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LEC1 (NF-YB9) directly interacts with LEC2 to control gene expression in seed

C. Boulard[a], J. Thévenin[a], O. Tranquet[b], V. Laporte[b], L. Lepiniec[a], B. Dubreucq[a,*]

[a] Institut Jean-Pierre Bourgin (IJPB), INRA, AgroParisTech, ERL-CNRS, Saclay Plant Sciences (SPS), Université Paris-Saclay, RD10, F-78026 Versailles, France
[b] UR1268 BIA, INRA Angers, Nantes Rue de la Geraudiere, 44316 Nantes Cedex 3, France

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ABSTRACT

The LAFL transcription factors LEC2, ABI3, FUS3 and LEC1 are master regulators of seed development. LEC2, ABI3 and FUS3 are closely related proteins that contain a B3-type DNA binding domain. We have previously shown that LEC1 (a NF-YB type protein) can increase LEC2 and ABI3 but not FUS3 activity. Interestingly, FUS3, LEC2 and ABI3 contain a B2 domain, the function of which remains elusive. We showed that LEC1 and LEC2 partially co-localised in the nucleus of developing embryos. By comparing protein sequences from various species, we identified within the B2 domains a set of highly conserved residues (*i.e.* TKxxARxxRxxAxxR). This domain directly interacts with LEC1 in yeast. Mutations of the conserved amino acids of the motif in the B2 domain abolished this interaction both in yeast and in moss protoplasts and did not alter the nuclear localisation of LEC2 in planta. Conversely, the mutations of key amino acids for the function of LEC1 *in planta* (D86K) prevented the interaction with LEC2. These results provide molecular evidences for the binding of LEC1 to B2-domain containing transcription factors, to form heteromers, involved in the control of gene expression.

1. Introduction

During seed development, the embryo passes through a series of stages that are under tight transcriptional controls. Several transcription factors that form a complex and intricate network (for review see [1–3]) have been characterized. Among them, the LAFLs are encoded by 4 genes, namely *LEAFY COTYLEDON 1* (*LEC1*) [4], *ABSCISSIC ACID INSENSITIVE 3* (*ABI3*) [5], *FUSCA3* (*FUS3*) [6] and *LEAFY COTYLEDONS 2* (*LEC2*) [7]. Genetic and molecular analyses have demonstrated that they control many aspects of seed development such as the accumulation of reserves [8–11], the acquisition of tolerance to desiccation [12,13], the inhibition of trichomes growth on the cotyledons or the repression of anthocyanin synthesis [14]. Besides their implication in seed development, these genes are involved in somatic embryogenesis [15,16] and, more broadly, in the initiation and maintenance of embryonic fate [16,17].

LEC2, ABI3 and FUS3 are members of the plant specific family of B3 transcription factors [18]. They contain a conserved DNA binding domain (the B3 domain) and are of variable amino acid length. ABI3 contains four domains (A1, B1, B2 and B3), LEC2 and FUS3 two (B2 and B3) [19]. It has been proposed that LEC2 and FUS3 derive from an ABI3-like common ancestor [19]. The B3 domain is involved in binding to a core DNA cis-element (CATG) called RY motif, with variable extension such as CGCATGCG for FUS3, and GCATGC for LEC2 and ABI3 [20]. The B2 domain was shown necessary to mediate the interaction of ABI3 at the Abscisic Acid Response Elements (ABRE) of cis regulatory sequences [21]. Previous analyses also suggested that the B2 domain could act as putative nuclear localisation signal, but mutations in this domain do not alter ABI3 localisation [22].

LEC1 is a member of the NF-YB protein family that is highly conserved among eukaryotes [23,24]. It has no specific DNA binding activity but interacts together with NF-YC and NF-YA, which possesses the DNA binding capabilities, to form a complex that binds the CCAAT cis-element involved in the initiation of transcription [25]. LEC1 and LEC1 like (L1L), its closest paralog possess specific amino acid residues compared to other NF-YB [4]. They can interact with other transcription factors such as bZIPs [26,27] but also modify chromatin accessibility and facilitate active histone modifications. LEC1 was shown to be involved in the long term reprogramming (*i.e.* from embryonic state to flowering plant) of embryonic chromatin state in plants [28].

In a previous work, we have shown that LEC1 and LEC2 cooperate *in vivo* to activate a target promoter and can co-immunoprecipitate in the presence of NF-YC2 and a target DNA sequence [20]. Nevertheless, little is known on the molecular interactions between those key transcription factors and biochemical data remain elusive. Here, we have demonstrated the partial co-localisation of LEC1 and LEC2 *in planta* and

* Corresponding author.
E-mail address: bertrand.dubreucq@inra.fr (B. Dubreucq).

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their direct interactions involving a specific motif in the B2 domain of LEC2 and specific residues in LEC1, in two hybrid experiments. Taken together, our results allow drawing a model for the cooperation of LEC1 and LEC2, possibly increasing the activity of the complex on target DNA sequences.

2. Materials and methods

2.1. Antibodies production and purification

The antibodies were produced from recombinant N term histidine tagged protein expressed in DE3 rosetta™ bacteria transformed with the pETG-10A vector. LEC1 and LEC2 production were induced with 0.5 mM IPTG, at 37 °C for 3 h. The bacteria were pelleted by centrifugation (5000 g, 15 min at 4 °C), resuspended in a lysis buffer (1 × PBS, 1% triton) and lysed with a sonicator (Sonics, USA) at 60% power over 1 min through steps of 10 s of sonication followed by 10 s of incubation on ice. After centrifugation of the lysate (45 min 15,000*g* at 4 °C), the pellet containing the inclusion bodies was washed with (i) PBS, 2% triton, (ii) PBS 1 × 1 M NaCl, 1 M urea and (iii) 1 × PBS, successively.

Antibody production was carried out on the technical platform of the Biopolymers Interaction Assemblies of INRA (https://www6.angers-nantes.inra.fr/bia_eng/Technical-Platforms/Protein-Antibodies). Animal experiments were carried out at the INRA facilities, which are authorized by the Local Veterinary Department (authorization no. 44502). Two “New Zealand” rabbits were immunized for LEC1 and eight “Wistar” rats were immunized for LEC2 by repeated intraperitoneal injections with 300 µg of LEC1 and 100 µg of LEC2 respectively, emulsified in Freund's adjuvant (complete for the initial injection, incomplete for the others). Immunizations were repeated four times with two weeks interval. One week after the last injection, animals were bled by cardiac puncture under generalized anaesthesia. Serum were prepared by centrifugation (2000*g*–20 min) and stored at − 20 °C.

The antibodies obtained were purified by immuno-affinity. Twenty-five micrograms of the recombinant proteins required for immunization were separated on a SDS-PAGE 8% gel, transferred onto a PVDF membrane and stained with red culvert (P7767, Sigma) The strips corresponding to the recombinant proteins were cut out and incubated in the saturation buffer TBST 5% milk (20 mM tris, 150 mM NaCl, pH 7,4, 0,5% tween, 5% milk) for 1 h at 4 °C. The serum was incubated with the membranes overnight at 4 °C. After 4 washings (15 min) of the membranes with TBST buffer (20 mM tris, 150 mM NaCl, pH 7,4, 0,5% tween), the bound antibodies were eluted several times with 500 mM glycine, 500 mM NaCl, 0.1% tween 20, 1% BSA, pH 3.0. The eluates were immediately neutralized with 0,1 volume of 1 M tris base solution. The purified antibodies were concentrated on Amicon Ultra 10,000 MWCO Millipore™ filtration units and stored at − 20 °C until further use.

2.2. Cytological analyses

2.2.1. Preparation of the samples

The developing siliques of Arabidopsis plants were fixed and wax embedded according to [29]. The slides were de-waxed and samples were rehydrated in successive decreasing (100% to 30% in 1 × PBS buffer) ethanol solutions, and then treated with 1 M sodium citrate pH 6.0 until boiling in the microwave for epitope unmasking and rinsed twice with 1 × PBS buffer. Slides were saturated 1 h with 1% BSA, 1 × PBS, and incubated overnight at 4 °C in a humid atmosphere with the specific antibody diluted 1:500th in the saturation solution. After 4 washings of 3 min in PBST (1 × PBS, 0.1% Tween 20), the slides were incubated 3H at 37 °C in a humid atmosphere with the 1:100th goat Alexa labelled secondary antibody (Anti/rabbit Alexa 488 ref: A11008, Anti/rabbit Alexa 647 ref: A21245, Anti/mouse Alexa 488 ref: A11001, Anti/rat, Alexa 488 ref: 11006). After two 10-minute wash in a 1 × PBS, the slides were mounted in H1000 vectashield (Vector laboratories INC) mounting medium containing 1 µg/ml DAPI and then sealed with varnish. The slides were stored at 4 °C until observation.

2.3. Confocal observations

The developing siliques of the pLEC2:GFP-LEC2 lines were harvested from 3 to 8 DAP. The embryos were extracted from the seeds under the binocular and placed on slides in a mounting medium (50% glycerol). Embryos were observed using a LEICA SP8 TCP spectral confocal laser microscope (Leica Microsystems, Wetzlar, Germany) equipped with an argon laser, 405 nm and 633 nm laser diodes and hybrid photodetectors. Emissions bands were 415–459 nm for DAPI, 503–527 nm for Alexa 488, 659–675–459 nm for Alexa 647 for fixed observations, emissions bands of 426–464 nm for DAPI, 490–522 nm for GFP for *in vivo* observations. The same equipment was used for observations of immunohisto detection of LEC1 and LEC2.

2.3.1. Construction of GFP: LEC2 lines

The *GFP* and *LEC2* cDNAs were amplified by PCR with the Phusion High-Fidelity polymerase (New England Biolabs, Ipswich, UK). The oligonucleotides were designed to position double Gateway recombination cassettes (Thermo Fisher) according to the following pairs: (AttB1 AttB5r), (AttB5, attB2) respectively.

The cDNA were BP-gateway recombined in p221 donor vectors according to the manufacturer's recommendations and then LR-gateway recombined into the desired destination vector.

2.3.2. Construction of pGWB2:pLEC2 (− 1 kb) destination vector

In order to express the tagged proteins in plants, the cDNA were placed under the control of the endogenous promoter, in the modified pGWB2 vector. The p35S promoter, initially present in the vector, was replaced by the 1 kb length *LEC2* promoter by enzymatic digestion. Enzyme restriction sites *Hin*dIII (R014S, Biolabs) and *Xba*I (R0145S, Biolabs) were placed at the 5′ and 3′ ends, respectively, of the *LEC2* promoter amplified by PCR, and then inserted into the Topo Blunt vector (Thermo Fisher Scientific). PCR product and the vector pGWB2 were digested successively by 2 units of each enzymes and then purified on gel using the Promega Wizard SV clean UP system kit. The ligation reaction was carried out according to a 9:1 PCR/vector product ratio with 800 units of T4 ligase (New England Biolabs), at 16 °C, overnight. DB3.1 bacteria were transformed with the ligation product and colonies obtained PCR checked with primers located in the LEC2 promoter sequence.

2.4. Plasmids constructions

LEC1, *LEC1D86K* and *LEC2 cDNAs* in pDONR207 were directly LR-gateway recombined in pCor104 PMDC140 R1R2. *lec2m1:* $T_{105}G$, $K_{106}S$, $A_{108}T$, $R_{109}N$, $I_{110}C$, $K_{113}S$ and, *lec2m12:* $T_{105}G$, $A_{108}T$, $R_{109}N$, were *de novo* synthesized (Geneart, DNA strings, Thermo Fisher, Regensburg, Germany). The sequences were PCR amplified to introduce attB1 attB2 Gateway recombination sequences at 5 “and 3” end of the cDNA respectively (see Sup. Table 1 for oligonucleotides sequence) BP-Gateway in pDONR207 after verification of the sequence and then recombined LR-recombined in pCor104 PMDC140 R1R2. For Yeast two-Hybrid analyses, the cDNAs of *LEC1* and *LEC1D86K* contained in pDONR207 were directly LR-Gateway recombined in pDEST22 and pDEST32 vectors (Thermo Fisher Waltham, USA). LEC2 was mutated in the B2 domain by site-directed mutagenesis according to the Agilent quick exchange protocol according to the manufacturer's instructions. LEC2 and LEC2*ΔB3* (*R168 STOP*), deleted from domain B3, were PCR amplified (see Sup. Table 1 for oligonucleotides used), verified by sequencing, BP-Gateway recombined in pDONR207. The inserts were then LR-Gateway recombined in the double hybrid vectors pDEST22 or pDEST32.