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ORIGINAL PAPER

Dissection of a QTL reveals an adaptive, interacting gene complex associated with transgressive variation for flowering time in rice

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Abstract A *days to heading* QTL (*dth1.1*) located on the short arm of rice chromosome 1 was sub-divided into eight sub-introgression lines (SILs) to analyze the genetic basis of transgressive variation for flowering time. Each SIL contained one or more introgression(s) from *O. rufipogon* in the genetic background of the elite *Oryza sativa* cultivar, Jefferson. Each introgression was defined at high resolution using molecular markers and those in the *dth1.1* region were associated with the presence of one or more flowering time genes (*GI*, *SOC1*, *FT-L8*, *EMF1*, and *PNZIP*). SILs and controls were evaluated for flowering time under both short- and long-day growing conditions. Under short-day lengths, lines with introgressions carrying combinations of linked flowering time genes (*GI/SOC1*, *SOC1/FT-L8*, *GI/SOC1/FT-L8* and *EMF1/PNZIP*) from the late parent, *O. rufipogon*, flowered earlier than the recurrent parent, Jefferson, while recombinant lines carrying smaller introgressions marked by the presence of *GI*, *SOC1*, *EMF1* or *PNZIP* alone no longer flowered early. Under long-day length, lines carrying *SOC1/FT-L8*, *SOC1* or *PNZIP* flowered early, while those carrying *GI* or *EMF1* delayed flowering. Across all experiments and in the field, only SIL_SOC1/FT-L8 was consistently early. A preliminary yield evaluation indicated that the transgressive early flowering observed in several of the SILs was also associated with a measurable and positive effect on yield. These SILs represent a new source of variation that can be used in breeding programs to manipulate flowering time in rice cultivars without the reduction in yield that is often associated with early maturing phenotypes.

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Introduction

The initiation of flowering is one of the most important transitions in the life cycle of a plant. It is regulated by a complex genetic network that integrates intrinsic developmental signals with environmental cues such as day length and temperature. The regulation of flowering time by both endogenous and exogenous signals ensures that flowering coincides with appropriate environmental conditions and leads to the successful reproduction of the species (Izawa et al. 2003; Turck et al. 2008).

Garner and Allard (1920) classified plants into three categories depending on their developmental response to specific day/night lengths: long day (LD), short day (SD) and day neutral (DN). SD plants are induced to flower when the day length is shorter than a particular duration, called the critical day length. In contrast LD plants flower when day length exceeds this critical value (inductive conditions). DN plants tend to flower similarly under both LD and SD. Obligate SD or LD plants require exposure to the critical day length before they flower, while facultative SD or LD plants are more likely to flower under the appropriate light/dark conditions, but will eventually flower regardless of day or night length.

In a previous study, 11 flowering time QTLs were mapped in an interspecific cross between the early flowering *Oryza sativa* cultivar, Jefferson, and a wild accession of *O. rufipogon* (Thomson et al. 2003). A QTL on the short arm of chromosome 1, *days to heading 1.1* (*dth1.1*; LOD = 9.0; R^2 = 0.08–0.15), promoted early flowering in the recurrent parent (cv. Jefferson) despite the fact that *O. rufipogon* flowers much later than Jefferson (Thomson et al. 2006). The QTL was originally detected in BC_2F_2 populations in two field environments as well as under greenhouse conditions. The shape of the interval plot for *dth1.1* was very broad: for the field environment the QTL plot was significant (LOD > 3.0) across approximately 54 cM, while the plot for the greenhouse environment showed a significant QTL across 38 cM of the short arm of chromosome 1 (Thomson et al. 2006). A similar region had been previously associated with flowering time in other interspecific populations of rice (Cai and Morishima 2002; Doi et al. 1998; Kohn et al. 1997; Xiao et al. 1998).

Using substitution mapping, Thomson et al. (2006) dissected *dth1.1* into two, non-overlapping separate QTLs, *dth1.1a* and *dth1.1b*, and demonstrated that *O. rufipogon* alleles across both regions independently conferred earliness under both short-day (SD = 10 h) and long-day (LD = 14 h) lengths in the Jefferson genetic background. However, the lines used for substitution mapping retained several *O. rufipogon* introgressions on other chromosomes, leaving open the possibility that genes outside the *dth1.1* region may also have contributed to transgressive early flowering.

Six flowering time genes were identified within the *dth1.1* QTL interval, based on sequence similarity to known flowering time genes from *Arabidopsis* [*GIGANTEA* (*OsGI*), *FLOWERING TIME LOCUS T* (*OsFTL* and *OsFT-L8*), *SUPPRESSOR OF CONSTANS 1* (*OsSOC1*), *EMBRYONIC FLOWER 1* (*OsEMF1*)] and from *Pharbitis nil* [*Leucine Zipper* (*OsPNZIP*)] (Thomson et al. 2006). The flowering time orthologs under *dth1.1* were all involved in the photoperiodic pathway. This pathway controls flowering in response to day length and involves photoreceptors (including phytochromes and cryptochromes), the circadian clock and several output pathways (Blazquez 2000; Izawa et al. 2002; Park et al. 1999). While these genes are all loosely linked within the 38 cM QTL region on the short arm of rice chromosome 1, their homologous counterparts in *Arabidopsis* show no linkage and are distributed on four different chromosomes (Supplementary Fig. 1). Rice and *Arabidopsis* are estimated to have diverged from a common ancestor ~200 MYA, and the average size of conserved syntenic regions is <3 cM (Paterson et al. 2009; Salse et al. 2002). This suggests that the evolutionary process would have disrupted any extensive gene complexes that existed in a common ancestor, and raises interesting questions about the origin of the array of flowering time genes found on rice chromosome 1.

Comparative studies in *Arabidopsis*, rice and *P. nil* have demonstrated that there is a highly conserved network of genes involved in the photoperiod pathway in both LD and SD plants (Izawa et al. 2003; Kojima et al. 2002). The basis for the differential flowering responses to photoperiod derive from duplications and changes in the expression of gene family members (Chardon and Damerval 2005; Komiya et al. 2008), development of alternative flowering induction pathways that are unique to each species (Doi et al. 2004; Kim et al. 2007; Komiya et al. 2008; Matsubara et al. 2008; Tsuji et al. 2008) and a reversal in the function of at least three genes in rice and *Arabidopsis*, *GIGANTEA* (*GI*), *CONSTANS* (*CO* or *HD1*), and *FLOWERING TIME LOCUS T* (*FT* or *HD3A*) (Hayama and Coupland 2004; Hayama et al. 2003; Kojima et al. 2002; Putterill et al. 2004). In rice, *GI* resides within the cluster of flowering time candidates in the *dth1.1a* QTL region on rice chromosome 1, while *HD1* and *HD3A* are located within 6.4 Mb of each other on the short arm of chromosome 6.

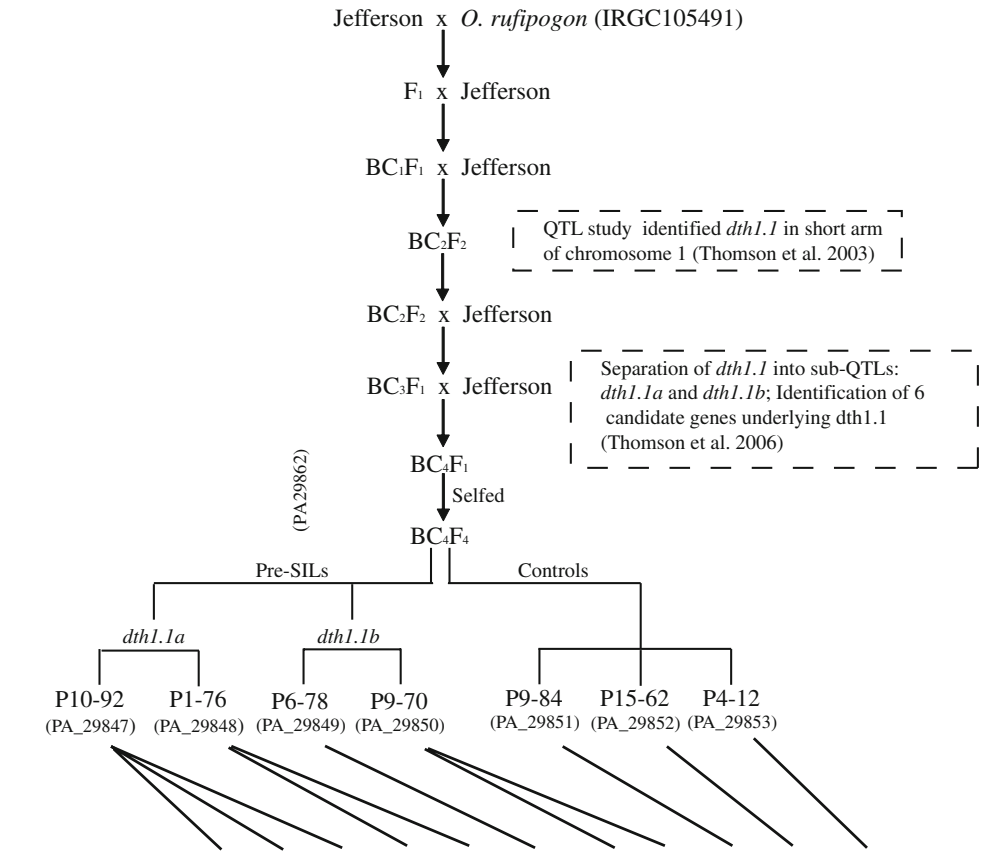
Although tremendous progress has been achieved in understanding which genes are involved in the regulation of flowering time and how different genes and gene families interact at the molecular level, our understanding of how flowering time is regulated under field conditions and how allelic variation and copy number variation in natural populations affects the flowering response of our major crop plants remains largely unknown. The introgression lines in our study provide an opportunity to investigate how allelic variation within the *dth1.1* QTL region contributes to variation in flowering time in the presence of either *O. rufipogon* or Jefferson DNA across the region on chromosome 6, known to contain a second cluster of flowering time genes.

The objectives of the present study were to (1) construct a set of sub-introgression lines (SILs) in the genetic background of the USA *tropical japonica* cultivar, Jefferson, each containing a well-defined introgression from *O. rufipogon* carrying one or more candidate genes for flowering time, (2) evaluate the SILs for flowering time under controlled conditions in both long and short days, and (3) evaluate the SILs for yield and flowering time under field conditions. We were interested in dissecting the *dth1.1* QTL to better understand the genetic basis of transgressive variation for flowering time and to determine whether creating novel haplotypes via recombination within the QTL region was capable of producing novel transgressive phenotypes that could be of use in plant improvement.

Materials and methods

Plant material

All of the introgression lines in this study are derived from BC_4F_4 families [Jefferson/*O. rufipogon* (IRGC #105491)//// Jefferson] as described by Thomson et al. (2006). The term "SIL" refers to BC_4F_8 SILs that each contained a defined introgression from *O. rufipogon* that sub-divides the original 38 cM *dth1.1* QTL target region. "Pre-SILs" are selected BC_4F_4 lines that served as initial materials from which the SILs were derived (Fig. 1). SILs were named according to the candidate gene(s) contained in each *O. rufipogon* introgression, and for ease of reading, will hereafter be referred to simply by the gene name, written without italics.



Selected SIL lines / Introgressions		GI (PA_29854)	GI/SOC1 (PA_29855)	GI/SOC1/FT-L8 (PA_29856)	SOC1 (PA_29857)	SOC1/FT-L8 (PA_29859)	EMF1 (PA_29860)	PNZIP (PA_29861)	EMF1/PNZIP (PA_29862)	P9-84 (PA_29851)	P15-62 (PA_29852)	P14-12 (PA_29853)
dth1.1 candidate genes	*GI*	■	■	■						■	■	■
	SOC1		■	■	■	■				■	■	■
	FT-L8			■		■				■	■	■
	FTL										■	■
	EMF1						■		■		■	■
	PNZIP							■	■		■	■
Chr. 6 candidate genes	*RFT1*									■		
	Hd3a									■		
	Hd1									■		
Background Introgression	Chr. 2										■	■
	Chr. 9									■		
Total genes in *dth1.1* Introgression		92	199	216	83	100	112	412	608	N.A	N.A	N.A

Fig. 1 Genetic scheme and pedigree showing development of introgression lines (*SILs*) and controls. Four segregating BC_4F_4 pre-SILs, P10-92, P1-76, P6-78 and P9-70, were selected as source materials for SIL development. Each line has been assigned a Pedigree Accession number (PA_29847-PA_29862) for seed tracking purposes. Summary of candidate flowering time gene(s) and background introgression(s) in each line are indicated in table below

Four pre-SILs (P10-92, P1-76, P6-78 and P9-70 in Fig. 1) were selected as the starting material for this study. Each contained a heterozygous introgression from *O. rufipogon* across the *dth1.1* region and demonstrated transgressive variation for early flowering under SDs in the study by Thomson et al. (2006). When selfed, these lines gave rise to SILs containing homozygous introgressions in the target region(s), heterozygous offspring, and "revertant" SIL controls containing no *O. rufipogon* DNA in the target regions. These revertants, P10-92R, P1-76R, P6-78R and P9-70R, were included as controls and enabled us to evaluate the phenotypic effect of spurious *O. rufipogon* introgressions remaining in the genetic background of the SILs. Lines P9-84, P4-12 and P15-62 were also included as controls in all flowering time experiments. Based on SSR results from the study by Thomson et al. (2006), line P9-84 was known to contain an *O. rufipogon* introgression across the *dth1.1a* sub-QTL on chromosome 1, as well as background introgressions on chromosomes 6 and 9; it had been identified as a late-flowering line under both SD and LD conditions (Thomson et al. 2006). Lines P4-12 and P15-62 were known to contain *O. rufipogon* DNA across the entire *dth1.1* region and a background introgression on chromosome 2; these lines flowered significantly earlier than the recurrent parent Jefferson as described in Thomson et al. (2006). The inclusion of these controls is necessary when dissecting natural variation in near isogenic lines because background introgressions from a donor may significantly affect phenotypic performance due to G × G and G × E interactions (Yamamoto et al. 2009).

To sub-divide the original *dth1.1* introgression, selfed progenies derived from the pre-SILs were grown in 50-mm-wide × 178-mm-deep plastic pots in the Guterman Greenhouse at Cornell University. DNA was extracted from 6 to 8-week old seedlings using the Matrix Mill method (Paris and Carter 2000) and marker-assisted selection was performed using SSR markers to identify recombinants containing single or multiple candidate genes.

PCR conditions and SSR genotyping across the *dth1.1* region

PCR was performed in 15-ml reactions containing 0.2 mM of each SSR or indel primer, 200 mM dNTP mix, 50 mM KCl, 10 mM TRIS–Cl, pH 8.3, 1.5 mM MgCl, 0.01% gelatin, and 1 unit of *Taq* polymerase. The PCR profile was: 94°C for 5 min for initial denaturation followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min for final extension. The PCR reaction was performed in a PTC-225 tetrad (MJ Research, Watertown, MA) or Mastercycler epgradient (Eppendorf, Westbury, NY) thermocycler.

A set of 35 SSRs markers distributed uniformly across the *dth1.1* target region (Fig. 2; Supplementary Table 1) was used to determine recombination break points in selfed progenies derived from the pre-SILs. SSR markers were detected using silver stained gels. PCR products were run on 4% denaturing polyacrylamide gels using a manual sequencing gel apparatus followed by silver staining, as described by Panaud et al. (1996). SSR markers were multiplexed three to five times per gel, depending on the length polymorphisms for the Jefferson and *O. rufipogon* alleles at individual loci.

Flowering time gene-specific markers

Primers were designed around indel polymorphisms within flowering time genes on chromosome 1 and on chromosome 6 (*HD1*, *HD3A* and *RFT1*) to distinguish the Jefferson (*tropical japonica*) and *O. rufipogon* alleles (Table 1). To identify regions likely to contain indel polymorphisms in the parents of our SILs (cv. Jefferson and *O. rufipogon*), we aligned candidate gene sequences from the two fully sequenced rice genomes, cv. Nipponbare (*japonica*) (http://dev.gramene.org/db/cmap/map_set_info?map_set_acc=grjp2008a) and cv. 93-11 (*indica*) (http://www.gramene.org/db/cmap/map_set_info?map_set_acc=bgi2005) and used them as proxies. This strategy was helpful because of the close genetic relationship between Jefferson and Nipponbare, on the one hand, and the relative similarity of *O. rufipogon* (IRGC #105491) and 93-11 on the other. The sequences were aligned using the SeqMan program of DNAstar (GeneCodes) and insertions/deletions were identified. Primers flanking the indels were designed using the Primer3 program and tested on the SIL parents. Primers were designed to have a common annealing temperature of 60°C and to generate amplicons ~80 to 240 bp in length. Using BLAST (Altschul et al. 1990), primer sequences were aligned to the sequenced Nipponbare genome to confirm their location and copy number in the rice genome. Indel markers were designed so that the size of the indel polymorphism represented ~10% of the total length of the amplicon to facilitate allele calling on agarose gels. Indel amplicons were size separated on 2% v/v agarose gels and stained using SYBR green® (http://www.introgen.com).

Background detection using SNP, SSR and RFLP markers

Historical data from 49 RFLP and 103 SSR markers previously mapped onto BC_2F_2 ancestral materials (Thomson et al. 2003) were used initially to select lines that contained as little *O. rufipogon* DNA in the genetic background as possible. This marker data was supplemented with data from an Illumina Golden Gate SNP assay (http://www.illumina.com)

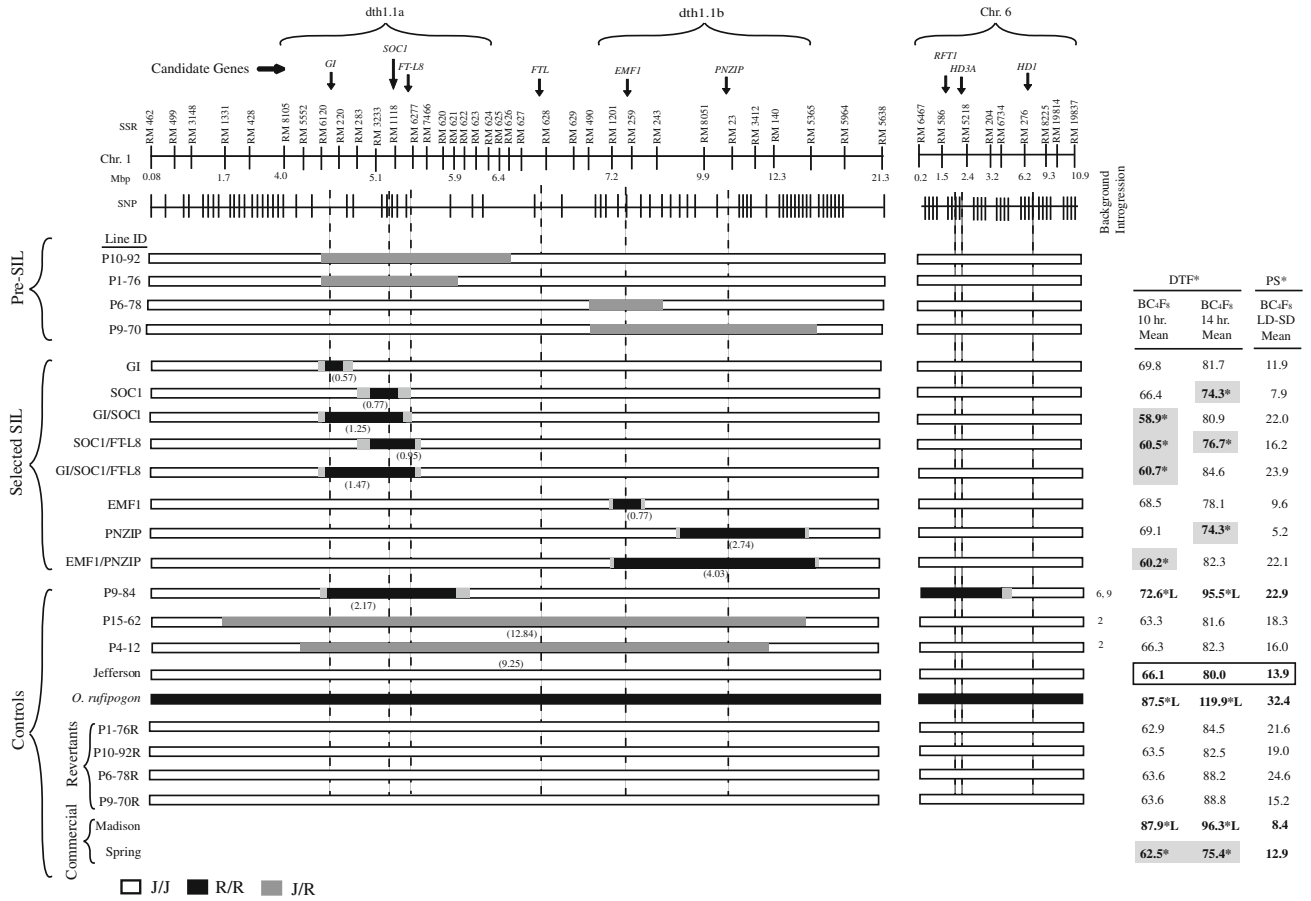


Fig. 2 Graphical genotypes of the SILs and controls showing regions of *O. rufipogon* introgression (*black rectangle* = homozygous; *gray* = heterozygous; *stippled* = region of recombination) across the *dth1.1a* and *dth1.1b* region of chromosome 1 and the *RFT1/HD3A/HD1* region of chromosome 6. Position of candidate flowering time genes indicated by *vertical arrows* across *top* in relation to SSR and SNP markers. Background introgressions indicated to *right*. Table summarizes days to flowering (*DTF*) under short day (10 h) and long day (14 h) and photosensitivity (*PS*). Lines that flowered significantly earlier than Jefferson (highlighted in *white box*) are indicated by *light gray rectangles* and "*"; late lines indicated by "*L*"

Table 1 Flowering time gene specific markers

Marker reagent	Forward primer	Reverse primer	Amplified band size (bp)		MSU gene model	Start (bp)	End (bp)
			Jefferson	*O. rufipogon*		MSU Assembly, version 6	
OsGI	tgaactccatcatgagccacta	acttccagctttgtgcagttg	230	190	LOC_Os01g08700	4,326,087	4,335,288
OsSOC1	tcggcagtggtagagtttga	aaacagaccttgccaccatt	100	70	LOC_Os01g08700	5,466,921	5,469,815
FT-L8	cgacatccttagtgggacaga	ttccttcggtagcatacaacg	160	150	LOC_Os01g10520	5,575,556	5,582,069
FTL	ggctgaaggttttgttttgg	tcatgggttacatgccaattt	190	180	LOC_Os01g11930	6,488,336	6,488,558
OsEMF1	gggggaatttatttcttggt	ggttcgtctacaccagcttc	240	235	LOC_Os01g12890	7,154,582	7,161,187
PNZIP	ttttgaccgaatccatcctt	catcaccttaatggccctgt	90	140	LOC_Os01g17170	9,871,104	9,873,729
RFT1	tggcaagtgagtaaatgaggaa	caaacaccacttttttcatgctt	120	131	LOC_Os06g06300	2,925,824	2,927,475
HD3A	tgctcgatcatatcccatctc	ttcggaaagctttctcttttg	90	110	LOC_Os06g06320	2,939,005	2,941,453
HD1	tcgacttgacacccccttac	gcatggctcttgtggaattt	240	205	LOC_Os06g16370	9,335,361	9,337,634

providing information on ~1,300 SNPs well distributed in the rice genome (K. Zhao, Cornell University, personal communication) (SNP assay developed as part of NSF Plant Genome Award 0606461 to SMc). SNPs included in the Illumina assay were expected to provide enough resolution to estimate the number and size of any spurious

introgression(s) remaining in the genetic background of the SILs.

Growth chamber evaluation

Sub-introgression lines, parental lines, and early and late-flowering controls (including the commercial cultivars, Spring (early) and Madison (late) were evaluated in growth chambers (Conviron, Pembina, ND). Plants were evaluated under both LD (14 h light) and SD (10 h light) conditions, with temperatures of 28°C during the light period and 25°C during the dark period. Light was provided at an intensity of 100 μmol m^{-2} s^{-1}. A total of 20 plants per line, organized in a randomized complete block design (RCBD), were grown in 50-mm-wide × 178-mm-deep plastic pots in growth chambers and all plants were sub-irrigated at a constant water level.

Field evaluation

Sub-introgression lines, parental lines, pre-SILs, revertants and commercial controls [(cv. Jefferson (RA8824), Madison (RA8826), Spring (RA8825), Cocodrie (RA8828) and Wells (RA8827)] were evaluated for flowering time under field conditions during the 2007 and 2008 growing seasons at the USDA-ARS Rice Research Unit in Beaumont, TX and the USDA-ARS Rice Research Unit in Stuttgart, AR. Yield data was based on four repetitions in Beaumont, TX during 2007 and in Stuttgart, AR during 2008. Yield data in Stuttgart, AR in 2007 were lost due to extensive bird damage and in Beaumont, TX in 2008 due to hurricane-induced flooding. Nursery-type plots were established by planting 75 g/line in six rows in a complete randomized block design (CRBD).

Trait evaluation

Days to flowering (DTF), defined as the number of days from seedling emergence until 50% of the main tillers had spikelets with extruded stigmas, was evaluated in both controlled and field environments, and field-grown plants were additionally evaluated for plant stand (plants/m^2), yield/plant, and yield/plot.

Statistical analysis

Analysis of variance for all phenotypic characters was performed using the JMP statistical package, version 7.0 for Windows (SAS Institute Inc., Cary, NC). Data from all experiments were normalized by eliminating extreme values and all assumptions of the least square model (LSM) for controlled environments and restricted maximum likelihood (REML) model for field data were tested to fit phenotypic traits to a linear model and to estimate the variance components. The LSM included the following fixed effects: genotypes (G), environment (E), replications nested within environments (rep(E)) and genotype by environment interaction (G × E). The REML for flowering time included the following fixed effects: genotypes (G) and random effects: environment (E), rep(E) and G × E interaction. The yield model included fixed effects: genotype (G) and random effects: environment (E), rep(E) and G × E interaction.

Multiple means comparisons of all lines for flowering time and yield were done using Dunnet's test with Jefferson as a control ($P < 0.05$). Correlation coefficients for plant height, plant stand, yield, panicle length, tiller number, and flowering time were calculated using the same software and the density ellipse command by which Pearson's correlation coefficients were calculated.

Results

Development of SILs

After four generations of marker-assisted selection from segregating pre-SIL progeny, eight SILs containing subdivided *O. rufipogon* introgressions across the *dth1.1* QTL region were selected for further study (Fig. 1). Using a combination of gene-specific markers and SSRs across the *dth1.1* region, four lines were selected that each contained an introgression carrying a single known flowering time gene (*GI*, *SOC1*, *EMF1* or *PNZIP*), three carried introgressions containing a pair of linked flowering time genes (*GI/SOC1*, *SOC1/FT-L8*, or *EMF1/PNZIP*), and one contained a combination of three flowering time genes (*GI/SOC1/FT-L8*) (Fig. 2). None of the lines in this study carried an *O. rufipogon* introgression containing only *FTL-8* or *FTL* (Figs. 1, 2).

The previously reported late-flowering line, P9-84, was shown to contain *O. rufipogon* DNA in the *dth1.1a* region containing *OsGI*, *SOC1* and *FT-L8* as well as two background introgressions, one on the short arm of chromosome 6 containing *HD3A* and *RFT1* (*FT-L2*) and one on chromosome 9 that contained neither an FT homolog nor any known flowering time genes (Fig. 2). Pre-SILs P4-12 and P15-62 both contained a homozygous introgression across the entire *dth1.1* region and were confirmed to carry *O. rufipogon* alleles at all five flowering time genes, as well as a background introgression on chromosome 2 that did not carry any identifiable flowering time genes (Figs. 1, 2).

Four "revertant" pre-SIL controls were selected: P10-92R, P1-76R, P6-78R and P9-70R (Fig. 2). These lines

were derived by selfing from the backcross populations and contained homozygous Jefferson DNA across the *dth1.1* QTL region (identical to the parental control), but they retained a random array of background introgressions inherited from the SIL families (Fig. 1). The revertant lines allowed us to separate the effect of the target *O. rufipogon* introgressions in the *dth1.1* region from the effect of background introgressions on flowering time and phenotypic performance.

Estimates of introgression size

As shown in Fig. 2, recombination break points across the *dth1.1* region were mapped using 35 SSR and 86 SNP markers that sub-divided the *dth1.1* region into roughly 700 kb sections ($\sim$2.4 cM). By aligning the SSR and SNP positions along the physical map of the sequenced Nipponbare genome (http://www.gramene.org), we were able to estimate the expected size of each introgression in the SILs. Assuming that the physical size of the *O. rufipogon* genome is roughly equivalent to Nipponbare across the *dth1.1* region, we estimate that the introgressions ranged from 0.57 Mb in GI to 4.03 Mb in EMF/PNZIP line. Similar marker data was used to estimate the size of background introgressions in each of the pre-SILs, which ranged from 260 kb in chromosome 4–8.58 Mb in chromosome 6. The Nipponbare genome sequence was also examined to identify the number of annotated genes that mapped within the regions of introgression in each SIL. The number of genes averaged 227, and varied from 92 (in the 0.57 Mb introgression containing GI) to 604 (in the 4.03 Mb introgression containing EMF1 and PNZIP) (Fig. 1).

Flowering of SILs under growth chambers conditions

Under SD conditions, the flowering time of the recurrent parent, cv. Jefferson was 21.4 days earlier than the *O. rufipogon* donor parent (66.1 vs. 87.5 days, respectively; $P < 0.001$) (Fig. 2). Transgressive variation for early flowering was observed only in SILs containing more than one flowering time gene: GI/SOC1 flowered 7.2 days earlier than Jefferson, SOC1/FT-L8 flowered 5.6 days earlier, GI/SOC1/FT-L8 flowered 5.4 days earlier and EMF1/PNZIP flowered 5.9 days earlier. Flowering of the lines harboring single flowering time genes was not significantly different than Jefferson under SD (Fig. 2).

Under LD, the flowering time of both Jefferson and *O. rufipogon* was delayed, while Jefferson flowered 40 days earlier than *O. rufipogon* ($P < 0.0001$). Three SILs showed transgressive variation for earliness under LD; SOC1/FT-L8 flowered 3.4 days earlier and was the only line that flowered earlier than Jefferson under both SD and LD, while both SOC1 and PNZIP flowered 5.7 days earlier (Fig. 2). Lines carrying either *GI* or *EMF1*, alone or in combination, were not significantly different than Jefferson. Because combinatorial SILs containing either of these genes were transgressive for early flowering under SD, these results suggest that *O. rufipogon* alleles at one or more of the genes contained in the *GI* and/or *EMF1* introgressions suppress the early flowering response under LDs and are thus critical determinants of the differential in flowering time between LD and SD. None of the revertant controls flowered significantly earlier than Jefferson under either SD or LD in growth chamber conditions, indicating that the background introgressions had little effect on flowering time (Fig. 2). These results were confirmed in field evaluations where revertant controls flowered similarly to Jefferson with the exception of revertant P10-92R which flowered slightly earlier than Jefferson in Beaumont, TX, but not in Stuttgart, AR.

Photoperiod sensitivity

Photoperiod sensitivity (PS) is defined as the difference between DTF under LD and SD. Photosensitivity was detected in all lines tested under growth chamber conditions (Fig. 2). Examination of the performance of SILs and controls reveals a wide range of responses to photoperiod. The least PS was detected in the lines carrying an introgression with a single flowering time gene, SOC1 (7.9 days), EMF1 (9.6 days) and PNZIP (5.2 days), and in the commercial, late-flowering control variety, Madison (8.4 days), while high sensitivity to photoperiod was observed in lines EMF1/PNZIP (22.1 days), GI/SOC1 (22.0 days), GI/SOC1/FT-L8 (23.9 days) and the late, pre-SIL P9-84 (22.9 days). Jefferson showed 13.9 days difference, while donor parent *O. rufipogon* showed the most extreme PS, with 32.4 days difference in flowering under SD versus LD conditions (Fig. 2). The degree of photosensitivity was significantly correlated with the number of flowering time genes identified in the *O. rufipogon* introgressions present in the SILs ($P < 0.001$). When two or more flowering time genes were present, photosensitivity of SILs increased by almost 8 days compared with the presence of a single flowering time gene. This trend was consistent with the fact that SILs containing two or more flowering time genes tended to be earlier under SD and later under LD than SILs containing a single flowering time gene, which were not significantly different than Jefferson.

Field evaluation of SILs

A summary of the agronomic performance of eight *dth1.1*-derived SILs, two pre-SIL, four revertant controls and five commercial rice varieties, including the Jefferson recurrent parent, is presented in Fig. 3. Fitted models for

flowering time and yield were highly significant ($P < 0.0001$) and explained between 90 and 95% (R^2 values) of the phenotypic variation for flowering time and ~75% for yield (Table 2B, C).

Under field conditions, the average number of DTF for cv. Jefferson was 66 days in Beaumont, TX (Lat: 34:13:24 N; Long: 91:31:15 W) and 69 days in Stuttgart, AR (Lat: 30:04:16 N; Long: 94:06:11 W) (Fig. 3). This is consistent with the expected difference of ~26 min per day in flowering time based on the difference in latitude between the two sites. Among the SILs, SOC1/FT-L8 flowered significantly earlier than Jefferson in both locations while GI/SOC1 flowered 2 days earlier than Jefferson in Beaumont, TX but was similar in Stuttgart, AR. The other five SIL revertants and other controls flowered similarly to Jefferson in both environments with the exception of revertant P10-92R which flowered earlier than Jefferson in Beaumont, TX (Fig. 3). Pre-SIL P9-84 was consistently later than Jefferson in both environments, and there was a difference of ~10 days between the latest and the earliest

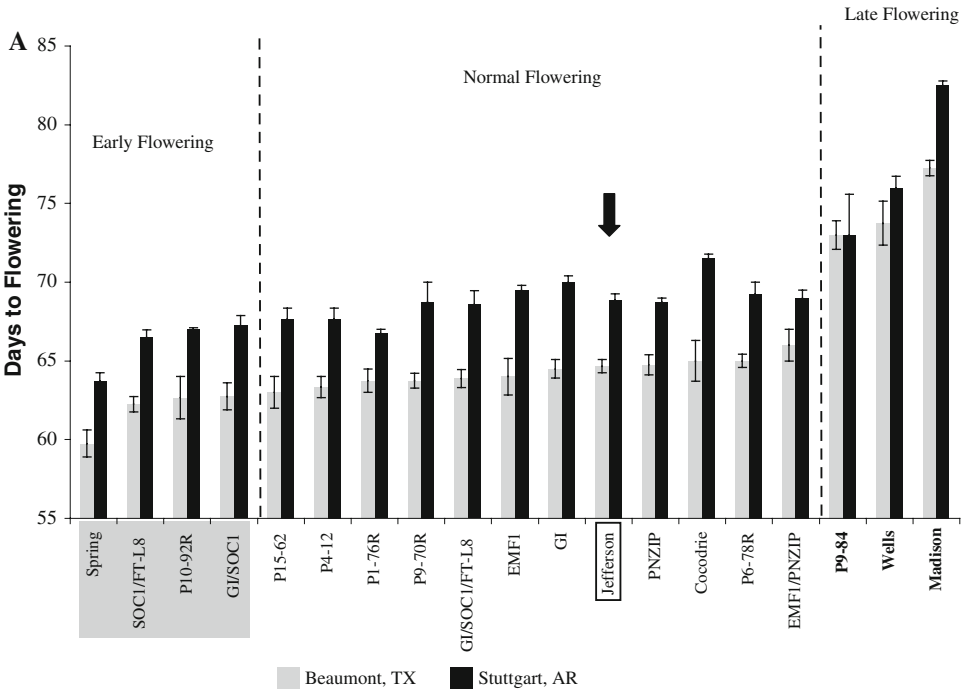


Fig. 3 **a** *Bar* graph showing days to flowering of SILs and controls in relation to recurrent parent, Jefferson (indicated by *black arrow*), in Beaumont, TX and Stuttgart, AR. Early, normal and late-flowering groups based on mean flowering time across locations. **b** Dunnet's multiple means test ($P < 0.05$) of days to flowering in relation to recurrent parent Jefferson (highlighted in *white rectangle*). Significantly early flowering lines indicated with "*" in *gray rectangles*; late lines indicated by "*L"

B

Genotype	Days to Flowering (DAG)		
	Beaumont	Stuttgart	Average
Spring	59.8*	63.8*	61.8*
SOC1/FT-L8	62.3*	66.5*	64.4*
P10-92R	62.7*	67.0	64.8*
GI/SOC1	62.7*	67.3	65.0*
P15-62	63.0	67.7	65.3
P4-12	63.3	67.7	65.4
P1-76R	63.8	66.8	65.3
P9-70R	63.8	68.8	66.3
GI/SOC1/FT-L8	63.9	68.6	65.5
EMF1	64.0	69.5	66.8
GI	64.5	70.0	67.3
Jefferson	64.7	68.9	66.5
PNZIP	64.8	68.8	66.8
P6-78R	65.0	69.3	67.1
Cocodrie	65.0	71.5*L	68.8
EMF1/PNZIP	66.0	69.0	67.7
P9-84	73.0*L	75.0*L	73.2*L
Wells	73.8*L	76.0*L	74.3*L
Madison	77.3*L	82.5*L	79.9*L

Table 2 ANOVA of (A) days to flowering (DTF) evaluated in growth chambers; (B) days to flowering in the field in Beaumont, TX and Stuttgart, AR; (C) yield in Beaumont, TX (2007) and Stuttgart, AR (2008)

(A) Variance component	DTF in controlled environments			
	SS	% Total	*F* value	Prob (*F*)
δ^2_G	41,199.3	26.78	123.3	<0.0001
δ^2_{PP}	74,699.5	48.55	4249.0	0.0001
$\delta^2_{R(PP)}$	4,705.3	3.06	7.9	<0.0001
$\delta^2_{G\times PP}$	6,320.4	4.11	18.9	<0.0001
(B) Variance component	DTF in field conditions			
	Estimate	% Total	*F* value	Prob (*F*)
δ^2_G	11.88	58.01	92.15	<0.0001
δ^2_E	7.54	36.81	588.42	<0.0001
$\delta^2_{R(E)}$	0.14	0.705	3.97	0.0011
$\delta^2_{G\times E}$	−0.08	−0.41	20.74	0.6129
(C) Variance component	Yield (kg/ha)			
	Estimate	% Total	*F* value	Prob (*F*)
δ^2_G	0.18	3.16	2.76	0.0008
δ^2_E	4.42	77.14	77.02	<0.0001
$\delta^2_{R(E)}$	0.25	2.75	2.15	0.0202
$\delta^2_{G\times E}$	0.13	2.27	1.55	0.0914

Jefferson-derived lines in this experiment (P9-84 and SOC1/FT-L8, respectively).

The most extreme flowering under field conditions was observed in the commercial cultivars. The earliest flowering line was Spring, which flowered two days earlier than the earliest SIL, SOC1/FT-L8. The latest lines were Wells and Madison, which flowered 1–2 weeks later than Jefferson, respectively, and 1–2 days later than the latest line in this study, P9-84. Cocodrie, the highest yielding line, was significantly later than Jefferson in Stuttgart, AR, but flowered similarly to Jefferson in Beaumont, TX (Fig. 3).

Agronomic performance of SILs

A preliminary evaluation of yield performance in Beaumont, TX and Stuttgart, AR showed that yield was negatively correlated with flowering time in these SILs ($R = -0.39$, $P < 0.0001$) (Fig. 4). These data provide evidence that earliness in these lines does not automatically incur a yield penalty. SILs GI/SOC1, GI/SOC1/FT-L8 and EMF1/PNZIP yielded significantly more than Jefferson as well Madison, Wells and Spring during the 2007 season in Beaumont and were also the highest yielding SILs in Stuttgart in 2008 (Fig. 4b). The only commercial cultivar that consistently out-yielded the SILs was Cocodrie, the highest yielding variety in the trials. No differences were observed between the early SILs and the Jefferson recurrent parent for plant height, tiller number, panicle length or panicle number (Supplementary Table 2). These results suggest that the *O. rufipogon* alleles at *dth1.1* affect flowering time and yield, but not plant stature, panicle traits or tillering ability.

Discussion

Development of SILs to dissect natural variation

Substitution mapping and phenotypic evaluation of lines carrying non-overlapping *O. rufipogon* introgressions across the *dth1.1* region were previously resolved into at least two separate QTLs (*dth1.1a* and *dth1.1b*) that contributed to earliness (Thomson et al. 2006). In this study, we further dissected the sub-QTLs by generating recombinant SILs, each carrying a single well-defined *O. rufipogon* introgression identified by the presence of one or more flowering time genes. These lines allowed us to observe the effects on flowering time and yield of *O. rufipogon* introgressions containing one or more linked flowering time genes in the Jefferson genetic background. Further dissection of these lines will allow us to fine map the genes underlying the effects on flowering time and yield and to determine how many genes are involved, and whether specific genes are acting pleiotropically on both traits, as in the case of *GHD7* (Xue et al. 2008), or whether genes associated with each trait are simply co-inherited due to linkage.

The individual introgressions in the SILs varied in size between 0.5 and 4.0 Mb and each was predicted to carry between ~90 and 600 genes, based on the annotated Nipponbare gene models. In this study, we used known flowering time genes as sentinels to define the introgressions in the SILs. Using allele-specific gene markers, we confirmed that individual SILs carried the expected donor alleles at each gene.

Our analysis was structured to describe the effect on flowering time of recombinationally defined segments of DNA where each SIL carried a different combination of *O. rufipogon* and Jefferson alleles across the target regions. This work provided an opportunity to ask not only whether *O. rufipogon* alleles in specific sub-regions of the *dth1.1* QTL were capable of generating useful transgressive variation for flowering time, but which specific combinations of Jefferson and *O. rufipogon* alleles across the chromosome 1 QTL and the *HD3A-RFT1*-containing region on chromosome 6 contributed optimally to both flowering time and yield in the southern USA. Prior to the use of molecular markers, it was virtually impossible to analyze whether a particular array of linked genes should be

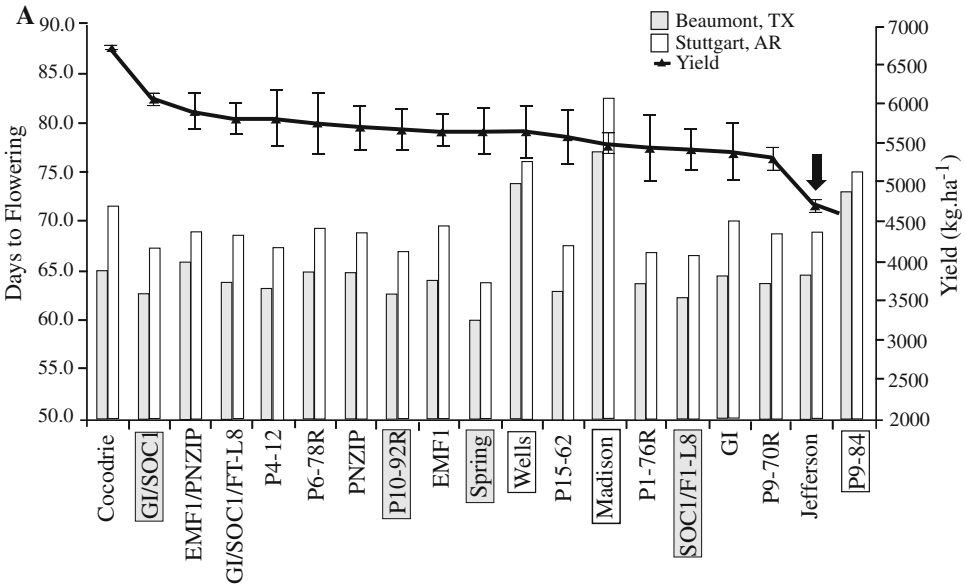


Fig. 4 **a** Line graph showing average yield (kg/ha) of SILs and controls in Beaumont, TX and Stuttgart, AR overlaid on *bar graph* showing days to flowering in both locations. Early flowering lines indicated by *light gray box*, normal-flowering lines with *no box*; and late-flowering lines in *white boxes*. **b** Summary of yield performance (kg/ha) of SILs and controls in relation to recurrent parent Jefferson (highlighted in *white rectangle*) using Dunnet's multiple means test ($P < 0.05$)

B

	Yield (kg.ha^{-1})		
Genotype	Beaumont	Stuttgart	Average
P9-84	3613.6	5486.7	4550.0
Jefferson	3788.2	5681.8	4735.0
GI	4273.0	6572.7	5422.8
SOC1/F T-L8	4643.8	6276.2	5460.0
P1-76R	4300.9	6650.5	5475.7
Madison	4196.8	6828.0	5512.4
P9-70R	5076.1	5595.9	5336.0
P15-62	5216.7	6015.5	5616.1
Wells	4680.4	6674.0	5677.2
Spring	5001.3	6365.3	5683.3
EMF1	5071.7	6314.5	5693.1
P10-92R	4880.7	6528.5	5704.6
PNZIP	5249.0	6212.9	5730.9
P6-78R	5424.8	6153.3	5789.1
P4-12	4921.7	6762.7	5842.2
GI/SOC1/F T-L8	5342.8	6355.0	5848.9*
EMF1/PNZIP	4917.8	6962.8	5940.3*
GI/SOC1	5738.4	6455.5	6097.0*
Cocodrie	6176.1	7334.5	6755.3*

selected intact in a plant breeding program, or whether it should be recombined to achieve maximum advantage. This study offers an example of how recombination within a defined QTL region may be advantageous and how molecular breeding strategies can help identify optimal recombinational profiles.

Promoting fine-scale recombination across the genome is likely to be particularly useful when wild or exotic donors are used as parents in crosses with elite breeding lines. Recombination helps to break up existing linkage blocks related to adaptation and fitness and to generate novel allelic combinations that underlie heterosis and transgressive variation. Because wild and exotic materials are generally highly diverged from elite breeding lines, they provide access to a wider array of allelic variation than do adapted x adapted crosses, but they are often ill-adapted to agronomic environments as evidenced by the fact that interspecific progeny often succumb to biotic or environmental stress, fail to flower or demonstrate a lack of vigor (fitness) in the new environment. In these cases, genome-wide recombination provides a way of shuffling the genomic deck to generate novel variation and better characterize the value of the exotic materials. Recombination and backcrossing makes it possible to evaluate small regions of donor chromosomes in the context of an adapted genetic background and to reveal the breeding value of the unadapted materials. Targeted, local recombination further helps to mitigate the hitch-hiking effect of deleterious alleles and can fortuitously give rise to valuable new haplotypes that can provide a distinctive advantage in a new genetic background or environment.

Effect of *O. rufipogon* sub-introgressions within *dth1.1*

The most noteworthy result from the dissection of *dth1.1* was the discovery that lines containing introgressions that sub-divided either the *dth1.1a* region containing *GI*, *SOC1* and *FT-L8*, or the *dth1.1b* region containing *EMF1* and *PNZIP*, significantly altered the performance of the lines under SD and LD. Our work strongly suggests that interaction among linked *O. rufipogon* alleles in the introgressed regions contributed positively to early flowering under SD and that *O. rufipogon* alleles contained within the *GI* and *EMF1* introgressions repressed early flowering under LD in the Jefferson background.

Pre-SIL P9-84 contains the same *O. rufipogon* introgression as GI/SOC1/FT-L8, but in addition, contains introgressions on chromosomes 6 (containing *HD3A* and *RFT1* but not *HD1*) and 9 (containing no known flowering time genes). P9-84 was both later flowering and lower yielding than GI/SOC1/FT-L8. We infer that the late flowering and depressed yields of pre-SIL P9-84 is a consequence of *O. rufipogon* alleles on chromosome 6 and/or 9. P9-84 offers an opportunity to further dissect the gene network governing both yield and flowering time, based on the fact that when *O. rufipogon* alleles are substituted for Jefferson alleles in the introgressed regions on chromosome 6 and/or 9, the positive transgressive variation for both flowering time and yield conferred by *O. rufipogon* alleles at *dth1.1a* is disrupted.

SOC1/FT-L8 was the only line that flowered earlier than Jefferson in all environments tested, including SD and LD in the growth chamber and field plots in Beaumont, TX and Stuttgart, AR. This line provides a model for studying the genetic control of transgressive variation for flowering time in rice. In this line there are $\sim$100 predicted genes in the $\sim$950 kb introgressed region and it will be interesting to further analyze them to determine which genes/allele(s) from the late-flowering *O. rufipogon* parent are necessary to promote early flowering in the Jefferson background and how they function in the context of a genetic network.

Neither of the genes underlying *dth1.1b*, *EMF1* and *PNZIP* have been extensively studied in rice. Both EMF1 and PNZIP SILs flowered similarly to Jefferson, but the combinatorial line EMF1/PNZIP flowered significantly earlier under SD conditions in the growth chamber and outyielded Jefferson in Beaumont, TX (where days are shorter than in Stuttgart, AR during the summer). Further dissection of the *dth1.1b* region will be needed to better define the genetic factors and functional interactions that promote yield and regulate flowering in these lines.

Role of flowering time genes

The early flowering observed in the SILs cannot be predicted based on the presence or absence of *O. rufipogon* alleles at any one of the flowering time genes investigated here. This argues against the use of a simple "candidate gene" approach to identify the gene(s) responsible for the transgressive phenotype in this study. Instead, we conclude that earliness can result from several different combinations of parental alleles across the *dth1.1a* region, with particular focus on the *O. rufipogon* introgressions marked by *GI/SOC1, SOC1/FTL-8* or *GI/SOC1/FTL-8*. While each introgression contains many genes (making it impossible to conclude that any one of the flowering time genes is functionally responsible for the phenotype), it is clear that an introgression containing either *SOC1* or *GI* alone does not confer earliness in the Jefferson background, but introgressions containing combinations of these genes, along with *FT-L8* do. Further, we know that an *O. rufipogon* introgression in the *HD3a* region of chromosome 6 can override the effects of the introgressions on chromosome 1, making early lines flower late. In future work, we will examine molecular models that have been developed to predict flowering time in rice to determine whether differences in the expression of the various flowering time genes are predictive of the phenotype, and whether they can provide insight into the genetic basis of transgressive variation for the trait.

Impact of *dth1.1* introgressions on photoperiod sensitivity

Of all the lines and pre-SILs evaluated in this study, SOC1, EMF1 and PNZIP showed the least photosensitivity in growth chamber conditions. Their degree of photosensitivity was similar to the late cultivar, Madison. However, all the SILs were earlier than Madison in the field, and they significantly out-yielded Madison. While both lines EMF1 and PNZIP showed low levels of photosensitivity (9.6 and 5.2 days, respectively), the combinatorial EMF1/PNZIP was one of the most photosensitive of all the lines in this study (22.1 days). Similarly, the single candidate lines, GI, SOC1 and SOC1/FT-L8, had relatively low levels of photosensitivity (11.9, 7.9 and 16.7 days, respectively), but it was greatly exaggerated in GI/SOC1 (22.0 days) and in GI/SOC1/FT-L8 (23.9 days). This result suggests that gene(s) located within the GI and EMF1 introgressed regions are associated with enhanced photosensitivity and helps explain why *O. rufipogon* introgressions that include these regions appear to repress early flowering under LD. For example SILs GI/SOC1, GI/SOC1/FT-L8 and EMF1/PNZIP, which are early under SD, are no longer early under LD, while SOC1/FT-L8 is early under both SD and LD. Using the candidate flowering time genes as reference points, our data fit a linear model that predicts an 8-day increase in photosensitivity when a SIL contains two or more flowering time genes from *O. rufipogon*, rather than a single gene.

Effect of background introgressions on flowering time and yield

SSR and SNP marker surveys detected and defined background introgressions as summarized in Fig. 1. Only the introgression on chromosome 6 carrying *HD3A* and *RTF1* had a significant impact on phenotype and it negatively impacted both flowering time and yield in control line P9-84 in all environments evaluated. Interestingly, no flowering time QTL was identified anywhere on chromosome 6 in the original study (Thomson et al., 2003), despite the fact that this region also contains *HD1 (OsCO),* known to be a major determinant of photosensitivity and flowering time in rice (Hayama et al. 2003; Takahashi et al. 2009; Yano et al. 2000). The results of this study indicate that *O. rufipogon* alleles in the *HD3A/RTF1*-containing region of chromosome 6 can negate the positive effects of introgressions in the *dth1.1* region and while we did not evaluate the role of an *O. rufipogon* introgression containing *HD1* (*OsCO*) (located only 6.4 Mb away from HD3A), this will be investigated in future studies. It is of interest to confirm whether *O. rufipogon* alleles at *HD3A* and *RFT1* are specifically responsible for the late-flowering phenotype in P9-84 and, if so, to investigate how they interact with other genes to simultaneously delay flowering and depress yield.

Potential use of *dth1.1*-derived SILs

The main rice-producing region of the USA includes the southern states, Arkansas, Louisiana, Texas, Missouri and Mississippi, which account for 60–80% of national production. In this region, early flowering and early maturity are desirable due to the practice of ratoon cropping where two harvests are obtained from a single planting. Rice is normally planted in this region in March–April and grows vegetatively during the longest days of the year. The crop matures in late August and after the first harvest, plants are left in the field to produce secondary growth, and a ratoon crop is normally harvested in late September. Plant growth late in the season is constrained by low temperatures, so early flowering during the summer is critical to crop productivity, allowing for extended growth duration of the ratoon crop. A general concern about early flowering is the potential reduction in productivity due to a shorter growing season. Thus, it is of interest that most of the *dth1.1*-derived lines yielded better than the elite recurrent parent, Jefferson, while flowering at the same time or slightly earlier. This study demonstrates that alleles coming from the late, low-yielding wild donor can enhance the performance of the early, high-yielding USA cultivar, Jefferson.

The transgressive variation captured in the SILs described in this study represents a form of heterosis that does not require the production of F_1 hybrids, as it can be fixed in inbred lines. Transgressive variation is frequently observed in offspring derived from genetically divergent parents and in naturally self-pollinating species where the load of deleterious recessives is low, it can be readily captured in inbred lines. These observations suggest that the transgressive variation for earliness and yield in this study is not due to overdominance. Rather, the underlying genetic mechanism is more likely to be a form of complementary dominance because early flowering was first observed in BC_2F_2 families where the mean family performance was earlier than the early parent, Jefferson (Thomson et al. 2003). We have now fixed the early trait in our SILs and demonstrated that specific combinations of *O. rufipogon* and Jefferson alleles within and between the introgressed regions are predictive of earliness under both LD and SD conditions. We further confirmed the presence of donor alleles at known flowering time genes in each of the target introgressions using allele-specific markers within the genes as "sentinels" during SIL development. This work suggested that *O. rufipogon* does not differ significantly from Jefferson in terms of the identity of genes or the linkage relationships among them in the introgressed regions. Nonetheless, it will be of interest to look carefully at the gene repertoire and gene order in the Jefferson and *O. rufipogon* parents to determine whether novel genetic elements or genome organization may underlie the expression of transgressive variation documented here. Significant differences in genome structure have been documented in both intra and interspecies comparisons in *Oryza* (Kim et al. 2007; Han and Xue 2003; Huang et al. 2008; Vitte et al. 2007). In addition, it would be of great interest to test our model and determine whether these *O. rufipogon* introgressions confer a similar advantage in different breeding backgrounds with known alleles at the sentinel flowering time genes, and whether F1 hybrid varieties developed using these SILs as one of the parents might further enhance the heterotic expression of earliness and yield.

A long-term objective of this project is to construct introgression lines that can be used as parents in applied plant breeding programs. Because we are working with the cv. Jefferson background, it is likely that any lines emerging from this work would be most immediately useful in a program working with *tropical japonica* germplasm and US grain quality. We are also interested in characterizing the gene repertoire found in the *dth1.1* region in the *O. rufipogon* donor used in this study (IRGC 105491), as well as in other wild/weedy accessions of *O. rufipogon*, to understand how the genome is structured across this region and how much variation exists in wild and exotic germplasm. A deeper understanding of how key genes and alleles interact to give rise to transgressive

variation for flowering time would allow plant breeders to more effectively manipulate this important reproductive trait in molecular breeding programs.

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