ORIGINAL ARTICLE

GmFtsH9 expression correlates with in vivo photosystem II function: chlorophyll *a* fluorescence transient analysis and eQTL mapping in soybean

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Abstract Filamentation temperature-sensitive H (FtsH) is an ATP-dependent zinc metalloprotease involved in diverse biological functions. There are 12 FtsH proteins in Arabidopsis, among which AtFtsH2 plays an important role in regulating the turnover of photosystem II (PSII) reaction center D1 protein and the development of the photosynthetic apparatus. Here, we have identified 11 FtsH genes in the soybean genome by a bioinformatics approach. These soybean FtsH genes corresponded to seven Arabidopsis FtsH genes, suggesting that the main characteristics of soybean FtsH genes were formed before the evolutionary split of soybean and Arabidopsis. Phylogenetic analyses allowed us to clone a soybean AtFtsH2-like gene designated as *GmFtsH9*. The predicted protein of *GmFtsH9* consists of 690 amino acids and contains three typical FtsH proteins conserved domains. The expression level of *GmFtsH9* was determined in a soybean recombinant inbred line population under a pot experiment conducted for measuring chlorophyll *a* fluorescence transient parameters, photosynthetic CO_2 fixation rate (P_N), and seed yield. Expression quantitative trait loci (eQTL) mapping revealed two *trans*-acting eQTLs for *GmFtsH9*. The significant correlation of gene expression level with chlorophyll *a* fluorescence transient parameters and the presence of overlapping eQTL (QTL) between gene expression level and chlorophyll *a* fluorescence transient parameters indicated that *GmFtsH9* could be involved in regulating PSII function. These results further lead to the understanding of the mechanism underlying FtsH gene expression, and contribute to the development of marker-assisted selection breeding programs for modulating soybean FtsH gene expression.

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Electronic supplementary material The online version of this article (doi:10.1007/s00425-011-1445-5) contains supplementary material, which is available to authorized users.

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Abbreviations

ABS/RC	Light energy absorbed by per RC
eQTL	Expression quantitative trait locus
ET_o/TR_o (ψ_{Eo})	Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-
F_v/F_m	Ratio of variable fluorescence to maximum fluorescence in the dark-adapted state, related to maximum quantum yield of PSII primary photochemistry
F_v'/F_m'	Ratio of variable fluorescence to maximum fluorescence in the light-adapted state,

related to the maximum quantum yield of PSII primary photochemistry

JIP-test	A procedure for quantification of prompt fluorescence transients
PI_{ABS}	Performance index on absorption basis, $PI_{ABS} = (RC/ABS)[\varphi_{Po}/(1 - \varphi_{Po})][\psi_{Eo}/(1 - \psi_{Eo})]$
P_N	Photosynthetic CO_2 fixation rate
ΦPSII	Actual quantum yield of PSII primary photochemistry in the light-adapted state
qN	Non-photochemical quenching coefficient
qP	Photochemical quenching coefficient
QTL	Quantitative trait locus
RC	Reaction center of PSII
RE_o/ET_o (δ_{Ro})	Probability that an electron beyond Q_A^- reduces photosystem I acceptors
RIL	Recombinant inbred line
TR_o/ABS (φ_{Po})	Flux ratio of trapping per absorption, $\varphi_{Po} \equiv 1 - F_0/F_M = F_V/F_M$

Introduction

Filamentation temperature-sensitive H (FtsH) protein is a membrane-anchored metalloprotease that belongs to the AAA (ATPase associated with diverse cellular activities) protein family (Ogura and Wilkinson 2001). It has a single AAA domain, two transmembrane helices in its N-terminus, and a zinc-binding site in its C-terminus. FtsHs are ubiquitous proteins found in prokaryotes and in the mitochondria and chloroplasts of eukaryotes. Since they were first identified in *E. coli* (Santos and De Almeida 1975), FtsH proteins have been identified in many prokaryotes and eukaryotes. The first FtsH protein in plants was reported in spinach chloroplasts (Lindahl et al. 1996), and FtsH genes were subsequently isolated and characterized in Arabidopsis (Lindahl et al. 1996), rice (Yu et al. 2005), tobacco (*Nicotiana tabacum*) (Seo et al. 2000), alfalfa (*Medicago sativa*) (Ivashuta et al. 2002), bell pepper (*Capsicum annum*) (Hugueney et al. 1995), pea (*Pisum sativum*) (Kolodziejczak et al. 2002), spinach (Yoshioka et al. 2006), tomato (Sun et al. 2006), and maize (Yue et al. 2010). One FtsH gene exists in bacterial genomes (Schumann 1999), 4 in cyanobacteria (Mann et al. 2000), 12 in *Arabidopsis thaliana* (Yu et al. 2004), and at least 9 in rice (Yu et al. 2005). It has been proposed that the greater number of FtsH genes in eukaryotes than prokaryotes correlates with the evolution of oxygenic photosynthesis (Yu et al. 2005).

The FtsHs identified thus far are involved in diverse biological functions. In *E. coli*, FtsH degrades unwanted and damaged membrane proteins, as well as soluble signaling factors, and thus is essential for cell division (Ito and Akiyama 2005). In chloroplasts, FtsH has a role in photosystem II (PSII) repair, where it degrades photooxidatively damaged D1 proteins (Kato and Sakamoto 2009). The deletion of one of the thylakoidal FtsHs in *Synechocystis* species PCC 6803 resulted in slower D1 degradation and impairment of the PSII repair cycle under high light conditions (Silva et al. 2003). Arabidopsis mutants lacking FtsH2 or FtsH5 are more prone to PSII photoinhibition, and D1 degradation is impaired (Kato et al. 2009). In spinach, FtsH is involved in D1 turnover in response to heat stress (Yoshioka et al. 2006). FtsHs also degrade the unassembled Rieske Fe–S proteins of the cytochrome *b6f* complex (Ostersetzer and Adam 1997) and the light-harvesting complex of PSII under conditions of high light and senescence (Zelisko et al. 2005). In addition, FtsHs have a role in phytochrome A-mediated signal transduction (Tepperman et al. 2001), the hypersensitive response against tobacco mosaic virus infection (Seo et al. 2000), thermotolerance (Chen et al. 2006), and chloroplast biogenesis (Zaltsman et al. 2005).

Expression patterns of FtsH genes in plants have been widely examined. Most FtsH genes in Arabidopsis respond to high light (Sinvany-Villalobo et al. 2004). An *FtsH*-like gene in alfalfa has been shown to be regulated either by low temperature or light (Ivashuta et al. 2002). An *FtsH*-like gene in tomato was shown to be induced by heat (Sun et al. 2006), and for the two identified *FtsH*-like genes in maize, one was markedly upregulated by water deficit stress and abscisic acid treatment, while the other was constitutively expressed under all tested stressful conditions (Yue et al. 2010). Transcript levels of genes can be influenced not only by the external environment, but also by genetic determinants. The approach of "genetical genomics" can identify the genetic determinants of gene transcript levels (Jansen and Nap 2001). This approach treats mRNA expression levels as quantitative traits in a segregating population and maps expression quantitative trait loci (eQTLs) that control expression levels in vivo. For almost any gene analyzed in a segregating population, eQTL analysis can identify the genomic regions influencing its expression level. The genetical genomics approach has been successfully employed for identifying eQTLs regulating gene expression (Potokina et al. 2006; Yin et al. 2010a).

Chlorophyll *a* fluorescence can provide qualitative and quantitative information about photosynthetic processes in chloroplasts, and has been used to investigate PSII function (Papageorgiou and Govindjee 2004). When dark-adapted photosynthetic samples are exposed to light, chlorophyll *a* fluorescence transient is observed, known as the Kautsky effect. For higher plants and algae, this transient shows a

fast increasing phase followed by a slow decreasing phase (Stirbet and Govindjee 2011). The fast phase is labeled as OJIP, where O is for origin, the first measured minimal level, J and I are intermediate levels, and P is the peak. The slow phase is called PSMT, where S stands for semi-steady state, M for a maximum, and T for a terminal steady-state level. Many empirical parameters have been derived from the chlorophyll *a* fluorescence transient (Paillotin 1976; Bilger and Schreiber 1986; Genty et al. 1989). These include the ratio of variable fluorescence to maximum fluorescence in the dark-adapted state (F_v/F_m) and the light-adapted state (F_v'/F_m'), the actual quantum yield of PSII primary photochemistry in the light-adapted state (ΦPSII), the non-photochemical quenching coefficient

(qN), and the photochemical quenching coefficient (qP). Except for F_v/F_m, which only extracts data from the OJIP transient, the other empirical parameters mentioned above need to incorporate the information from the PSMT transient (for an explanation of these empirical parameters, see Table 1).

Based on several assumptions, some theoretical chlorophyll fluorescence parameters that correlate with the biophysical processes taking place in photosynthetic apparatus, namely the so-called JIP parameters, have been developed using a procedure called the JIP-test (Strasser et al. 2004). These parameters include ABS/RC (light energy absorbed per reaction center, RC), TR_o/ABS (φ_{Po}) (flux ratio of trapping per absorption), ET_o/TR_o (ψ_{Eo})

Table 1 Equations and definitions of the chlorophyll *a* fluorescence parameters (Paillotin 1976; Bilger and Schreiber 1986; Genty et al. 1989; Strasser et al. 2004) measured and analyzed in this study

Data extracted from the fluorescence transient OJIP measured with Handy-PEA and the so-called JIP parameters	
$F_{20\mu s}$	Minimal reliable recorded fluorescence at 20 μs
$F_{300\mu s}$	Fluorescence intensity at 300 μs
$F_J \equiv F_{2ms}$	Fluorescence intensity at the J-step (2 ms) of OJIP
$F_I \equiv F_{30ms}$	Fluorescence intensity at the I-step (30 ms) of OJIP
F_P	Maximal recorded fluorescence intensity at the peak P of OJIP
$F_0 \cong F_{20\mu s}$	Minimal fluorescence (all PSII RCs are assumed to be open)
$F_M (=F_P)$	Maximal fluorescence, when all PSII RCs are closed (equal to F_P when the actinic light intensity is above 500 μmol photons m^{-2} s^{-1} and provided that all RCs are active as Q_A reducing)
$F_V \equiv F_M - F_0$	Maximal variable fluorescence
$M_0 \equiv 4(F_{300\mu s} - F_{50\mu s})/(F_M - F_{50\mu s})$	Approximated initial slope (in ms^{-1}) of the fluorescence transient normalized on the maximal variable fluorescence F_V
$V_t \equiv (F_t - F_0)/(F_M - F_0)$	Relative variable fluorescence at time *t*
$\varphi_{Po} \equiv TR_0/ABS = F_V/F_M$	Maximum quantum yield for primary photochemistry
$ABS/RC = M_0(1/V_J)(1/\varphi_{Po})$	Absorption flux (of antenna chlorophylls) per RC
$\psi_{Eo} \equiv ET_0/TR_0 = (1 - V_J)$	Efficiency/probability for electron transport (ET), i.e. efficiency/probability that an electron moves further than Q_A^-
$\delta_{Ro} \equiv RE_0/ET_0 = (1 - V_I)/(1 - V_J)$	Efficiency/probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side (RE)
$PI_{ABS} = (RC/ABS)[\varphi_{Po}/(1 - \varphi_{Po})][\psi_{Eo}/(1 - \psi_{Eo})]$	Performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors
Data extracted from the fluorescence transient measured with PAM2100 and the derived empirical parameters	
F_o	Initial fluorescence in dark-adapted leaf
F_m	Maximal fluorescence in dark-adapted leaf
F_s'	Steady-state fluorescence in light-adapted leaf
F_o'	Minimal fluorescence in light-adapted leaf
F_m'	Maximal fluorescence in light-adapted leaf
$F_v/F_m = (F_m - F_o)/F_m$	Ratio of variable fluorescence to maximum fluorescence in dark-adapted state, related to maximum quantum yield of PSII primary photochemistry
$F_v'/F_m' = (F_m' - F_o')/F_m'$	Ratio of variable fluorescence to maximum fluorescence in light-adapted state, related to the maximum quantum yield of PSII primary photochemistry
$qP = (F_m' - F_s')/(F_m' - F_o')$	Photochemical quenching coefficient
$qN = 1 - [(F_m' - F_o')/F_o']/[(F_m - F_o)/F_o]$	Non-photochemical quenching coefficient
$\Phi PSII = (F_m' - F_s')/F_m'$	Actual quantum yield of PSII primary photochemistry in light-adapted state

(probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-), RE_o/ET_o (δ_{Ro}) (probability that an electron beyond Q_A^- reduces photosystem I acceptors), and PI_{ABS} (performance index on absorption basis). These JIP parameters only extract data from the OJIP transient (for an explanation of these JIP parameters, see Table 1).

The assumptions for the derivation of the JIP parameters were recently presented and discussed in detail by Stirbet and Govindjee (2011). Briefly, the variation of chlorophyll fluorescence intensity is mainly caused by the reduction of Q_A. PSII units are homogeneous, active, and not connected to each other. In the same physiological state, an open PSII center has a low fluorescence emission, and a closed PSII has a high fluorescence emission. The PSII antenna chlorophyll content of the measured sample approximates to the absorbed photon flux, and the phenomenological absorbed photon flux approximates to the F_o or F_m values. During the course of the OJIP transient, no significant change of physiological state occurs in the sample, the number of electrons transferred to Q_A is equal to the number of excitons trapped by PSII RCs, and the dissipated energy flux is directly proportional to the measured fluorescence. Furthermore, Q_A undergoes only a single turnover at the O–J part, as in the 3-(3′,4′dichlorophenyl)-1,1′dimethylurea (DCMU) treated sample, and at the initial part of the J-I phase the PQ-pool begins to be reduced. The inverse of the initial slope of the relative variable curve of a DCMU treated sample approximates the normalized area over the fluorescence transient (Stirbet and Govindjee 2011).

Soybean [Glycine max (L.) Merr.] is an essential source of seed protein and oil, with numerous uses in food, feed, and industrial applications, playing an important economic role in international trade. However, to date, no information concerning FtsH genes in soybean is available. In Arabidopsis, the most highly expressed FtsH gene, VAR2 (AtFtsH2), has been reported to be involved in the turnover of the PSII reaction center D1 protein, as well as in other processes required for the development and maintenance of the photosynthetic apparatus (Zaltsman et al. 2005; Kato et al. 2009; Liu et al. 2010; Zhang et al. 2010). In this study, we cloned a soybean FtsH gene, designated as GmFtsH9, which is homologous to AtFtsH2. To examine the modulation and function of GmFtsH9, eQTLs for this gene were mapped in a soybean recombinant inbred line (RIL) population and compared with the quantitative trait loci (QTLs) for several chlorophyll fluorescence parameters, photosynthetic CO_2 fixation rate (P_N), and seed yield that we had previously identified in the same population (Yin et al. 2010a, b). Our results show that the expression of GmFtsH9 is controlled by two trans-acting eQTLs and this gene could play an important role in regulating PSII function in soybean.

Materials and methods

Plant material and plant growth conditions

The RIL population used to map eQTLs was derived from a cross between soybean varieties Kefeng No.1 and Nannong1138-2 (see also Yin et al. 2010a, b). This population consists of 184 F7:11 lines derived via single-seed descent at the National Center for Soybean Improvement of China. The RILs were grown in pots in 2007. The two parental lines, Kefeng No.1 and Nannong1138-2, were grown in pots in 2007, and in both pots and the field in 2008.

The planting experiment was conducted under natural conditions at the Jiangpu Experimental Station, Nanjing Agricultural University. To control environmental effects on phenotypic evaluation, the RILs and the two parental lines were sown at different times, according to their maturity observed from three previous years (data not shown), such that when leaves were collected for gene expression determination, the lines were at a similar growth stage.

For the pot experiments, plants were individually grown in plastic pots containing 3.0 L of soil. The genotypes were grown in a completely randomized design with six replications (each consisting of one plant per pot). In 2007, the RILs and the two parental lines were sown on 8, 15, and 22 May; in 2008, the two parental lines, Nannong1138-2 and Kefeng No.1, were sown on 12 and 19 May, respectively. For the field experiment, the two parental lines were grown in hill plots in a completely randomized design with five replications (each consisting of a hill of three plants). Hills were planted every 40 cm along rows spaced 60 cm apart. Sowing was carried out on 12 and 19 June 2008 for Nannong1138-2 and Kefeng No.1, respectively. In this study, the environment for pot experiment in 2007 was designated as E1, this in 2008 as E2, and this for field experiment in 2008 as E3.

Sufficient nutrition and water was supplied throughout the experiments to avoid potential nutrient and drought stresses. The daily mean temperature and daily light global radiation throughout the experiments are shown in the supplemental Fig. S1, Fig. S2 and Fig. S3 (data obtained from the local meteorological station of Nanjing, China). The average temperatures throughout the experiments of E1, E2, and E3 were 25.8, 25.7, and 26.6°C, respectively. The global light radiation levels were 15.0, 16.3, and 15.1 MJ m^{-2} day^{-1}, respectively. The average temperatures 14 days before leaves were collected (see below) were 29.2°C (E1), 28.2°C (E2) and 24.0°C (E3). The average global light radiation levels 14 days before leaves were collected were 18.0, 15.3, and 13.9 MJ m^{-2} day^{-1} for the three experiments, respectively. At the R6 stage of development, the mature upper third leaves were collected

individually from three plants of each RIL or parental line in the morning (9:00–11:30 a.m.) on a sunny day, frozen immediately in liquid nitrogen, and stored at −80°C until further use. In E1, the temperature of the day (6 August 2007) was 29.6°C and the global light radiation level was 23.7 MJ m^{-2}. In E2, the temperature of the day (5 August 2008) was 29.8°C and the global light radiation level was 24.9 MJ m^{-2}. In E3, the temperature of the day (17 September 2008) was 26.2°C and the global light radiation level was 16.6 MJ m^{-2}.

Bioinformatic and phylogenetic analysis of soybean FtsH genes

The sequences of 12 Arabidopsis FtsH proteins (Yu et al. 2004) were downloaded from http://www.ncbi.nlm.nih.gov/. To identify FtsH genes in soybean, BLASTP searches targeted to the soybean genome sequence were performed with the Arabidopsis FtsH proteins as queries using Phytozome Search Tools (http://www.phytozome.net). FtsH-like proteins were downloaded, and then Pfam (Finn et al. 2010) was used to predict the three diagnostic signatures of FtsH proteins: two N-terminal transmembrane domains, a single AAA cassette, and a zinc-binding site (HEXXH) (Yu et al. 2004). If a protein sequence contained all the three signatures, it was thought to be soybean FtsH protein. The new FtsH sequences detected in soybean were used reiteratively to search the soybean proteome databases. The deduced nucleotide and protein sequences of soybean FtsH genes were downloaded from the Phytozome database (http://www.phytozome.net). EST searches with the soybean FtsH full-length cDNA sequences as queries were performed using the BLASTN tool against the soybean EST database in NCBI. A phylogenetic tree was constructed using the MEGA program (version 4.1).

Isolation and sequence analysis of a full-length *GmFtsH9* gene in soybean

Based on the information from in silico cloning, gene-specific primers (5′-GAGGTTCTCTTCTTTATACGGA-3′, 5′-TGTGGAGAGCTACACTACATTG-3′) for *GmFtsH9* were designed for PCR amplification. The collected leaf samples from the two parents of the RIL population, Kefeng No.1 and Nannong1138-2, were used for gene cloning. PCR products obtained from cDNA prepared from leaves were separated on 1% agarose gels and purified with a gel extraction kit (HuaShun, Shanghai), according to the manufacturer's protocol. The purified product was cloned into the pGEM-T vector (Promega) and sequenced (Invitrogen, Shanghai). Sequence analysis was performed using DNAMAN software (http://www.lynnon.com). The

molecular weight of the predicted protein was calculated using the BioXM program (version 2.6) (http://www.bio-soft.net/format/bioxm.htm). The sequence alignment was performed using ClustalX program (version 1.81) and viewed with GeneDOC software (version 2.6).

Determining *GmFtsH9* expression levels

The expression level of *GmFtsH9* was determined using real-time RT-PCR assays using the ABI 7500 system (Applied Biosystems). Each reaction contained 50 ng first-strand cDNAs, 0.5 μL 10 μmol/L gene-specific primers (Q1 and Q2), and 10 μL real-time PCR SYBR MIX (QPK-201 TOYOBO). Amplification conditions were 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The soybean endogenous reference gene *tubulin* was used as a control, with primers E1 and E2, to test for sample-to-sample variation in the amount of cDNA (Potokina et al. 2006; Yin et al. 2010a). The cDNA from soybean Kefeng No.1, a common experimental sample, was used as the calibrator in each RT-PCR plate. Two technical replicates of each reaction were performed, and data were analyzed using the ABI 7500 system v1.4.0. Normalized expression for each line was calculated as $\Delta\Delta CT = (C_{T,Target} - C_{T,tubulin})_{genotype} - (C_{T,Target} - C_{T,tubulin})_{calibrator}$. The primers used were as follows: Q1 5′-CCTCCTGGAACTGGGAAGACAC-3′, Q2 5′-CGAGAAGCACCAACACCAACAA-3′, E1 5′-GGAGTTCACAGAGGCAGAG-3′ and E2 5′-CACTTACGCATCACATAGCA-3′. Two samples from two plants of each genotype were used to determine the relative expression level of *GmFtsH9*. The results presented are the means of two biological replicates for each genotype.

Statistical analysis and eQTL mapping

In the same soybean RIL population grown in E1, as described above, we had measured TR_o/ABS, ET_o/TR_o, RE_o/ET_o, ABS/RC, PI_{ABS}, F_v/F_m, F_v'/F_m', qP, qN, $\Phi PSII$, and P_N at the R6 growth stage as well as seed yield at the final growth stage (Yin et al. 2010a, b). These collected data were re-analyzed to calculate correlation coefficients between these traits and the expression level of *GmFtsH9*. All phenotypic data were analyzed using SAS V9.0 (for Windows). SAS PROC CORR was used to calculate the Pearson phenotypic correlations between traits.

The mean value of the *GmFtsH9* expression level for each RIL and a genetic linkage map (Fu et al. 2006) were used for eQTL analysis. The linkage map of this RIL population covered 2,625.9 cM of the soybean genome, converged into 24 linkage groups consisting of 221 simple sequence repeat markers and one R gene

(resistance to soybean mosaic virus). The average distance between markers was 11.8 cM. The eQTL analysis was performed using QTL Cartographer version 2.5 (Wang et al. 2005). Model 6 of composite interval mapping was deployed for mapping eQTLs and estimating their effects. The control marker number and window size were set at 5 and 10 cM, respectively. The genome was scanned at 2 cM intervals, and the forward regression method was selected. The log of the odds (LOD) score for declaring a significant eQTL was 2.5 by permutation test analyses (1,000 permutations, 5% overall error level). To find as many putative eQTLs as possible, and to obtain a clearer understanding of the relationships among examined traits, a threshold of 2.0 for declaring an eQTL was employed. Low thresholds may not be useful in plant breeding programs, but they have been shown to help in understanding the relationships among various traits (Thumma et al. 2001).

The maximum LOD score along the interval was taken as the position of the eQTL, and the region in the LOD score within two LOD units of the maximum was taken as the confidence interval. Additive effects of detected eQTLs were estimated from composite interval mapping results as the mean effect of replacing both Nannong1138-2 alleles at the locus of interest by Kefeng No.1 alleles. Thus, for an eQTL to have a positive effect, the Kefeng No.1 allele must increase the trait value. The contribution of each identified eQTL to total phenotypic variance (R^2) was estimated by variance component analysis. eQTL nomenclature was adapted as follows: starting with 'q', followed by an abbreviation of the trait name, the name of the linkage group, and the number of eQTLs affecting the trait on the linkage group.

Results

Eleven structurally different FtsH genes are present in soybean

After surveying the soybean genome sequence (http://www.phytozome.net/), we identified 11 FtsH homologs, designated as *GmFtsH1* through *GmFtsH11* (Supplemental Table S1 and Fig. 1). The length of FtsH proteins in soybean ranged from 674 amino acids (AA) to 810 AA. Except for *GmFtsH11* detected on scaffold_28, the FtsH genes were distributed on nine of the 20 soybean chromosomes. Searching the EST database of soybean in NCBI showed that all these soybean FtsH genes matched at least one significant hit (score >600, *e* value equal to zero) (data not shown), indicating they are expressed and functional in soybean. Comparison of the exon/intron structure showed differences among the *GmFtsH* genes in terms of exon numbers and lengths of introns or exons (Fig. 1). The number of exons ranged from 4 to 15; however, most *GmFtsHs* had 4 or 5 exons, except for *GmFtsHs 1*, *6*, *8*, and *11*, which had 15, 8, 14, and 13 exons, respectively.

Figure 2 shows the phylogenetic relationships of the 11 *GmFtsH* genes compared to the 12 Arabidopsis *AtFtsH* genes and 4 *Synechocystis* FtsH genes. Full-length protein sequences were used in the analyses. We divided the FtsH proteins into five major groups (A–E) based on the phylogenetic tree. Group A and B contained FtsH proteins from Arabidopsis, soybean, and *Synechocystis*, suggesting that the main characteristics of GmFtsH genes in these two groups might have been established before the evolutionary split of the three examined species. Similarly, group D contained proteins from both Arabidopsis and soybean and
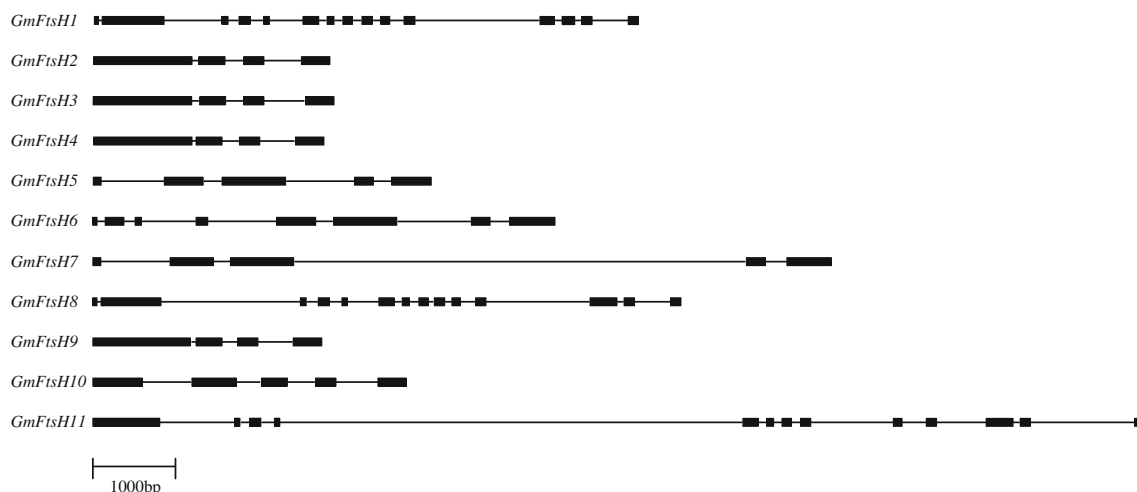

Fig. 1 Exon/intron structures of the soybean FtsH genes. *Boxes* and *lines* represent exons and introns, respectively. The *lengths of the boxes or lines* are proportional to the length of the corresponding exon or intron

might represent the main characteristics of GmFtsH before the evolutionary split of these two examined species. In contrast, the FtsH proteins in groups C and D came only from Arabidopsis, indicating that these two groups might have been independently formed in Arabidopsis from the other two species. Alternatively, soybean may have lost members of this group during the long period of evolution. Four pairs of paralogous proteins in soybean, GmFtsH2/3, GmFtsH4/9, GmFtsH1/8, and GmFtsH6/7, and only one pair of orthologous proteins for soybean and Arabidopsis, GmFtsH6/10, were found at the terminus of the phylogenetic tree, indicating that most of the FtsH genes in soybean had expanded in a species-specific manner, and probably only a few members originated from the common ancestral genes that existed before the evolutionary split of Arabidopsis and soybean. *GmFtsH6* and *GmFtsH7* are arrayed in tandem on the chromosome 12 (Supplemental Table S1), suggesting that they arose by a duplication event; in Arabidopsis, a single locus (*AtFtsH5*) corresponds to these two genes.

Cloning and sequence analysis of *GmFtsH9*

Among the 12 Arabidopsis FtsH genes, *AtFtsH2* is the most highly expressed FtsH gene and plays an important role in regulating PSII function (Zaltsman et al. 2005; Kato et al. 2009; Liu et al. 2010; Zhang et al. 2010). Among the 11 soybean FtsH genes, *GmFtsH9*, *GmFtsH4*, *GmFtsH2*, and *GmFtsH3* were most homologous to *AtFtsH2*; these 4 genes showed about 85% identity to *AtFtsH2* at the amino acid level, whereas the others showed much less identity to *AtFtsH2*, ranging from 50–70%. In this study, we focused on *GmFtsH9*. Based on the results of in silico cloning, a pair of gene-specific primers was designed to clone *GmFtsH9*, and one DNA fragment between 2,000 and 3,000 base pairs (bp) was amplified from soybean leaf cDNA. Sequencing results showed that the *GmFtsH9* cDNA consisted of 2,134 bp including a complete open reading frame (ORF) and partial untranslated sequences (GenBank accession number: JF780916). The predicted protein encoded by *GmFtsH9* was 690 AA, with a calculated molecular mass of 73.97 kDa. The protein sequence contains all the characteristic elements of ATP-dependent membrane-bound FtsH protease, such as the transmembrane domain, the AAA domain, and the zinc-binding site (Fig. 3).

Correlation between gene expression and chlorophyll *a* fluorescence parameters, P_N, and seed yield

Gene-specific primers were designed to determine the expression level of *GmFtsH9*. The inherent variation in the expression of housekeeping genes makes the use of a
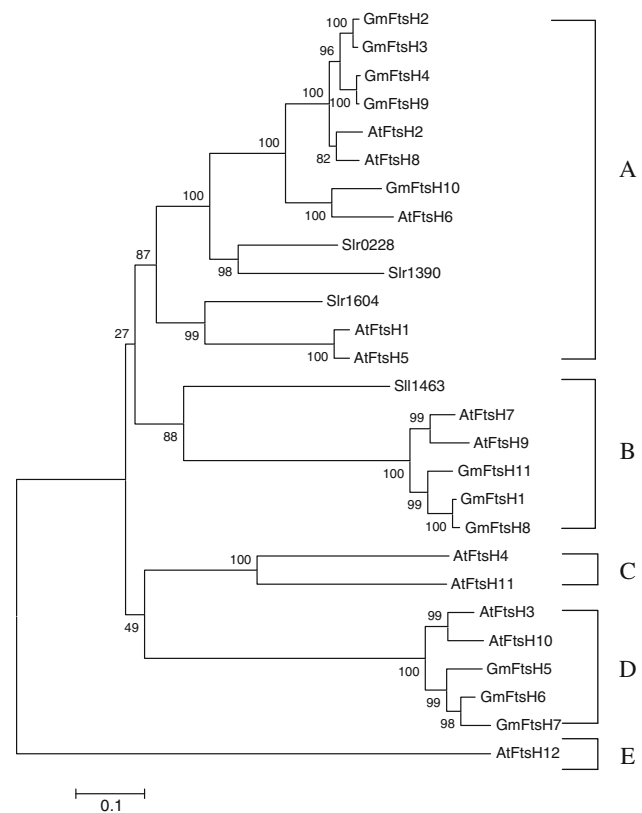

Fig. 2 Phylogenetic tree for Arabidopsis, Synechocystis and soybean full-length FtsH proteins. The tree was constructed by the neighbor-joining method using MEGA software (version 4.1). *Numbers* at branches represent percentage of bootstrap support from 1,000 resampling replicates, and the *scale bar* indicates branch length in terms of substitutions per site. The groups of homologous genes identified are indicated. GmFtsH1-11 (this report); AtFtsH1 (NP_564563); AtFtsH2 (O80860); AtFtsH3 (Q84WU8); AtFtsH4 (NP_565616); AtFtsH5 (Q9FH02); AtFtsH6 (NP_568311); AtFtsH7 (Q9SD67); AtFtsH8 (Q8W585); AtFtsH9 (Q9FIM2); AtFtsH10 (Q8VZI8); AtFtsH11 (Q9FGM0); AtFtsH12 (Q9SAJ3); Sll1463 (NP_440797); Slr0228 (NP_442160); Slr1390 (NP_440525); Slr1604 (NP_440330)

proper endogenous reference gene indispensable for accurate normalization of mRNA samples. For reliable estimation of the relative expression level of a target gene, the amplification efficiency of RT-PCR for an endogenous reference gene should be equal to that for the target gene. In this study, the soybean *tubulin* gene was used as an endogenous reference to examine the sample-to-sample variation in the amount of cDNA (Potokina et al. 2006; Yin et al. 2010a), because its amplification efficiency was similar to *GmFtsH9* (reflected by the slope of the lines shown in Fig. 4).

Gene expression level of *GmFtsH9* was measured in a set of RILs derived from soybean varieties Kefeng No.1 and Nannong1138-2. This trait showed a continuous distribution in the RIL population (data not shown), indicating that it was quantitatively inherited. Correlation analysis
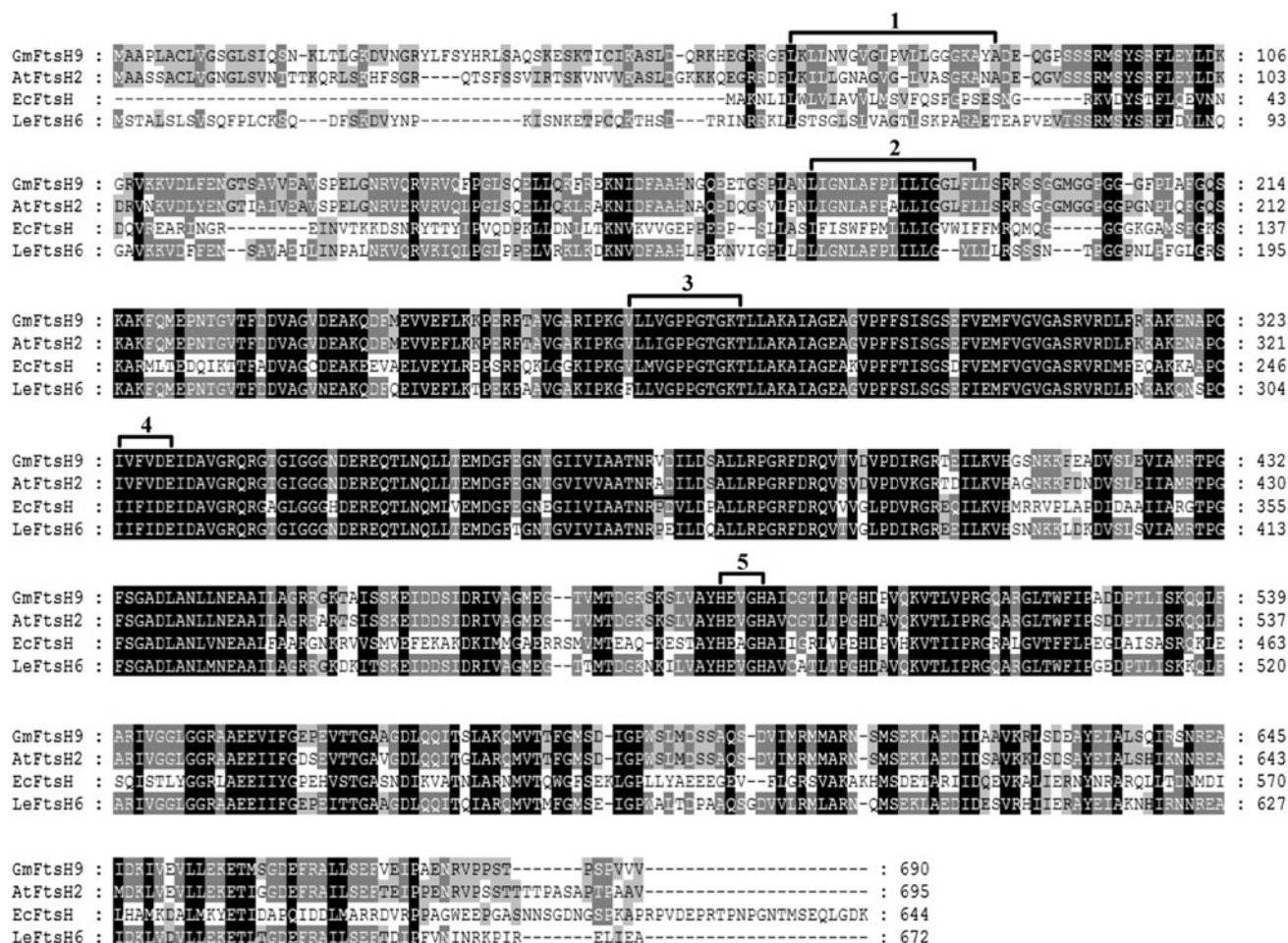
Fig. 3 Amino acid sequence alignment of FtsH proteins. Identical amino acids (single-letter code) are shown in white characters on black and similar amino acids are shaded in *gray*. The following sequences were aligned using the ClustalX program (version 1.81), and viewed by GeneDOC program (version 2.6). GmFtsH9, Soybean (this report: JF780916); AtFtsH2, *Arabidopsis thaliana* (O80860); EcFtsH, *E. coli* (P28691); LeFtsH6; *Solanum lycopersicum* (AB217916). The numerals indicate transmembrane regions (1 and 2), two regions of the ATP biding motif (3 and 4), and a Zn^{2+} binding motif (5)

between the expression level of *GmFtsH9* and chlorophyll *a* fluorescence parameters, P_N, and seed yield was performed (Table 2). The expression level of *GmFtsH9* showed a significantly positive correlation with TR_o/ABS, ET_o/TR_o, PI_{ABS}, F_v/F_m, F_v'/F_m', qN, and seed yield. However, there was no significant correlation between the expression level of *GmFtsH9* and RE_o/ET_o, ABS/RC, qP, ΦPSII, and P_N (see "Abbreviations").

eQTL mapping of *GmFtsH9* gene

We measured the expression level of *GmFtsH9* gene in the two varieties Nannong1138-2 and Kefeng No.1 at the R6 growth stage grown in E1, E2, and E3, using both semi-quantitative RT-PCR and real-time RT-PCR assay. The results showed that, although the expression level of

GmFtsH9 gene fluctuated in the different environments for each line, the difference in gene expression level between the two varieties Kefeng No.1 and Nannong1138-2 were stable, with the former having a higher level than the latter in all tested environments (Supplemental Fig. S4 and Fig. S5). This remarkable difference in *GmFtsH9* expression level between Kefeng No.1 and Nannong1138-2 provided a good genetic background for the subsequent eQTL mapping for this gene in the RIL population derived from these two varieties.

Two eQTLs were detected for expression level of *GmFtsH9*, on linkage groups A2 and D1b, respectively (Table 3; Fig. 5). eQTL *qFt9A2.1* explained 5.80% of the total phenotypic variance, with the alleles of Nannong1138-2 showing increased *GmFtsH9* expression level at this locus. eQTL *qFt9D1b.1* explained 7.76% of the
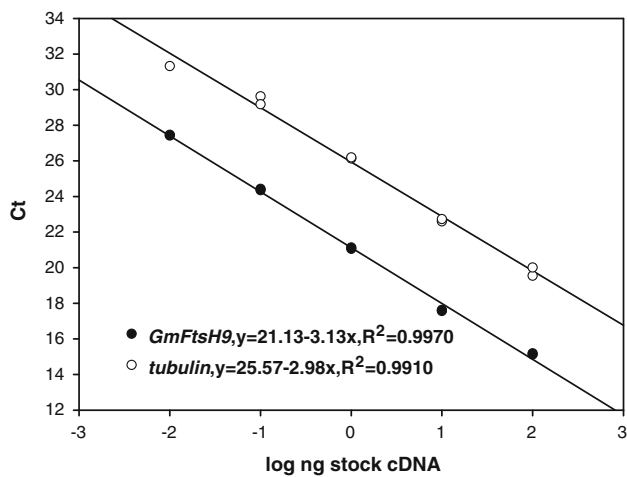
Fig. 4 Standard curves generated for the amplification of *GmFtsH9* and the endogenous reference gene *tubulin*. The plot of the log of target amount in five dilutions of the standard cDNA stock versus Ct represents a *straight line*, which can be described by the corresponding regression formulae. The slope of the curves reflects the amplification efficiency of the corresponding genes

phenotypic variance, with positive alleles from Kefeng No.1.

To examine the effect of *GmFtsH9* expression on chlorophyll fluorescence parameters, P_N, and seed yield, the eQTL data of the present study were compared with those QTLs identified previously for corresponding traits in the same material (Yin et al. 2010a, b). None of the eQTLs for *GmFtsH9* expression were co-localized with any of the QTLs for RE_o/ET_o, F_v/F_m, F_v'/F_m', qN, qP, $\Phi PSII$, P_N (see "Abbreviations"), and seed yield. In contrast, this analysis revealed the presence of an overlapping eQTL (QTL) between *GmFtsH9* expression and TR_o/ABS, ET_o/TR_o, ABS/RC, and PI_{ABS}, on the linkage group A2 (Fig. 5). eQTL *qFt9A2.1* was possibly co-localized with the QTLs for TR_o/ABS, ET_o/TR_o, ABS/RC, and PI_{ABS}, due to their overlapping confidence intervals on linkage group A2. In addition, the involved eQTL and QTLs showed the same additive direction, with Nannong1138-2 alleles at this chromosome region increasing the value of *GmFtsH9* expression, TR_o/ABS, ET_o/TR_o, ABS/RC, and PI_{ABS}.

Table 2 Correlation coefficients and significance of correlations between the expression level of *GmFtsH9* and chlorophyll *a* fluorescence parameters, P_N, and seed yield in the soybean recombinant inbred line (RIL) population

TR_o/ABS	ET_o/TR_o	RE_o/ET_o	ABS/RC	PI_{ABS}	F_v/F_m
0.370**	0.167*	−0.090	−0.095	0.230**	0.390**
F_v'/F_m'	qP	qN	$\Phi PSII$	P_N	Seed yield
0.208**	−0.130	0.238**	0.007	0.122	0.181*

TR_o/ABS flux ratio of trapping per absorption, *ET_o/TR_o*, probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-, *RE_o/ET_o* probability that an electron beyond Q_A^- reduces photosystem I acceptors, *ABS/RC* light energy absorbed by reaction center (RC), *PI_{ABS}* performance index on absorption basis, *Fv/Fm* the ratio of variable fluorescence to maximum fluorescence in the dark-adapted state, *Fv'/Fm'* the ratio of variable fluorescence to maximum fluorescence in the light-adapted state, *qP* photochemical quenching coefficient, *qN* non-photochemical quenching coefficient, *$\Phi PSII$* actual quantum yield of PSII primary photochemistry in the light-adapted state, *P_N* photosynthetic CO_2 fixation rate

* $P < 0.05$; ** $P < 0.01$

Table 3 Principal characteristics of eQTLs for the expression level of *GmFtsH9* in soybean

eQTL[a]	Linkage group[b]	Marker interval[c]	Position (cM)[d]	Confidence intervals[e]	LOD peak[f]	R^2 (%)[g]	Additive effects[h]
qFt9A2.1	A2	BE820148–sat_162	0.01	0–15.8	2.47	5.80	−0.0695
qFt9D1b.1	D1b	satt634–satt558	238.71	218.2–246.7	3.22	7.76	0.0795

[a] eQTLs are named: starting with 'q', followed by an abbreviation of the trait name, the name of the linkage group, and the number of eQTLs affecting the trait on the linkage group. *Ft9*, *GmFtsh9* expression level

[b] Linkage group (LG) on which eQTLs were mapped

[c] Marker intervals within which eQTLs were mapped

[d] Position from the first marker on each linkage group

[e] Confidence intervals were set as the map interval corresponding to a 2-LOD decline on either side of the LOD peak

[f] Maximum LOD score at the eQTLs position; LOD (Log10 of the likelihood odds ratio), the probability associated with the most likely location of the detected eQTLs

[g] Coefficient of determination or the percentage of the phenotypic variance that is explained by the detected eQTLs

[h] Estimated phenotypic effect of substituting both Nannong1138-2 alleles with Kefeng No.1 alleles at eQTLs
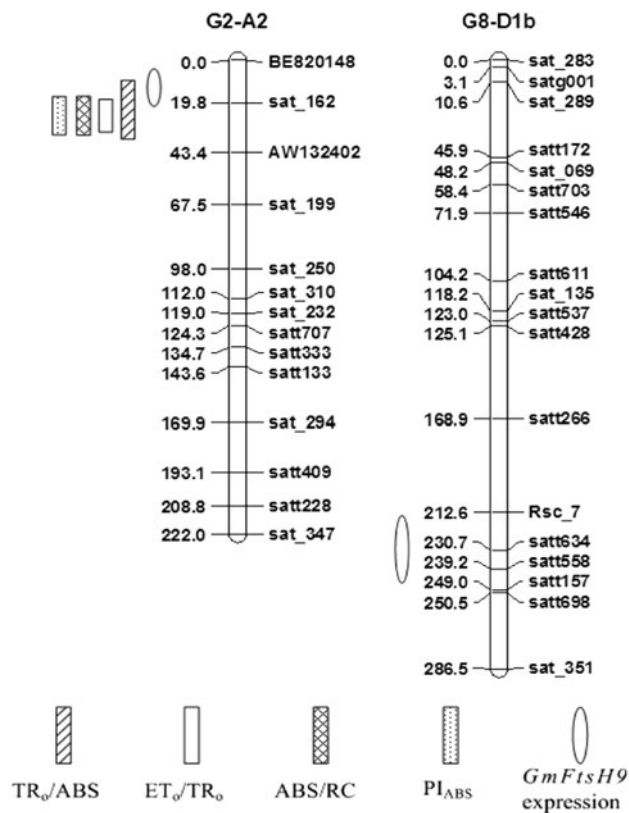
Fig. 5 Position of QTLs on soybean linkage groups (chromosomes) for TR_o/ABS, ET_o/TR_o, ABS/RC, and PI_{ABS} (Yin et al. 2010b) as compared to eQTLs for *GmFtsH9* expression levels. Markers are shown on the right of the linkage groups (chromosomes) and cumulative distances in cM are on the left. eQTLs (QTLs) represented by bars are shown on the left of the linkage groups, close to their corresponding markers. The lengths of the bars are proportional to the confidence intervals of the corresponding eQTLs (QTLs)

Discussion

Evolution of FtsH genes in soybean

E. coli has one FtsH gene (Schumann 1999), cyanobacteria have four (Mann et al. 2000), rice has at least nine, and Arabidopsis has 12 (Yu et al. 2004). By investigating the soybean genome (http://www.phytozome.net/), we identified 11 FtsH genes, *GmFtsH1* through *GmFtsH11*, on 9 chromosomes in soybean (Supplemental Table S1 and Fig. 1). The existence of multiple FtsH genes in soybean is not surprising, given that soybean is a paleopolyploid (Shoemaker et al. 2006). Similar to rice and Arabidopsis, soybean is the product of an ancient genome duplication event, with multiple neopolyploid speciation events having been superimposed on this paleopolyploid genome within the past 50,000 years (Shoemaker et al. 2006). The region-specific duplication or genome-wide duplication during the long period of genome evolution is expected to create gene duplication.

Soybean and Arabidopsis diverged from a common ancestor about 90 million years ago (Grant et al. 2000). Genomic conservation and divergence between these two species have been reported (Grant et al. 2000). In the present study, for 7 of the 12 Arabidopsis FtsH genes (*AtFtsH2*, *AtFtsH3*, *AtFtsH6*, *AtFtsH7*, *AtFtsH8*, *AtFtsH9*, and *AtFtsH10*), there is at least 1 corresponding gene in soybean (Fig. 1), indicating that the main characteristics of these genes were formed before the evolutionary split of soybean and Arabidopsis. This conservation of FtsH genes was also observed in Arabidopsis and rice (Yu et al. 2005). For the remaining Arabidopsis FtsH genes, *AtFtsH1*, *AtFtsH5*, *AtFtsH4*, *AtFtsH11*, and *AtFtsH12*, no corresponding genes were found in soybean, suggesting that after the evolutionary split of soybean and Arabidopsis, these genes evolved in a species-specific manner. This type of divergence of a gene family in different species was also observed in other studies (Yang et al. 2008).

During long-term genome evolution, levels of retention for gene duplicates can vary among different plant species (Zhang et al. 2007). This could explain the varied number of FtsH genes between soybean and Arabidopsis, as well as other plant species, though, compared to many other species, such as rice and Arabidopsis, soybean has experienced additional genome duplications within the past 5 million years (Adams and Wendel 2005). We have reported five Rubisco activase (RCA) genes in soybean (Yin et al. 2010a). Compared with RCA genes, we found many more FtsH genes in soybean, suggesting that during the same genome evolution, different genes could have different levels of retention. A higher number of FtsH genes in soybean may provide a higher variability of the gene product assemblies that can react to the changing environment.

The combination of chlorophyll *a* transient and eQTL mapping provides evidence for the involvement of *GmFtsH9* in modulating PSII function in soybean

Empirical chlorophyll transient parameters, such as F_v/F_m, F_v'/F_m', $\Phi PSII$, qN, and qP (for an explanation of these parameters, see Table 1), that are used in this study, have been widely accepted to reflect the structure and function of the photosynthetic apparatus (Papageorgiou and Govindjee 2004). The so-called JIP parameter, such as TR_o/ABS, ET_o/TR_o, RE_o/ET_o, ABS/RC, and PI_{ABS} (for an explanation of these parameters, see Table 1), could theoretically correlate with the biophysical processes taking place in the photosynthetic apparatus (Strasser et al. 2004). Although some of the assumptions used as the basis for the derivation of JIP parameters might be not accurate, the JIP-test is still an attractive and practical tool, because of its quick quantification of the PS II architecture and behavior

(Stirbet and Govindjee 2011). For example, the statement that Q_A undergoes only a single turnover during the O-J part of the OJIP transient is inaccurate [it has been shown that a non-negligible fraction of Q_A during this part is reduced a second time (Lazár 2009)]. With field and pot experiments, we have shown that some genetic variation of the empirical chlorophyll fluorescence parameters and P_N could be explained by the QTLs for JIP parameters (Yin et al. 2010b). Based on the above considerations, we included both the empirical chlorophyll fluorescence parameters and JIP parameters in this study to examine the function of *GmFtsh9* gene.

The chlorophyll fluorescence parameters derived only from the OJIP transient are measured during a 1 s or less exposure of a dark-adapted leaf to light; thus, the steady state of photosynthesis is not achieved at this stage and they mainly reflect the intrinsic function and structure of the photosynthetic apparatus (Strasser et al. 2004; Stirbet and Govindjee 2011). The parameters that require data from the PSMT transient are measured when the light-adapted steady state is reached, where both light and dark reactions of photosynthesis play a role; therefore, they are affected not only by the intrinsic features of the photosynthetic apparatus, but also by many light-regulated biochemical or physiological processes (Papageorgiou and Govindjee 2004; Stirbet and Govindjee 2011). AtFtsH2 is involved not only in chloroplast development (Zhang et al. 2010), but also in regulating the turnover of the D1 protein (Kato et al. 2009; Kato and Sakamoto 2009; Liu et al. 2010). The D1 protein is one of the major components of the PSII reaction center. If the turnover of the D1 protein is impaired, the photosynthetic process could be inactivated in the photosynthetic apparatus. GmFtsH9 is very similar to AtFtsH2 at the amino acid level; therefore, it is likely that this gene might act in similar ways in vivo. Thus, the expression level of this gene might affect the function of photosynthetic apparatus, which was directly demonstrated by the various parameters obtained from chlorophyll *a* fluorescence measurements.

In the present study, the significant correlation between *GmFtsh9* expression with several chlorophyll fluorescence parameters, TR_o/ABS, ET_o/TR_o, PI_{ABS}, F_v/F_m, F_v'/F_m' and qN, indicated that this gene could play a role in regulating PSII function (Table 2). Furthermore, one eQTL for *GmFtsh9* expression coincided with the QTLs for several JIP parameters on linkage group A2 (Fig. 5), and this common eQTL (QTL) showed the expected additive direction predicted by correlations using phenotypic data. In contrast, no common eQTL (QTL) was found between *GmFtsh9* expression and the parameters related to the PSMT transient. The coincidence of QTLs for two traits, with allelic differences corresponding to the expected relationship between the traits, is strong evidence that the two traits are causally related (Thumma et al. 2001). Thus, we concluded that *GmFtsh9* expression level is more important in determining the parameters related only to the OJIP transient than those related to the PSMT transient. The amount of protein, as well as the activity of AtFtsH2, has been proved to be important for the function of FtsH heterocomplexes (Zhang et al. 2010). In addition, several studies have shown that AtFtsH expression is closely related to F_v/F_m in various plant mutants (Zaltsman et al. 2005; Chen et al. 2006; Kato et al. 2009).

We also investigated the relationship between *GmFtsH9* expression and P_N and seed yield. The correlation coefficients between gene expression and P_N and seed yield were relatively small (Table 2), and no coincident QTL (eQTL) was found between gene expression levels and these two traits (Fig. 5). This result suggests that *GmFtsH9* is not a limiting factor for P_N and seed yield in the soybean RIL population. P_N and seed yield are complex traits regulated by a number of elementary factors, and not every elementary factor is equally effective in determining the phenotype. Only the limiting factors would have a high correlation with the phenotype, and coincident QTLs would be detected. Thus, we must conclude that genes other than *GmFtsH9* limit P_N and seed yield in RIL population used in this study.

The expression of *GmFtsH9* is controlled by *trans*-acting eQTLs

eQTLs can be classified as *cis*- or *trans*-acting, depending on whether the eQTL influencing gene expression is located close to the gene transcript (Doss et al. 2005). If the position of one gene and its eQTL are congruent, then *cis*-acting eQTLs could be inferred, suggesting that the allelic polymorphism of the gene itself, or closely linked regulatory elements, directly influence the gene's expression. Such a pattern was observed for the serine carboxypeptidase I gene, *Cxp1*, where the *Cxp1* eQTL coincided with its structural gene on the chromosome (Potokina et al. 2006). *Trans*-acting eQTLs are inferred when the expression level does not involve DNA variation of the gene in question, but rather, is secondary to alterations of other genetic variants. In the present study, *GmFtsH9* is on chromosome 15, corresponding to linkage group E (Supplemental Table S1), while the eQTLs for this gene were identified on linkage group A2 and D1b (Table 3; Fig. 5). This result suggests that the observed differences in *GmFtsH9* expression in the soybean RIL population are mainly regulated by *trans*-acting factors. A similar phenomenon has been observed for two soybean RCA genes; the expression of each was controlled by two *trans*-acting eQTLs (Yin et al. 2010a).

Breeding implication of eQTLs mapping for *GmFtsH9*

Under environmental stresses, the PSII function of plants is prone to damage. For example, the D1 protein can be damaged by even low light intensities (Tyystjärvi and Aro 1996). In the present study, we have shown that *GmFtsH9* could play an important role in regulating plant PSII function. The identified eQTLs for this gene make it possible to improve *GmFtsH9* expression, and, consequently, PSII function, by marker-assisted breeding methods, such as QTL pyramiding, which is a process of assembling several QTLs for a specific trait from different loci to produce superior genotypes (Xu 1997). However, our study constitutes only first-order knowledge about the genetic determinism of *GmFtsH9* expression levels in soybean. Considering that only those QTLs (eQTLs) detected in different materials and under multiple environments are the most valuable ones for breeding, further eQTL mapping of *GmFtsH9* in a range of soybean materials under different environments is warranted. In addition, there are at least 11 FtsH genes in soybean; however, only the modulation and function of *GmFtsH9* was analyzed in the present study. Future studies of the remaining soybean FtsH genes are also required.

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