1   **The conserved *PFT1* tandem repeat is crucial for proper flowering in *Arabidopsis thaliana***

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1   **Running title:** *PFT1* STR is crucial for flowering.

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14   **SECTION: GENETICS OF COMPLEX TRAITS**

2

1   **ABSTRACT**

2   It is widely appreciated that short tandem repeat (STR) variation underlies substantial phenotypic

3   variation in organisms. Some propose that the high mutation rates of STRs in functional genomic

4   regions facilitate evolutionary adaptation. Despite their high mutation rate, some STRs show

5   little to no variation in populations. One such STR occurs in the *Arabidopsis thaliana* gene *PFT1*

6   (*MED25*), where it encodes an interrupted polyglutamine tract. Though the *PFT1* STR is large

7   (~270 bp), and thus expected to be extremely variable, it shows only minuscule variation across

8   *A. thaliana* strains. We hypothesized that the *PFT1* STR is under selective constraint, due to

9   previously undescribed roles in PFT1 function. We investigated this hypothesis using plants

10   expressing transgenic *PFT1* constructs with either an endogenous STR or with synthetic STRs of

11   varying length. Transgenic plants carrying the endogenous *PFT1* STR generally performed best

12   across adult PFT1-dependent traits. In stark contrast, transgenic plants carrying a *PFT1* transgene

13   lacking the STR entirely phenocopied a *pft1* loss-of-function mutant for flowering time

14   phenotypes, and were generally hypomorphic for other traits, establishing the functional

15   importance of this domain. Transgenic plants carrying various synthetic constructs occupied the

16   phenotypic space between wild-type and *pft1*-loss-of-function mutants. By varying *PFT1* STR

17   length, we discovered that *PFT1* can act as either an activator or repressor of flowering in a

18   photoperiod-dependent manner. We conclude that the *PFT1* STR is constrained to its

19   approximate wild-type length by its various functional requirements. Our study implies that there

20   is strong selection on STRs not only to generate allelic diversity, but also to maintain certain

21   lengths pursuant to optimal molecular function.

22

23   **INTRODUCTION**

1   Short tandem repeats (STRs, microsatellites) are ubiquitous and unstable genomic elements that

2   have extremely high mutation rates (Subramanian *et al.* 2003; Legendre *et al.* 2007; Eckert and

3   Hile 2009), leading to STR copy number variation within populations. STR variation in coding

4   and regulatory regions can have significant phenotypic consequences (Gemayel *et al.* 2010). For

5   example, several devastating human diseases, including Huntington's disease and

6   spinocerebellar ataxias, are caused by expanded STR alleles (Hannan 2010). However, STR

7   variation can also confer beneficial phenotypic variation and may facilitate adaptation to new

8   environments (Fondon *et al.* 2008; Gemayel *et al.* 2010). For example, in *Saccharomyces*

9   *cerevisiae* natural polyQ variation in the FLO1 protein underlies variation in flocculation, which

10 is important for stress resistance and biofilm formation in yeasts (Smukalla *et al.* 2008). Natural

11 STR variants of the *Arabidopsis thaliana* gene *ELF3*, which encode variable polyQ tracts, can

12 phenocopy *elf3* loss-of-function phenotypes in a common reference background (Undurraga *et*

13 *al.* 2012). Moreover, the phenotypic effects of *ELF3* STR variants differed dramatically between

14 the divergent backgrounds Col and Ws, consistent with the existence of background-specific

15 modifiers. Genetic incompatibilities involving variation in several other STRs have been

16 described in plants, flies, and fish (Peixoto *et al.* 1998; Scarpino *et al.* 2013; Rosas *et al.* 2014).

17 Taken together, these observations argue that STR variation underlies substantial phenotypic

18 variation, and may also underlie some genetic incompatibilities.

19   The *A. thaliana* gene *PHYTOCHROME AND FLOWERING TIME 1* (*PFT1, MEDIATOR*

20 *25, MED25*) contains an STR of unknown function. In contrast to the comparatively short and

21 pure *ELF3* STR, the *PFT1* STR encodes a long (~90 amino acids in PFT1, vs. 7-29 for ELF3),

22 periodically interrupted polyQ tract. The far greater length of the *PFT1* STR leads to the

23 prediction that its allelic variation should be greater than that of the highly variable *ELF3* STR

1   (Legendre *et al.* 2007, http://www.igs.cnrs-mrs.fr/TandemRepeat/Plant/index.php). However, in

2   a set of diverse *A. thaliana* strains, *PFT1* STR variation was negligible compared to that of the

3   *ELF3* STR (Supp. Table 1). Also, unlike ELF3, the PFT1 polyQ is conserved in plants as distant

4   as rice, though its purity decreases with increasing evolutionary distance from *A. thaliana*. A

5   glutamine-rich C-terminus is conserved even in metazoan MED25 (File S1). A recent survey of

6   coding STRs suggested that there may be different classes of STR. Specifically, STRs that are

7   conserved across large evolutionary distances appear in genes with substantially different

8   functions than those coding STRs that are not strongly conserved in species (Schaper *et al.*

9   2014). Consequently, *PFT1/MED25* polyQ conservation may functionally differentiate the *PFT1*

10   STR from the *ELF3* STR.

11   *PFT1* encodes a subunit of Mediator, a conserved multi-subunit complex that acts as a

12   molecular bridge between enhancer-bound transcriptional regulators and RNA polymerase II to

13   initiate transcription (Bäckström *et al.* 2007; Conaway and Conaway 2011). *PFT1/MED25* is

14   shared across multicellular organisms but absent in yeast. In *A. thaliana*, the PFT1 protein binds

15   to at least 19 different transcription factors (Elfving *et al.* 2011; Ou *et al.* 2011; Çevik *et al.*

16   2012; Chen *et al.* 2012) and has known roles in regulating a diverse set of processes such as

17   organ size determination (Xu and Li 2011), ROS signaling in roots (Sundaravelpandian *et al.*

18   2013), biotic and abiotic stress (Elfving *et al.* 2011; Kidd *et al.* 2009; Chen *et al.* 2012), PHYB-

19   mediated-light signaling, shade avoidance and flowering (Cerdán and Chory 2003; Wollenberg

20   *et al.* 2008; Iñigo, Alvarez, *et al.* 2012; Klose *et al.* 2012).

21   PFT1 was initially identified as a nuclear protein that negatively regulates the PHYB

22   pathway to promote flowering in response to specific light conditions (Cerdán and Chory 2003;

23   Wollenberg *et al.* 2008). Recently, Iñigo and colleagues (2012) showed that PFT1 activates

1   CONSTANS (CO) transcription and FLOWERING LOCUS T (FT) transcription in a CO-

2   independent manner. Specifically, proteasome-dependent degradation of PFT1 is required to

3   activate FT transcription and to promote flowering (Iñigo, Giraldez, *et al.* 2012). The wide range

4   of PFT1-dependent phenotypes is unsurprising given its function in transcription initiation, yet it

5   remains poorly understood how PFT1 integrates these many signaling pathways.

6   Given the conservation of the PFT1 polyQ tract and the known propensity of polyQ tracts

7   for protein-protein and protein-DNA interactions, we hypothesized that this polyQ tract plays a

8   role in the integration of multiple signaling pathways and is hence functionally constrained in

9   length. We tested this hypothesis by generating transgenic lines expressing *PFT1* with STRs of

10 variable length and evaluating these lines for several PFT1-dependent developmental

11 phenotypes. We show that the *PFT1* STR is crucial for *PFT1* function, and that PFT1-dependent

12 phenotypes vary significantly with the length of the *PFT1* STR. Specifically, the endogenous

13 STR allele performed best for complementing the flowering and shade avoidance defects of the

14 *pft1-2* null mutant, though not for early seedling phenotypes. Our data indicate that most assayed

15 *PFT1*-dependent phenotypes require a permissive *PFT1* STR length. Taken together, our results

16 suggest that the natural *PFT1* STR length is constrained by the requirement of integrating

17 multiple signaling pathways to determine diverse adult phenotypes.

18

19 **RESULTS**

20 We used amplification fragment length polymorphism analysis and Sanger sequencing to

21 evaluate our expectation of high *PFT1* STR variation across *A. thaliana* strains. However, we

22 observed only three alleles of very similar size (encoding 88, 89 and 90 amino acids, Table S1),

23 in contrast to six different alleles of the much shorter *ELF3 STR* among these strains, some of

6

1 which are three times the length of the reference allele (Undurraga *et al.* 2012). These data

2 implied that the *PFT1* and *ELF3* STRs respond to different selective pressures. In coding STRs,

3 high variation has been associated with positive selection (Laidlaw *et al.* 2007), though some

4 basal level of neutral variation is expected due to the high mutation rate of STRs. We

5 hypothesized that the *PFT1* STR was constrained to this particular length by *PFT1*'s functional

6 requirements.

7 To test this hypothesis, we generated transgenic *A. thaliana* carrying *PFT1* transgenes

8 with various STR lengths in an isogenic *pft1*-2 mutant background. These transgenics included

9 an empty vector control (VC), 0R, 0.34R, 0.5R, .75R, 1R (endogenous *PFT1* STR allele), 1.27R,

10 and 1.5R constructs. All STRs are given as their approximate proportion of WT STR length – for

11 instance, the 1R transgenic line contains the WT STR allele in the *pft1*-2 background (Table S2).

12 We used expression analysis to select transgenic lines with similar *PFT1* expression levels

13 (Table S3).

14

15 **The *PFT1* STR length is essential for wild-type flowering and shade avoidance:** We first

16 evaluated the functionality of the different transgenic lines in flowering phenotypes. Removing

17 the STR entirely substantially delayed flowering under long days (LD, phenotypes days to

18 flower, rosette leaf number at flowering; Figure 1A). In LD, any STR allele other than 0R was

19 able to rescue the *pft1-2* late-flowering phenotype. Indeed, one allele (1.5R) showed earlier

20 flowering than WT (Figure 1B, 1C), whereas other alleles provided a complete or nearly

21 complete rescue of the *pft1-2* mutant (Figure 1D).

22 In short days (SD), we observed an unexpected reversal in rosette leaf phenotypes

23 (compare SD and LD rosette leaves, Figures 1B, 1D). Rather than flowering late (adding more

1   leaves) as in LD, the loss-of-function *pft1-2* mutant appeared to flower early (fewer leaves at

2   onset of flowering). Only the endogenous STR (1R) fully rescued this unexpected phenotype

3   (Figure 1D). We observed the same mean trend for days to flowering in SD, although differences

4   were not statistically significant, even for *pft1-2* (Figure 1D). This discrepancy may be due to

5   insufficient power, or to a physiological decoupling of number of rosette leaves at flowering and

6   days to flowering phenotypes in *pft1-2* under SD conditions. Regardless, our results indicate that

7   *pft1-2*'s late-flowering phenotype is specific to LD conditions. Our observation of this reversal in

8   flowering time-related phenotypes appears to contradict previous data (Cerdán and Chory 2003).

9   However, a closer examination of this data reveals that the previously reported rosette leaf

10   numbers in SD for the *pft1-2* mutant show a similar trend. *PFT1* STR length shows an

11   approximately linear positive relationship with the SD rosette leaf phenotype, forming an allelic

12   series of phenotypic severity. This allelic series strongly supports our observation of either

13   slower growth rate (*i.e.* delayed addition of leaves) or early flowering of *pft1-2* as measured by

14   SD rosette leaves at flowering.

15   *PFT1* genetically interacts with the red/far-red light receptor *PHYB*, which governs

16   petiole length through the shade avoidance response (Cerdán and Chory 2003; Wollenberg *et al.*

17   2008). We measured petiole length at bolting for plants grown under LD to evaluate the strength

18   of their shade avoidance response, and thus whether the genetic interaction is affected by repeat

19   length. Like the flowering time phenotypes, we found that the 1R allele most effectively rescued

20   the long-petiole phenotype of the *pft1-2* null among all STR alleles (Figure 2), though some

21   alleles (*e.g.* 1.5R) show a rescue that is nearly as good.

22   In summary, plants expressing the 1R transgene most closely resembled wild-type plants

23   across a range of adult phenotypes. In contrast, the other STR alleles showed inconsistent

1 performance across these phenotypes, rescuing only some phenotypes or at times out-performing
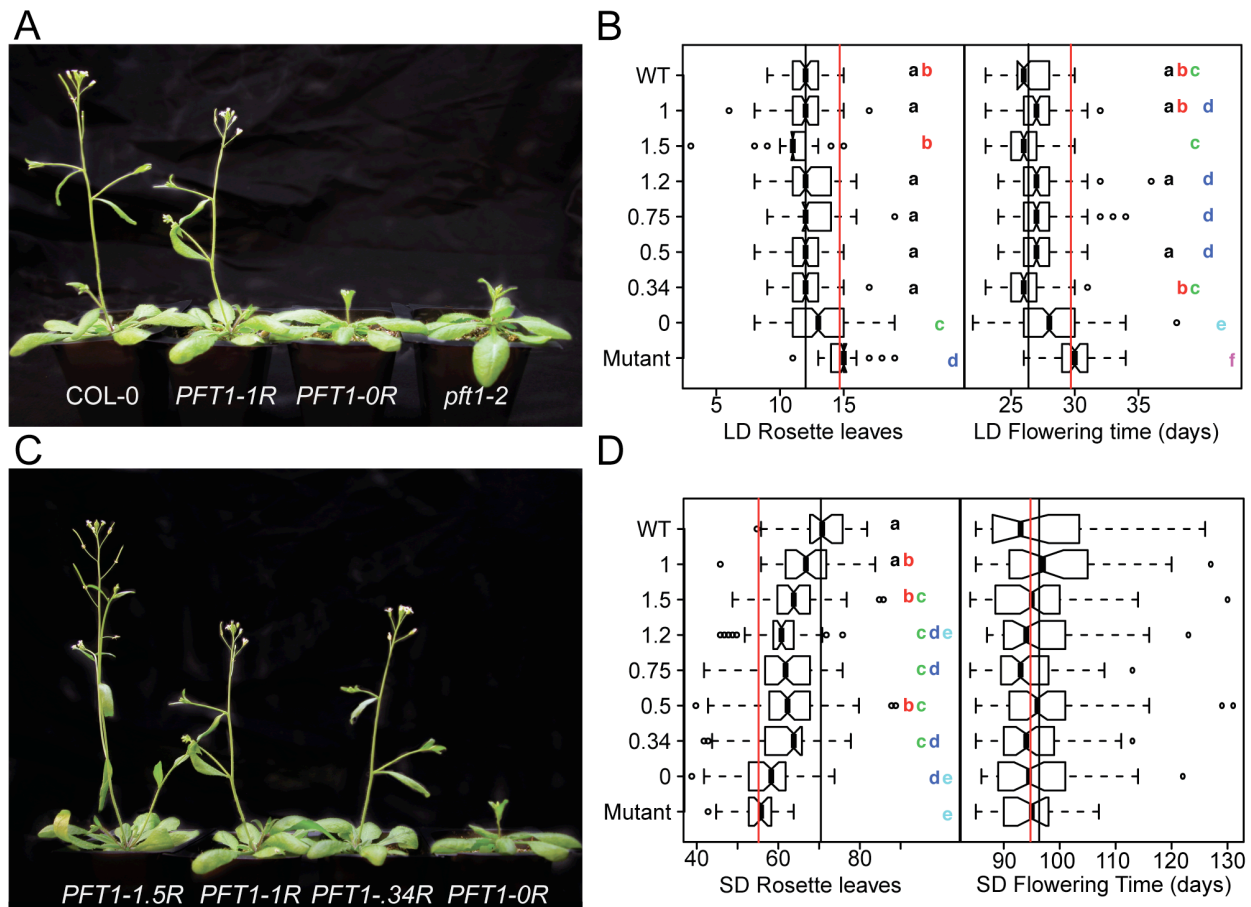
2 wild-type.

3
4 **Figure 1. *PFT1* STR alleles differ in their ability to rescue a *pft1* loss-of-function mutant for**
5 **flowering phenotypes. A, C**) Transgenic plants carrying different *PFT1* STR alleles. Plants
6 were grown under LD for 31 days and photographed. Background was removed in Adobe
7 Photoshop CS 6.0. **B, D**) Strains sharing letters are not significantly different by Tukey's HSD
8 test. Black lines represent WT means, red lines represents *pft1-2* means for each phenotype. Each
9 STR allele is represented by at least two independent transgenic lines (Table S3), with N>20 for
10 SD phenotypes and N>35 for LD phenotypes, $\alpha = 0.05$. LD=long days, SD=short days. In SD
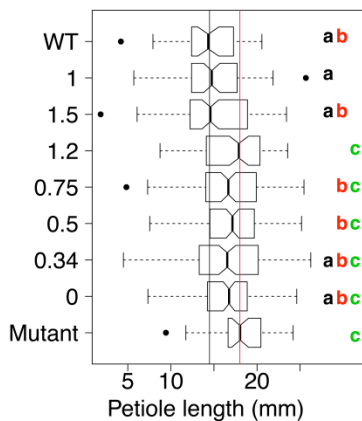11 flowering time (days), no groups are significantly different.

Figure 2. *PFT1* STR alleles differ in their ability to rescue a *pft1* loss-of-function mutant for petiole length in long days. Strains sharing letters are not significantly different by ANOVA with Tukey's HSD test. Black lines represent WT means, red lines represent *pft1-2* means for each phenotype. Each allele is represented by at least two independent transgenic lines, N>35 for each allele, α = 0.05.

***PFT1* STR alleles fail to rescue early seedling phenotypes:** We next assessed quantitative phenotypes in early seedling development, some of which had been previously connected to *PFT1* function. Specifically, we measured hypocotyl and root length of dark-grown seedlings and examined germination in the presence of salt. The *pft1-2* mutant showed the previously reported effect on hypocotyl length as well as a novel defect in root length (Figure 3A). None of the transgenic lines, including the one containing the 1R allele, effectively rescued these *pft1-2* phenotypes (Figure 3A). Similarly, 1R was not able to rescue the germination defect of *pft1-2* on high-salt media. However, both the 1.5R and 0.5R alleles were able to rescue this phenotype (Figure 3B). In summary, no single STR allele, including the endogenous 1R, was consistently able to rescue the early seedling phenotypes of the *pft1-2* mutant. One explanation for the failure of the endogenous STR (PFT1-1R) to rescue early seedling phenotypes is that the *PFT1* transgene represents only the larger of two splice forms. The smaller *PFT1* splice form, which we did not test, may play a more important role in early seedling development. To explore this hypothesis, we measured mRNA levels of the two splice forms in pooled 7-day seedlings grown

10

1   under the tested conditions and various adult tissues at flowering in Col-0 plants. However, we

2   found that both splice forms were expressed in all samples, and in all samples the larger splice

3   form was the predominant form (data not shown). The possibility remains that downstream

4   regulation or tissue-specific expression may lead to a requirement for the smaller splice form in
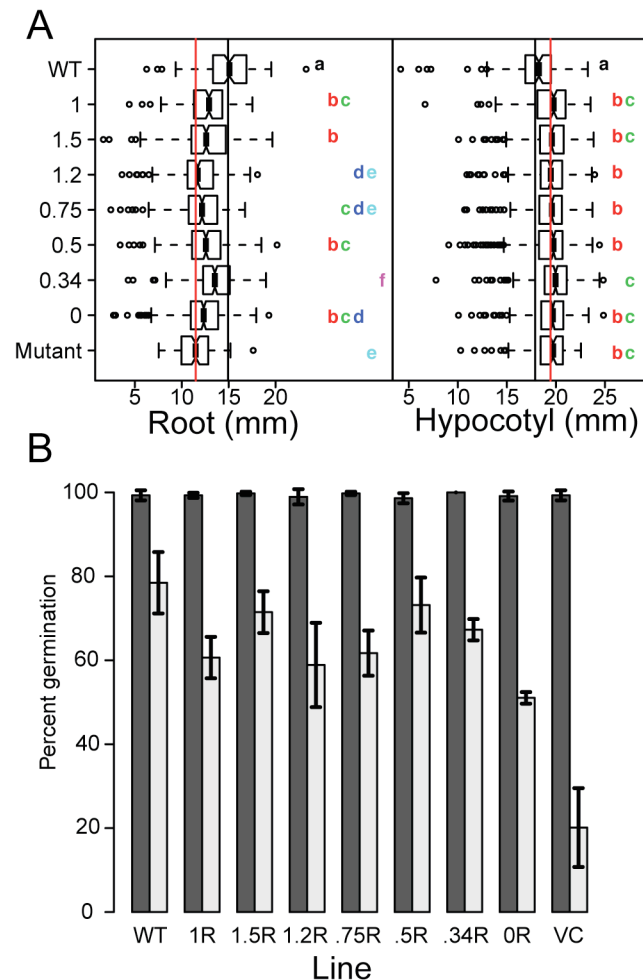
5   early seedlings.

6

Figure 3. *PFT1* STR alleles differ in their ability to rescue a *pft1* loss-of-function mutant for early seedling phenotypes. A) Strains sharing letters are not significantly different by ANOVA with Tukey's HSD test. Black lines represent WT means, red lines represent *pft1-2* means for each phenotype. Each allele is represented by at least two independent transgenic lines, N>100 for all phenotypes for each allele, pooled across at least two experiments; $\alpha = 0.05$. Hypocotyl length and root length were assayed in 7d seedlings grown in dark conditions. **B)** Dark and light bars represent mean germination across 3 biological replicates on 0 mM NaCl and 200 mM NaCl, respectively. N = 36 for each replicate experiment. Error bars represent standard error across these three replicates.

1

2 **Summarizing *PFT1* STR function across all tested phenotypes:** Given the complex

3 phenotypic responses to *PFT1* STR substitutions, results were equivocal as to which STR allele

4 demonstrated the most 'wild-type-like' phenotype across traits, as measured by its sufficiency in

5 rescuing *pft1-2* null phenotypes. To summarize the various phenotypes, we calculated the mean

6 of each quantitative phenotype for each allele, and used principal component analysis (PCA) to

7 visualize the joint distribution of phenotypes observed.

8 All STR alleles were distributed between the *pft1-2* null and wild-type (WT) in PC1,

9 which was strongly associated with adult traits and represented a majority of phenotypic

10 variation among lines (Figure 4). PC1 showed that 1R was the most generally efficacious allele

11 for adult phenotypes. However, 1R showed incomplete rescue in early seedling phenotypes such

12 as hypocotyl length, which drove PC2. All STR alleles showed substantial rescue in adult

13 phenotypes, and even the 0R allele without an STR showed some partial rescue in some

14 phenotypes; however, rescue of early seedling phenotypes was generally poor for all alleles. The

15 first principal component also captured our observation that the *pft1-2* flowering defect reversed

16 sign in SD vs. LD: according to Figure 4, SD and LD quantitative phenotypes are both strongly

17 represented on principal component 1, but they show opposite directionality. We take this

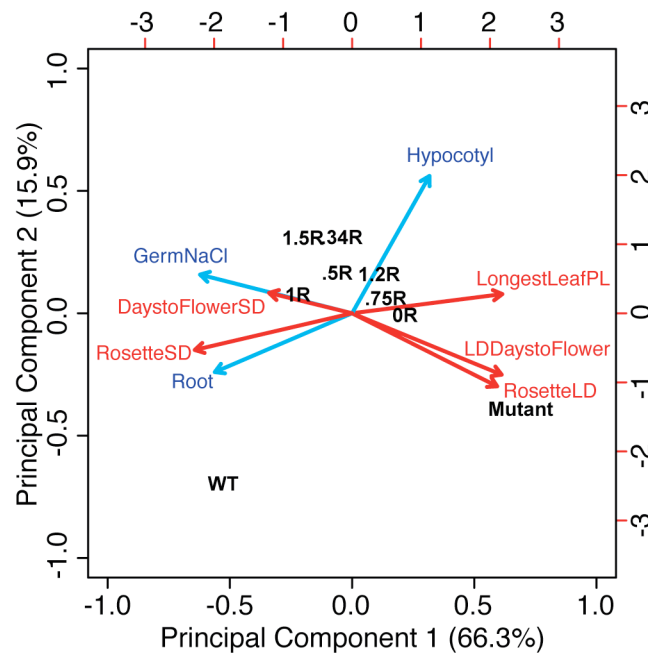18 observation as support of this hitherto-unknown complexity in *PFT1* function.

1
2 **Figure 4. Distribution of *PFT1* STR allele performance across all phenotypes, relative to**
3 **wild-type and *pft1-2* mutants.** Biplot representation of PCA on all phenotypes across all tested
4 *PFT1* STR alleles. Percentages on axes are the % variance in the overall data contributed by that
5 principal component. Contributions of specific phenotypes to these axes are shown by size and
6 direction of arrows. Red arrows represent adult phenotypes, blue arrows represent early seedling
7 phenotypes; adult phenotypes are in red, whereas early seedling phenotypes are in blue.
8 "RosetteSD": number of rosette leaves under SD, "RosetteLD": number of rosette leaves under
9 LD, "LongestLeafPL": petiole length of the longest leaf of rosette, "GermNaCl": proportion of
10 germinants on 200 mM NaCl, "Hypocotyl" and "Root" refer to lengths of the specified organs in
11 dark-grown seedlings. Transgenic STR alleles are indicated by their proportion of the wild-type
12 (WT) repeat, i.e. "1.5R". Top and right axes provide a relative scale for the magnitude of
13 phenotype vectors (blue and red arrows).
14

15 **DISCUSSION**

16 STR-containing proteins pose an intriguing puzzle –they are prone to in-frame mutations, which

17 in many instances lead to dramatic phenotypic changes (Gemayel *et al.* 2010). Although STR-

18 dependent variation has been linked to adaptation in a few cases, the presence of mutationally

19 labile STRs in functionally important core components of cell biology seems counterintuitive.

20 PFT1, also known as MED25, is a core component of the transcriptional machinery across

21 eukaryotes and contains an STR that is predicted to be highly variable in length. Contrary to this

22 prediction, we found PFT1 STR variation to be minimal, consistent with substantial functional

13

1   constraint. The existing residual variation (~2% of reference STR length, as opposed to >100%

2   for the *ELF3* STR in the same *A. thaliana* strains) suggests that the *PFT1* STR is mutationally

3   labile like other STRs despite its interrupted nature. In fact, several of the synthetic *PFT1* alleles

4   examined in this study arose spontaneously during cloning. Strong functional constraint,

5   however, may select against such deviations in STR length *in planta*.

6   Here, we establish the essentiality of the full-length *PFT1* STR and its encoded polyQ

7   tract for proper PFT1 function in *A. thaliana*. We found that diverse developmental phenotypes

8   were altered by the substitution of alternative STR lengths for the endogenous length.

9   Leveraging the support of the *PFT1* STR allelic series, we report new aspects of PFT1 function

10   in flowering time and root development.

11

12   **The *PFT1* STR is required for PFT1 function in adult traits:** The *PFT1* 0R lines did not

13   effectively complement *pft1-2* for adult phenotypes, suggesting a crucial role of the *PFT1* STR

14   in regulating the onset of flowering and shade avoidance. Generally, *PFT1*-1R was most

15   effective in producing wild-type-like adult phenotypes. The precise length of the STR, however,

16   seemed less important for the onset of flowering in LD. With exception of *PFT1*-0R, all other

17   STR alleles were also able to rescue the loss-of-function mutant to some extent, suggesting that

18   as long as some repeat sequence is present, the PFT1 gene product can fulfill this function.

19   Under other conditions, and for other adult phenotypes, requirements for *PFT1* STR length

20   appeared more stringent. Specifically, under SD, the rosette leaf number phenotype of the *pft1-2*

21   mutant can only be rescued by *PFT1*-1R, while STR alleles perform worse with increasing

22   distance from this length "optimum".

23

1   ***pft1-2* mutants are late-flowering in LD but not SD:** *pft1-2* plants had fewer rosette leaves at

2   flowering in SD, but more rosette leaves in LD, consistent with previous, largely undiscussed

3   observations (Cerdán and Chory 2003). Under LD conditions, *pft1-2* null mutants flowered late,

4   as described in several previous studies (Cerdán and Chory 2003; Wollenberg *et al.* 2008), but

5   we observe no such phenotype under SD conditions, contradicting at least one prior study

6   (Cerdán and Chory 2003). These data suggest that while PFT1 functions as a flowering activator

7   under LD, its role is more complex under SD.

8   One recent study showed that PFT1 function in LD is dependent upon its ability to bind

9   E3 ubiquitin ligases (Iñigo, Giraldez, *et al.* 2012). Inhibition of proteasome activity also prevents

10   PFT1 from promoting FT transcription and thus inducing flowering, suggesting that degradation

11   of PFT1 or associated proteins is a critical feature of PFT1's transcriptional activation of

12   flowering in LD. If this degradation is somehow down-regulated in SD, PFT1 could switch from

13   a flowering activator to a repressor, through decreased Mediator complex turnover at promoters.

14   Recent studies raised the possibility that different PFT1-dependent signaling cascades have

15   different requirements for PFT1 turnover (Ou *et al.* 2011; Kidd *et al.* 2009), which may

16   contribute to the condition-specific PFT1 flowering phenotype we observe. Conservatively, we

17   conclude that the regulatory process that mediates the phenotypic reversal between LD and SD

18   depends on the endogenous *PFT1* STR allele, suggesting that the polyQ is crucial to PFT1's

19   activity as both activator and potentially as a repressor of flowering.

20

21

22   **Incomplete complementation of germination and hypocotyl length by the PFT1 constructs:**

23   Whereas *pft1-2* adult phenotypes were rescued by the *PFT1*-1R allele, most of our transgenic

15

1 lines could not fully rescue *pft1-2* early seedling phenotypes of 1) germination under salt, 2)

2 hypocotyl length, and 3) root length. The *PFT1* gene is predicted to have two different splice

3 forms, the larger of which was used to generate our constructs (both splice forms contain the

4 STR). Several studies have shown that, under stress conditions, different splice forms of the

5 same gene can play distinct roles (Yan *et al.* 2012; Leviatan *et al.* 2013; Staiger and Brown

6 2013). We note that the conditions under which *PFT1*-1R fails to complement are also

7 potentially stressful conditions (artificial media, sucrose, high salt, dark). The shorter splice form

8 of PFT1 may be required in signaling pathways triggered under stress conditions. We presume

9 that the failure to complement results from a deficiency related to this missing splice form.

10 However, hypocotyl length was the only trait in which all examined STR alleles resembled the

11 *pft1-2* mutant. The significant functional differentiation among the STR alleles for root length

12 and germination suggests that the large splice form does retain at least some function in early

13 seedling traits.

14

15

16 **Implications for STR and *PFT1* biology:**

17 Coding and regulatory STRs have been previously studied and discussed as a means of

18 facilitating evolutionary innovation (Smukalla *et al.* 2008; Vinces *et al.* 2009; Gemayel *et al.*

19 2010; Undurraga *et al.* 2012; Persi and Horn 2013; Schaper *et al.* 2014). However, this means of

20 innovation is based upon the same sequence characteristics that promote protein-protein and

21 protein-DNA binding (Escher *et al.* 2000; Schaefer *et al.* 2012), such that STR variability must

22 be balanced against functional constraints. This balance has recently been described for a set of

23 18 coding dinucleotide STRs in humans, which are maintained by natural selection even though

1   any mutation is likely to cause frame-shift mutations (Haasl and Payseur 2014). Considering

2   again the possibility that more conserved coding STRs have distinct functions from non-

3   conserved STRs (Schaper *et al.* 2014), we suggest that *PFT1* and *ELF3* can serve as models for

4   these two selective regimes, and that the structural roles of their respective polyQs underlie the

5   differences in natural variation between the two. In some cases, such as *ELF3*, high variability is

6   not always inconsistent with function, even while holding genetic background constant

7   (Undurraga *et al.* 2012). In *PFT1*, we have identified a STR whose low variability reflects strong

8   functional constraints. We speculate that these constraints are associated with a structural role for

9   the PFT1 polyQ in the Mediator complex, either in protein-protein interactions with other

10   subunits or in protein-DNA interactions with target promoters. Given that a glutamine-rich C-

11   terminus appears to be a conserved feature of MED25 even in metazoans (File S1), we expect

12   that our results are generalizable to Mediator function wherever this protein is present. Future

13   work will be necessary in understanding possible mechanisms by which the MED25 polyQ

14   might facilitate Mediator complex function and contribute to ontogeny throughout life.

15   Moreover, attempts must be made to understand the biological and structural characteristics

16   unique to polyQ-containing proteins that tolerate (or encourage) polyQ variation, as opposed to

17   those polyQ-containing proteins (like PFT1) that are under strong functional constraints.

18

19   **METHODS**

20   **Cloning:** A 1000 bp region directly upstream of the *PFT1* coding region was amplified and

21   cloned into the pBGW gateway vector (Karimi *et al.* 2002) to create the entry vector pBGW-

22   *PFT1*p. A full-length *PFT1* cDNA clone, BX816858, was obtained from the French Plant

23   Genomic Resources Center (INRA, CNRGV), and used as the starting material for all our

1   constructs. The *PFT1* gene was cloned into the pENTR4 gateway vector (Invitrogen) and the

2   repeat region was modified by site-directed mutagenesis with QuikChange (Agilent

3   Technologies), followed by restriction digestions and ligations. The modified *PFT1* alleles were

4   finally transferred to the pBGW-*PFT1*p vector via recombination using LR clonase (Invitrogen)

5   to yield the final expression vectors. Seven constructs expressing various polyQ lengths (Table

6   S2), plus an empty vector control, were used to transform homozygous *pft1-2* mutants by the

7   floral dip method (Clough and Bent 1998). Putative transgenics were selected for herbicide

8   resistance with Basta (Liberty herbicide; Bayer Crop Science) and the presence of the transgene

9   was confirmed by PCR analysis. Homozygous T_3 and T_4 plants with relative *PFT1* expression

10   levels between 0.5 and 4 times the expression of Col-0 were utilized for all experiments

11   described. A minimum of two independent lines per construct was used for all experiments.

12

13   **Expression Analysis:** All protocols were performed according to manufacture's

14   recommendations unless otherwise noted. Total RNA was extracted from 30mg of 10-days-old

15   seedlings with the Promega SV Total RNA Isolation System (Promega). 2 μg of total RNA were

16   subjected to an exhaustive DNaseI treatment using the Ambion Turbo DNA-free Kit (Life

17   Technologies). cDNA was synthesized from 100-300 ng of DNase-treated RNA samples with

18   the Roche Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative Real-Time PCR

19   was performed in a LightCyler® 480 system (Roche) using the 480 DNA SYBR Green I Master

20   kit. Three technical replicates were done for each sample. RT-PCR was performed under the

21   following conditions: 5 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 20 s at 55 °C, and

22   20s at 72 °C. After amplification, a melting-curve analysis was performed. Expression of *UBC21*

23   (At5g25760) was measured as a reference in each sample, and used to calculate relative *PFT1*

1   expression. All expression values were normalized relative to WT expression, which was always

2   set to 1.0. To measure splice forms, the protocol was the same but reactions were carried out in a

3   standard thermal cycler and visualized on 2% agarose stained with ethidium bromide. For

4   primers, see Table S4.

5

6   **Plant Materials and Growth Conditions:** Homozygous plants for the T-DNA insertional

7   mutant SALK_129555, *pft1-2*, were isolated by PCR analysis from an F_2 population obtained

8   from the Arabidopsis Stock Center (ABRC) (Alonso *et al.* 2003). Plants were genotyped with the

9   T-DNA specific primer LBb1 (http://signal.salk.edu/tdna_FAQs.html) and gene-specific primers

10 (Table S4).

11   Seeds were stratified at 4°C for 3 days prior to shifting to the designated growth

12 conditions, with the shift day considered day 0. For flowering time experiments, plants were

13 seeded using a randomized design with 15-20 replicates per line in 4x9 pot trays. Trays were

14 rotated 180° and one position clockwise everyday in order to further reduce any possible position

15 effect. Plants for LD were grown in 16 hours of light and 8 hours of darkness per 24 hour period.

16 Bolting was called once the stem reached 1 cm in height.

17   Full strength MS media containing MES, vitamins, 1% sucrose, and 0.24% phytagar was used

18 for hypocotyl experiments. For germination experiments, half-strength MS media was used,

19 supplemented with 1% sucrose, 0.5 g/L MES, and 2.4 g/L phytagel containing 200 mM NaCl or

20 H_2O mock treatment with the pH adjusted to 5.7. All media was sterilized by autoclaving with 30

21 minutes of sterilization time. Seeds for tissue culture were surface sterilized with ethanol

22 treatment prior to plating and left at 4°C for 3 days prior to shifting to the designated growth

23 conditions. Plants for hypocotyl experiments were grown with 16 hours at 22°C and 8 hours at

1   20°C in continuous darkness following an initial 2 hour exposure to light in order to induce

2   germination. Germination experiments were scored on day 4 under LD at 20-22°C. ImageJ

3   software was utilized to make all hypocotyl and root length measurements.

4

5   **Statistical Analysis:** All statistical analyses were performed in R version 2.15.1 with $\alpha = 0.05$

6   (R Development Core Team 2012). Principal component analysis was performed using the

7   *prcomp()* function after scaling each phenotypic variable to a standard normal distribution

8   (phenotypes are not measured on the same quantitative scale).

9

10   **Sequence Analysis:** Length of *ELF3* and *PFT1* STRs were determined by Sanger (dideoxy)

11   sequencing. PFT1 and MED25 reference amino acid sequences were obtained from KEGG

12   (Ogata *et al.* 1999) and aligned with Clustal Omega v1.0.3 with default options (Sievers *et al.*

13   2011).

14

15   **ACKNOWLEDGMENTS**

23

AUTHOR CONTRIBUTIONS

C.Q. and S.U. designed the research. P.R., J.B., T.G., M.O.P., and S.U. performed research. J.B. generated the transgenic lines. M.O.P., J.B. and S.U. analyzed data. S.U., M.O.P., P.R., J.B. and C.Q. wrote the paper.

REFERENCES

Alonso, J. M., A. N. Stepanova, T. J. Leisse, C. J. Kim, H. Chen *et al.*, 2003 Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science (New York, N.Y.) 301: 653–7.

Bäckström, S., N. Elfving, R. Nilsson, G. Wingsle, and S. Björklund, 2007 Purification of a plant mediator from Arabidopsis thaliana identifies PFT1 as the Med25 subunit. Molecular cell 26: 717–29.

Cerdán, P. D., and J. Chory, 2003 Regulation of flowering time by light quality. Nature 423: 881–5.

Çevik, V., B. N. Kidd, P. Zhang, C. Hill, S. Kiddle *et al.*, 2012 MEDIATOR25 acts as an integrative hub for the regulation of jasmonate-responsive gene expression in Arabidopsis. Plant physiology 160: 541–55.

Chen, R., H. Jiang, L. Li, Q. Zhai, L. Qi *et al.*, 2012 The Arabidopsis mediator subunit MED25 differentially regulates jasmonate and abscisic acid signaling through interacting with the MYC2 and ABI5 transcription factors. The Plant cell 24: 2898–916.

Clough, S. J., and A. F. Bent, 1998 Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant Journal 16: 735–43.

Conaway, R. C., and J. W. Conaway, 2011 Function and regulation of the Mediator complex. Current opinion in genetics & development 21: 225–30.

Eckert, K. A., and S. E. Hile, 2009 Every microsatellite is different: Intrinsic DNA features dictate mutagenesis of common microsatellites present in the human genome. Molecular carcinogenesis 48: 379–88.

Elfving, N., C. Davoine, R. Benlloch, J. Blomberg, K. Brännström *et al.*, 2011 The Arabidopsis thaliana Med25 mediator subunit integrates environmental cues to control plant development. Proceedings of the National Academy of Sciences of the United States of America 108: 8245–50.

Escher, D., M. Bodmer-Glavas, A. Barberis, and W. Schaffner, 2000 Conservation of Glutamine-Rich Transactivation Function between Yeast and Humans. Molecular and Cellular Biology 20: 2774–2782.

Fondon, J. W., E. A. D. Hammock, A. J. Hannan, and D. G. King, 2008 Simple sequence repeats: genetic modulators of brain function and behavior. Trends in neurosciences 31: 328–34.

Gemayel, R., M. D. Vinces, M. Legendre, and K. J. Verstrepen, 2010 Variable tandem repeats accelerate evolution of coding and regulatory sequences. Annual review of genetics 44: 445–77.

Haasl, R. J., and B. A. Payseur, 2014 REMARKABLE SELECTIVE CONSTRAINTS ON EXONIC DINUCLEOTIDE REPEATS. Evolution; international journal of organic evolution.

Hannan, A. J., 2010 Tandem repeat polymorphisms: modulators of disease susceptibility and candidates for "missing heritability". Trends in genetics 26: 59–65.

Iñigo, S., M. J. Alvarez, B. Strasser, A. Califano, and P. D. Cerdán, 2012 PFT1, the MED25 subunit of the plant Mediator complex, promotes flowering through CONSTANS dependent and independent mechanisms in Arabidopsis. The Plant Journal 69: 601–12.

Iñigo, S., A. N. Giraldez, J. Chory, and P. D. Cerdán, 2012 Proteasome-mediated turnover of Arabidopsis MED25 is coupled to the activation of FLOWERING LOCUS T transcription. Plant Physiology 160: 1662–73.

Karimi, M., D. Inzé, and A. Depicker, 2002 GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends in plant science 7: 193–5.

Kidd, B. N., C. I. Edgar, K. K. Kumar, E. A. Aitken, P. M. Schenk *et al.*, 2009 The mediator complex subunit PFT1 is a key regulator of jasmonate-dependent defense in Arabidopsis. The Plant cell 21: 2237–52.

Klose, C., C. Büche, A. P. Fernandez, E. Schäfer, E. Zwick *et al.*, 2012 The mediator complex subunit PFT1 interferes with COP1 and HY5 in the regulation of Arabidopsis light signaling. Plant physiology 160: 289–307.

Laidlaw, J., Y. Gelfand, K.-W. Ng, H. R. Garner, R. Ranganathan *et al.*, 2007 Elevated Basal Slippage Mutation Rates among the Canidae. Journal of Heredity 98: 452–460.

Legendre, M., N. Pochet, T. Pak, and K. J. Verstrepen, 2007 Sequence-based estimation of minisatellite and microsatellite repeat variability. Genome Research 17: 1787–96.

Leviatan, N., N. Alkan, D. Leshkowitz, and R. Fluhr, 2013 Genome-wide survey of cold stress regulated alternative splicing in Arabidopsis thaliana with tiling microarray. PloS one 8: e66511.

Ogata, H., S. Goto, K. Sato, W. Fujibuchi, H. Bono *et al.*, 1999 KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic acids research 27: 29–34.

Ou, B., K.-Q. Yin, S.-N. Liu, Y. Yang, T. Gu *et al.*, 2011 A high-throughput screening system for Arabidopsis transcription factors and its application to Med25-dependent transcriptional regulation. Molecular plant 4: 546–55.

Peixoto, a a, J. M. Hennessy, I. Townson, G. Hasan, M. Rosbash *et al.*, 1998 Molecular coevolution within a Drosophila clock gene. Proceedings of the National Academy of Sciences of the United States of America 95: 4475–80.

Persi, E., and D. Horn, 2013 Systematic analysis of compositional order of proteins reveals new characteristics of biological functions and a universal correlate of macroevolution. (J. S. Fetrow, Ed.). PLoS computational biology 9: e1003346.

R: A language and environment for statistical computing. R Development Core Team., 2012.

Rosas, U., Y. Mei, Q. Xie, J. A. Banta, R. W. Zhou *et al.*, 2014 Variation in Arabidopsis flowering time associated with cis-regulatory variation in CONSTANS. Nature communications 5: 3651.

Scarpino, S. V, P. J. Hunt, F. J. Garcia-De-Leon, T. E. Juenger, M. Schartl *et al.*, 2013 Evolution of a genetic incompatibility in the genus Xiphophorus. Molecular biology and evolution 30: 2302–10.

Schaefer, M. H., E. E. Wanker, and M. A. Andrade-Navarro, 2012 Evolution and function of CAG/polyglutamine repeats in protein-protein interaction networks. Nucleic acids research 40: 4273–87.

Schaper, E., O. Gascuel, and M. Anisimova, 2014 Deep Conservation of Human Protein Tandem Repeats within the Eukaryotes. Molecular biology and evolution 31: 1132–1148.

Sievers, F., A. Wilm, D. Dineen, T. J. Gibson, K. Karplus *et al.*, 2011 Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology 7: 539.

Smukalla, S., M. Caldara, N. Pochet, A. Beauvais, S. Guadagnini *et al.*, 2008 FLO1 is a variable green beard gene that drives biofilm-like cooperation in budding yeast. Cell 135: 726–37.

Staiger, D., and J. W. S. Brown, 2013 Alternative splicing at the intersection of biological timing, development, and stress responses. The Plant cell 25: 3640–56.

1 Subramanian, S., R. Mishra, and L. Singh, 2003 Genome-wide analysis of microsatellite repeats
2   in humans: their abundance and density in specific genomic regions. Genome Biology 4:
3   R13.

4 Sundaravelpandian, K., N. N. P. Chandrika, and W. Schmidt, 2013 PFT1, a transcriptional
5   Mediator complex subunit, controls root hair differentiation through reactive oxygen
6   species (ROS) distribution in Arabidopsis. The New phytologist 197: 151–61.

7 Undurraga, S. F., M. O. Press, M. Legendre, N. Bujdoso, J. Bale *et al.*, 2012 Background-
8   dependent effects of polyglutamine variation in the Arabidopsis thaliana gene ELF3.
9   Proceedings of the National Academy of Sciences 1211021109–.

10 Vinces, M. D., M. Legendre, M. Caldara, M. Hagihara, and K. J. Verstrepen, 2009 Unstable
11   tandem repeats in promoters confer transcriptional evolvability. Science 324: 1213–6.

12 Wollenberg, A. C., B. Strasser, P. D. Cerdán, and R. M. Amasino, 2008 Acceleration of
13   flowering during shade avoidance in Arabidopsis alters the balance between FLOWERING
14   LOCUS C-mediated repression and photoperiodic induction of flowering. Plant physiology
15   148: 1681–94.

16 Xu, R., and Y. Li, 2011 Control of final organ size by Mediator complex subunit 25 in
17   Arabidopsis thaliana. Development (Cambridge, England) 138: 4545–54.

18 Yan, K., P. Liu, C.-A. Wu, G.-D. Yang, R. Xu *et al.*, 2012 Stress-induced alternative splicing
19   provides a mechanism for the regulation of microRNA processing in Arabidopsis thaliana.
20   Molecular cell 48: 521–31.

21

22

23 **Table S1:** Number of repeat units in two polyglutamine regions encoded by trinucleotide repeats
24 across eight *A. thaliana* strains.

A. thaliana strain	Number of *PFT1* trinucleotide repeats	Number of *ELF3* trinucleotide repeats
C24	90	9
Can-0	90	20
Col-0	88	7
Cvi-0	89	9
Ler-1	90	17
Mt-0	88	21
Tamm-2	88	9
Ws-2	88	16

25

26 **Table S2.** Encoded amino acid sequence of the repeat regions across *PFT1* constructs used in
27 this study, named for their approximate proportion of the length of the endogenous repeat.

24

Allele name	Protein sequence in repeat region
0R	NLQ
0.34R	NLQQQQQQQQQQQQQQHQLTQLQHHHQQQQQ
0.5R	NQQQQQQQQLHQQQQQQQQIQQQQQQQQQHLQQQQMPQLQHHHQQQQQ
0.75R	NQQQQQQQQLHQQQQQQQQIQQQQQQQQQHLQQQQMPQLQQQQQQQHQQQ QQQQHQLTQLQHHHQQQQQ
1R*	NQQQQQQQQLHQQQQQQQQIQQQQQQQQQHLQQQQMPQLQQQQQQQHQQQ QQQQHQLSQLQHHQQQQQQQQQQQQQQHQLTQLQHHHQQQQQ
1.27R	NQQQQQQQQLHQQQQQQQQIQQQQQQQQQHLQQQQMPQLQQQQQQQHQQQ QQQQHQLTQLQHHHQQQQQQQQQQQQQQHQLTQLQHHHQQQQQQQQQQ QQQHQLTQLQHHHQQQQQ
1.5R	NLQHHHQQQLQQQQQQQQLHQQQQQQQQIQQQQQQQQQHLQQQQMPQLQQ QQQQQLHQQQQQQQQIQQQQQQQQQHLQQQQMPQLQQQQQQQHQQQQQQQ HQLSQLQHHQQQQQQQQQQQQQQHQLTQLQHHHQQQQQ

1   *: endogenous PFT1 polyglutamine sequence. All other sequences are synthetic (not observed in
2   *A. thaliana*).
3
4   **Table S3.** Transgenic T_3 and T_4 *A. thaliana* lines used in this study.

Repeat Unit	Line Name	Repeat Region PCR Confirmed	Expression Relative to Col-0*	Standard Error*
1.5	1.5R3-3	Yes	0.96	0.12
1.5	1.5R4-4	Yes	3.21	0.07
1.5	1.5R8-1	Yes	2.32	0.19
1.27	1.27R6-2	Yes	1.78	0.10
1.27	1.27R13-2	Yes	0.89	0.05
1.27	1.27R14-2	Yes	1.49	0.46
1	1R1-2	Yes	0.90	0.05
1	1R8-3	Yes	1.10	0.22
0.75	0.75R1-4	Yes	1.92	0.19
0.75	0.75R2-1	Yes	0.69	0.21
0.75	0.75R10-2	Yes	0.92	0.27
0.5	0.5R5-1	Yes	2.70	0.09
0.5	0.5R6-4	Yes	0.66	0.29
0.5	0.5R7-3	Yes	0.98	0.13
0.34	0.34R1-2	Yes	0.63	0.05
0.34	0.34R2-2	Yes	0.82	0.02
0.34	0.34R9-1	Yes	2.85	0.49
0	0R4-2	Yes	1.33	0.01

0	0R8-2	Yes	0.94	0.06
V	V3-1	NA	0.36	0.04

1 *: Expression level estimate and standard error for each line are estimated from three biological
2 replicates.
3

4 **Table S4. Primers used in this study.**

Target	Forward primer	Reverse primer
pft1-2 allele	ATTATTTGGGTGCTTTCTCATGGCC	TGGGCTTTCCCTGCATTTAAAACAG
UBC	GACCAAGATATTCCATCCTA	GTTAAGAGGACTGTCCG
PFT1 (cloning)	ATCAACAGGAATGGCTACATC	TTGTTTGAGGACTAAAGGCATTAT
PFT1 (splice forms)	GCAAACCATCGTCTCCGACTATC	ccactccgttgtaccaagcaa
PFT1.1 (large splice form only)	CAGGTCTTTCTGTGGCAGTGA	ccactccgttgtaccaagcaa
PFT1.2 (small splice form only)	CAGAGGAACCCTGTTTCTACT	ccactccgttgtaccaagcaa

5

6 **File S1.** Alignment of PFT1 and MED25 amino acid sequences from diverse eukaryotes.