Plant Science 236 (2015) 116–125

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Role of B3 domain transcription factors of the AFL family in maize kernel filling

Aurélie Grimault[a,b,c,1], Ghislaine Gendrot[a,b,c], Sandrine Chaignon[a,b,c], Françoise Gilard[d], Guillaume Tcherkez[d,2], Johanne Thévenin[e,f], Bertrand Dubreucq[e,f], Nathalie Depège-Fargeix[a,b,c], Peter M. Rogowsky[a,b,c,*]

[a] Université de Lyon, Ecole Normale Supérieure de Lyon, Université Lyon 1, Unité Reproduction et Développement des Plantes, F-69364 Lyon, France
[b] INRA, UMR 879, Reproduction et Développement des Plantes, F-69364 Lyon, France
[c] CNRS, UMR 5667, Reproduction et Développement des Plantes, F-69364 Lyon, France
[d] CNRS, UMR 9213, Institute of Plant Sciences Paris-Saclay, F-91405 Orsay, France
[e] INRA, UMR 1318, Institut Jean-Pierre Bourgin, Saclay Plant Sciences, Versailles, France
[f] AgroParisTech, Institut Jean-Pierre Bourgin, Saclay Plant Sciences, Versailles, France

ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 26 March 2015
Accepted 27 March 2015
Available online 6 April 2015

Keywords:
AFL network
Endosperm
Maize
Starch
Transcription factor

ABSTRACT

In the dicot *Arabidopsis thaliana*, the B3 transcription factors, ABA-INSENSITIVE 3 (ABI3), FUSCA 3 (FUS3) and LEAFY COTYLEDON 2 (LEC2) are key regulators of seed maturation. This raises the question of the role of ABI3/FUS3/LEC2 (AFL) proteins in cereals, where not only the embryo but also the persistent endosperm accumulates reserve substances. Among the five *ZmAFL* genes identified in the maize genome, *ZmAFL2* and *ZmAFL3/ZmVp1* closely resemble *FUS3* and *ABI3*, respectively, in terms of their sequences, domain structure and gene activity profiles. Of the three genes that fall into the *LEC2* phylogenetic subclade, *ZmAFL5* and *ZmAFL6* have constitutive gene activity, whereas *ZmAFL4*, like *LEC2*, has preferential gene activity in pollen and seed, although its seed gene activity is restricted to the endosperm during reserve accumulation. Knock down of *ZmAFL4* gene activity perturbs carbon metabolism and reduces starch content in the developing endosperm at 20 DAP. ZmAFL4 and ZmAFL3/ZmVp1 *trans*-activate a maize oleosin promoter in a heterologous moss system. In conclusion our results suggest, based on gene activity profiles, that the functions of *FUS3* and *ABI3* could be conserved between dicot and monocot species. In contrast, LEC2 function may have partially diverged in cereals where our findings provide first evidence of the specialization of *ZmAFL4* for roles in the endosperm.

1. Introduction

In maize, as in many cereal crops, the reserve substances necessary for efficient germination of the embryo, and thus successful propagation, are stored both within the embryo (mainly oil and protein) and in a surrounding nourishing tissue, the endosperm (mainly starch and protein). Whereas the entire maize seed is cherished by mankind as a major source of food and animal feed, the non-food use of the maize seed is largely limited to the endosperm and more precisely to starch extraction for industrial products or hydrolysis for biofuels.

In maize, the accumulation of reserve substances in the embryo and endosperm occurs during the filling stage of seed development [1,2]. This stage is preceded by developmental events such as pattern formation, morphogenesis and differentiation, and followed by seed dehydration, which allows the seed to become quiescent. Distinct transcriptome profiles suggest that the three developmental stages (early, filling and desiccation) have dedicated genetic programs which are controlled mainly at the transcriptional level [3,4]. Despite passing through functionally equivalent stages, the embryo and endosperm clearly execute different genetic programs to elaborate their characteristic morphology and to accumulate distinct reserve substances [5,6].

Abbreviations: ABI3, aba-insensitive 3; AFL, ABI3/FUS3/LEC2; AGPase, ADP-glucose pyrophosphorylase; ANOVA, analysis of variance; bZIP, basic leucine zipper; DAP, days after pollination; FUS3, fusca 3; G3P, glycerol-3-phosphate; LEC, leafy cotyledon; qRT-PCR, quantitative reverse transcription-PCR; RNAi, RNA interference; ZmVp1, *Zea mays* Viviparous1.

* Corresponding author at: RDP, ENS de Lyon, 46 allée d'Italie, F-69364 Lyon, France. Tel.: +33 4 72 72 86 07; fax: +33 4 72 72.
E-mail address: peter.rogowsky@ens-lyon.fr (P.M. Rogowsky).
[1] Present address: Carnegie Institution of Science, 260 Panama Street, Stanford, CA 94305, USA.
[2] Present address: Research School of Biology, ANU College of Medicine, Biology and Environment, Australian National University, 2601 Canberra, ACT, Australia.

http://dx.doi.org/10.1016/j.plantsci.2015.03.021
0168-9452/

The control of seed filling has been well characterized in Arabidopsis, where the endosperm is largely transient and reserve substances therefore principally accumulate in the embryo. The three B3 domain transcription factors *A*BA-INSENSITIVE 3 (ABI3) [7], *F*USCA3 (FUS3) [8] and *L*EAFY COTYLEDON 2 (LEC2) [9] form the so-called "AFL network" in association with LEAFY COTYLEDON 1 (LEC1) [10], which is homologous to the HAP3 subunits of the CAAT box-binding factor family [11,12]. Mutations in these genes cause pleiotropic but distinct effects on seed maturation, including a lack of both storage reserve accumulation and desiccation tolerance [13–15]. *AFL* genes have distinct temporal and spatial gene activity patterns during Arabidopsis seed development. All three genes are active in the embryo and *LEC2* and *FUS3* also are active in the endosperm [2,16]. Temporally, *LEC2* is the first gene to become active, with a peak at the heart stage. The activity of the *FUS3* gene peaks during early seed maturation, and finally *ABI3* is active during desiccation [17,18]. Despite a certain overlap in their gene activity patterns, each *AFL* gene has a distinct function. Networking is indicated by interactions among *AFL* genes. For example, LEC2 activates *ABI3* and *FUS3* gene activity whereas ABI3 and FUS3 auto-regulate themselves and interact through mutual activation [17,19,20]. In addition, interactions between *LEC1* and *AFL* genes have been shown by genetic and transcriptome analyses: LEC1 can activate *ABI3* and *FUS3* activity whereas *LEC1* activity is up-regulated by LEC2 [21–23].

AFL transcription factors are considered master regulators since they trigger a regulatory cascade by activating secondary transcription factors, which in turn govern multiple metabolic and developmental pathways. For example *WRI1*, which encodes the main regulator of lipid biosynthesis in the seed, is a direct target of LEC2 [24]. However LEC2 also acts directly on genes involved in reserve accumulation including *OLE1*, encoding an oleosin [25] and *At2S1–S4* and *2S-like*, which encode seed storage proteins [17,26]. Direct targeting by LEC2 is mediated by its B3 domain, which binds specifically to RY-motifs such as CATGCA [19,26].

The functions of *AFL* genes have been extensively studied in Arabidopsis, and putative orthologs have been described in several monocot and dicot species [2]. However, it remains unclear to what extent their functions and targets are conserved. The best characterized *AFL* gene in cereals is *ZmViviparous* (*ZmVp1*), the maize ortholog of *ABI3* [27–29]. However, the extensive characterization of the *Zmvp1* mutant has focused largely on a single aspect, the role of *ZmVp1* in ABA-mediated regulation of kernel dormancy [30,31]. Indications for functional conservation also exist for *ZmLEC1* and *ZmWri1*, since their over-expression increases seed oil content in maize [32,33]. *ZmWri1a* and *ZmWri1b* both complement their putative co-ortholog *WRI1* in the Arabidopsis *wri1* mutant, despite minor qualitative changes in the oil of *wri1* mutants complemented with *ZmWri1a* and *ZmWri1b* [32,34]. However, since in maize oil accumulates principally in the scutellum of the embryo [35] and since dormancy also concerns primarily the embryo, these data do not shed light on the question of a potential control of endosperm reserve substances by members of the AFL network, which cannot be satisfactorily addressed in the exalbuminous Arabidopsis seed.

The identities of the transcriptional regulators of well-known structural genes needed for endosperm starch synthesis in cereals remain surprisingly elusive despite their potential as targets for the modification of important crop traits. Recent work has shown that the rice basic leucine zipper (bZIP) transcription factor bZIP58 directly regulates *Starch synthase IIa* and *Starch branching enzyme 1* gene activity [36]. In addition, seed storage protein gene activity in the maize endosperm is known to be regulated by the bZIP transcription factor Opaque2 and interacting proteins such as the zinc finger transcription factor PBF [37]. Here we address the important question of whether members of the AFL family could also participate in the regulation of seed storage product accumulation. We present the identification, phylogenetic analysis and gene activity analysis of putative maize orthologs of *ABI3*, *FUS3* and *LEC2*. Our results indicate a previously unexplored function for *ZmAFL4* in kernel metabolism and the regulation of starch accumulation in the maize endosperm.

2. Materials and methods

2.1. Plant material and plant culture

Maize plants were grown in a greenhouse with a 16-h photoperiod (400 μmol m^{-2} s^{-1}) at 24 °C/19 °C (day/night) and without control of the relative humidity, as described in Pouvreau et al. [32]. All plants were propagated by hand pollination. Maize genotype B73 was used for temporal and spatial gene activity analyses of *ZmAFL* genes during kernel development, and genotype A188 for maize transformation and for gene activity analyses of *ZmAFL* genes in maize organs. Seed of the *Zmvp1* mutant allele *vp1-Mum1::Mu* was obtained from the Maize Genetics Cooperation Stock Center (stock 326BH).

2.2. T-DNA constructs and plant transformation

The coding sequences of *ZmAFL* genes were PCR amplified from cDNA from appropriate kernel stages (genotype B73), using primers reported in supplementary Table S3. The PCR products were cloned into the vector pDONRZeo (Invitrogen), and the resulting entry vectors were sequenced prior to LR recombination. The final plasmids L1238 (*ZmAFL2*-RNAi), L1240 (*ZmAFL4*-RNAi) and L1242 (*ZmAFL5/6*-RNAi) were used for maize transformation as described previously [32]. The plasmids contained the backbone of vector pSB11, a Basta resistance cassette (rice *Actin* promoter and intron, *Bar* gene and *Nos* terminator) next to the right border, a GFP cassette (*CsVMV* promoter and *FAD2* intron, *GFP* gene and *Nos* terminator) and the respective *ZmAFL* gene fragment separated by the rice *Tubulin* intron in a head to head configuration under the control of the constitutive rice *Actin* promoter and intron.

2.3. Sequence analysis

Protein sequences similar to *AtAFL* proteins were retrieved by using the BlastP program to query the maize genome database (http://blast.gramene.org/Multi/blastview) and the protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The protein sequence alignments were generated using the programs ClusalW2 or Omega available at http://www.ebi.ac.uk/Tools/msa/. Gene models were downloaded from release AGPv3 of the B73 maize (*Zea mays*) genome assembly (http://ensembl.gramene.org/Zea_mays/Info/Index). Functional B3 domains were identified using the Pfam HMM database (http://pfam.xfam.org/). A1, B1, B2 and C-terminal were defined as conserved blocks in multiple sequence alignments [7].

2.4. Phylogenetic analysis

Amino acid sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). Conserved blocks were selected manually with the Seaview program (http://doua.prabi.fr.fr/software/seaview.html) and phylogenetic trees were generated using PHYML with the WAG substitution model and 1000 bootstrap replicates.