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Positional Cloning of the Responsible Genes for Maturity Loci *E1, E2* and *E3* in Soybean

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1. Introduction

The change from vegetative to reproductive growth is a critical developmental transition in the life of plants. Various external cues, such as photoperiod and temperature, are known to initiate plant flowering under the appropriate seasonal conditions. Endogenous cues include a system of juvenile to adult transition that affects competence to flower. To understand the molecular mechanism of flowering, extensive studies have been performed using model plants, *Arabidopsis thaliana* and rice (*Oryza sativa*), and these have revealed the numerous regulatory network components associated with flowering (Jung & Muller, 2009; Amasino, 2010). The general concept of the photoperiodic induction of flowering (photoperiodism) and the range of response types among plant species was established by Garner and Allard (1920). Among the external cues, light is the most important, being received by several photoreceptors including phytochromes, cryptochromes and phototropins. The role of phytochromes, that is the R-light- and FR-light- absorbing photoreceptors, in flowering has been investigated in several plant species. In *Arabidopsis*, a quantitative long-day (LD) plant, a phyA mutant flowered later in either long-day or short-day (SD) conditions with a night break (Johnson et al., 1994; Reed et al., 1994). In rice, a SD plant, the phyA monogenic mutant exhibited the same flowering time as the wild type under LD conditions, while, in the phyB and phyC mutant backgrounds, the flowering was greatly accelerated relative to phyB and phyC monogenic mutants (Takano et al., 2005). In pea, a LD plant, loss- or gain-of-function phyA mutants displayed late or early flowering phenotypes, respectively (Weller et al., 1997, 2001). Day length is found to be perceived by leaves by Knott (1934). Because flowering occurs in the shoot apical meristem (SAM), the leaves must transmit a signal to the SAM and this signal is referred to as florigen (Chailakhyan, 1936). In *Arabidopsis*, three genes, *CONSTANS* (*CO*), *GIGANTEA* (*GI*) and *FLOWERING LOCUS T* (*FT*) were found to be involved in the production of a flowering promoter in LD conditions (Koornneef et al., 1991; Kardailsky et al., 1999). FT protein is now known to be florigen, and CO and GI are key players in the activation of FT expression. CO is a zinc-finger protein that

functions as a transcription factor (Putterill et al., 1995), and GI is a large protein involved in circadian clock function (Fowler et al., 1999; Park et al., 1999). FT is a small protein with some resemblance to RAF kinase inhibitors (Kardailsky et al., 1999; Kobayashi et al., 1999) that is produced in leaves and moves to the SAM (Corbesier et al., 2007; Jaeger & Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007; Notaguchi et al., 2008). The rice orthologs of *Arabidopsis CO* and *FT* genes, *Heading date 1* (*HD1*) and *Heading date 3a* (*Hd3a*), respectively, have been identified (Yano et al., 2000; Kojima et al., 2002; Hayama et al., 2003). The promotion of flowering in *Arabidopsis* in LD conditions results from activation of *FT* by *CO*, while the delay in flowering in rice in LD conditions results from repression of *Hd3a* by *Hd1* (Izawa et al., 2000; Kojima et al., 2002; Roden et al., 2002; Hayama et al., 2003). A *CO*/*FT* module is likely to be conserved throughout the plant kingdom. CYCLING DOF FACTORS (CDFs) exhibit circadian cycling and bind to *CO* promoter and repress *CO* expression. The abundance of CDFs is controlled by FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN1 (FKF1) that appears to be involved in the ubiquitin-mediated degradation of CDFs. GI protein physically interacts with FKF1 and stabilizes it promoting CDF degradation and subsequent *CO* expression (Imaizumi et al., 2005.; Sawa et al., 2007; Fornara and Coupland, 2009; Imaizumi, 2009). Despite the conserved functions of *FT* orthologs, their expression may be controlled by different systems in different species. Non-*CO*/*FT* pathways have been proposed for several plants, such as morning glory (*Pharbitis nil*) (Hayama et al., 2007) and tomato (Ben-Naim et al., 2006; Lifschitz et al., 2006). In rice, *Early heading date 1* (*Ehd1*) has been found to promote flowering by inducing *FT*-like gene expression only under SD conditions independently of *Hd1* (Doi et al., 2004). There is no *Ehd1* ortholog in *Arabidopsis*.

Soybean is a typical SD plant whose photoperiodic sensitivity was discovered by Garner and Allard in 1920. Compared to the model plants, photoperiodic control of flowering in soybean is far less understood. The eight loci, *E1* to *E8*, conditioning flowering has been genetically identified (Bernard, 1971; Buzzel, 1971; Buzzel and Voldeng, 1980; McBlain and Bernard, 1987; Bonato and Vello, 1999; Cober and Voldeng, 2001; Cober et al., 2010). At each of these loci, two alleles have been identified, and except for *E6*, the recessive alleles at the *E* loci condition early flowering under both LD and SD conditions. The partially dominant alleles at the *E* loci delay flowering under LD conditions. Near-isogenic lines (NILs) for *E* loci have been developed and used for studies to elucidate the flowering in soybean (Saidon et al., 1989a,b; Upadhyay et al., 1994a,b; Cober et al., 1996a). Among these *E* loci, *E1*, *E3*, *E4* and *E7* are known to be involved in the response to the phtoperiod (Buzzell, 1971; Buzzell and Voldeng, 1980; McBlain et al., 1987; Cober et al., 1996b; Cober and Voldeng, 2001; Abe et al., 2003). The *E3* locus was first identified with the use of fluorescent lamps to extend day length. The *e3e3* recessive homozygote can initiate flowering under LD conditions where the day length was extended to 20 hr using fluorescent lamps (FLD) with a high red to far-red (R: FR) ratio (Buzzell, 1971). The *E4* locus was identified by extending the natural day length to 20 hr with incandescent lamps with a low R: FR ratio (Buzzell and Voldeng, 1980). The insensitivity of *e4e4* genotype to LD conditions with a low R: FR ratio is necessary of *e3e3* background (Buzzell and Voldeng, 1980; Saindon et al., 1989b; Cober et al. 1996b). The *E1* and *E7* loci are involved in the control of insensitivity to artificially induced LD conditions in the *e3* and *e4* backgrounds (Cober et al., 1996b; Cober and Voldeng 2001). Of the known *E* loci, the *E1* locus is considered to have the largest effect on time to flowering under field conditions (Stewart et al., 2003).

Flowering time is a very important trait which is related to productivity, adaptability and domestication. Soybean breeders have attempted to modify flowering and maturity to expand growing areas for soybean. Molecular identification of *E* loci and flowering network of soybean is useful for efficient breeding to control adaptability and increase yield of soybean. We have identified flowering-time quantitative loci (QTL), *FT1*, *FT2* and *FT3*, and found to correspond to *E1*, *E2* and *E3*, respectively (Yamanaka et al., 2001). We successfully identified the responsible genes for the *E1* (Xia et al., unpublished), *E2* (Watanabe et al., 2009) and *E3* (Watanabe et al., in press) by positional cloning strategy. In this chapter, we will describe the process of identification of responsible genes for the *E1*, *E2* and *E3* loci with variation of alleles and propose a tentative major flowering time pathway in soybean.

2. Strategy for fine mapping and positional cloning

As flowering time is a quantitative trait, we employed QTL analysis (Tanksley, 1993) to dissect the genetic factors for flowering time into individual components by using recombinant inbred lines (RIL) derived from Misuzudaizu, a Japanese variety, and Moshidou Gong 503, a weedy line from China. To identify the underlying molecular basis for each QTL, map-based cloning method was performed because molecular or biochemical information for soybean flowering was very few or totally not available. Although NILs are usually used for fine mapping of each QTL, developing NILs is time-consuming and laborious process especially in soybean. Alternatively, we have proposed fine mapping using residual heterozygous lines (RHLs) (Yamanaka et al., 2005). An RHL selected from an RIL population harbors a heterozygous region where the target QTL is located but contains a homozygous background for most other regions of the genome. The progenies of the RHL are expected to show a simple phenotypic segregation based on the effects of the target QTL at the heterozygous region (Fig. 1). A similar term, heterogeneous inbred family (HIF), was used by Tuinstra et al. (1997) to identify the QTL associated with seed weight in sorghum. The RHL strategy has already been used to identify loci underlying pathogen resistance in soybean (Njiti et al., 1998; Meksem et al., 1999; Triwitayakorn et al., 2005). Genotypes of a trait in recombinants identified in the progenies of RHL, could be determined in the next generation.

The probability of discovering RHLs for a target QTL depends on the heterozygosity ratio in a population and the size of the population. If p is the ratio of hetrozygosity of any population with size n, then the probability of detecting k individuals with a heterozygous genotype is supposed as ${}_nC_k\ p^k\ (1\text{-}p)^{n-k}$ based on a binomial distribution. In the case of an F_7 generation of RILs, the ratio of heterozygosity (p) is 0.0156 and with a population size of 200 (n), the probability of detecting at least one RHL is more than 0.95. We propose that QTL analysis using the F_6-F_8 RIL population in combination with the RHL strategy is useful for dissecting genetic factors for an agronomic trait into each QTL where the homozygous ratio is sufficiently high to evaluate traits with replication and the heterozygosity ratio is not so low and will allow the identification of a sufficient number of RHLs.

In progenies of an RHL, we can identify NILs for the target QTL. New DNA markers in the heterozygous region were developed using NILs, bulked segregant analysis (BSA) in progenies of the RHL, and sequences of bacterial artificial chromosome (BAC) clones covering the target QTL. We usually developed amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and sequence characterized amplified region (SCAR) markers. Genetic analyses of flowering phenotypes and DNA markers were performed in the

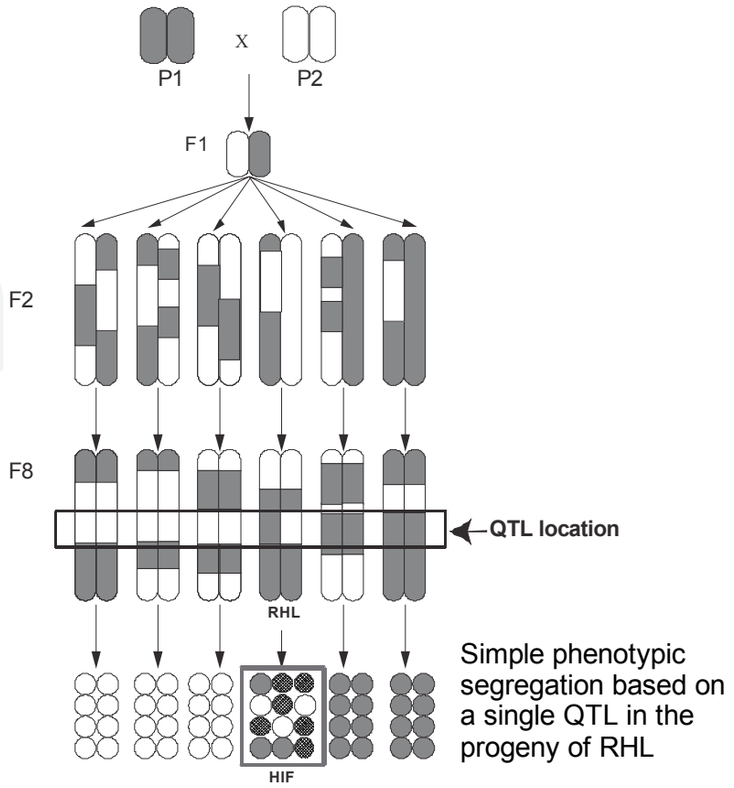

Fig. 1. A schematic representation of RHL. An RHL harbors a heterozygous region where the target QTL is located but contains a homozygous background for most other regions of the genome. Meshed circles show heterozygous individuals.

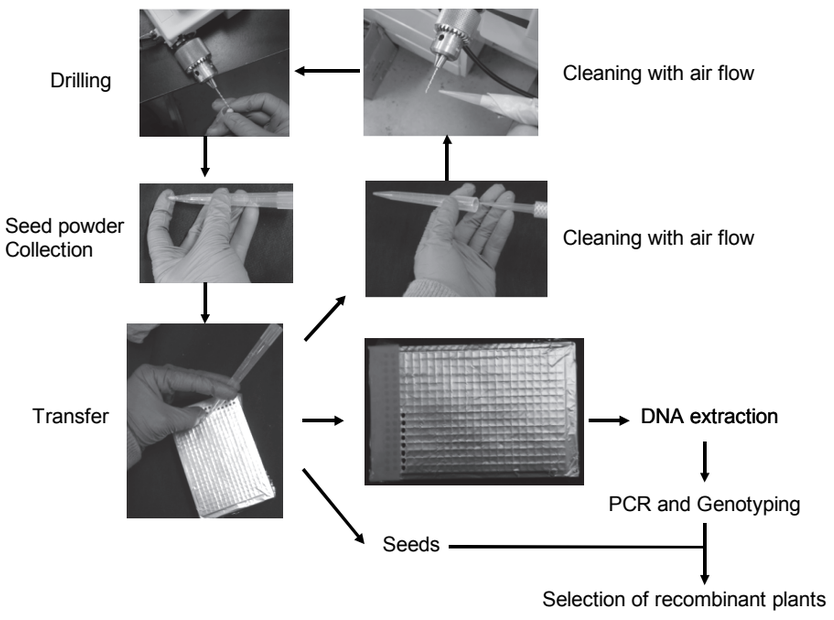

Fig. 2. A procedure for seed genotyping.

progenies of RHLs with a large population. Recombinants of DNA markers were identified in the population and the genotypes of flowering time of recombinants were confirmed by progeny test. The cosegragated region of DNA markers with genotypes of flowering time, and BAC contig covering the region were identified. Sequencing of BAC clones covering the target region and annotation of sequences were performed. Confirmation of a candidate gene was carried out by association of phenotypes and sequence polymorphism of several alleles and gene disruption by induced mutation.

Population size of progenies of RHL for fine mapping depends on recombination frequency, that is, the position of a QTL. We usually used about 1,000 individuals but more than 10,000 plants are necessary when the target locus is located in the peri-centromeric or centromeric region. For high throughput genotyping, the cotyledon flour was obtained by drilling a hole on the surface of seed without any damage to the embryonic axis (Fig. 2). The initially drilled material was discarded to eliminate any possible contamination from the seed coat. Collected materials were transferred into wells in 384-well plate. The drill and tube were cleaned by air flow.

3. Positional cloning of the responsible genes for the *E1, E2* and *E3* loci

A population of 156 RILs ($F_{8:10}$) derived from a cross between Misuzudaizu and Moshidou Gong 503 was used for QTL analysis of flowering. Three QTLs for flowering time, *FT1*, *FT2* and *FT3* were identified at LG C2 (Chr. 6), LG O (Chr. 10) and LG L (Chr. 19), respectively (Fig. 3). The *FT1*, *FT2* and *FT3* were considered to correspond to *E1*, *E2*, *E3*, respectively, based on their map positions (Yamanaka et al., 2001; Watanabe et al., 2004). The late-flowering alleles *FT1*, *FT2* and *FT3* are partially dominant over the early-flowering alleles, *ft1*, *ft2* and *ft3*, respectively. Misuzudaizu harbored the late-flowering allele of the *FT1* and *FT3* loci, whereas Moshidou Gong 503 carried the late-flowering alleles of the *FT2* locus.

3.1 Positional cloning of the responsible gene for the *E2* locus

The line RIL6-8 was found to be heterozygous for the *FT2* locus and was designated as RHL6-8 (Fig. 4). DNA marker analysis showed that RHL6-8 harbored a heterozygous region covering approximately 10 cM including the *FT2* locus. The RHL6-8 generated NILs6-8-*FT2* and –*ft2* among its progenies. Using BSA, a polymorphic AFLP marker, E7M19, was detected between the early-flowering bulk and late-flowering bulk derived from the progeny of RHL6-8. This marker was located close to the LOD peak position of the QTL assigned *FT2* (Fig. 5). We developed additional DNA markers tightly linked to the *FT2* locus using NILs6-8. Among the products amplified from all possible 4,096 primer pair combinations, only five polymorphic bands showed constant polymorphism between the contrasting genotypes of *FT2*/*FT2* and *ft2*/*ft2* in NILs6-8. These polymorphic bands were excised from the gel, sequenced and converted to SCAR markers. Three SCAR markers, originating from five AFLP bands, were developed and used for screening of 10 BAC clones from two independent BAC libraries. A contig covering the *FT2* region was constructed based on the results of PCR analysis using the BAC end sequences. Five of the 10 BAC clones were then subjected to shotgun sequence analysis. Each BAC clone was separately analyzed and assembled, and the sequence information then combined using overlapping sequences. The total length covered by the five clones was approximately 430 Kb. A total of three DNA markers, including one AFLP-derived marker (marker 2) and two PCR-based markers developed from BAC sequences (markers 1 and 3), were used in the fine mapping to minutely restrict the *FT2* locus (Table 1). The positions of these markers are shown in Fig. 6.

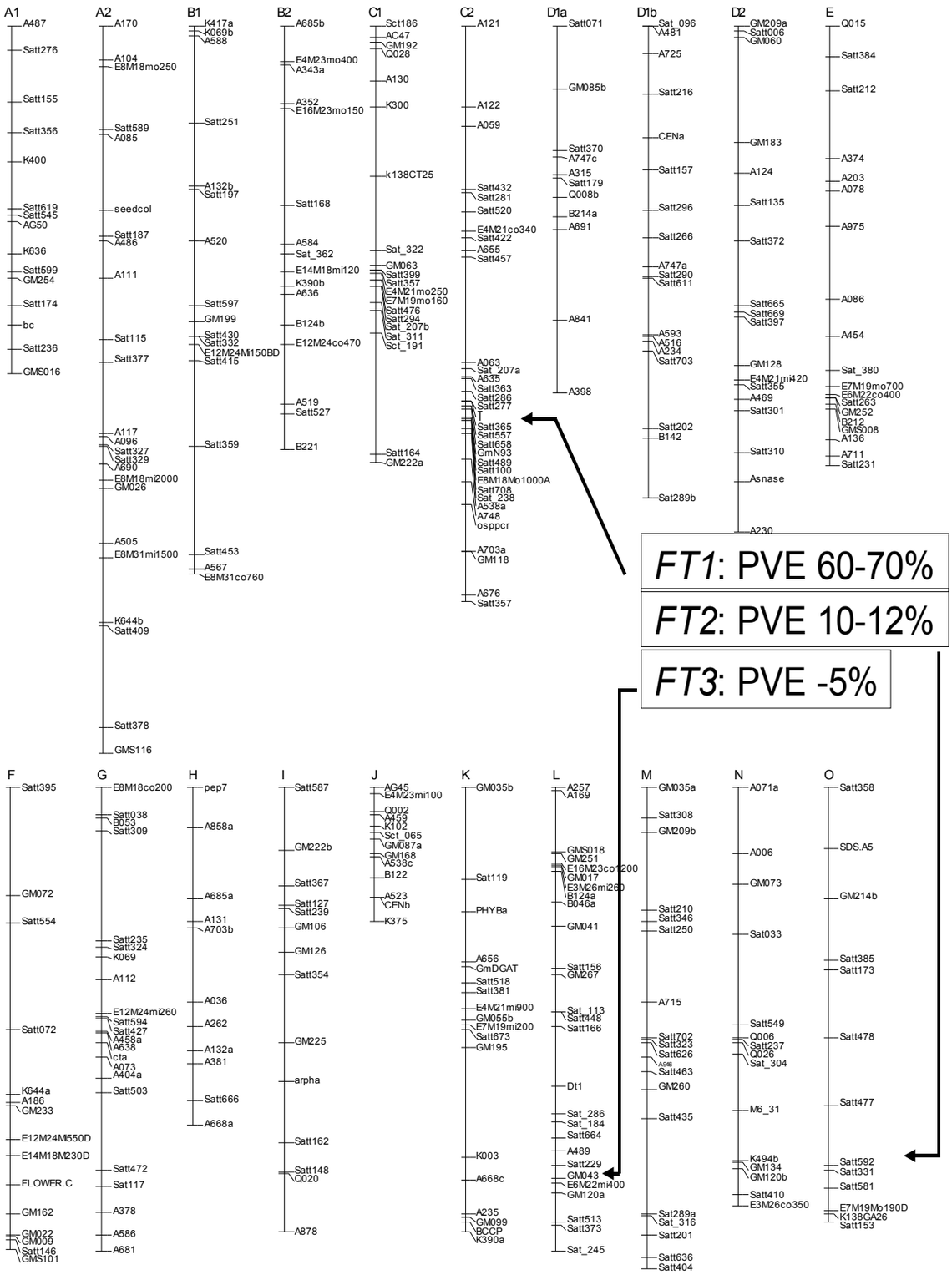

Fig. 3. QTLs for flowering time identified in the RIL population. PVE: phenotypic variance explained by each QTL.

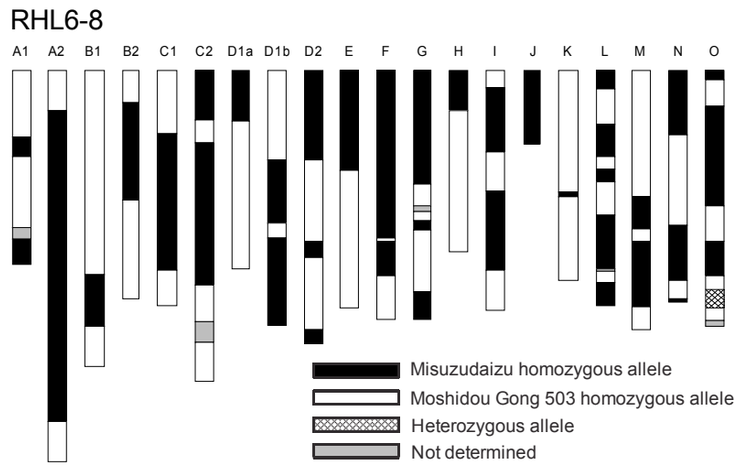

Fig. 4. Graphical genotype of RHL6-8. Solid bars, open bars and meshed bar indicate Misuzudaizu homozygous, Moshidou Gong homozygous genotypes and heterozygous genotype, respectively.

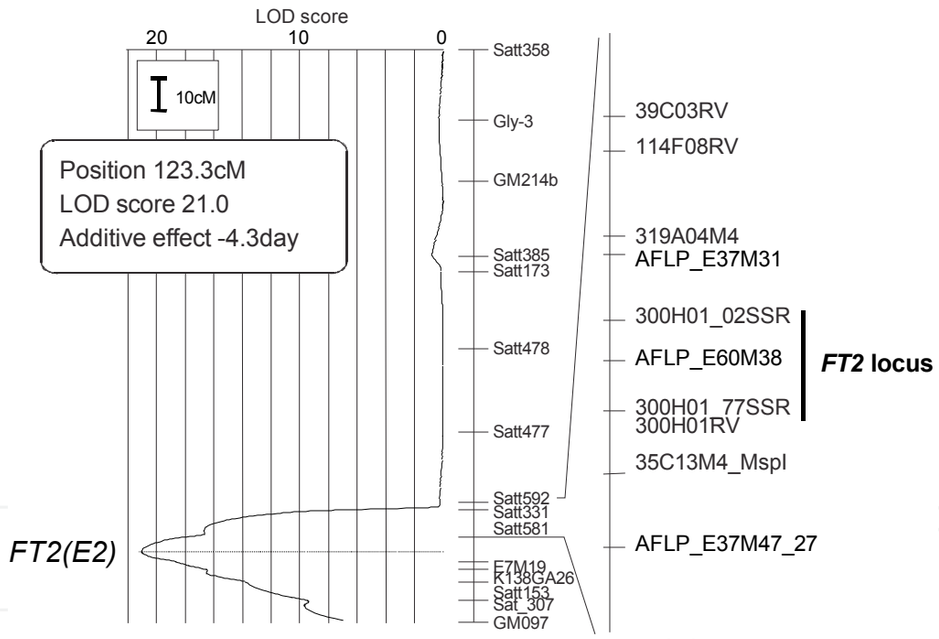

Fig. 5. QTL analysis for the *FT2* locus in the RIL population. The LOD scores for the *FT2* locus calculated by composite interval mapping and displayed in the left panel. DNA markers closely linked to the *FT2* locus are shown in the right panel.

A population consisting of 888 plants, derived from several RHL6-8 plants, was used for fine mapping of the *FT2* locus. Recombination between in this region was found in 21 plants among 843 plants. The remaining 45 individuals were omitted from the analysis because of missing data for phenotypes or genotypes. The number of *FT2* homozygous late-flowering genotypes (n=213), heterozygous (n=420), and *ft2* homozygous early-flowering genotypes (n=210) fitted well with a 1: 2: 1 segregation ratio. The additive effect and dominant effect of

this QTL were estimated to be -5.17 days and 0.57 days, respectively. The ratio of genetic variance explained by the *FT2* locus accounted for 87.9 % of the total variance, indicating that the variation observed in this population was largely controlled by the single QTL effect. The genotypes of the selected 3 markers and flowering genotypes confirmed by progeny test are shown in Fig. 6. The genotypes of marker 2 cosegregated with flowering genotypes indicating that the QTL was close to this marker. Among the recombinants, line 6-8_501 rec had a recombination point between marker 1 and marker 2. Another lines, 6-8_452rec_A, 528rec_B and 6-8_120 rec, generated a recombination between marker 2 and marker 3. Marker 1 and 3 originated from the end sequences of a BAC clone MiB300H01. Considering the recombination points in each line and their flowering genotypes, this indicated that the *FT2* locus was restricted to the single BAC clone, MiB300H01. To identify the responsible gene for this QTL, the nucleotide sequence of this BAC clone was determined.

Marker name	Type of marker	Clone name	Direction	Sequence (5'-3')	Glyma1.0 (Gm10) [b]
Marker 1	BAC end	GMJMiB300H01RV	Fw	CATAGCCGACCTTCTCCAAA	44,787,669
			Rv	AGCCCAATATGGCAGCATAC	44,787,287
Marker 2 [a]	AFLP(SCAR)	E60M38	Fw	CAGTGTTCGCCAGGCTTAGT	44,726,500
			Rv	GCTTGGGTAAACATCCCAAA	44,726,011
Marker 3	BAC end	GMJMiB300H01fw	Fw	GAGAGCAGGGTTATTGGATGA	44,696,157
			Rv	GCCACTGTGCCACATTACAC	44,696,810

a) The digestion with the restriction enzyme *Eco*RI was needed to detect polymorphism.

b) Physical position at Gm10 in Glyma1.0 (http://www.phytozome.net/).

Table 1. List of DNA markers used for fine mapping of the *FT2* locus.

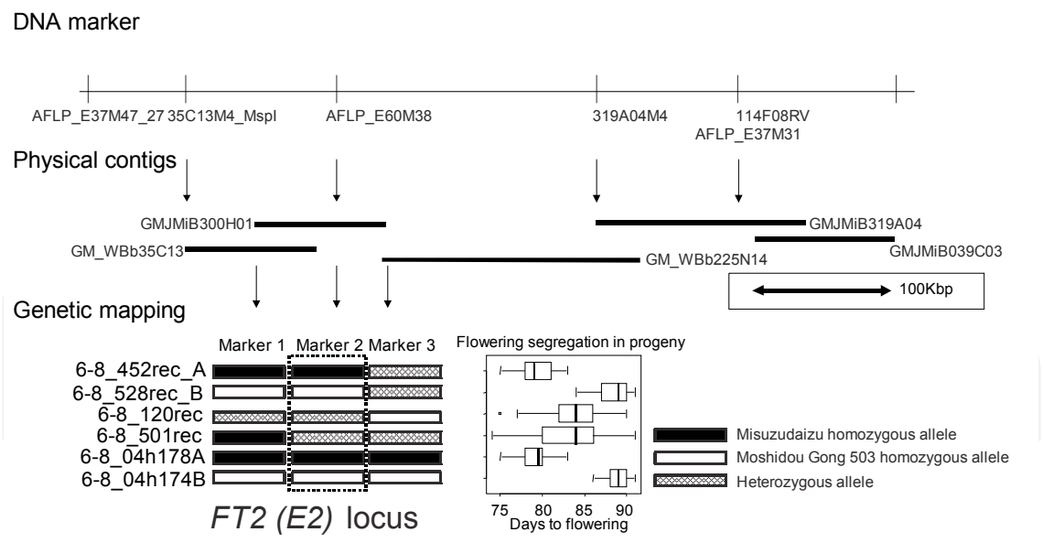

Fig. 6. Fine mapping of the *FT2* locus. The genotypes of each DNA marker of recombinants are shown in the left panel and segregation of flowering in the progenies is displayed in the right panel at the bottom of the figure. The interquatile region, median, and range are indicated by a box, vertical line, and horizontal line, respectively.

In the 94 Kb sequence of MiB300H01, nine annotated genes were predicted. One of these genes, Glyma 10g36600 (assigned in phytozome ver. Glyma 1.0 http:// www.phytozome.net/), with a high level of similarity to *GIGANTEA* (*GI*) gene, was

considered a strong candidate for the *FT2* locus. We isolated the complete predicted coding region using an RNA sample extracted from leaves of NILs6-8-*FT2*. We refer to this gene as *GmGIa*, since another *GI* gene, *GmGIb*, was also obtained from the same RNA sample. The coding sequence of *GmGIa*-Mo from Moshidou Gong 503 was extended to a 20Kb genomic region and contained 14 exons (Fig. 7A). Marker 2, which cosegragated with the *FT2* genotypes and originated from the AFLP marker, E60M38, was located in the 5th intron (Fig. 7). Compared to *GmGIa*-Mo, the Misuzudaizu early flowering allele, *GmGIa*-Mi, showed four single nucleotide polymorphisms (SNPs) in its coding sequence. One of these SNPs, detected in the 10th exon, introduced a premature stop codon mutation that led to a truncated 521 amino acids GI protein in the *GmGIa*-Mi allele (Fig. 7B). This stop codon mutation was considered a candidate for a functional nucleotide polymorphism in *GmGIa*. A derived amplified polymorphic sequence (dCAPs) marker was developed to examine the identity of this stop codon mutation in other NILs originating from Harosoy (*e2*/*e2*). The genotypes of all NILs tested coincided well with the genotype of this diagnostic dCAPs marker. This result indicated that the responsible gene for the *FT2* and *E2* loci was identical to each other, and that a conserved mutation might have caused the early flowering phenotype in the recessive alleles. To validate the significance of the mutation in the *GmGIa*, we screened a mutant line from X-ray irradiated and ethyl methanesulfonate (EMS) treated libraries by targeting-induced local lesions in genomes (TILLING) (McCallum et al., 2000). The sequence of *GmGIa* in the wild type Bay cultivar was completely identical to that of the *E2* allele. One mutant line harboring a deletion in the 10th exon that caused a truncated protein (735 amino acids) (Fig. 7B) showed a significant earlier (8days) flowering phenotype than the wild type under natural day-length conditions. These results indicate that *GmGIa* is the gene responsible for the *E2* locus.

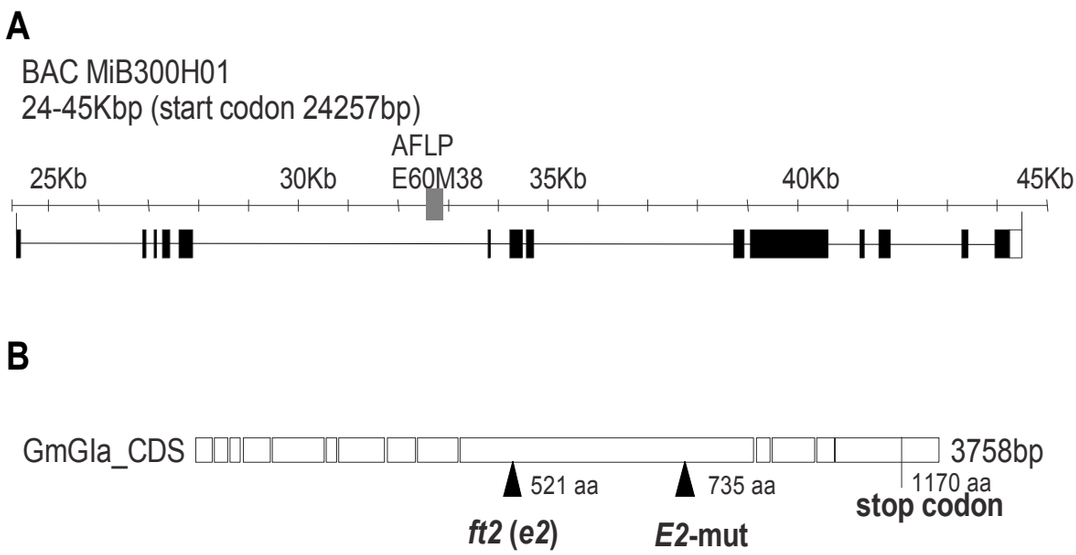

Fig. 7. Variation of gene structure of *GmGIa*. A: Exons, a part of the 3'UTR, and introns of the *GmGIa* gene in the 24-45 Kb region of MiB3300H01 are indicated by bold boxes, open boxes and lines, respectively. The location of marker 2, originating from AFLP marker E60M38, is presented in the 5th intron by the gray box. B: The truncated sites of amino acid sequences in *ft2* (*e2*) and the mutant allele (*E2*-mut) are indicated by the solid triangles.

3.2 Identification and variation analysis of the responsible gene for the *E3* locus

The line RIL1-146 was found to be heterozygous for the *FT3* locus. One other line, RIL6-22, showed segregation for growth habit. This trait is controlled by the *Dt1* locus and is linked to the *FT3* locus at a distance of about 25 cM. The segregating region of RIL6-22 included both the *Dt1* and the *FT3* loci. A single plant with a genotype of *dt1dt1 FT1FT1 ft2ft2 FT3ft3* was selected from RIL1-146, and 5plants with a genotype of *Dt1dt1 ft1ft1 ft2ft2 FT3ft3* were selected from RIL6-22 and designated as RHL1-146 and RHL6-22, respectively. From both progenies of these RHLs, two NILs, 1-146-*FT3* and –*ft3*, and 6-22-*FT3* and –*ft3* were selected. Using BSA analysis, a polymorphic AFLP marker, E6M22, was detected between the early-flowering bulk and the late-flowering bulk derived from the progeny of RIL1-146. This marker was located at the LOD peak position of the *FT3* (Fig. 8).

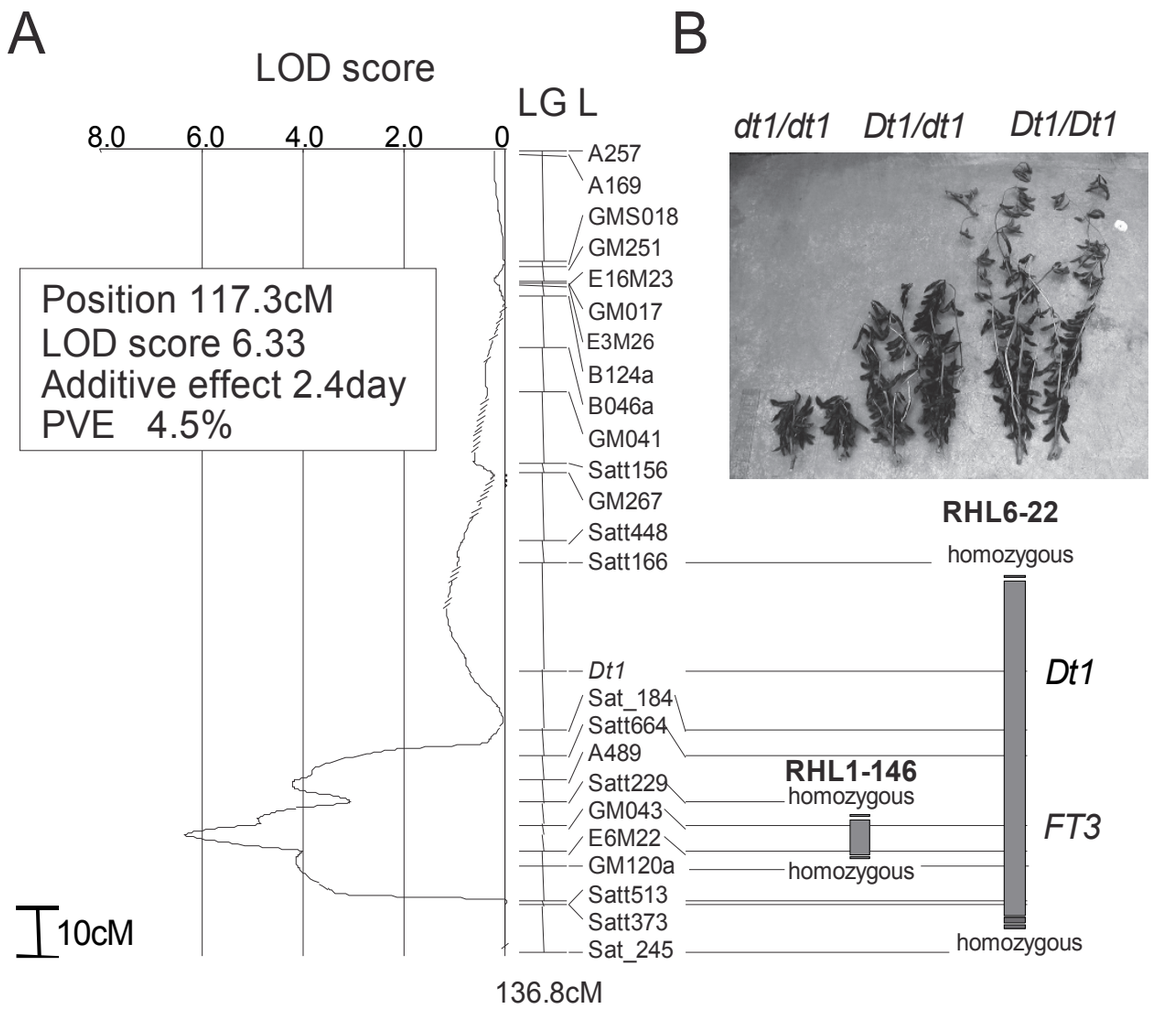

Fig. 8. LOD scores for the *FT3* locus and heterozygous regions of RHLs. The location of the *FT3* locus and the segregating regions of two RHLs, 6-22 and 1-146 are shown. Solid line indicates the LOD scores calculated by composite interval mapping for the QTL (A). Shaded bars indicate the heterozygous regions of two RHLs (B).

As a result of marker analysis, the heterozygous region in RHL1-146 extended for about 5 cM including the *FT3* locus. In contrast, the heterozygous region in RHL6-22 extended for about 40 cM including the *FT3* and *Dt1* loci. Two groups of NILs, NILs1-146 and NILs6-22, were used to develop the AFLP markers tightly linked to the *FT3* locus. Of all possible 4096 primer pairs, only six fragments showed constant polymorphism between the genotypes of *FT3*/*FT3* and *ft3*/*ft3* in NILs1-146 and NILs6-22. These polymorphic bands were excised from the gel, then sequenced, and converted to codominant SCAR markers. Several BAC and transformation-competent bacterial artificial chromosome (TAC) clones were screened using the SCAR markers. The nucleotide sequences of a BAC clone, GMJMiB242F01, and a TAC clone, GM_TMiH_H17D12, were determined. These BAC/TAC sequences were used to develop new PCR-based markers. A total of six DNA markers, including three AFLP-derived markers (markers 1, 3, and 6) and three PCR-based markers developed from the BAC/TAC sequences (markers 2, 4, and 5) were used for fine mapping of the *FT3* locus (Table 2).

A population of 897 plants derived from seven RHL1-146 plants was used for precise mapping of the *FT3* locus. No recombination between these markers was found in 883 plants. The numbers of *FT3* homozygous late-flowering genotype (n=208) and heterozygous (n=441) and *ft3* homozygous early flowering genotypes (n=234) fitted a 1: 2: 1 segregation ratio. These results suggested the presence of a single QTL for flowering time within a small heterozygous region in RHL1-146. The additive effect and the dominant effect of this QTL were estimated to be 3.0 and 0.98 days, respectively. The ratio of genetic variance explained by the *FT3* locus accounted for 70.7 % of the total variance. On the other hand, 14 plants showed recombination between these markers (Fig. 9) and the recombination points were determined by the genotype of markers 2-5. The *FT3* genotypes in each recombinant completely coincided with the genotypes of marker 3 that originated from the closest AFLP marker E6M22 to the LOD peak position (Fig. 8). Moreover, recombination points occurred on both sides of marker 3 and corresponded to both sides of the TAC clone, GM_TMiH_H17D12. These results suggested that the gene responsible for the *FT3* locus was restricted to the physical region covered by GM_TMiH_H17D12 (Fig. 9).

Name of marker	Origin of marker [a]	Direction	Primer sequence (5'-3')	Glyma1.0 (Gm19) [e]
Marker1	AFLP	FW	GAATGTGCTTGTGTTTGTGTC	47,172,164
	(SCAR_E54/56M59)	RV	TAAAGAACCCAGATACAGTCC	47,171,779
Marker2 [b]	TAC_end sequence	FW	CACACAGAAAGCCACAGCAT	47,441,801
	(GM_TMiH_H17D12-RV)	RV	GCTGATCCTTGTGCTGATGA	47,442,133
Marker3 [c]	AFLP	FW	TGGGTCTTCAGTTCAGTTGG	47,516,419
	(SCAR_E6M22)	RV1	TGCTTCCTTTCACTTTCTGATG	47,519,757
		RV2	AGGAAAGGTGGAAGGCGTAT	47,518,322
Marker4 [d]	BAC internal sequence	FW	AATTGAGCTCAGGGAACAGC	47,536,043
	(GMJMiB242F01)	RV	GGATAGAGATGGCGAAGCAA	47,536,374
Marker5	BAC_end sequence	FW	GCCATGGAAGAGAGGAAGATT	47,549,930
	(GMJMiB 242F01-M4)	RV	CACCGTCGTTTTCCTTCTTC	Not found with BLASTsearch
Marker6	AFLP	FW	CAGTAAAATTGATGGGTGCC	47,672,769
	(SCAR_E30M47)	RV	CTGATACACCCAAAGGAAAC	47,672,997

a) Code of AFLP markers and the clone name are indicated in parentheses.
b) Digestion using the restriction enzyme *Mse*I was applied to detect polymorphism.
c) The two combinations of PCR (FW-RV1 and FW-RV2) enabled to distinguish the each allele as a co-dominant marker.
d) The amplified region by marker 4 was included in GM_TMiH_H17D12 and was located 1.5 Kbp from the end of this clone.
e) Physical position at Gm19 in Glyma1.0 (http://www.phytozome.net/).

Table 2. List of DNA markers used for fine mapping of the *FT3* locus.

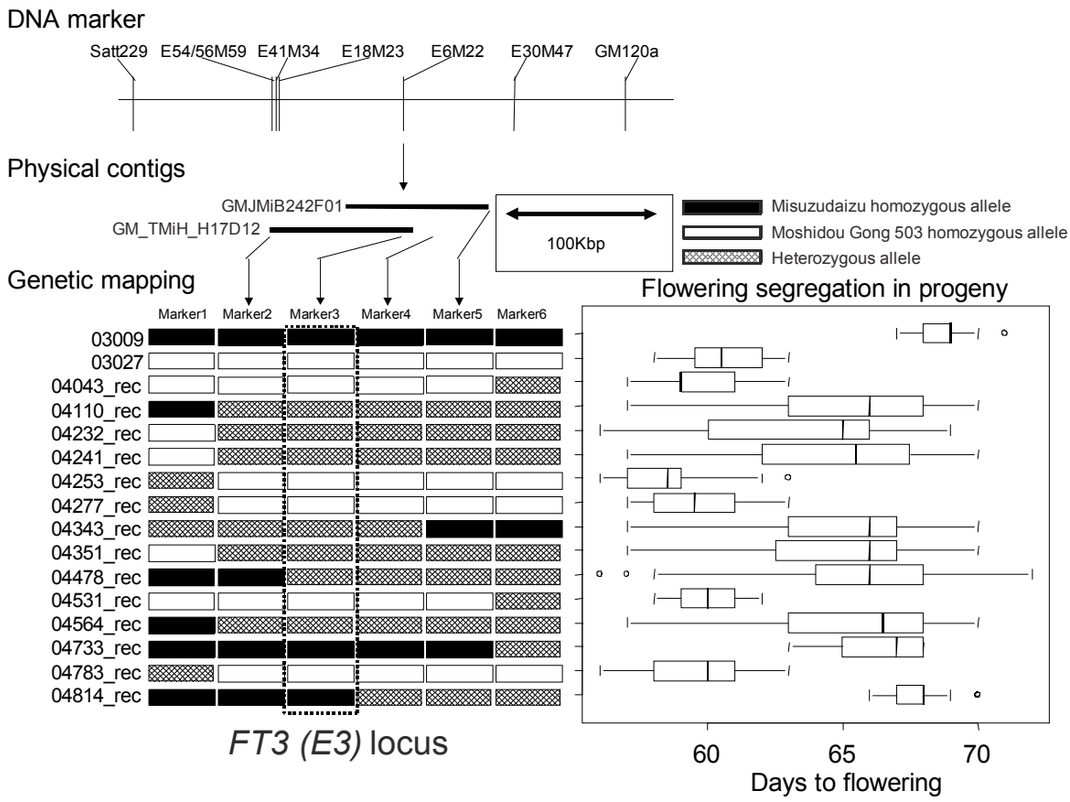

Fig. 9. Fine mapping of the *FT3* locus. The genotypes of each recombinant are shown in the left panel. Misuzudaizu homozygous, Moshidou Gong 503 homozygous and heterozygous genotypes are indicated by solid, open and meshed boxes, respectively. The phenotypic segregation in the progenies of each recombinant was shown in the right panel. The interquartile region, median, and range are indicated by a box, bold vertical line, and horizontal line, respectively.

A total of 11 genes were predicted in the sequence of GM_TMiH_H17D12. Previous studies had suggested that the *FT3* locus may be identical to the maturity locus *E3* (Yamanaka et al., 2001) and that the *E3* gene which showed a large effect on flowering time under FLD conditions had some association with a photoreceptor (Cober et al., 1996b). Considering these findings, one gene highly similar to that encoding phytochrome A was considered to be the gene responsible for the *FT3* locus. To confirm this assumption, differences in this gene between the parental lines were investigated. This phytochorome gene was referred to as *GmPhyA3*, since two other phytochrome A genes had been previously designated as *GmPhyA1* and *GmPhyA2* by Liu et al. (2008). *GmPhyA3* obtained from Misuzudaizu (*GmPhyA3*-Mi) was found to encode a protein composed of 1130 amino acids. A BLAST search found that *GmPhyA3*-Mi displayed normal features of phytochrome A, including a chromophre-attached domain, two PAS domains, and a histidine kinase domain as conserved domains. Compared to *GmPhyA3*-Mi, the *GmPhyA3* gene of Moshidou Gong 503 (*GmPhyA3*-Mo) showed a large insertion in the fourth intron and one SNP for a nonsynonymous amino acid substitution (glycine to arginine) in the third exon (Fig. 10). This SNP corresponded to the polymorphism detected by the AFLP marker E6M22. The inserted sequence was 2.5 Kb in length and a part of this sequence was found to be highly

similar to that of the non-long-terminal-repeat (LTR) retrotransposon reverse transcriptase element, but did not resemble the Ty1/copia or Ty1/gypsy sequences in the *e4* allele (Liu et al., 2008). Moreover, this inserted sequence showed a similar short sequence on both sides of the inserted position. To collect allelic information about *GmPhyA3*, the genes from Harosoy and Harosoy-*e3* were also isolated and designated as *GmPhyA3-E3* and *GmPhyA3-e3*, respectively. While a large retrotransposon-like insertion sequence was observed in *GmPhyA3-E3*, similar to that in *GmPhyA3*-Mo, the amino acid sequences encoded by *GmPhyA3*-Mi and –*E3* were identical (Fig. 10). On the other hand, a large deletion of 13.33 Kb at a position after the third exon was detected in *GmPhyA3-e3* (Fig. 10). Additionally, one mutant (*GmPhyA3*-mut), with a 40-bp deletion in the middle of the first exon of the *GmPhyA3* gene was screened from the mutant libraries of Bay by TILLING (Fig. 10). The sequence of *GmPhyA3* from Bay was identical to that of *GmPhyA3-E3*.

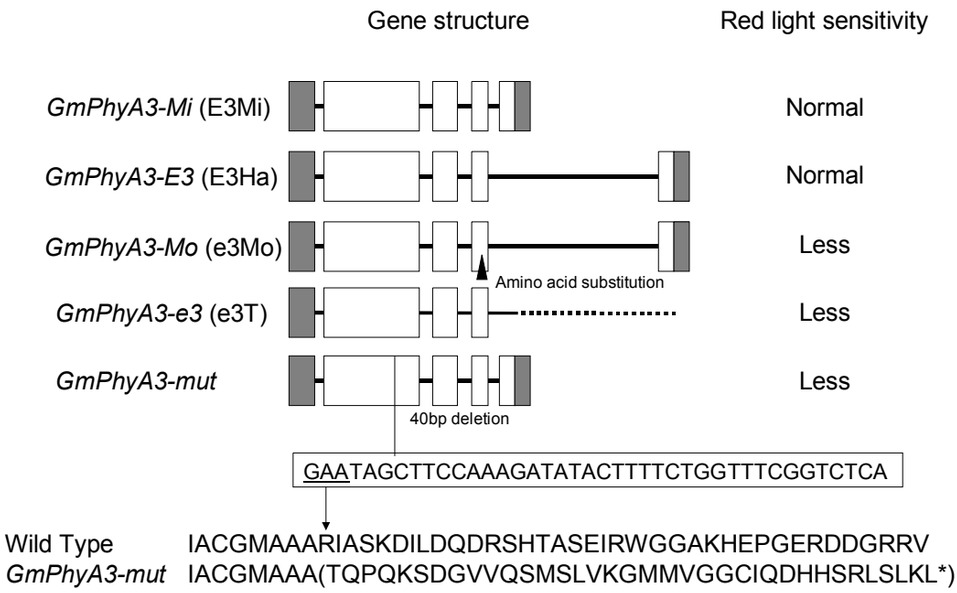

Fig. 10. Variation of gene structure of *GmPhyA3* and red light sensitivity. Open boxes, shaded boxes, and horizontal lines indicate exons, UTRs, and introns, respectively. The deleted region detected in Harosoy-*e3* is denoted by a dotted line. The deleted region in the middle part of the first exon of the mutant is shown at the bottom of the figure. The sequence of 40-bp deletion and the corresponding translated amino acid sequence in the wild-type plant are displayed. As a result of the deletion, a stop codon following the 36 amino acids at the deletion site appears in the mutant.

For allelism test among the *E3*, *FT3*, and *ft3* alleles, two population from crosses between Harosoy (*Dt1Dt1 e1e1 e2e2 E3E3*) and 6-22-*FT3* (*Dt1Dt1 ft1ft1 ft2ft2 FT3FT3*) and 6-22-*ft3* (*Dt1Dt1 ft1ft1 ft2ft2 ft3ft3*) were developed. Genetic analysis revealed that only the crossing population of Harosoy and 6-22-*ft3* showed a significant difference in genetic effect on flowering time. This indicated that the *E3* and *FT3* alleles had the same effect. The large insertion-like retrotransposon observed in *GmPhyA3-E3* and –Mo therefore might have no effect on the phenotype, whereas the one-amino-acid substitution observed in the *GmPhyA*-Mo might have weakened the effect of the *FT3* allele.

Since Cober's study (1966b) indicated that the *E3* allele exerted a large effect under FLD, the sensitivity to FLD conditions between the three NILs (Harosoy and *–e3*, 6-22-*FT3* and *–ft3*, 1-146-*FT3* and *–ft3*) and the mutant line for the *GmPhyA3* gene were evaluated. While the flowering days of each line varied because of their different genetic backgrounds, the effect of the *E3*/*FT3* allele was enhanced under FLD conditions in all the NILs. The mutant line with *GmPhyA3*-mut flowered 15 days earlier than the original variety Bay under extended mercury-vapor lamp with high red/far-red (R/FR) conditions like FLD.
These results strongly suggest that *GmPhyA3* is the gene responsible for the locus *E3*/*FT3*.
We designated the type of gene structure of *GmPhyA3*-Mi, *GmPhyA3-E3*, *GmPhyA3*-Mo and *GmPhyA3-e3* as E3Mi, E3Ha, e3Mo and e3T, respectively, hereafter. Distribution of these alleles was investigated using several cultivars and lines covering all the maturity groups in Japan. Three primer pairs were designed for discrimination among E3Mi, E3Ha/e3Mo and e3T. The sequences of these primers are shown in Table 3 and the positions of these primers are indicated in Fig. 11. The e3Mo type was distinguished from E3Ha type by *Mse1* digestion of a PCR product using specific primers, E3_07666FW and E3_08417RV. PCR products or digested fragments were separated by 1% agarose gel electrophoresis. Among the 80 accessions randomly selected from Genebank of the National Institute of Agrobiological Sciences (NIAS) in Japan, the E3Mi and e3T types were equally abundant, while the E3Ha and e3Mo types seldom occurred.

Marker type	Primer name	5'-sequence
STS	E3_08557FW	TGGAGGGTATTGGATGATGC
	E3_09908RV	CTAAGTCCGCCTCTGGTTTCAG
	E3Ha_1000RV	CGGTCAAGAGCCAACATGAG
	e3T_0716RV	GTCCTATACAATTCTTTACGACG
CAPS_*MseI*	E3_07666FW	CTCGGATCTTGACAGCATCA
	E3_08417RV	CAACTGAACTGAAGACCCACAA

Table 3. The DNA markers for genotype analysis of the *E3* locus.

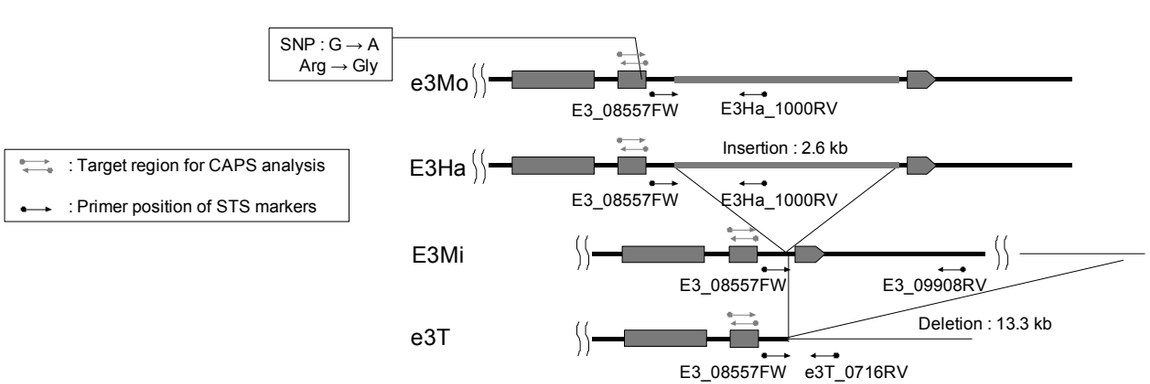

Fig. 11. Variation of *GmPhyA3* gene structure with the position and orientation of PCR primers.

The *E3* region were amplified with four pairs using the total DNA of 30 varieties, and four PCR products were designated as E3f1 to E3f4 (Table 4). The positions of these primers and PCR fragments are indicated in Fig. 12. Sequencing primers were constructed at intervals of

approximately 500 bases on the fragments. The PCR products were sequenced and alignment of the sequences was carried out. Days from sowing to the first flowering and alleles at the *E3* locus of 30 accessions are listed in Table 5. The results showed that E3Mi and e3T types were abundant, followed by the E3Ha type, while the e3Mo seldom occurred. No other type has been detected so far. The E3Ha type was detected in the accessions from China and North America. The latest flowering group harbored the E3Mi type, while the earliest flowering group, the e3T type. There was no clear relationship between the flowering time and the alleles at the *E3* locus in the other groups, because the flowering time depends on the combination of alleles at many loci.

Fragment	Primer name	5'-Sequence
E3f1	E3_00527FW	TCATGTCCAGCAACGCGTAGCATATT
	E3_03552RV	GAGACACTGCCATGCCATAA
E3f2	E3_03384FW	GGCTGCAATCATCATCACAT
	E3_06355RV	TCACTGCATCCAGTTCTTGC
E3f3	E3_05879FW	AACAAGGTGTGGCGATTAGG
	E3_08417RV	CAACTGAACTGAAGACCCACAA
E3f4(E3Mi/E3Ha/e3Mo)	E3_08115FW	TTGCATGAAGTTTTGGTTGC
	E3_09908RV	CTAAGTCCGCCTCTGGTTTCAG
E3f4(e3T)	E3_08115FW	TTGCATGAAGTTTTGGTTGC
	e3T_3544RV	AACTGGCCAAATCAAAGTGC

Table 4. Anchor primers for sequence analysis at the *E3* locus.

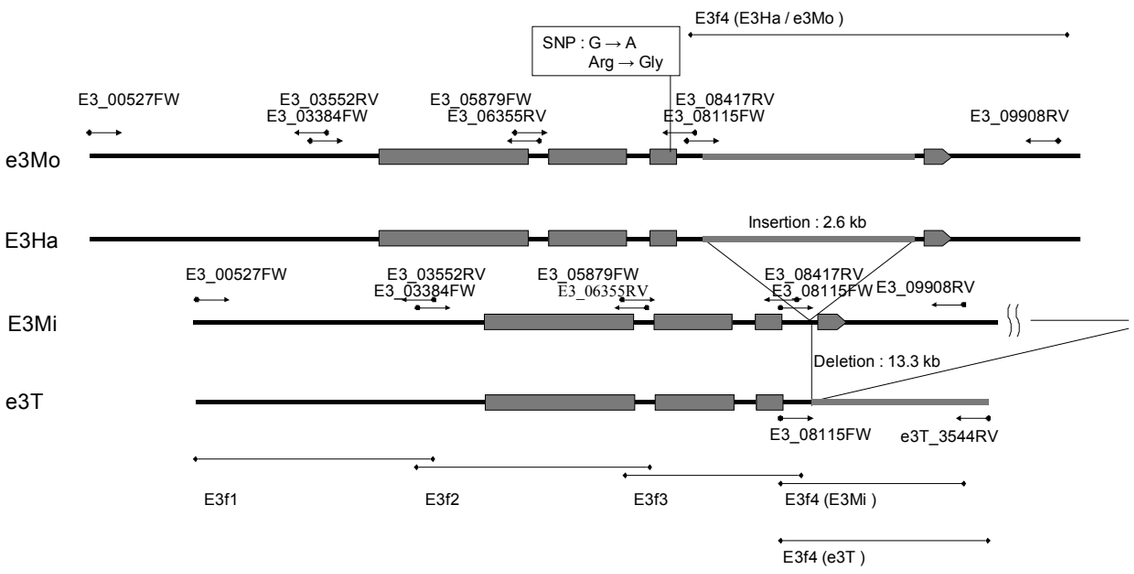

Fig. 12. Variation of *GmPhyA3* gene structure with the position and orientation of primers for PCR walking. The PCR products (E3fi, E3f2, E3f3 and E3f4) are shown at the bottom of the figure. As the e3T type lacked a portion of the third intron and the downstream region, the reverse primer for E3f4 was different from that for other alleles.

Accsession	Days of flowering	Genotype	Origin
Akisengoku	69.6	E3Mi	Japan
Tamanishiki	64.4	E3Mi	Japan
Oushokuakidaizu	62.2	E3Mi	Japan
Fukuyutaka	58.8	E3Mi	Japan
Hyoukeikuro 3	58.6	E3Mi	Japan
Akishirome	55.6	E3Mi	Japan
Misuzudaizu	52.6	E3Mi	Japan
Peking	52.2	E3Ha	China
akasaya(Nagano)	50.8	E3Mi	Japan
Ani	48.0	e3T	Japan
Enrei	46.0	e3T	Japan
Tamatsukuri	45.0	e3T	Japan
Norin 2	44.6	e3T	Japan
Harosoy_E1	44.4	E3Ha	Canada
Aohigu	43.3	e3T	Japan
Kin	42.2	e3T	Japan
Matsuura	42.0	E3Mi	Japan
Wasekin	41.2	e3T	Japan
NIL-13-E1	38.4	e3T	Japan
Harosoy_E2	37.8	E3Ha	Canada
Tokachinagaha	37.6	e3T	Japan
Moshidou Gong 503	36.8	e3Mo	China
Kingen 1	36.4	E3Ha	China
Toyomusume	36.2	E3Mi	Japan
Harosoy	34.8	E3Ha	Canada
NIL-11-e4	33.6	e3T	Japan
Harosoy_e4	33.6	E3Ha	Canada
NIL-13-e2	33.4	e3T	Japan
Harosoy_e3	31.2	e3T	Canada
Sakamotowase	28.6	e3T	Japan

Table 5. Days from sowing to the first flowering and alleles at the *E3* locus. These accessions were sown on June 10, 2008 at the NIAS

3.3 Toward the positional cloning of the *E1* gene

Among the the 156 RILs, a single line was identified as being heterozygous around the *FT1* locus (approximately 17 cM) based on the genotypes of the DNA markers, and was named RHL1-156 (Fig. 13). A population of 1,006 plants derived from RHL1-156 was used for fine mapping of the *FT1* locus. The *FT1* locus mapped between tightly linked DNA markers, Satt365 and GM169 (Fig. 14).

As it was difficult to find AFLP markers around this region in this population, we used mapping populations derived from a cross between Harosoy-*E1* (*E1E1 e2e2 E3E3*) and Harosoy (*e1e1 e2e2 E3E3*). The *E1* locus was mapped proximate to Satt557 between Satt365 and Satt289 using the F_2 population (117 plants). In a $F_{2:4}$ population (mixed progenies from F2 heterozygotes at Satt557 locus) with 1,442 individuals, seven recombinants were identified between Satt365 and Satt289. The flowering genotypes for each recombinant are confirmed by the progeny segregation pattern. With these recombinants, we were able to delimit the *E1* region to approximately 289 kb between markers A and E5 (Fig. 15). No recombination was found between markers S8 and Satt557, despite a physical distance of 133 kb. Because more than 40 genes were identified in the 289 kb region, more intense fine

mapping was conducted by using more than 13,000 plants with a protocol for large-scale genotyping of soybean seeds (Fig. 2) and a candidate gene was identified (Xia et al., unpublished).

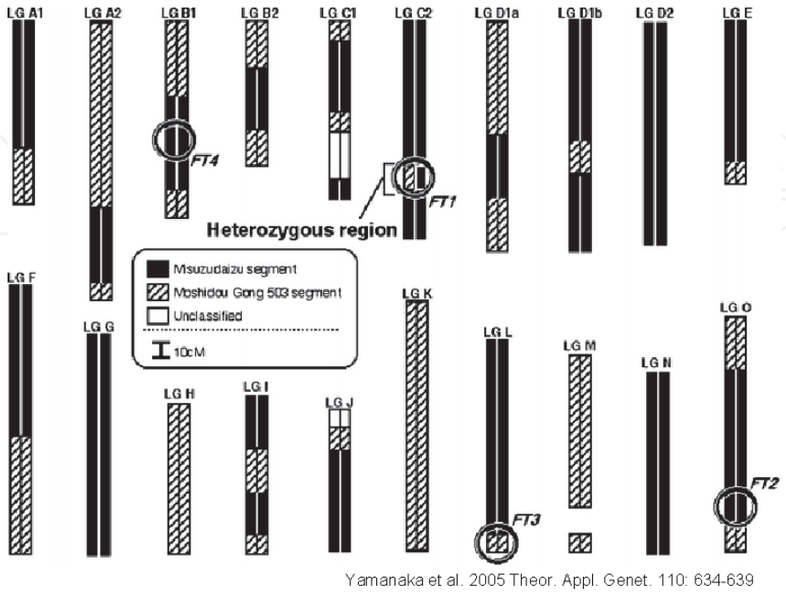

Fig. 13. Graphical genotype of RHL1-156. Solid bars and bars with slanted lines represent Misuzudaizu and Moshidou Gong 503 homozygous segments, respectively. Open bars represent unclassified segments. Putative location of each QTL is circled.

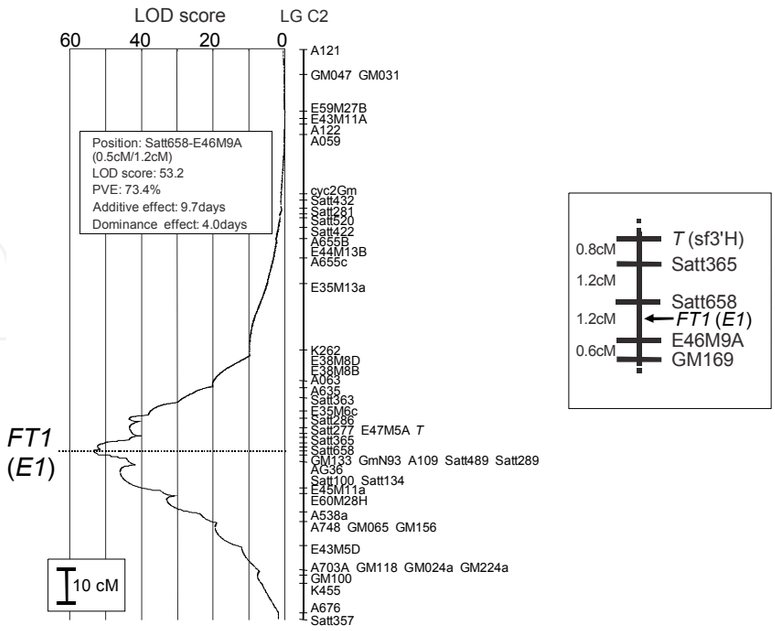

Fig. 14. QTL analysis for the *FT1* locus. LOD scores calculated by interval mapping are shown in the left panel. Close-up of the *FT1* region is highlighted in the right panel.

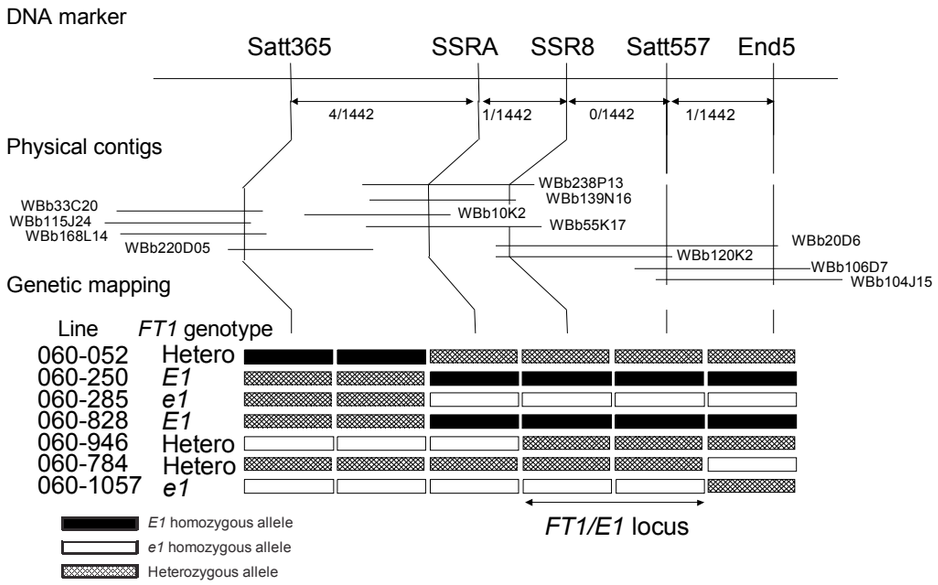

Fig. 15. Fine mapping of the *FT1/E1* locus. *E1* homozygous, *e1* homozygous and heterozygous genotypes are shown by solid, open and meshed boxes, respectively. The *FT1* genotype of each recombinant was identified by progeny test.

The *FT1* locus was genetically mapped into the semi-central domain of linkage group C2 (Fig. 3) and was included in the pericentromeric region of chromosome 06 (http://www.phytosome.net/). In the heterochromatic regions, the ratio of physical to genetic distance is 3.5Mb/cM in comparison of 197 Kb/cM in euchromatic regions (Schmutz et al., 2010). The responsible gene for *FT1/E1* locus is characterized by relatively lower mRNA abundance. In fact, no EST data of the *FT1/E1* gene could be retrieved from public databases. The gene encodes a novel small protein and is unique in the sense of no apparent orthologs in model plants *Arabidopsis* or rice. We are analyzing the ligands of this protein and the interaction with DNA sequences.

4. Putative pathway of flowering time in soybean

The responsible gene for the *E4* locus was identified as *GmPhyA2* through the candidate gene approach (Liu et al., 2008). At the *e4* allele, a Ty1/copia-like retrotransposon was inserted in exon 1 of the gene, which resulted in dysfunction of the gene and photoperiod insensitivity. Similarly, natural and artificial mutations of *GmPhyA3* resulted in weak or complete loss of photoperiod sensitivity (Watanabe et al., 2009). The *FT* homologs in soybean have been identified (Kong et al., 2010) and two of them, *GmFT2a* and *GmFT5a*, were highly upregulated under SD conditions and showed diurnal expression patterns with the highest expression 4h after dawn. Under LD conditions, expression of *GmFT2a* and *GmFT5a* was downregulated and did not follow a diurnal pattern. Ectopic expression analysis in *Arabidopsis* confirmed that both *GmFT2a* and *GmFT5a* had the same function as *Arabidopsis FT*. A double-mutant (*e3e3 e4e4*) for *GmPhyA2* and *GmPhyA3* expressed high levels of *GmFT2a* and *GmFT5a* under LD conditions (18-h light) with an R: FR ratio of 1.2, and it flowered slightly earlier under LD than the wild type (*E3E3 E4E4*) grown under SD. The expression levels of *GmFT2a* and *GmFT5a* were regulated by PHYA-mediated

photoperiodic regulation system, and the *GmFT5a* expression was also possibly regulated by photoperiod-independent system in LD.

GI have the conserved function of controlling the expression of the *FT* gene in *Arabidopsis*, rice and pea (Hayama et al., 2003; Mizoguchi et al., 2005; Hecht et al., 2007). We analyzed the expression of *GmFT2a* and *GmFT5a* at 9:00 a.m. 4 weeks after sowing under natural day-length conditions using *E2* (*FT2*) NILs in which photoperiod changed from LD to SD. A clear association between the *GmFT2a* expression level and early flowering phenotype was observed in both NILs. However, there was no significant difference in the *GmFT5a* expression levels between these NILs. These results suggested that *GmGIa* probably controlled flowering time through the regulation of *GmFT2a*. The recessive alleles of the *E2* (*FT2*) locus were perhaps unable to suppress *GmFT2a* expression and resulted in the early flowering phenotype.

There are strong interaction among the effects of *E1* (*FT1*) and *E2* (*FT2*), *E1* (*FT1*) and *E3* (*FT3*) (Yamanaka et al. 2000; Watanabe et al. 2004). The *e3e3* recessive homozygote can initiate flowering under R-enriched LD, but the *e3e3* genotype is necessary for plants with *e4* mutant allele to flower under FR-enriched LD. In the mapping population with *e3* background, photoperiodic insensitivity could occur in either genotypes of *e1E4*, *E1e4* or *e1e4* (Abe et al., 2003). These results suggest that *E1*, *E2*, *E3* and *E4* might concurrently mediate photoperiodic flowering in a shared pathway. The expression of the candidate gene for the *E1* locus was found to be repressed under SD. Under SD conditions, *E3*/*E4*-mediated photoperiodic regulation system up-regulates the expression of *GmFT2a* and *GmFT5a* possibly through the repression of the *E1* gene (Fig. 16). The *E2* locus also might control the *GmFT2a* expression through the *E1* gene.

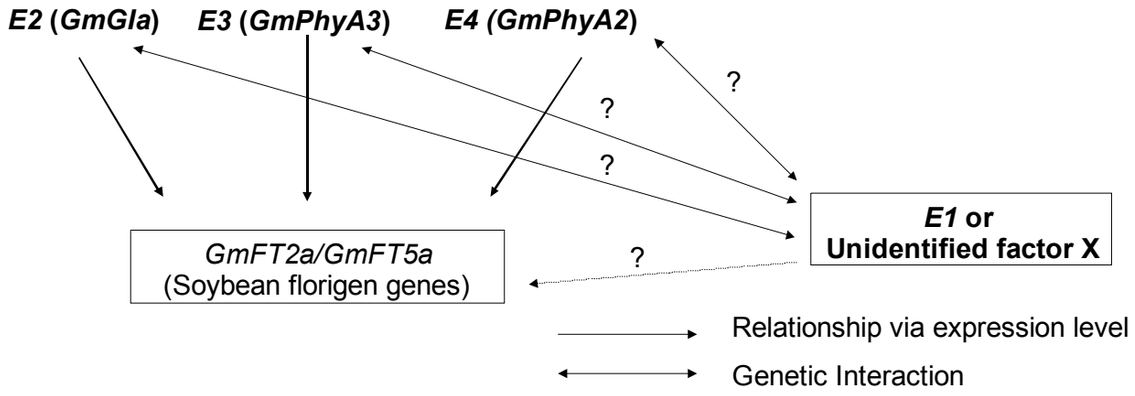

Fig. 16. A putative network of flowering time genes in soybean.

5. Conclusion

We successfully identified the responsible genes for the *E1*, *E2* and *E3* by positional cloning strategy and proposed a tentative flowering time gene network in soybean based on interaction of these genes. We used RHLs derived from RIL for fine mapping a single QTL effectively. An RHL harbors a heterozygous region where the target QTL is located and a homozygous background in most other regions of the genome. Novel DNA markers tightly linked to the locus were developed based on AFLP between the NILs of the locus derived from an RHL. A large-scale population derived from RHLs was used to locate the target

locus precisely. We developed manual large-scale genotyping of seeds, in which powdered cotyledon was obtained by drilling a hole on the surface of seed without any damage to the embryonic axis. Recombinants carrying crossovers in the target region were selected based on genotypes of DNA markers around the region. Genotypes of the flowering time locus of recombinants were determined by progeny test and identified the cosegregated region based on these genotypes. Physical contigs were constructed with BAC/TAC clones screened by SCAR markers converted from these AFLP fragments. By sequencing the BAC contig covering the cosegregated region, we identified the candidate genes. Confirmation of the responsible gene was performed by investigation of association between natural and induced variation of the candidate gene structures and flowering time. Mutant screening was carried out with TILLING using X-ray irradiated or EMS treated mutant libraries. The interactions between the identified genes were analyzed using several NILs and segregating population for the *E* loci. A tentative flowering time network in soybean was proposed taking into consideration the possible functions of responsible genes for *E1*, *E2*, *E3* and *E4* loci and *GmFT*s. Further characterization of other *E* loci is necessary to reveal the molecular mechanism of flowering in soybean.

Recently, soybean genome sequence has been reported (Shumutz et al., 2010) and a large number of SSR (Song et al., 2010) and SNP (Hyten et al., 2010a; Lam et al., 2010) markers has been developed. New high-throughput sequencing technologies, and multiplex assays for genotyping a huge number of SNPs have become available. These technologies and information will accelerate the identification of responsible genes for agriculturally important loci. But methods and materials to precisely locate the target loci in the genome are still important. Moreover, variation of regional genome structure and gene content (Kim et al., 2010 ; William et al., 2010; Xia et al., unpublished) will need the sequencing of genome clones covering the target region.

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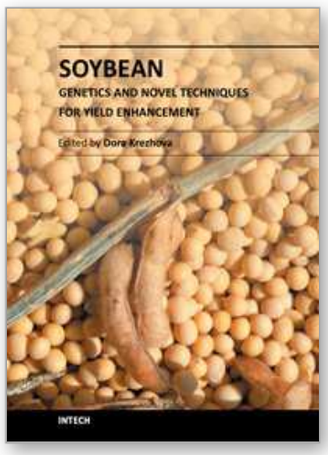

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This book presents the importance of applying of novel genetics and breading technologies. The efficient genotype selections and gene transformations provide for generation of new and improved soybean cultivars, resistant to disease and environmental stresses. The book introduces also a few recent modern techniques and technologies for detection of plant stress and characterization of biomaterials as well as for processing of soybean food and oil products.

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