STEPWISE ORIGIN AND FUNCTIONAL DIVERSIFICATION OF THE AFL SUBFAMILY B3 GENES DURING LAND PLANT EVOLUTION

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The AFL genes (*ABI3/VP1*, *FUS3* and *LEC2*) belong to the plant-specific B3 superfamily, playing important roles in regulating seed development and maturation. It is unclear, however, whether these genes appeared at the same time as the origin of seed plants and if all these genes are necessary and sufficient for seed development for all seed plants. By conducting a genome-wide comparative analysis of the putative AFL genes in various plant species, we found that the *ABI3* homologous genes existed in all land plant genomes, but the *FUS3* homologous were present only in seed plant genomes and the *LEC2*-like sequences only in dicot genomes. Phylogenetic analysis indicated that the AFL genes had undergone successive rounds of gene duplication and subsequent diversification during land plant evolution, resulting in the stepwise origin of the *ABI3*, *FUS3* and *LEC2* genes. Comparison of gene structure of the AFL genes revealed a trend of decreasing in the number of conserved domains from *ABI3* to *FUS3* and *LEC2*.

Keywords: AFL B3 genes; genome-wide comparative analysis; stepwise origin; functional diversification.

1. Introduction

B3 genes encode plant-specific transcription factors, which play a variety of roles in plant growth and development. More than one hundred B3 genes have been identified from various plant genomes. Proteins encoded by B3 genes vary in sequence similarity and domain structure but all contain a highly conserved B3 domain, forming the B3 superfamily. This superfamily consists of several distinct families, including the LAV, ARF, RAV and REM families, with a few subfamilies to be identified in each family.^{1,2} The AFL subfamily belongs to the LAV family, containing three members: *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *FUSCA 3* (*FUS3*) and

LEAFY COTYLEDON 2 (*LEC2*). The three members of the AFL subfamily play important roles in regulating seed development and maturation.^{3–5} Loss-of-function mutants in *ABI3*, *FUS3* and *LEC2* exhibit defects in seed maturation and desiccation intolerance.^{6,7} *ABI3*, *FUS3* and *LEC2* are thus considered master genes in controlling seed development, especially for seed maturation.^{8–11}

Seed formation is one of the key innovations during land plant evolution,¹² which endows plants with higher adaptability toward unfavorable environment and strengthens the ability of dispersal and competition. The process of seed development can be divided into two phases, i.e. the early embryo morphogenesis phase and the late maturation phase.^{13,14} During the late maturation phase, the embryo accumulates various storage compounds and acquires desiccation tolerance, and eventually enters dormancy.^{15,16} The AFL B3 genes act in a partially redundant manner during seed maturation.⁹ It remains unclear, however, whether all these genes are necessary and sufficient for induction of the maturation phase of seed development for all seed plants. The questions such as whether these genes appeared at the same time as the origin of the seed plants and how they originated remain unanswered.

The *ABI3* gene was first identified from the maize genome,¹⁷ and is now found to be widely distributed in different plant genomes. There is currently limited information on the distribution patterns of *FUS3* and *LEC2*, though their functions have been thoroughly studied in *Arabidopsis*.^{4,5} *ABI3*, *FUS3* and *LEC2* are closely related with each other in gene sequences and functions. However, the evolutionary relationships among *ABI3*, *FUS3* and *LEC2* are still ambiguous. We have ever explored the origin and evolution of another master regulatory gene for seed development, *LEC1*,¹⁸ and revealed that, although *LEC1* is crucial to seed maturation, it is not seed-plant specific. The *LEC1* gene originated in non-seed vascular plant genomes and was inductively expressed under drought stress in non-seed plants. This gene was recruited to a novel regulatory network in the early stages of seed plant evolution and steadily expressed during seed development and maturation.^{9–11} It is thus intriguing to know whether this pattern of evolution is also applicable to the AFL B3 genes.

In this study, genome-wide screens for the AFL genes were performed based on currently available plant genome sequences. By conducting a comprehensive analysis of the distribution patterns, gene structures and phylogenetic relationships of *ABI3*, *FUS3* and *LEC2*, we intend to address questions, such as when these genes originated, how they duplicated and diverged during evolution, and whether all these genes are indispensable components of the regulatory network involved in the control of seed development for all seed plants. We found evidence that the AFL genes experienced independent gene duplication events at different stages of land plant evolution. The *FUS3* and *LEC2* genes appeared in the gymnosperm and angiosperm genomes in a stepwise pattern via neo- and/or sub-functionalization of duplicated genes.

2. Methods

2.1. Genome-wide screen for putative AFL genes

Two BLAST ways, BLAST via a web interface and stand-alone BLAST, were used to search for putative AFL genes in various land plant genomes. Since the structure and function of *Arabidopsis thaliana* *ABI3*, *FUS3* and *LEC2* genes have been most thoroughly characterized, we first downloaded the CDS and protein sequences of *Arabidopsis* AFL genes from TAIR (<http://www.arabidopsis.org/>), and then conducted extensive similarity searches through the public BLAST interfaces at the NCBI website and elsewhere, using *Arabidopsis* AFL sequences as query sequences. Multiple BLASTN and TBLASTN searches were carried out against the complete or nearly complete plant genome sequences in GenBank (<http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html>) and other public trace archives (<http://www.plantgdb.org/>, <http://www.phytozome.net/>), with a cutoff e-value of e^{-10} . Since it has been revealed that sequences containing more than one structural/functional domain are problematic when used in homology searches, e.g. they can cause an explosion of a search to common domains, all sequences retrieved from GenBank and other databases were subjected to domain structure analyses using the SMART (<http://smart.embl-heidelberg.de/>) and Inter-ProScan (<http://www.ebi.ac.uk/InterProScan/>) platforms, and compared with the structure of *Arabidopsis* AFL genes. Sequences with clearly distinct domain compositions from *Arabidopsis* AFL genes were excluded from the dataset, though possessing the highly conserved B3 domain.

To search for putative AFL genes in plants that are important for detecting the phylogenetic patterns of the AFL genes but their complete genomes are currently unavailable, e.g. *Pinus taeda* and *Picea abies*, BLAST searches were also performed against the available EST databases at <http://plantta.jcvi.org/> and <http://compbio.dfci.harvard.edu/tgi/>. The conserved domain sequences of *Arabidopsis* AFL genes were used as query seeds to conduct BLASTN and TBLASTN searches. The acquired EST clusters were assembled using CAP3²² to form consensus sequences, and then translated into putative peptide sequences using a 6-frame translation scheme. By matching similarity of translation products to *Arabidopsis* target proteins, we identified potential homologous AFL sequences from plants without genome sequence information.

For conducting more thorough homology searches and refining the BLAST hits obtained from homology searches via web interfaces, stand-alone BLAST was also performed against the local databases (see Supplementary Material). The Formatdb program implemented in stand-alone BLAST 2.2.13 software²³ was used to construct a BLAST database for each plant. BLAST searches were then performed on the stand-alone BLAST server, using *Arabidopsis* AFL sequences as queries.

2.2. Sequence alignment and phylogenetic analysis

Multiple sequence alignments were generated with Clustal X 2.0.12 (24, 25) with default parameters: Gonnet series as the protein weight matrix and IUB as the DNA weight matrix, gap opening 10.00, gap extension 0.20, delay divergence sequences 30%, DNA transition weight 0.50, and adjusted manually with BioEdit.²⁶ The AFL subfamily comprises diverse multi-domain genes. While the functional/structural domains are well conserved, the N/C-terminal extensions and/or internal insertions are highly variable among members of the family. We thus initially performed a global alignment of all sequences of interest, and conducted a preliminary phylogenetic analysis by constructing a neighbor-joining (NJ) tree using MEGA 4.1²⁷ to determine the number and composition of different groups. The sequences of each subgroup were then aligned again separately, and the sequences that could not be aligned unambiguously were removed. The final alignment was generated using Gblocks 0.91b,²⁸ allowing smaller final blocks.

Phylogenetic analyses were conducted using the neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. NJ and MP trees based on amino acid sequences were built using MEGA 4.1 and PAUP4.0b8,²⁹ respectively. The Jones-Taylor-Thornton (JTT) model³⁰ was employed for distance calculations in NJ analyses with pairwise deletion option selected. Heuristic tree search under parsimony was conducted using the tree-bisection-reconnection (TBR) swapping algorithm. The ML tree was constructed using RAxML,³¹ which is a program for sequential and parallel maximum likelihood-based inference of phylogeny. GTRCAT_GAMMA and PROTCAT_GAMMAIWAG were used as the models of nucleotide substitution and amino acid substitution, respectively. The robustness of the tree topology was assessed by bootstrap analysis, with 100 replicates for the ML method.

3. Results

3.1. Phylogenetic distribution of the AFL genes in different lineages

BLAST results showed that the AFL subfamily genes are widely distributed in various plants (Table 1). Three sequences homologous to the AFL B3 genes were identified from *Physcomitrella patens* and *Selaginella moellendorffii* genomes, respectively. Gene structure and phylogenetic analyses indicated that all these sequences are homologues of *ABI3*. Of them, three *P. patens* genes (*PpABI3A*, *PpABI3B* and *PpABI3C*) have been functionally clarified.³² There is currently no complete genome sequence for gymnosperms. However, there exist a few databases that contain plenty of ESTs and incomplete genome sequences from various gymnosperms. We thus searched for the gymnosperm AFL genes against all the databases available. An *ABI3* homologous gene was identified from *Pinus taeda* and *Picea abies*, respectively, which showed high similarity in gene sequence and

Table 1. Identification and distribution patterns of AFL subfamily members of various plants. Numbers in the table stand for the copy number of each gene in various plant genomes. The last column summarizes total numbers of AFL subfamily members. As for *Picea abies* and *Pinus taeda*, because of the lack of EST data, some of the genes are not applicable (N/A).

Species name	Classification	<i>ABI3</i>	<i>FUS3</i>	<i>LEC2</i>	<i>IDEF1</i>	Total
<i>Physcomitrella patens</i>	Bryophytes	5	0	0	0	5
<i>Selaginella moellendorffii</i>	Lycophytes	4	0	0	0	4
<i>Picea abies</i>	Gymnosperm	1	N/A	0	0	N/A
<i>Pinus taeda</i>	Gymnosperm	1	1	0	0	N/A
<i>Arabidopsis thaliana</i>	Dicot	1	1	1	0	3
<i>Populus trichocarpa</i>	Dicot	1	2	2	0	5
<i>Glycine max</i>	Dicot	2	2	1	0	5
<i>Manihot esculenta</i>	Dicot	2	2	1	0	5
<i>Oryza sativa</i>	Monocot	1	1	0	3	5
<i>Sorghum bicolor</i>	Monocot	1	1	0	2	4
<i>Zea mays</i>	Monocot	1	1	0	3	5

structure with other annotated *ABI3* genes. We also found an incomplete homologue of *FUS3* in *P. taeda*. The homologous *ABI3* and *FUS3* genes were present in all angiosperm genomes. However, the *LEC2*-like sequences were only found in the dicot genomes. BLAST searches of the cereal genomes also detected another group of conserved genes in *Oryza sativa*, *Zea mays* and *Sorghum bicolor*, that share sequence similarity with the angiosperm *FUS3* and *LEC2* but formed an independent clade in the phylogenetic tree. According to the genome annotation of *O. sativa*, these genes were homologues of *IDEF1*, encoding the iron deficiency-responsive *cis*-acting element binding factors.^{33,34}

3.2. Phylogenetic relationship of AFL genes

The evolutionary relationships among various AFL genes were inferred by NJ, MP and ML analyses. Complete gene sequences and the B3 domain sequences were used to construct phylogenetic trees, respectively. The tree topologies produced by different methods were similar on the overall structure but not perfectly match. Figure 1 shows the phylogenetic tree reconstructed by ML using the B3 domain sequences. It highlights three important features in all inferred trees. First, the *ABI3* genes of seed plants formed a well-supported group in the phylogenetic tree and showed a close relationship with those from non-seed plant genomes. Second, the *FUS3* and *LEC2* genes, as well as the *IDEF* homologous genes of angiosperms grouped into three separate clades, with the position of the *IDEF* group varying relative to other two groups in different trees. Third, the homologous *FUS3* gene of *P. taeda* did not cluster together with the angiosperm *FUS3* genes but formed an independent branch ancestral to the angiosperms genes except *ABI3*.

Fig. 1. (a) Phylogenetic analysis of protein sequences of B3 domain from AFL family of 23 species representing all plant groups from moss to seed plants. The species is indicated by the first two letters of the gene names. Ah: *Arachis hypogaea*; At: *Arabidopsis thaliana*; Bd: *rachypodium distachyon*; Brapa: *Brassica rapa*; Bnapus: *Brassica napus*; Chnoot: *Callitropsis nootkatensis*; Fp: *Festuca pratensis*; Gm: *Glycine max*; Hv: *Hordeum vulgare*; Selmo: *Selaginella moellendorfii*; Kd: *K.daigremontianus*; Lj: *Lotus japonica*; Mes: *Manihot esculenta*; Mt: *Medicago truncatula*; Os: *Oryza sativa*; Pabies: *Picea abies*; Pp: *Physcomitrella patens*; Pt: *Populus trichocarpa*; Ptaeda: *Pinus taeda*; Rc: *Ricinus communis*; Sb: *Sorghum bicolor*; Sl: *Solanum lycopersicum*; Vv: *Vitis vinifera*; Zm: *Zea mays*. (b) Domain position and composition of each AFL genes. Different domains are indicated with different colors. Yellow block: A (serine-rich) domain; Purple block: B1 domain; Orange block: B2 domain; Light blue block: *FUS3*-specific conserved sequence of monocots; Dark blue block: *FUS3*-specific conserved sequence of dicots.

Fig. 2. (a) Alignment of FUS3 proteins from dicots. (b) Alignment of FUS3 proteins from monocots. Conserved sequences covered with green lines are B3 domains. Conserved sequences covered with dark blue lines are FUS3-specific fragments of dicots. Conserved sequences covered with light blue lines are FUS3-specific fragments of monocots.

(b)

Fig. 2. (Continued)

3.3. Analysis of conserved domains in AFL genes

Comparison of the domain structure of proteins encoded by *ABI3*, *FUS3* and *LEC2* demonstrated that the homologous genes shared similar number and type of conserved domains. We also detected a decreasing trend in the number of domains contained in *ABI3*, *FUS3* and *LEC2*. All *ABI3* genes contain four domains, namely A1, B1, B2, and B3. Of them, the A1 domain is located at the N-terminus of the protein, which plays an important role in the function of these proteins as transcription activators.³² The B1 domain of *ABI3* binds to the N-terminal charged domains of *ABI5*, and is critical for the physical interaction between *ABI3* and *ABI5*.³⁵ The B2 domain is required for trans-activation of the Em and 2S promoter,^{36,37} thus being essential for regulation of other proteins.³⁸ The B3 domain is a common conserved domain existing in all AFL subfamily members. Comparison of gene structure revealed that the A1 and B1 domains have been lost in both *FUS3* and *LEC2* sequences. However, we detected a novel *FUS3*-specific domain at the C-terminal of the sequences that was highly conserved but showed significant difference between

monocot and dicot plants (Fig. 2). Blast searches against Pfam 24.0 using the *FUS3*-specific fragment as a query sequence returned no hits. Furthermore, only a single B3 domain was found in the *LEC2* and *IDEF* homologous genes. The B3 domains were mostly located in the middle part of the *LEC2* sequences but in the proximal region of the C terminus of *IDEF* homologues.

4. Discussion

Much attention has been paid in recent years to the evolution of the B3 superfamily.^{1,2} The role of the AFL B3 domain transcription factors in controlling seed development has also been studied extensively.¹⁰ However, the evolutionary relationships of the AFL members have not yet been clearly assessed due to only a few sequences from model plants used in previous studies. We explored the evolutionary history of the AFL subfamily based on sequences collected from all available databases, covering all major lineages of land plants. The data shows clearly that the AFL genes underwent a series of independent duplications and diversifications during land plant evolution. The duplicate *ABI3* genes of non-seed plants seem to have structurally and functionally diverged prior to the diversification of seed plants,^{39,40} leading to the reduction of the numbers of *ABI3* in the seed plant genomes, and giving rise to the appearance of the *FUS3* gene. In accompaniment to the divergence of gymnosperm and angiosperm^{41,42} about 300 million years ago,⁴³ the *FUS3* gene experienced a new round of duplication with subsequent diversification through neo- and/or sub-functionalization. As a result, one copy retained the original function of *FUS3* in both gymnosperm and angiosperm genomes and the other copy diverged into the ancestors of *LEC2* and *IDEF*. Present data suggest that the *LEC2* gene appeared initially and limitedly distributed in the dicot genomes,^{5,44,45} while the functionally diverged *IDEF* gene limited to the monocot genomes.³⁴ Therefore, the *ABI3*, *FUS3* and *LEC2* genes have appeared in the land plant genomes in a stepwise pattern.

There is a clear trend toward the reduction of the conserved domain contained in the *ABI3*, *FUS3* and *LEC2* genes. Moreover, functional diversification of *ABI3*, *FUS3*, *LEC2* and *IDEF* seems to be correlated with the variation in domain structure. It has been reported that *ABI3* plays an important role in regulation of abscisic acid-responsive genes. Mutants of *AtABI3* showed low sensitivity to ABA, chlorophyll breakdown and desiccation intolerance.^{3,6,8} Mutations of *FUS3* and *LEC2* lead to similar pleiotropic effects on seed phenotype, including desiccation intolerant and viviparous.^{9,46,47} In contrast, *IDEF* functions as the early response factor toward iron deficiency in cereals because it can recognize the CATGC sequence within *IDE1* (Iron-Deficiency Element).^{33,34,48,49} Compared to the wide range of expression patterns of *ABI3*, *FUS3* and *LEC2* exhibit restricted spatial and temporal expression.⁵⁰ *ABI3* was found to express in the whole embryo throughout the maturation phase in Arabidopsis,⁵¹ and it also has been detected in vegetative organs.^{9,52} *FUS3* was found to be highly expressed in siliques⁴ and protoderm⁵³

before the maturation phase,⁵⁴ while *LEC2* was mainly expressed in the embryo axis.⁹ The restricted spatial and temporal expression of *FUS3* and *LEC2* possibly resulted from the reduction of conserved domains in these genes, which increases specialization of gene function.

ABI3, *FUS3* and *LEC2* are currently considered the master regulatory genes for seed maturation.^{9,10} Based on the currently available data, the *LEC2* genes are only distributed in the dicot genomes. It is thus doubtful whether all the *ABI3*, *FUS3* and *LEC2* genes are indispensable components of the regulatory network controlling seed development for all seed plants. To answer this question, we need more complete genome sequences, especially the genomic information of gymnosperms. Meanwhile, the evolutionary relationships between *LEC2* and *IDEF* remain to be further assessed. It is currently unsure when *LEC2* and *IDEF* diverged. These two genes probably descended from different ancestors that appeared at the early stage of angiosperm diversification, with the *LEC2* and *IDEF* homologues sequences lost in the monocot and dicot genomes, respectively. But we also cannot exclude the possibility that *IDEF* was derived from *LEC2* through duplication and subsequent diversification during the monocots-dicots split. To investigate when these genes originated and how they diverged during evolution will provide crucial insights into the molecular mechanisms controlling seed formation and evolution.

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