and auxillary meristems throughout the growth of the rice plant, but it appears to have a different expression profile compared to other grasses [85]. RFL affects flowering time and acts upstream of OsMADS50 (osSOC1) and RPT1 to promote flowering [85]. This contrasts with Arabidopsis where LFY functions downstream of SOC1 (Figure 1). The protein is well conserved and a Brachypodium homologue was found (Bradi5g203400) so differences in function may reflect changes in expression profile.

3. Vernalization pathways

In contrast to photoperiod, the control of flowering by exposure to extended periods of cold (vernalization) is generally thought to have evolved independently in Arabidopsis and grasses (reviewed by [1,12,16,17,18,19]). However, in both cases there is repression of FT expression (Figure 1) and there are common components of epigenetic regulation [86]. Three genes determining vernalization requirement (VERNALIZATION-1 (VRN-1), VRN-2 and VRN-3) have been identified in wheat and barley using mutations that distinguish “winter” types (requiring vernalization) from “spring” types (no vernalization requirement). (N.B. the cereal VRN genes are unrelated to the VRN genes of Arabidopsis).

3.1 VERNALIZATION-1 (VRN-1). The grass VRN-1 gene is related to the FRUITFUL (FUL)/APETALA1 (API) subgroup of the MADS-box transcription factors in Arabidopsis [87,88]. API is expressed in the shoot apex but the role of the grass VRN-1 is more complex as it is expressed in vegetative tissues (leaf) as well as in the apex and may have different roles in these tissues [88].
In contrast to the Arabidopsis major flowering repressor FLC (section 5), VRN-1 increases in expression during vernalization as plants move towards flowering competence [12,18,19,97]. In cereals, mutations in the promoter or first intron of VRN-1 create "spring" alleles that do not require vernalization, suggesting that they remove regulatory domains normally required for repressing gene expression [summarized in [16,18]]. In polyploid wheats, the presence of a single spring allele is sufficient to allow flowering without vernalization. Plants of diploid wheat (*T. monococcum*) which are homozygous for a deletion of VRN-1 cannot flower, suggesting that VRN-1 is essential for the transition from vegetative to reproductive growth [89], although it is not clear whether additional genes are also deleted. A VRN-1 homologue is present in Brachypodium (*BdVRN1* or *BdFUL1*) in a colinear position with respect to rice (*OsMADS14*) and wheat (*Vrn1*) genes (Figure 10B).

The FUL/API clade includes four genes in the grasses (Figure 10A). The *VRN-1/FUL1/OsMADS14* gene is a paralogue of the *FUL2/OsMADS15* gene arising from a segmental duplication [Figure 10B [88]]. Both genes have been identified as controllers of flowering in rice but only *VRN-1* is currently known to have a role in temperate grasses. There is likely to be some diverged function for the two genes as they have differences in expression pattern [88]. The third gene and fourth rice genes, *OsMADS18* and *OsMADS20* both had temperate grass orthologues.

### 3.2 VERNALIZATION-2 (VRN-2).

VRN-2 is a dominant repressor of flowering that behaves genetically in a similar way to the Arabidopsis FLC gene [90]. In contrast to FLC (encoding a MADS-box protein), VRN-2 encodes a zinc-finger and CCT domain protein also called ZCCT1 [90]. VRN-2 is a member of the group IV subfamily of CC-like CCT domain genes [51]. This subfamily is not found in Arabidopsis [51,90]. Cereals have two or three ZCCT genes arranged in tandem (Figure 11A) but experimental evidence suggest that ZCCT1 plays the important role in vernalization [90]. In contrast to FLC, VRN-2 is controlled by day length as well as temperature and is only expressed in long days [91,92]. The group IV subfamily of CCT genes also contains a long-day repressor in rice (*Ghd7*) [93] suggesting that long-day repression might be an ancestral feature and that regulation by cold was recruited to the cereal flowering pathway at a later date. Brachypodium Bd21 resembled rice in having no VRN-2 gene. Flanking genes from sequenced wheat BAC clones were present in collinear order suggesting that the gene is deleted (Figure 11A). This could mean that Brachypodium does not use vernalization as a flowering control in the same way as temperate cereals. However, Brachypodium accessions vary in flowering characteristics and Bd21 is a rapid flowering form with no requirement for vernalization [94]. This suggests that Bd21 resembles the *vrn-2* mutant of spring barley in having a complete deletion of the gene and that a VRN-2 orthologue will be present in Brachypodium accessions with a vernalization requirement. Genetic mapping and sequencing of additional accessions will show if this is the case.

Although no VRN-2 gene was found, Brachypodium did have two group IV CCT genes (Figure 11B); Bradi3g33340 is orthologous to *HeCO9*. A role for *CO9* in flowering has not been shown but is plausible as this gene is a paralogue of VRN-2 arising from segmental duplication (Figure 11A). The absence of VRN-2 in Bd21 and the segmental relationship to *HeCO9* has also been described by Cockram et al. [95]. The second Brachypodium gene (Bradi3g10010) was intermediate between *HeCO9* and VRN-2 in the phylogenetic tree (Figure 11B) and had a diverged first exon compared to the latter. Brachypodium had no *Ghd7* homologue although genes flanking *Ghd7* in rice were well conserved and in collinear order (Figure 11C).

### 3.3 VERNALIZATION-3 (VRN-3).

VRN-3 is the *FT1* gene (section 2.1). The spring mutation in wheat is a transposon insertion in the promoter but the basis in barley is less clear. However, in both cases it is likely that there is loss of a regulatory region that normally allows repression by the vernalization pathway [96].

### 4. Meristem identity genes

A key stage in flowering is the transition of the apical meristem from the vegetative phase (the production of leaves) to the reproductive phase (the production of flowers). In Arabidopsis the activation of *LFY*, *FUL* and *API* is important in this change (Figure 1). A protein complex containing FT and the basic leucine zipper (bZIP) domain protein FD activates *API* expression [68,69] and this is likely to be conserved in other species including temperate grasses [97]. The bZIP clade (Figure 12) was identified using an online interrogatory tree [98] with Arabidopsis FD as the query sequence. This clade has several gene duplication events, complicating the interpretation of relationships.

*Delayed flowering1* (DFL1) in maize encodes a bZIP protein that acts downstream of *ID1* and mediates floral inductive signals at the shoot apex [99]. DFL1 is closely related to Arabidopsis FD in the phylogenetic tree; putative orthologues of DFL1 are present in Brachypodium and rice (Figure 12).

Five FD-like proteins have been identified in wheat of which TaFDL2 has been identified as a functional homologue of Arabidopsis FD because it interacts with both TaFT and the promoter of the wheat meristem identity gene *VRN-1* and is expressed in vegetative and reproductive apices [97]. In contrast to DFL1, TaFDL2 is not among the temperate grass proteins closely related to FD (Figure 12). These results suggest temperate grass genes more closely related to FD and DFL1 would be of interest for study.

VRN-1 and its parologue *FUL2* (section 3.1) are likely to have roles in meristem identity [88]. In Arabidopsis, the *LFY*, *FUL* and *API* genes are activated by *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3)* (Figure 1) which is regulated by *FT* and microRNA156 [100]. This control might also apply to VRN-1 and/or FUL2 in grasses. However, SPL3 is a member of a large gene family in Arabidopsis and this was also the case in rice and Brachypodium, making identification of likely orthologues difficult (Figure S8).

### 5. The Arabidopsis vernalization and autonomous pathways

#### 5.1 FLOWERING LOCUC C (FLC).

In Arabidopsis, the major flowering repressor in the vernalization pathway is the MADS-box gene *FLC* which represses *FT* and *FD* (Figure 1A; reviewed by [5]). Arabidopsis also has five MAIDS AFFECTING FLOWERING (MAF) genes which are homologous to *FLC* (Figure 13A). Vernalization represses *MAF1* (*FLM*), *MAF2* and *MAF3* but induces *MAF4*. *MAF1* is a flowering repressor and *MAF2* functions in preventing short cold periods from inducing the vernalization response [1]. *FLC* and the MAFs formed a MADS-box subgroup lacking rice or Brachypodium members (Figure 13A), consistent with previous findings in the grasses [reviewed by [11,12,16,17,18,19]]. The *FLC/MAF* group of MADS-box genes is therefore likely to have evolved after the dicot/monocot divergence.

#### 5.2 AGAMOUS-LIKE 19 (AGL19).

In Arabidopsis, *AGL19* (related to *SOC1*) is regulated by vernalization (Figure 1A) but in contrast to *FLC* it is upregulated and acts as a floral promoter (reviewed by [1]). Rice and Brachypodium genes related to *SOC1*
Figure 10. Phylogenetic relationship of FUL/AP1/VRN1 type MADS-box proteins and physical maps of the FUL1 and FUL2 regions. A) NJ tree of VRN1 and related proteins (whole protein alignment; Dataset S17). The FUL/AP1 clade selected from Figure S7 is shown. B) Structure of the segmental duplication containing the FUL1 (VRN1; OsMADS14) and FUL2 (OsMADS15) genes of the grasses.

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Figure 11. Phylogenetic relationship of VRN2/ZCCT proteins and physical maps of the VRN2 region. A) Structure of the segmental duplication containing the ZCCT genes. B) NJ tree of ZCCT and related proteins (whole protein alignment; Dataset S18). C) Colinear regions of rice chromosome 7 and Brachypodium chromosome 1 showing the absence of a Ghd7 homologue from Brachypodium.
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and AGL19 were found and are described in section 2.3 (Figure 9B).

5.3 SHORT VEGETATIVE PHASE (SVP) and AGAMOUS-LIKE 24 (AGL24). In Arabidopsis, the MADS-box transcription factor SVP has a role in repressing FT, TSP and SOC1 (Figure 1 [102]) and is likely to do this by interaction with FLC [103]. SVP also has a role in ambient temperature regulation [104]. SVP protein level is affected in lhy/aal1 double mutants, suggesting that the clock also influences flowering by an additional route (not shown in Figure 1 [105]) which is also thought to involve ELF3 [106]. AGL24 is upregulated by vernalization and acts as a flowering promoter (reviewed by [1]).

SVP/AGL24 like genes were found in rice and Brachypodium (Figure 13B) and have also been described in barley and wheat. In view of the lack of an FLC homologue in grasses the potential for SVP/AGL24-like genes to be involved in vernalization in grasses needs to be considered. When VEGETATIVE TO REPRODUCTIVE TRANSITION 2 (TaVRT2) is a member of this group that has been reported to be down regulated by vernalization and to interact with VRN2 protein to repress FVE1 [107,108]. However, other researchers disagree. Trevaskis et al. [109] did not find VRT2 to be downregulated by vernalization and their studies of transgenic barley showed SVP-like genes to have roles in meristem development. VRT2 is therefore shown in two possible positions in Figure 1C.

5.4 FRIGIDA (FRI). FRI, FRI-like (FRL1, FRL2), FRIGIDA ESSENTIAL 1 (FES1) and SUPPRESSOR OF FRIGIDA4 (SUF4) act to establish FLC expression (Figure 1A [110]). Genes with the closest homology to FRI in rice and Brachypodium were Os03g63440 and Bradi1g01520, respectively (Figure 13C). However, the sequences are considerably diverged from the Arabidopsis protein and it is unclear whether they are functionally related. For example, Arabidopsis FRI has two predicted coiled-coil domains [111] but neither was found in the rice or Brachypodium proteins. The relationships of the FRI-like genes are therefore ambiguous and functional assays of the rice and Brachypodium genes are needed to assess their function.

5.5 Autonomous pathway genes. In Arabidopsis, FLC is downregulated by genes of the autonomous pathway (Figure 1A). In contrast to FLC and FRI, autonomous pathway genes are well conserved and are found in rice and Brachypodium (Table 1). FCA and FY interact and FCA transcript levels are controlled by alternative splicing which is conserved in grasses [112,113]. Genes of the autonomous pathway are not solely concerned with the regulation of FLC and have functions in RNA-mediated chromatin silencing that affect other developmental processes [114,115,116]. These are plausible functions in other species and may include roles in flowering. FCA has also been shown to affect the level of miRNA172, providing a link to the photoperiod pathway in Arabidopsis [56]. FCA, FY, FLOWERING LOCUS D (FLD), FPA, FVE and LUMINIDEPENDENS (LD) were represented by orthologous single copy genes in rice and Brachypodium while FLD had two orthologues in rice and in Brachypodium (Table S1).

5.6 Other genes repressing FLC. In Arabidopsis, FLC is repressed by vernalization in two phases, first by a repression of expression by cold and secondly by a stable maintenance of repression that persists after the plant returns to warm temperatures [5,114]. These processes overlap and several genes function in both. The initial downregulation is thought to involve VERNALIZATION INSENSITIVE 3 (VIN3) and VERNALIZATION 5 (VRN5) which are related PHD-FNIII-VID (PHD) domain genes. Two additional members of this family, VERNALIZATION5/VIN3-LIKE (VEL) and VEL2 are also found in Arabidopsis. Four PHD genes from rice and three from T. monococcum were described previously, with the T. monococcum genes shown to be upregulated by vernalization [117]. Five genes were found in Brachypodium, four corresponding to the known rice genes with the fifth being related to OsVIL2 (Figure 13D). As previously found [117], the grass genes are most closely related to VRN5 and could play roles in gene regulation through chromatin
changes. There were no genes highly homologous to VFN3, VEL1, VEL2 or VEL3.

Stable repression of FLC involves a protein complex containing VRN5, VFN3, VEL1 and polycrype repressive complex 2 (PRC2) components VERNALIZATION 2 (VRN2; polycrype group protein), SWINGER (SWN; SET domain protein), FERTILIZATION INDEPENDENT ENDOSPERM 1 (FIE1; polycrype group) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1; WD repeat domain proteins) [118,119]. Arabidopsis VERNALIZATION 1 (VRN1; a class VI REM type B3 domain protein [120]) was not found in rice or Brachypodium. Genes related to the PRC2 components were found in grasses and may have roles in gene regulation that involve changes to chromatin structure. This could include vernalization which, as in Arabidopsis, has an epigenetic “memory”. However, other flowering processes may also be regulated since FLC has also been shown to have PRC2 control [121].

No convincing homologue of Arabidopsis VRN2 was found in rice. Two genes of the polycrype type were found (Os09g13630 and Os04g08034) but they were more closely related to Arabidopsis EMBRYONIC FLOWER 2 (EMF2). One gene was found in Brachypodium (Figure 13F). This was also EMF2 like and was orthologous to Os09g13630. A second Brachypodium gene may be present on chromosome 4 but is not currently annotated.

Two SET domain genes SWINGER (SWN) and CURLY LEAF (CLF) have a role in vernalization in Arabidopsis; putative orthologs were found in both rice and Brachypodium (Table S1). Two WD repeat genes resembling AtFIE1 were found in rice, positioned closely on chromosome 8 and separated only by a putative actin gene. Four possible genes were found in Brachypodium of which Bradi3g14520 was the closest relative. These results suggest that grasses contain chromatin regulatory genes related to PRC2 complexes, as might be expected, but lack convincing orthologues of key FLC regulators such as VRN2.

6. Ambient temperature pathways

In addition to control by vernalization, flowering in Arabidopsis is strongly affected by changes in ambient temperature. For example, a change in temperature from 23°C to 27°C (thermal induction) in short days promotes flowering as effectively as changing from short to long days [122]. Thermal induction is dependent on FT and FLD but does not depend on CRY1, CRY2, CO, GI or SOC1, showing that FT is activated independently of the photoperiod pathway. Thermal induction is suppressed by FLC and by mutations of FCA, FVE and FLD that increase FLC expression [122].

Recent work in Arabidopsis shows that an important component of temperature response is the presence of histone H2A.Z in nucleosomes at higher temperatures (17°C vs. 27°C [123]). H2A.Z was depleted from the FT promoter at higher temperature, explaining the photoperiod independent induction of expression. ACTIN-RELATED PROTEIN 6 (ARP6; Table 1) is part of the SWR1 complex that incorporates H2A.Z in nucleosomes. The arp6 mutant cannot incorporate H2A.Z, constitutively behaves as if at a higher temperature and is early flowering, especially in short days, consistent with higher FT expression [123]. SWR1 is also required for FLC expression suggesting that nucleosome changes may affect multiple flowering-time genes. Cereals show a degree-day response and it would be of interest to quantify effect of temperature in relation to FT expression. Also, given the lack of an FLC equivalent and the day length dependent expression of the flowering repressor VRN2 (ZCCT; section 3.2) it would be interesting to compare cereal temperature responses in short and long days.

Discussion

We analyzed the genome of Brachypodium distachyon to identify homologues of genes likely to be involved in the control of flowering time. As well as using flowering-time genes, we used flanking genes from rice or other cereals, where available, to clarify the relationships of putative orthologues. The use of flanking genes or “genomic context” was especially useful for understanding cases where a gene was absent from Brachypodium. A prominent example of this was the absence of the VRN2 gene which acts as a flowering repressor in the cereal vernalization pathway.

The FT hub

The core photoperiod pathway (circadian clock-GI-CO-FT) defined in Arabidopsis is well conserved as are components of the autonomous pathway. However, there is good evidence that additional components and regulatory pathways have been added during the evolution of different lineages and a common feature of these, illustrated in Figure 1, is that they provide additional promoting or repressing controls of the floral pathway integrator FT. As FT is a key signalling molecule moving from the leaves to the apical meristem (reviewed by [9]) this suggests that adjustment of FT levels provides a convenient evolutionary solution when alteration of flowering time is needed. However, care must be taken in this interpretation because FT is studied in many experiments and other significant hubs might emerge as additional genes, and other species, are studied in more depth.

Segmental duplications

The number of genes in a family is affected by the segmental duplications that are well characterized in rice and Brachypodium [124,125]. The overall structure of the duplications we examined is well conserved, consistent with the idea that duplication occurred in an ancestral grass genome and assumed much of the modern structure before the divergence of the rice, Brachypodium and temperate grass lineages. Segmental duplication accounts for the presence of two grass genes resembling Arabidopsis PRR7 (Figure 3), the presence of two CO-like genes (CO1, CO2) compared to one in rice (Hd1) (Figure 5) and the presence of two FUL/AP1 like genes (VRN1 and FUL2, Figure 10). Segmental duplication also shows the relationship between the grass VRN2 (ZCCT) genes and CO9 (Figure 11). Tandem duplications explain other differences such as the presence of one FT like gene in Brachypodium, barley and wheat (FTI) compared to two in rice (Hd3a and RFT1).

A significant feature of the segmental duplications is that multiple alleles conferring natural variation in flowering time in
cereals are associated with only one of the genes in each pair. Variation in photoperiod response is provided by the PRR57 gene in barley (Ppd-H1) and wheat (Ppd-A1, B1 and D1, chromosomes 2A, 2B and 2D) but no such effect is known for the PRR73 gene. Variation in vernalization requirement is provided by the VRN1 gene in barley and diploid, tetraploid and hexaploid wheat but no such effect is known for the FUL2 gene. Variation in vernalization requirement is also provided by the ZCC7 gene in barley (Vrn-H2) and diploid and tetraploid wheat (Vrn-A2 and Vrn-B2) but no such effect is known for its counterpart C09. This suggests that the gene pairs are diverged in function and that only one member can be mutated to provide useful variation, at least in an agricultural context. It would be interesting to explore this in wild grasses and to assess the function of the second member of each pair experimentally. Brachypodium would provide an excellent vehicle for this.

Clear versus fuzzy homology
Some genes such as CO and GI had convincing orthologues in all species, but other genes were harder to interpret. For example, the relationship between monocot and dicot members of the PRR, CDF1, TOE1, TEM and HAP genes is complex and is likely to reflect different patterns of expansion and contraction in these families since the divergence of Arabidopsis, rice and Brachypodium. However, even within these families, rice and Brachypodium genes were generally associated in pairs in the phylogenetic trees, suggesting that it will be possible to integrate knowledge of gene function between grasses if not between grasses and Arabidopsis.

The evolution of novel pathways
There are clear examples of distinct genetic controls in different lineages. The vernalization pathways are a prominent example, with FLC encoding the main repressor of flowering in Arabidopsis and VRN2 in cereals. The absence of VRN2 from Brachypodium was an unexpected finding but this may reflect the choice of accession used for sequencing. Analysis of additional Brachypodium accessions for the presence of VRN2 would be of great interest.

Other examples are Vgt1 from maize and Ehd1 and Ghd7 from rice which were not found in Brachypodium, and MADS51 and ID1 which were found in rice and Brachypodium but not in Arabidopsis. These genes are members of families and there is therefore the risk that “fuzzy” homology may be missed. This problem would be exacerbated by incorrect gene prediction. We therefore checked these examples carefully, especially as homologues of positive and negative regulators of Ehd1 and Ghd7 were found. We reanalyzed relevant regions using reciprocal TBLASTN searches of nucleotide sequence but were unable to find homologous sequences and we conclude that these genes are genuinely absent. As with VRN2, it would be interesting to explore further accessions to see if these genes are consistently absent from particular species or if gain or loss reflects local adaptation.

Our analysis used known flowering-time genes from species other than Brachypodium. As Brachypodium transformation is now established [126] it will be possible to experimentally test conservation of gene function. Novel flowering-time genes may be identified in Brachypodium itself from mutation screens or from the analysis of different ecotypes. The latter also provide an excellent system to test if flowering time variation between wild ecotypes involves the same genes that have been selected in cereals during domestication.

Materials and Methods

Sources of sequences
The following datasets were used for profile Hidden Markov Models (HMMs) analysis and BLAST searches: Arabidopsis TAIR9 (http://www.arabidopsis.org, TAIR9_pep_20090619), Rice MSU Osa1 Release 6.1 (http://rice.plantbiology.msu.edu, all.pep – June 2009) and Brachypodium v1 (http://www. modelcrop.org, Brd1_v1_Protein1).fa – May 2009. Updated annotations for Brachypodium are available in Brachypodium v1.2. The representative gene model was used; for most genes this is the ‘1’ version. If the alternative gene model version (‘2’, ‘3’) is used this is indicated in the phylogenetic trees (supplement) or in Table S1.

Protein sequences for wheat, barley and maize flowering-time genes were obtained from UNIPROT (http://www.uniprot.org) or NCBI (http://www.ncbi.nlm.nih.gov) and Sorghum sequences were obtained from (http://genome.jgi-psf.org/Sorbi1).

Homologue identification in rice and Brachypodium
Starting with Arabidopsis flowering-time genes (Table 1), BLASTP searches were carried out against rice protein sequences with an E-value threshold of 1e-20. The top ranked rice hit was used for BLASTP searches back into the Arabidopsis protein to confirm homology (reciprocal BLAST searching). Starting with both Arabidopsis and rice homologues, BLASTP and TBLASTN searches were carried out against the Brachypodium v1 protein predictions and genomic sequence, respectively. Putative orthologues were confirmed by reciprocal BLASTP searches of the Arabidopsis and rice protein sequence datasets and are summarized in Table S1. Genes that have been shown experimentally to have a role in flowering time in grasses were also included in the BLAST analyses. A ClustalW alignment (http://www.ebi.ac.uk/ Tools/clustalw2) was performed for each gene set and the clustal score recorded as a measure of protein sequence identity (Table 1).

For flowering-time genes that belong to large gene families where duplication and gene loss complicated the identification of homologues the following methods were used: 1) profile HMMs searches to identify all members of each family or subfamily followed by phylogenetic analysis, 2) genomic location. Specific strategies for finding homologues are discussed in the result section for the individual gene families.

Database searches using profile HMMs
For the larger transcription factor (TF) families (AP2 (TOE), ERF-B3 (TEM), bZIP (FD), NF-YB-like (HAP), Dof zinc finger (CDF), C2H2 zinc finger (ID), MADS-box) TCP (CHE), bHLH (CIB1), genes for particular subgroups were identified from Arabidopsis, rice and Brachypodium protein sequences by searches with profile HMMs using the HMMER suite of programs [24]. The profile HMM for each family was made from an alignment of the conserved region (centred around the DNA binding domain), seeded by protein sequences that were already known to play a role in the flowering time response and other close homologues (see main text for references). For the MADS-box family, the profile HMM was made from an alignment of all members of this family present in Arabidopsis, excluding sequences that did not align correctly. The full alignment was used in the case of the ERF-B3, CDF and ID families.

For some TF families, there was a cut off in the list of hits from the HMMSearch program below which it was clear that sequences belonged to different subgroups of the gene family (ERF-B3, Dof). For other families (AP2, C2H2 zinc finger, bZIP, TCP and bHLH), the boundary of the relevant subgroup was
found by adding the sequence hits as queries to an online interrogatory tree for each family [98]. It was necessary to identify and perform phylogenetic analysis on all members of the MADS box family to identify the members of each subgroup of interest in the MIRK-type MADS-box proteins. In the case of the NF-YB-like (HAP) family, an E-value cut off of 1.0 was applied to the HMMSEARCH results.

The above approach was also adopted to search for all members of the 14-3-3, FRIGIDA-like and SPL gene families. In these cases, an existing profile HMM from the pFAM website was used, PF00244, PF07899 and PF03110 respectively.

All sequence matches selected from the HMMSEARCH output were aligned using PRANK [25], except for the HAP family whose matches were aligned back to the HMM using the HMMALIGN program. The resulting alignments were used for phylogenetic analysis.

**Phylogenetic Analysis**

The alignment for each gene family data set (see supplementary data) was used for distance-based phylogenetic analysis with the PHYLIP [127] or MEGA4.0 [128] software packages. With both packages, identical methods were used to calculate distance estimates (the Jones Taylor Thornton matrix [JTT] model of evolution) and to construct phylogenetic trees (Neighbor-Joining method). Each tree was rooted using the midpoint rooting method.

To provide statistical support for each node, a consensus tree was generated from 100 (supplement) or 1000 bootstrap data sets.

From the larger trees shown as supplementary data, genes from particular subclades were taken and a second smaller tree generated for the main manuscript to illustrate the relationship of genes of interest and to include additional closely related genes from barley, wheat, maize or sorghum where relevant. Where appropriate, a longer alignment was used for the phylogenetic analysis (see supplementary data).

**Protein domains**

Protein domains and secondary structure were analysed using PROSITE at the ExPASY facility (http://us.expasy.org/).

**Genome Duplication Data**

Gene duplication data was obtained from three sources, the Plant Genome Duplication Database (PGDD - http://chibba.agtec.uga.edu/duplication/) [26], the Rice Genome MSU release 6.1 segmental duplications (http://rice.plantbiology.msu.edu/segmental_dup) and duplicated blocks in Arabidopsis [129]. Segmental duplications events were indicated in the phylogenetic tree diagrams for Arabidopsis and the grasses (rice and Brachypodium).

**Supporting Information**

**Table S1** Complete list of Arabidopsis, Rice, Brachypodium and Cereal flowering time related proteins. Extended version of Table 1 showing details of accession numbers, reciprocal blast hits and EST evidence.

Found at: doi:10.1371/journal.pone.0010065.s001 (0.08 MB XLS)

**Figure S1** The relationship between Arabidopsis CHE and closely related TCP proteins. The region of the alignment used to estimate the tree spanned the TCP domain. Although there was no significant bootstrap value leading to the clade that contained CHE, there was sequence conservation outside the DNA binding domain for all six proteins in the clade. The sequence in the C-terminal end showed that the Bradi3g60350 protein was mostly closely related to CHE and At5g23280 relative to the other proteins in the clade. This indicates that Brachypodium contains an orthologue of CHE that rice has lost.

Found at: doi:10.1371/journal.pone.0010065.s002 (0.09 MB PPT)

**Figure S2** The relationship between Arabidopsis TOE1 and other closely related proteins in the AP2 family. An alignment of both repeats that comprised the AP2 domain was used to estimate the tree.

Found at: doi:10.1371/journal.pone.0010065.s003 (0.09 MB PPT)

**Figure S3** The relationship between the monocot ID1 proteins and other ID domain proteins. The alignment for estimating the tree contained only the region of the four tandemly arranged zinc finger domains, excluding a small number of columns containing non-homologous amino acids. A neighbouring subgroup within a full C2H2 zinc finger family tree is also shown at the base of the tree, illustrating that the main subgroup shown is distinct from other proteins of this family. The monocot ID1 proteins within this subgroup form another distinct, internal subgroup.

Found at: doi:10.1371/journal.pone.0010065.s004 (0.08 MB PPT)

**Figure S4** The relationship between Arabidopsis CIB1 and other proteins in subgroup 12 of the bHLH family. The region of the alignment used to estimate the tree spanned the bHLH domain and adjacent regions that were also conserved in proteins belonging to this subgroup. Neither bootstrap analysis nor studying regions outside this region provided evidence for an orthologue of Arabidopsis CIB1 in Brachypodium.

Found at: doi:10.1371/journal.pone.0010065.s005 (0.08 MB PPT)

**Figure S5** The relationship between members of the NF-YB-like (HAP) family. A conserved region of eighty four amino acids was identified in an alignment of these proteins and used to estimate the tree. For this family, the alignment was created by aligning the sequences to a profile HMM of the conserved region.

Found at: doi:10.1371/journal.pone.0010065.s006 (0.11 MB PPT)

**Figure S6** The relationship between the rice GF14c protein and the rest of the 14-3-3 protein family. The full alignment was used to estimate the tree, except that the alignment ends were trimmed.

Found at: doi:10.1371/journal.pone.0010065.s007 (0.07 MB PPT)

**Figure S7** The relationship between flowering time genes that belong to the MADS-box family. The alignment was created by aligning all the sequences corresponding to this family to a profile HMM of the MADS-box domain. Nineteen poorly aligned proteins were removed from the data set before estimating the tree.

Found at: doi:10.1371/journal.pone.0010065.s008 (0.17 MB PPT)

**Figure S8** The relationship between SPL proteins. The region of the alignment used to estimate the tree corresponded to the pFAM profile HMM (PF03110) but excluded columns containing non-homologous amino acids.

Found at: doi:10.1371/journal.pone.0010065.s009 (0.08 MB PPT)

**Figure S9** The relationship between the FRIGIDA protein and related proteins. The region of the alignment used to estimate the tree corresponded to the pFAM profile HMM (PF07899) but excluded columns containing non-homologous amino acids.

Found at: doi:10.1371/journal.pone.0010065.s010 (0.07 MB PPT)

**Dataset S1** Fasta formatted protein sequences for HvPRR genes (Figure 3).

Found at: doi:10.1371/journal.pone.0010065.s011 (0.00 MB TXT)
**Dataset S2**  Fasta formatted alignment of CKIIa proteins used for tree in Figure 2A.  
Found at: doi:10.1371/journal.pone.0010065.s012  (0.01 MB TXT)

**Dataset S3**  Fasta formatted alignment of ZTL proteins used for tree in Figure 2B.  
Found at: doi:10.1371/journal.pone.0010065.s013  (0.01 MB TXT)

**Dataset S4**  Fasta formatted alignment of ELF3 proteins used for tree in Figure 2C.  
Found at: doi:10.1371/journal.pone.0010065.s014  (0.01 MB TXT)

**Dataset S5**  Fasta formatted alignment of ELF4 proteins used for tree in Figure 2D.  
Found at: doi:10.1371/journal.pone.0010065.s015  (0.00 MB TXT)

**Dataset S6**  Fasta formatted alignment of PRR proteins used for tree in Figure 3A.  
Found at: doi:10.1371/journal.pone.0010065.s016  (0.00 MB TXT)

**Dataset S7**  Fasta formatted alignment of CDF proteins used for tree in Figure 4.  
Found at: doi:10.1371/journal.pone.0010065.s017  (0.04 MB TXT)

**Dataset S8**  Fasta formatted alignment of CO proteins used for tree in Figure 5A.  
Found at: doi:10.1371/journal.pone.0010065.s018  (0.03 MB TXT)

**Dataset S9**  Fasta formatted alignment of TOE proteins used for tree in Figure 6A.  
Found at: doi:10.1371/journal.pone.0010065.s019  (0.03 MB TXT)

**Dataset S10**  Fasta formatted alignment of TEM proteins used for tree in Figure 7A.  
Found at: doi:10.1371/journal.pone.0010065.s020  (0.01 MB TXT)

**Dataset S11**  Fasta formatted alignment of ID domain proteins used for tree in Figure 7C.  
Found at: doi:10.1371/journal.pone.0010065.s021  (0.00 MB TXT)

**Dataset S12**  Fasta formatted alignment of FT proteins used for tree in Figure 8A.  
Found at: doi:10.1371/journal.pone.0010065.s022  (0.01 MB TXT)

**Dataset S13**  Fasta formatted alignment of HAP3 proteins used for tree in Figure 8B.  
Found at: doi:10.1371/journal.pone.0010065.s023  (0.01 MB TXT)

**Dataset S14**  Fasta formatted alignment of HAP5 proteins used for tree in Figure 8C.  
Found at: doi:10.1371/journal.pone.0010065.s024  (0.01 MB TXT)

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**Author Contributions**

Conceived and designed the experiments: JAH PCB DAL. Performed the experiments: JAH PCB DAL. Analyzed the data: JAH PCB DAL. Contributed reagents/materials/analysis tools: JAH PCB DAL. Wrote the paper: JAH PCB DAL.
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