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Research article

Role of protein farnesylation events in the ABA-mediated regulation of the *Pinoresinol–Lariciresinol Reductase 1* (*LuPLR1*) gene expression and lignan biosynthesis in flax (*Linum usitatissimum* L.)

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ABSTRACT

A *Linum usitatissimum LuERA1* gene encoding a putative ortholog of the *ERA1* (*Enhanced Response to ABA 1*) gene of *Arabidopsis thaliana* (encoding the beta subunit of a farnesyltransferase) was analyzed *in silico* and for its expression in flax. The gene and the protein sequences are highly similar to other sequences already characterized in plants and all the features of a farnesyltransferase were detected. Molecular modeling of LuERA1 protein confirmed its farnesyltransferase nature. *LuERA1* is expressed in the vegetative organs and also in the outer seedcoat of the flaxseed, where it could modulate the previously observed regulation operated by ABA on lignan synthesis. This effect could be mediated by the regulation of the transcription of a key gene for lignan synthesis in flax, the *LuPLR1* gene, encoding a pinoresinol lariciresinol reductase.

The positive effect of manumycin A, a specific inhibitor of farnesyltransferase, on lignan biosynthesis in flax cell suspension systems supports the hypothesis of the involvement of such an enzyme in the negative regulation of ABA action. In Arabidopsis, ERA1 is able to negatively regulate the ABA effects and the mutant *era1* has an enhanced sensitivity to ABA. When expressed in an Arabidopsis cell suspension (heterologous system) *LuERA1* is able to reverse the effect of the *era1* mutation. RNAi experiments in flax targeting the farnesyltransferase β-subunit encoded by the *LuERA1* gene led to an increase *LuPLR1* expression level associated with an increased content of lignan in transgenic calli.

Altogether these results strongly suggest a role of the product of this *LuERA1* gene in the ABA-mediated upregulation of lignan biosynthesis in flax cells through the activation of *LuPLR1* promoter. This ABA signaling pathway involving ERA1 probably acts through the ABRE box found in the promoter sequence of *LuPLR1*, a key gene for lignan synthesis in flax, as demonstrated by *LuPLR1* gene promoter–reporter experiments in flax cells using wild type and mutated promoter sequences.

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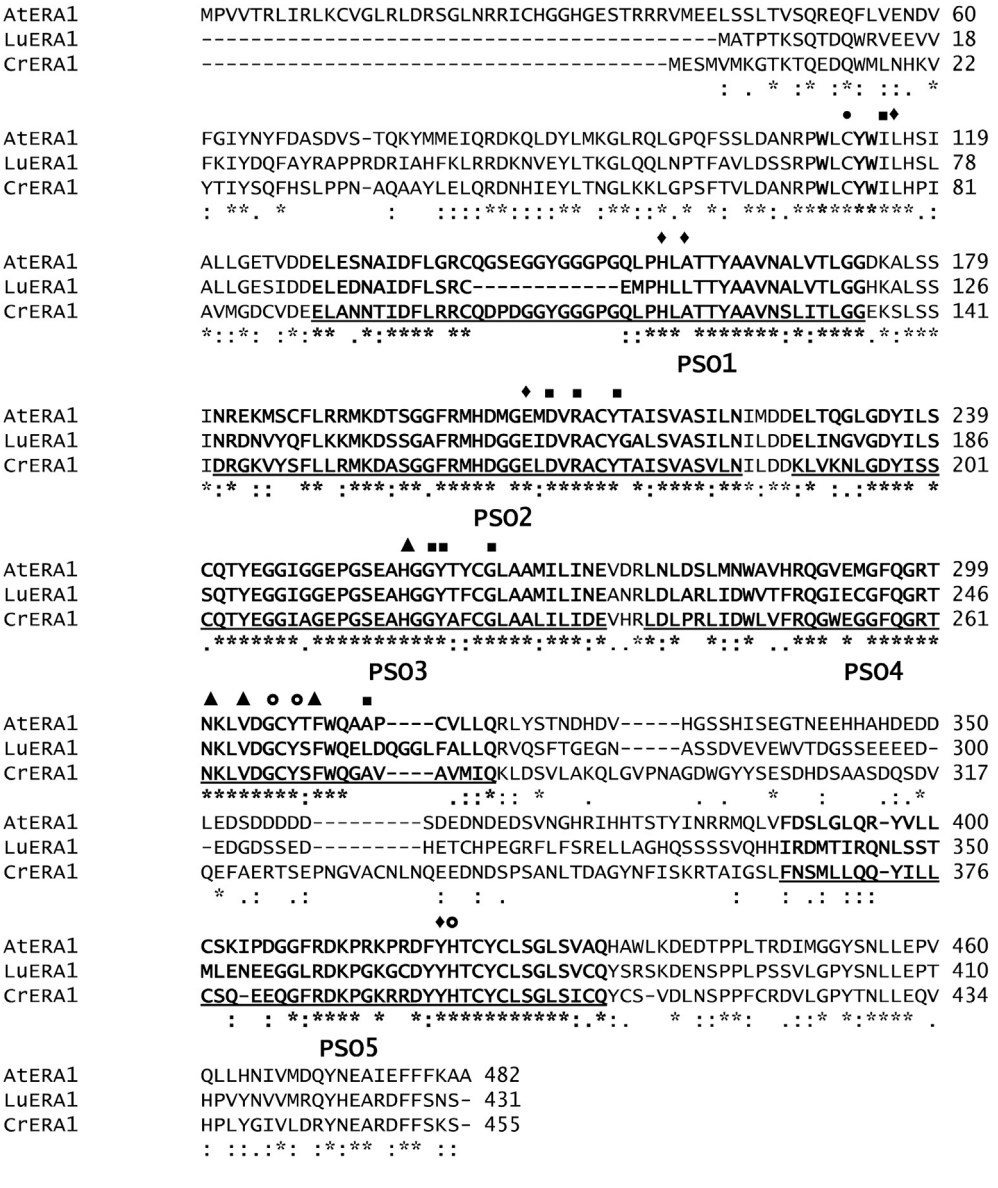
1. Introduction

Protein isoprenylation is a post-translational modification proposed to mediate protein–membrane and/or protein–protein interactions [1] and is a universal process conserved among protozoans, metazoans, fungi and plants [2]. In fact, this modification consists of the addition of a C15 farnesyl or a C20 geranylgeranyl moiety through a thioester bound to a conserved cysteine located in the C terminal end of the target protein [3]. This lipidation addition is catalyzed by two very similar cytosolic heterodimeric proteins, protein farnesyltransferase (PFT, EC 2.5.1.58) and protein geranylgeranyltransferase type I (PGGT1, EC 2.5.1.59) which share a common alpha subunit although they differ by their beta subunit. PFT and PGGT1 catalyze the prenylation of protein substrates with a conserved carboxy-terminal tetrapeptide sequence, the CaaX box, where C refers to cysteine, "a" is an aliphatic residue, and X typically refers to methionine, serine, alanine, or glutamine for PFT or to leucine for PGGT1 [4]. A third class of protein geranylgeranyltransferase, PGGT type II, is mainly responsible for the C20 geranyl moiety transfer on RAB protein. In



PSO Prenyltransferase and Squalene Oxidase Repeat
- ● Trp Residu FPP fixation site
- ■ FPP Fixation (farnesyl moiety)
- ♦ CAAX Fixation localized in the Cterm end of the target protein
- ▲ FPP Fixation (pyrophosphate moiety)
- o Zn^{2+} Fixation

Fig. 1. Amino acid sequence comparison of the ERA1 gene product encoding β-subunit of farnesyltransferase. PFT from *Arabidopsis thaliana* (AtERA1), *Linum usitatissimum* (LuERA1) and *Catharanthus roseus* (CrERA1); for details see box.

Arabidopsis thaliana the encoding genes and the corresponding mutants have been characterized [5]. The structural biology of the PFT proteins has already been described in detail by Lane and Beese [6]. As a reminder of structure, these are heterodimeric proteins composed of two subunits: an alpha- and a beta-subunit, of 48 and 46 kDa respectively. The PPTA (Protein prenyltransferase alpha subunit repeat, PF01239) domain consists of several repeats of a 31 residue motif folding into a right handed alpha hairpin that forms a two-layer banded structure referred to as the "crescent-shaped" superhelical domain. Together with the β-subunit, which folds itself into an α/β barrel leading to a groove formation right in the middle, the α-subunit forms a catalytic complex where both contribute to a strictly conserved active site. The groove is the site where both, the farnesyl diphosphate and (CaaX) peptide substrates are fused.

The beta-subunit of the *A. thaliana* PFT is encoded by the *ENHANCED RESPONSE TO ABSCISIC ACID 1* (*ERA1*, At5g40280) gene named due to the increased seed dormancy and stomatal closure in response to abscisic acid (ABA) as well as a drought tolerance of the corresponding mutant [7,8]. Brady et al. [9] have shown that in *A. thaliana* ERA1 acts upstream of ABI3 (ABSCISIC ACID INSENSITIVE 3, At3g24650), a plant-specific B3 domain transcription factor that regulates a set of events involved in seed development and which mediates abscisic acid (ABA) effect on transcription [10]. These observations suggest that at least one farnesylated protein functions as a negative regulator of ABA signaling [9].

ABA signaling plays a critical role in the regulation of many aspects of seed development and maturation [11,12]. In *A. thaliana*, around 10% of the genes are responsive to ABA, and among these genes a number are associated with embryo maturation and stress responses, such as those involved in the phenylpropanoid biosynthesis pathway [11]. Many of the effects of this phytohormone involve gene expression changes [13]. ABA-regulated genes are characterized by the presence of specific *cis*-acting elements, among which are the ABA response element (ABRE) and the MYB2 binding site [11–14].

Flax (*Linum usitatissimum* L.) seeds are the richest source of lignans [15] known to develop numerous beneficial effects on human health [16]. Their biosynthesis in flaxseed has been elucidated and starts with the dimerization of two E-coniferyl alcohol molecules to form (−)-pinoresinol, followed by its conversion into (−)-lariciresinol and (+)-secoisolariciresinol through the action of the enzyme pinoresinol lariciresinol reductase (PLR). In flaxseed (+)-secoisolariciresinol is then glucosylated intoits storage form, secoisolariciresinol diglucoside (SDG) [17]. The PLR enzymes, responsible for the two successive reduction steps from pinoresinol to secoisolariciresinol, have been proposed to be key enzymes for lignan biosynthesis in *Linum perenne* [18] and *Linum corymbulosum* [19]. In *L. usitatissimum*, two PLRs have been characterized, LuPLR1 was assigned to the formation of (+)-secoisolariciresinol the main lignan accumulated in seeds [20], whereas LuPLR2 was allocated to the formation of (−)-secoisolariciresinol mainly in vegetative tissues [21]. The study of *LuPLR1* gene expression using promoter–reporter transgenic plants during seed development confirmed these results in good accordance with the accumulation kinetic and location of SDG in flaxseed [22,23]. All these results put together clearly indicate the key role of LuPLR1 in the synthesis of flaxseed lignan (+)-secoisolariciresinol.

More recently it has been demonstrated that the transcriptional activation of *LuPLR1* gene in response to exogenous supply of ABA is accompanied by an increase of SDG accumulation in both flaxseed [24] and in flax cell cultures [25]. Several *cis*-acting elements involved in this ABA regulation have also been identified and characterized in the *LuPLR1* gene promoter sequence [22,24,25].

To date, the main flaxseed lignan, (+)-secoisolariciresinol, has been well studied due to its antioxidant, phytoestrogenic and cancer chemopreventive properties in humans [16]. However a number of important questions remain concerning the role of this compound in plants. The understanding of the transcriptional regulation of the genes related to lignan synthesis had little investigation although such data could yield information about their role *in planta*.

In the present study the protein farnesyltransferase (PFT) encoding gene, *LuERA1*, was isolated and functionally characterized to get insight into its role in the ABA-mediated regulation of (+)-secoisolariciresinol through *LuPLR1* gene expression control in flax. The structure of this putative PFT was also investigated *in silico* for further confirmation of its nature.

2. Results and discussion

2.1. Isolation and characterization of LuERA1, a gene encoding the β-subunit of farnesyltransferase in flax

2.1.1. LuERA1 gene structure

LuERA1 gene was cloned using primers designed from a BlastN search using partial cDNA on the Phytozome (www.phytozome.net) database [26] containing all recently released genome sequencing results from flax [27]. The annotated sequence *Lus10014536* matches the orthologs *AtERA1* (from *A. thaliana*) and *CrERA1* (from *Catharanthus roseus*) gene sequences. Cloning of the *LuERA1* complete cDNA was performed using an RT-PCR strategy on immature flax seed (developmental stage 4, [22]) and sequencing of this cDNA confirmed the latter *in silico* results. The structure of this putative 3471 pb long *LuERA1* gene sequence is composed of 13 exons (110, 87, 106, 83, 55, 53, 61, 63, 70, 87, 175, 100, 246 bp) and the mature transcript is 1296 bp long. Based on sequence comparison and confirmed by RT-PCR, *LuERA1* is close to the Arabidopsis orthologous gene (56.1% of sequence identity and 81.2% of similarity) and also to the *C. roseus* ortholog (51.6% of identity and 78.7% of similarity). These features suggest that this *LuERA1* gene belongs to the prenyltransferase gene family.

2.1.2. Molecular modeling of the LuERA1 protein

The *LuERA1* gene encodes a putative 473 amino-acid product (Fig. 1). A comparison of the LuERA1 encoded putative

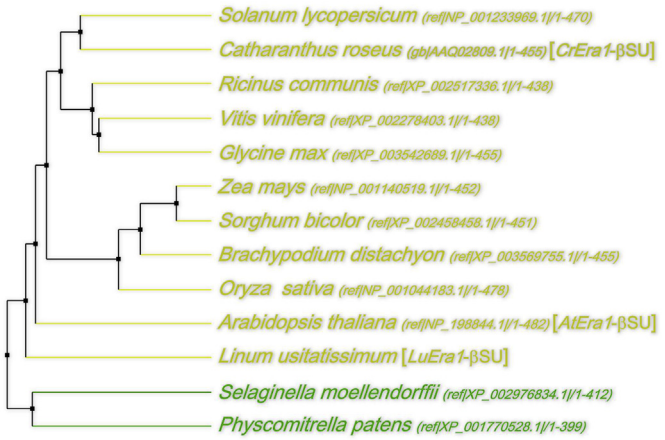


Fig. 2. Tree executed by Jalview based on a sequence alignment of farnesyl transferase-like proteins from the *plantae* kingdom sorted by their average distance based on a BLOSUM62 matrix (see ESM1). The given codes correspond to the NCBI Reference Sequence accession numbers with the exception of CrEra1-βSU for which the GenBank accession is given instead and of LuEra1-βSU not yet deposited.

farnesyltransferase β-subunit protein with those of *A. thaliana* and *C. roseus* shows the presence of five conserved Prenyltransferase and Squalene Oxydase repeat sequences (PSO) already mentioned in those species [28]. The amino-acids involved in the fixation to the CaaX box of the target proteins and those involved in the farnesylpyrophosphate substrate binding (Fig. 1) essential for catalytic reaction [28], were also found. The Zn^{2+} binding amino-acids were shown in this protein sequence [29]. These features and sequence alignments confirmed the nature of the encoded protein as a β-subunit of PFT (Fig. 2).

Given the conservation traits existing between LuERA1 and the well known PFT members and taking advantage of a fully piped tried and tested and straight forward meta-approach of @tome V2 [30], we were able, once a multiple alignment had been validated, to generate the expected coordinates by using the Modeller option [31] to attempt a modeling by homology in order to predict and to represent the plausible organization at the atomic scale of this new putative PFT member of the plant kingdom, the protein ERA1 of *L. usitatissimum*.

The evaluation program ProSA [32] returned a Z-score of −6.45 and of −7.5 conforming to what was expected with models of this size (Fig. 3A). The returned models of each subunit were combined into one quaternary structure by a single superimposition to a high resolution structure of a PFT (Fig. 3B). The given conservation of the key residues involved in the interface allowed reconstruction of the complex with less room left for doubt. Spatial correspondence of donors and acceptors of hydrogen bonds was respected (as controlled with PISA) and all the expected actors for the substrates acceptance and the catalysis were found exactly where they were expected (Fig. 3B–D) allowing placing the different substrates and the Zn^{2+} cofactor.

One peculiarity emerging from sequence alignments and from modeling (Figs. 3E–G and 4) is an acidic segment, forming a loop that might be part of an interacting site for a complementary basic patch borne by a putative partner.

To accomplish their function, transcription factors are necessarily basic at least at their DNA binding site or DNA Binding Domain, a functional module responsible for a direct DNA specific interaction. Such a segment is found in a number of plant homologs of PFT-like beta-subunits (e.g. *Glycine max*, *Solanum lycopersicum* and *Selaginella moellendorffii*), so it could suggest that this particular region might be a key of the localization regulation of transcription factors. This segment, which is constitutively water soluble, has to be exposed and easily accessible. An automatic screening executed by both GPS [33] and Motif Scan [34] gave it as a possible target for phosphorylases such as PKR or CK2 (Fig. 3F). Kinases like the latter trigger numerous processes in plants, and the disruption of sequestration of transcription factors by cytosolic proteins could be one of them. A related segment exists in the PFT beta-subunit of the yeast *Cryptococcus neoformans* (Fig. 3E). The latter protein had its structure solved by radiocrystallography and was recently published [35] in different kind of complexes.

2.1.3. LuERA1 gene promoter analysis

The promoter of the *LuERA1* gene was obtained by *in silico* screening of the publicly available database www.phytozome.net [26].

In silico analysis of the 5′ flanking 1500 bp sequence upstream of the translation initiation codon using the public databases, PLACE [36] and Plant CARE [37], confirmed the promoter nature of this sequence and revealed the presence of putative *cis*-acting elements (Fig. 5 and Table 1).

A TATA box (GAAT**TATAT**TGGG, Fig. 5) matching the consensus for plant TCAC**TATA**TATAG, is located 38 bp upstream of the putative transcription start determined as being the adenine with two cytosine residues downstream (+1, Fig. 5). This distance and these sequence characteristics are regular features of plant promoters [38]. The transcription start is located 179 bp upstream from the A of the translation start (ATG, Fig. 5). This 5′ untranslated region (5′ UTR, Fig. 5) is AT rich (around 63.1%), a common characteristic of the 5′ UTR sequence of the dicotyledons [39].

Several *cis*-acting elements involved in hormonal regulation and tissue such as root and seed specific expressions have been identified in the putative promoter region of the *LuERA1* gene [40–45], consistent with the role of the plant PFT [5,46].

Among the several *cis*-acting elements involved in the tissue-specificity expression located in this promoter sequence of the *LuERA1* gene GLU-box, E-box and SEF3 motifs (Fig. 5 and Table 1) have been previously implicated in the seed expression of several genes as they constitute important binding sites for transcription factor enhancing gene expression of several storage proteins in seeds [45], such as legumin [40], glutenin [40], napin [47] or beta-conglycinin [48]. The presence of such motifs is consistent with a potential role of ERA1 in the control of biological process associated with seed maturation and germination such as the B3 domain transcription factor ABI3 involved in seed maturation [5]. Interestingly a putative binding site for RAV1 [49], another well known B3 domain transcription factor, has been identified in the promoter sequence of the *LuERA1* gene (Fig. 5 and Table 1).

This putative promoter sequence also contains several regulatory motifs required for hormonal control such as the ABRE and MYB2 motifs involved in the response to ABA [14,50], GARE involved in response to GA [51,52] and motif involved in response to auxin [53].

The regulation of phytohormone signaling by protein isoprenylation and by the PFT in particular is well documented [1,9] and both ABA and GA were found to regulate the lignan synthesis in flax cells [25]. Interestingly a putative binding site for the AGAMOUS transcription factor [54] known to negatively regulate the *Arabidopsis* MADS box transcription factor APETALA1 (AP1) [55] which is involved in determination of the outer floral organ nature and is known to be a substrate for PFT [56], has been identified in the *LuERA1* gene promoter sequence (Fig. 5 and Table 1).

Other putative consensus binding sequences for transcription factors involved in biotic responses (Wbox involved in response to elicitors for example [57,58]) or abiotic responses (DRE involved in response to dehydration [59,60]) have also been identified in the promoter region of *LuERA1* gene.

Interestingly, the previously demonstrated hormonal control of the *LuPLR1* gene expression and lignan accumulation in flax by ABA [24] and GA [25] and the presence of some common putative *cis*-acting elements identified in the promoter sequences of the *LuERA1 and LuPLR1* genes, such as ABRE, MYB2, SEF3 or Wbox motifs [22,24,25], could explain, at least partly, the similarity between the expression pattern of these genes in flaxseed (Fig. 6).

2.1.4. Pattern of LuERA1 expression

LuERA1 transcripts were detected by RT-PCR in roots, leaves, cell suspensions and at greater levels in stem and seeds (Fig. 6A and B). *ERA1* transcript detection has previously been described in roots and leaves of *C. roseus* [28], in roots of *Pisum sativum* [61] and in the inner phloem of stems in transgenic tobacco transformed with a pea *ERA1* promoter–gus reporter [46].

Interestingly, the detection of *LuERA1* transcripts in seeds supports its potential role on ABA-signaling by controlling several processes in seed development and maturation [10]. *LuERA1* expression was also evidenced in maturing seeds as was also observed by Zhou et al. [46] for the orthologous *PsERA1* gene in pea. Cutler et al. [5] suggested that PFT plays a role in ABA signaling in Arabidopsis seed. In our immature seeds, *LuERA1* gene expression was observed mainly in the outer part of the tegument

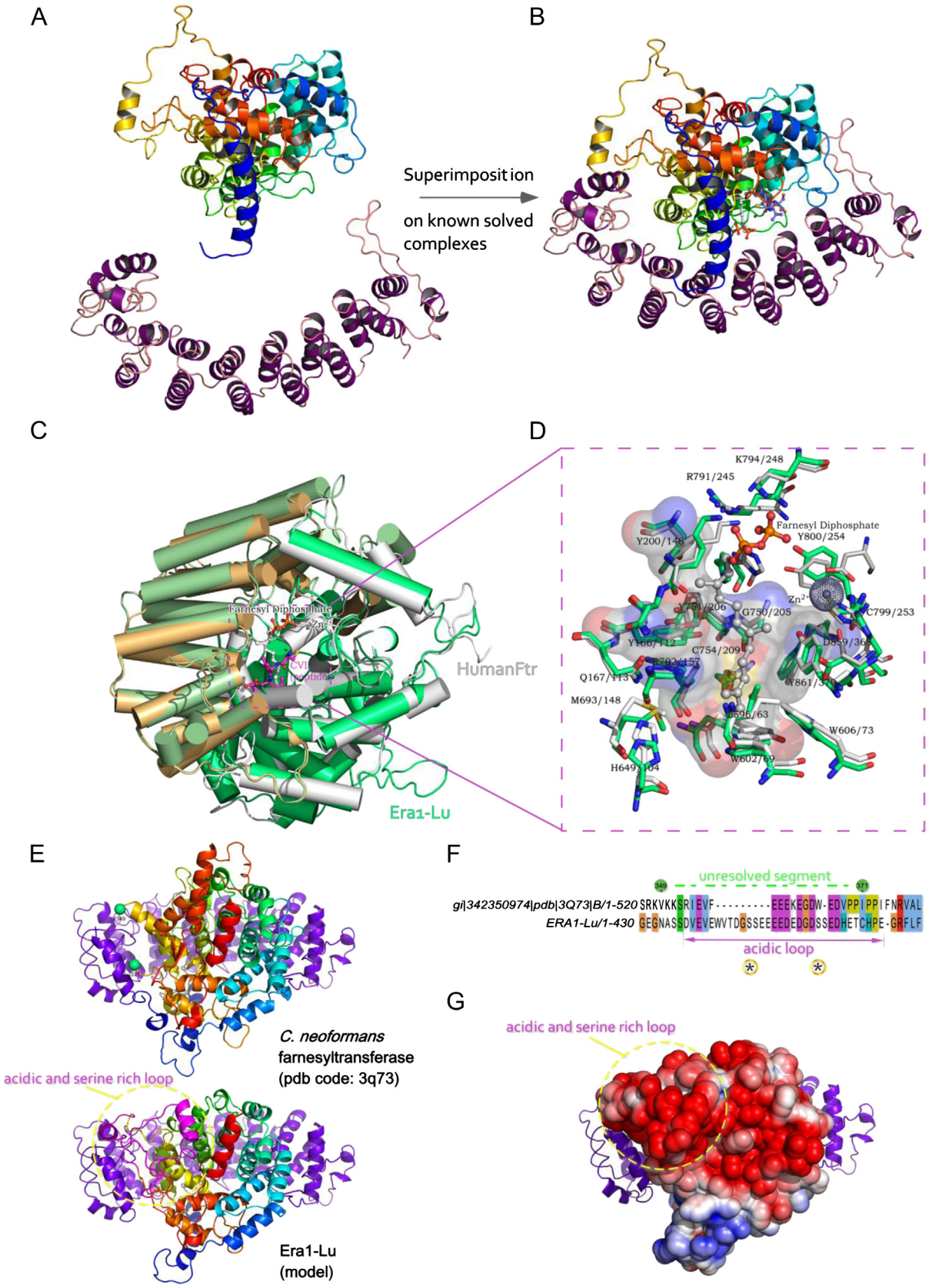


Fig. 3. Molecular modeling of LuERA1 protein. A – Cartoon representation of both subunit models of the farnesyl transferase from *Linum usitatissimum* (LuERA1) as returned by "@tome V2" metaserver [31]. The alpha subunit is colored purple while the beta subunit is colored rainbow style to easily follow the amino acid progression along the chain from the Nterminus (blue) to the Cterminus (red). B – Result of the minimization by GROMACS [71] of the superimposition of the previous models on the high resolution structure of the human farnesyl transferase (by keeping the same representation encoding). The ligands "in balls and sticks" were kept in place to locate, define and check the conformity of the binding sites. These are – from left to right – farnesyl-diphosphate, Zn^{2+} and the CVLS peptide. C – Schematic representation of the superimposition of LuERA1, in green, on the human farnesyl transferase [82], in

(Fig. 6B), a pattern similar to that of *LuPLR1* gene which is regulated by ABA [22]. This expression profile could suggest a potential involvement of LuERA1 in the *LuPLR1* expression regulation by ABA.

2.2. Role of LuERA1 and protein farnesylation events in the ABA-mediated regulation of the LuPLR1 gene expression and lignan biosynthesis

2.2.1. The protein farnesyltransferase inhibitor ManuA increases LuPLR1 gene expression and (+)-secoisolariciresinol accumulation in flax cell cultures

Manumycin A (ManuA), a specific inhibitor of PFT [62] targeting its β-subunit [61] was used to investigate the role of LuERA1 in ABA-mediated regulation of the *LuPLR1* gene expression and lignan biosynthesis. When adding ManuA to flax cell suspensions containing a promoter–reporter construct (*LuPLR1* gene promoter fused to the *uidA* reporter gene), an increase of *LuPLR1* expression is detected by monitoring the β-glucuronidase activity (Fig. 7A) and by RT-sqPCR (Fig. 7B) whatever the inhibitor concentration used. As a consequence, (+)-secoisolariciresinol concentration was also higher in ManuA-treated cell suspensions. Yet the highest secoisolariciresinol accumulation was not obtained with the highest ManuA concentration, perhaps due to an overall detrimental effect of this inhibitor on the metabolism caused by pleiotropic effects with 5 μM of this PFT inhibitor. Indeed, previous work conducted in our lab showed that exposure to high Manu A concentration for days affected the cells in a manner that suggested a deleterious effect (data not shown). Ali et al. [62] suggest such an effect could result from damage to the mitochondria.

ManuA is thus able to promote the expression the *LuPLR1* gene and the accumulation of (+)-secoisolariciresinol. This gene has been demonstrated to be regulated by ABA in flax [24,25]. The influence of ManuA is similar to that observed with ABA treatment [24] which strongly suggests that LuERA1 could modulate metabolism of lignans. The known role of negative regulator of the β-subunit of PFT *ERA1* in ABA-mediated effects [5,46] encouraged us to perform experiments to verify if such negative regulation does exist in flax.

2.2.2. Effects of ManuA and ABA supplementation on LuPLR1 gene expression and (+)-secoisolariciresinol accumulation in maturing flax seed

The study using cell suspension led us to hypothesize that LuERA1 is involved in the *LuPLR1* regulation and SDG accumulation. In flax plants, the seed coat constitutes the main tissue for expression of the *LuPLR1* gene and the accumulation site of (+)-secoisolariciresinol [17,22]. Using the same experimental system as used in Ref. [24] the effect of ManuA and ABA alone or in combination on *LuPLR1* gene expression was investigated using immature seed of transgenic plants with *LuPLR1* promoter fused to the *GUSint* reporter gene (Fig. 8A). Lignan accumulation was measured in immature capsules using wild type seed in order to confirm the results obtained at metabolite level (Fig. 8A). An up-regulation of *LuPLR1* gene expression was shown by GUS expression quantification in *pLuPLR1:GUS* seeds whatever the applied treatment (Fig. 8A). In parallel, the increased *LuPLR1* gene expression observed in treated seeds led to a 2.5-fold increase in SECO content per seed (Fig. 8B). These results are in accordance with the effect of ManuA observed with flax cell suspensions (Fig. 7) suggesting that in seeds *LuPLR1* expression as well as SDG content could also be regulated by LuERA1.

No additive effect of the ManuA and ABA treatment on *LuPLR1* gene promoter transcriptional activity and on lignan accumulation was observed (Fig. 8). This apparent discrepancy could be explained by the difference of the basal endogenous ABA concentration reported for these tissues (i.e. between 11.6 and 23.9 ng/g DW in whole seeds [24] and 5.9 ng/g DW in flax suspension cells [25]).

The ABA treatments have confirmed the previously shown enhancing effect of this phytohormone on *LuPLR1* gene expression and lignan accumulation in seeds [24]. The new major point is that the PFT inhibitor ManuA, also raises *LuPLR1* gene expression and lignan accumulation in flax seed (Fig. 8). These results suggest a possible role of ERA1 in ABA-signaling leading to *LuPLR1* stimulation and lignan accumulation in flax seed, while it is known that at least one farnesylated protein acts as a negative regulator of ABA signaling [4,5,7]. In *A. thaliana* protein farnesylation events impact the gene expression regulation mediated by the *ABI3* transcription factor [8] and therefore affect ABA-mediated gene expression regulation which involves this transcription factor.

2.2.3. Role of protein farnesylation events in the ABA-mediated regulation of LuPLR1 gene is conserved in Arabidopsis

In order to get a functional confirmation that *LuERA1* encodes a subunit of the farnesyltransferase, experiments have been performed on *A. thaliana* cell lines initiated from Columbia ecotype, either wild type (WT) or *era1* homozygote mutant (SALK_116854C; Fig. 9A). In these established cell lines the effect of ABA and ManuA and overexpression of *LuERA1* were monitored using a promoter–reporter designed to reproduce the effect on the key gene involved in lignan synthesis. This expression is upregulated by ABA in flax (Fig. 9).

The pLuPLR1:GUS construct (Fig. 9B) was introduced in cell suspension cultures of wild type Col-0 wild type and in *era1* mutant by *Agrobacterium*-based transient transformation. The reporter gene activity was measured in the absence or presence of 50 μM ABA, alone or in combination with 5 μM ManuA (Fig. 9D). The *LuPLR1* promoter was active in Col-0 cells and the expression driven by this promoter was higher in the *era1* mutant which shows that the lack of PFT activity enhances *LuPLR1* expression and confirms the probable involvement of ERA1 in lignan biosynthesis regulation. *LuPLR1* expression was increased 2-fold by the treatment with 50 μM ABA both in WT and in mutant cells. WT cells supplemented with ABA had an expression level similar to *era1* cells most likely because era1 failed to down regulate the effect of a low endogenous level of ABA (Fig. 9C). Adding 1 μM ManuA to the 50 μM ABA treatment results only in a slight but not significant increase in *LuPLR1* promoter activity in Col-0. Similarly no cooperative effect for the combined treatment on the mutant *era1* was observed. One could expect that Col-0 treated with ManuA and ABA would behave as the *era1* mutant does but in fact the significant difference

white and pale copper (humanFtr – pdb code: 1ld8), as processed by PyMOL [79,80]. The root-mean-square deviation (RMSD) returned by the program after 5 cycles of the 'align' command equals 0.772 Å for 526 atoms finally aligned. D – Ligplot+ [81] result for the binding of the farnesyl molecule inside its pocket within the quaternary structure of the humanFtr (pdb code 1ld8). The concerned residues are shown with carbons painted white and the corresponding residues from the LuERA1 model are shown with carbons painted green. This superimposition is in favor of a strict conversation of those key residues among phyla. E – Side by side comparison of the topology of the LuERA1 beta subunit model (down) with the high resolution crystallographic structure of the farnesyl transferase beta subunit from *Cryptococcus neoformans* (up) [36]. The unresolved loop implant is highlighted with labeled green balls on the mycete structure while the corresponding modeled loop is illustrated in magenta. F – Local highlight of a T-Coffee [78] alignment (of the LuERA1 beta subunit sequence with the farnesyl transferase beta subunit from *Cryptococcus neoformans* sequence) from which emerges the evidence of a common trait between the two taxa: an acidic motif. Besides, this acidic motif is predicted as a putative phosphorylation site by both GPS [34] and Motif Scan [35] on serine positions as designated by a circled *. G – Reported electrostatic surface potential calculated using sequentially PDB2PQR [83] and APBS [84], for the LuERA1 beta subunit model. The result is represented as a tri-color gradient going from blue (+3 kT) over white (neutral) to red (−3 kT). The acidic and serine-rich loop contributes to the negative surface displayed on the free side of the protein and could provide a docking site for a basic interactor (Fig. 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)00017171

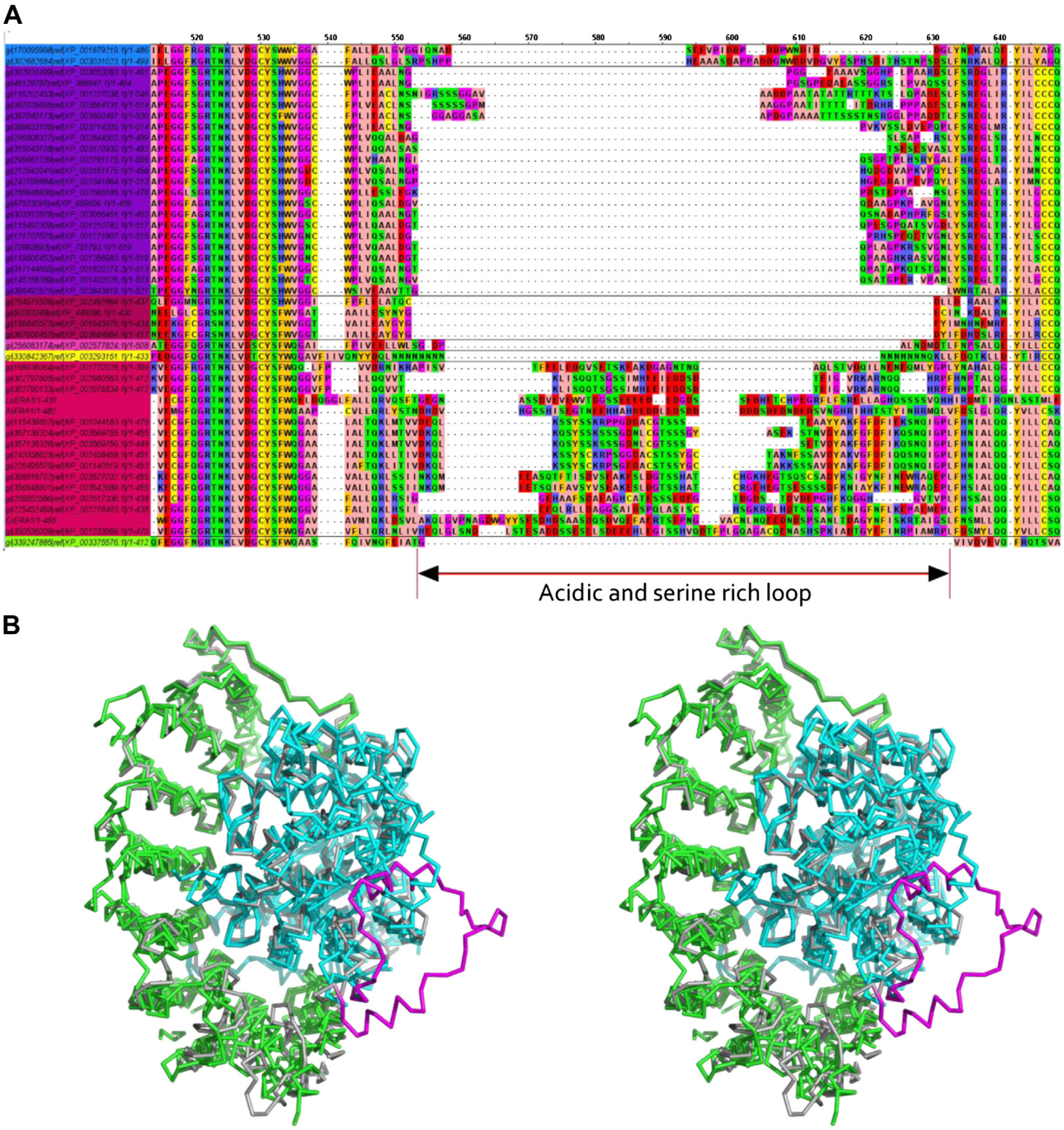


Fig. 4. Alignment profile PFT sequences and superimposition on the selected LuERA1 model. A – Zoom on a similar region concerning an inserted loop, the one is mostly acid and serine-rich (Zappo coloring scheme). This result comes from an alignment of 283 sequences of farnesyl transferase-like proteins among the different phyla as returned by a clustalw [76] comparison and as visualized in Jalview [75] (Clustalx coloring scheme) (For more detail see ESM2). B – Crossed-eyes stereoview superimposition on the selected LuERA1model (white) of the farnesyl transferases high resolution structures from human, rat and yeast (green or cyan depending on the subunit depicted) showing in magenta the peculiar acidic modeled loop relative location. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

between these cell cultures when ABA/ManuA treatment is applied suggests that ManuA doesn't inhibit the isoprenylation operated by the PFT totally. The higher β-glucuronidase activity in the *era1* mutant without exogenous ABA might indicate (Fig. 9D) that some endogenous ABA is already present; its action being counterparted by the farnesylation in the WT but not in the mutant.

These results indicate that in this heterologous system the upregulation of *LuPLR1* expression by ABA is conserved and that Arabidopsis ERA1 could be a negative regulator of this effect.

An effector construct was then introduced via *Agrobacterium* to transiently express the flax *LuERA1* in both WT and mutant cell lines. Expression of the *LuERA1* gene has no effect on the transcriptional activity of the *LuPLR1* gene promoter in wild type Col-0 line. On the contrary, it has resulted in a dramatic decrease of β-glucuronidase activity in *era1* mutant cells (Fig. 9D). The *era1* mutant expressing this heterologous coding sequence was clearly complemented as it exhibited a GUS activity level similar to the WT in the previous experiment. Expressing LuERA1 in the WT had no

Fig. 5. Promoter sequence analysis of the putative *LuERA1* encoding gene. The putative regulatory boxes identified with PLACE and Plant CARE are underlined, they are described further in Table 1. The putative transcription start is at the A with +1 and the translation start is underlined.

promoting effect, as expected given the presence and functionality of the endogenous ERA1 established by the previous experiments.

The Arabidopsis orthologs to *LuPLR1* (*AtPR1* and *AtPR2*) are encoding pinoresinol reductase which convert pinoresinol to lariciresinol [63] and not PLR able to catalyze two successive steps of reduction up to secoisolariciresinol as in flax. Thus, in Arabidopsis, the main lignan is the lariciresinol accumulated in the root. Despite this difference it appears that transcriptional regulation of lignan biosynthesis could be conserved between flax and Arabidopsis.

LuERA1 is able to complement the *era1* mutant cells of Arabidopsis and probably restore the control on the ABA-induction of the *LuPLR1* gene promoter through protein isoprenylation events. It can be hypothesized for flax that, as already observed in *A. thaliana* [7,9] at least one farnesylated protein functions as a negative regulator of ABA signaling.

2.2.4. The ABRE and MYB2 ABA-response elements located in the LuPLR1 promoter are both required for the LuPLR1 regulation by ABA but the regulation by ERA1 (involving protein farnesylation events) is operated only through the ABRE sequence

In order to identify the sequences that could be involved in the activation process by ManuA and ABA in the *LuPLR1* gene

Table 1
Putative *cis*-acting elements localized in the LuERA1 gene promoter sequence.

Elements	Sequences	Location	Roles	Ref
Tissue expression				
GLU-BOX	TGTAAAAGT	−583	Glutenin box for seed expression	[40]
SEF3	AACCCA	−73	Seed expression	[41,42]
OSEroot	AAAGAT	−226	Sequence motifs of organ-specific elements (OSE)/Root expression motif	[43,44]
ROOT-MOTIF	ATATT	−67	Root expression motif	[45]
		−446		
Hormone signaling				
ABRE box	(A)ACGT(T)	−1038	Transcriptional regulation of ABI3- and ABA-responsive genes	[50]
MYB2box	AAACCA	−1006	MYB recognition site found in the promoters of dehydration-responsive genes	[14]
		−1088		
NTBB	ACTTTA	−322	Root expression and auxin induction	[53]
GARE	TAACGTA	−987	GA-responsive element	[52]
	TAACAAA	−1164		[51]
Others				
AGAMOUS	CWWWWWWWWG	−196	Binding site for AGL15 (AGAMOUS-like 15)	[54]
RAV1	CAACA	−508	Binding consensus sequence of Arabidopsis transcription factor RAV1	[49]
ACGTcore	ACGT	−241	Required for expression of erd1 (early responsive to dehydration)	[59]
DRE	GTCGAC	−651	Dehydration-responsive, also involved in low temperature response	[60]
Wbox	AGTCA	−171	Plant defense binding site for WRKY	[57]
		−336		[58]

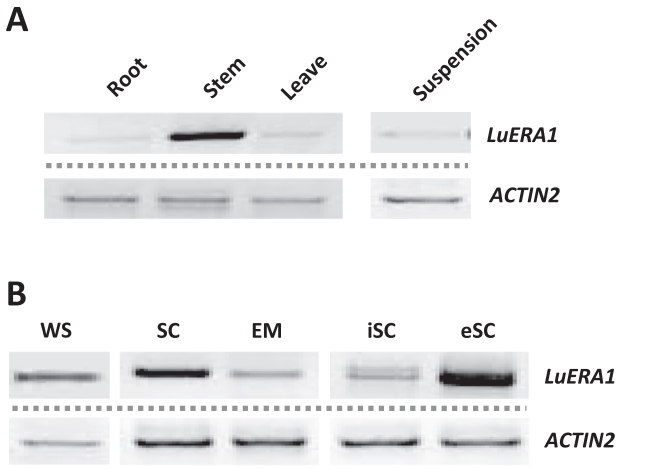


Fig. 6. *LuERA1* expression pattern in flax organs and in expression localization maturing flax seed. A – RT-sqPCR analysis of *LuERA1* gene expression in different plant organs and tissues (roots, stems, leaves, cell suspensions). B – RT-sqPCR analysis of *LuERA1* gene expression in maturing seed at developmental stage 3 [21] in whole seeds (WS) and in different parts of the seed: seedcoat (SC), embryo (E), internal seedcoat (iSC) and external seedcoat (eSC). Total RNAs isolated were subjected to semi quantitative RT-PCR analysis *ACTIN2* gene as internal control. Ten microliters of RT-PCR products were loaded on 1% (w:v) agarose gel. This figure is representative of three independent experiments performed in the same conditions.

promoter, the two *cis*-acting elements ABRE and MYB2, previously reported for their ABA-response ability [24,25] and present in *LuPLR1* promoter were functionally tested. To this aim, constructs of *LuPLR1* promoter–gus reporter with the WT promoter or with promoter harboring a single mutation in one of the regulatory boxes (mABRE and mMYB2) or a simultaneous disruption (mABRE/MYB2) were generated (Fig. 10A). They were expressed transiently in flax cells and the effect of ManuA treatment with or without additional ABA was investigated according to the β-glucuronidase activity.

In the control, ABA and ManuA supplementation of the flax cells was synergistic for the increase of the *LuPLR1* WT-promoter driven gene expression (Fig. 10B). This observation suggests a LuERA1 antagonistic role in the ABA-stimulation of *LuPLR1* expression in accordance with a negative role of PFT in ABA-signaling already proposed by Cutler et al. [5].

The ABRE sequence mutation abolished the positive effect of ManuA treatment on the *LuPLR1* promoter gene driven expression (Fig. 10B) which suggests an important role of the ABRE box in the regulation of *LuPLR1* gene expression by a protein farnesylation event. Moreover a reduced *LuPLR1* promoter activity was observed with the ManuA/ABA-treatment as compared to control. As the ABRE box is known to be involved in the ABA response, the present results suggest that this response might involve a protein farnesylation event. Nevertheless, the GUS activity monitored after the addition of ABA in transformed cells using the *LuPLR1* gene promoter with the ABRE sequence mutation (mABRE) was still significantly higher than when using the cells harboring a non mutated promoter (Fig. 10B). Thus ABA still positively modulates the promoter activity indicating either a regulation path that is not sensitive to the protein farnesylation or that does not require the ABRE box to be exerted.

On the opposite, the single mutation in the MYB2 *cis*-acting element did not suppress the enhancement of *LuPLR1* promoter activity by the ManuA supply, suggesting that ERA1 action is not mediated by this MYB2 box (Fig. 10B). As already observed with ABRE mutation, the MYB2 mutation reduced the upregulation by

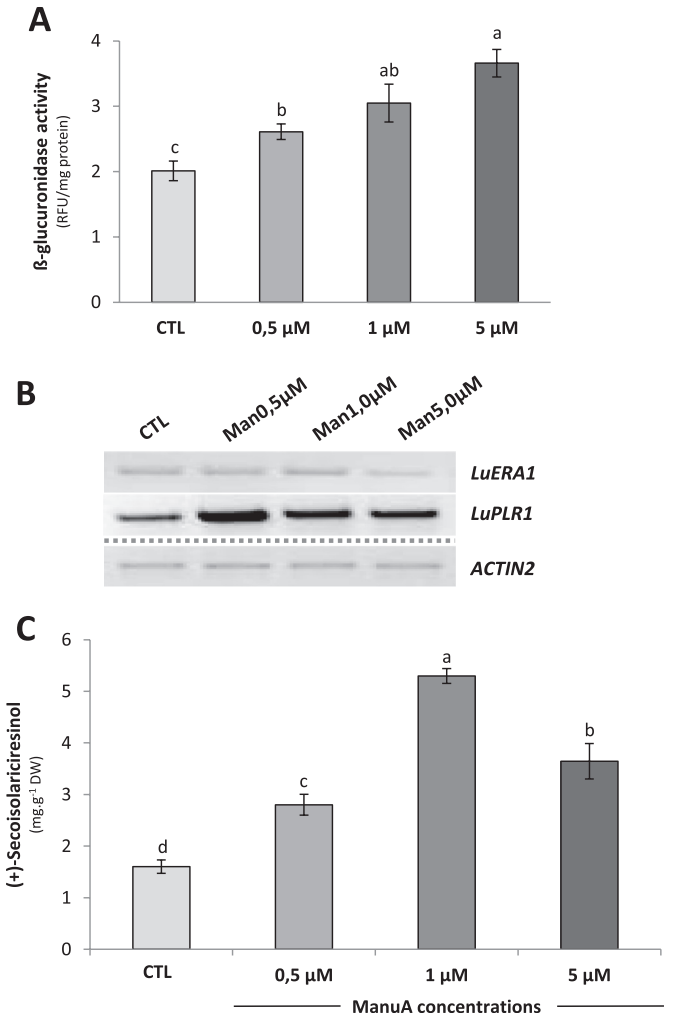


Fig. 7. Effects of ManuA supplementation (0.5 µM, 1 µM, 5 µM) on *LuPLR1* gene expression and lignan biosynthesis in flax cell suspensions. A – β-Glucuronidase activity in cell suspensions of stably transformed transgenic flax cell suspensions containing the pLuPLR1:GUS promoter–reporter construct and treated with ManuA. CTL is the control without ManuA. Data are expressed as the mean of 3 independent experimentations ± standard deviation of the mean and different letters indicate significant differences between conditions ($P < 0.05$). B – RT-sqPCR analysis of *LuERA1* and *LuPLR1* gene expression in flax cell suspensions treated with ManuA. Total RNAs isolated were subjected to semi quantitative RT-PCR analysis with *ACTIN2* gene as internal control. This figure is representative of three independent experiments performed in the same conditions. C – (+)-Secoisolariciresinol quantification in flax cell suspensions treated with ManuA. CTL is the control without ManuA. Data are expressed as the mean of 3 independent experimentations ± standard deviation of the mean and different letters indicate significant differences between conditions ($P < 0.05$).

the ManuA/ABA treatment but did not abolish it totally. This last result is in accordance with the involvement of MYB2 in the ABA-regulation of *LuPLR1* expression in flax which was previously reported [25]. Simultaneous disruption of ABRE and MYB2 sequence, in either ManuA or ManuA/ABA-treated flax cells, prevents any *LuPLR1* gene expression enhancement (Fig. 10B).

Altogether these results lead to the hypothesis that both ABRE and MYB2 boxes might play a role in ABA upregulation of *LuPLR1* expression but protein farnesylation event would operate only in the signaling way that require the ABRE box.

All these data are consistent with the involvement of LuERA1 in the ABA-mediated *LuPLR1* gene expression regulation that might be acting as negative regulator in ABA-signaling in flax cells.

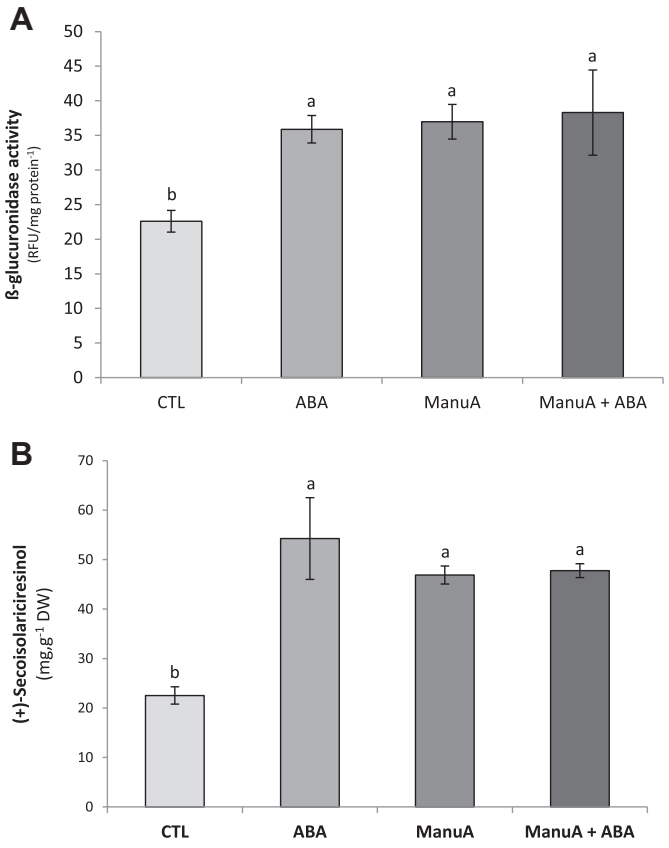


Fig. 8. Effects of ManuA combined with ABA supplementation on LuPLR1 gene expression and lignan accumulation in maturing seed. Seed (developmental stage 2 and 3 [22]) were treated with ManuA 5 μM only or ABA 50 μM or a combination of both. A – β-Glucuronidase activity in seeds of stably transformed transgenic flax cell suspensions containing the pLuPLR1:GUS promoter–reporter construct. CTL is the control without treatment. Data are expressed as the mean of 3 independent experimentations ± standard deviation of the mean and different letters indicate significant differences between conditions ($P < 0.05$). B – (+)-Secoisolariciresinol quantification in treated flax seed. CTL is the control without treatment. Data are expressed as the mean of 3 independent experimentations ± standard deviation of the mean and different letters indicate significant differences between conditions ($P < 0.05$).

2.2.5. Reduction of LuERA1 gene expression through RNA-interference leads to an enhanced lignan accumulation

RNA interference was used to reduce *LuERA1* transcript level in flax. A 517 bp long fragment in the N-terminal sequence was chosen to target the β-subunit of PFT specifically. Transformation of flax hypocotyls using *Agrobacterium tumefaciens* harboring the *LuERA1* RNAi-construction was performed to investigate effect of reduced *LuERA1* transcript level in a plant system. Transgenic buds were generated and screened for kanamycin resistance and *eGFP* gene expression and callus lines were initiated from the micro-propagated shoots generated from the buds in order to obtain enough biological material for (+)-secoisolariciresinol accumulation analysis.

The transgenic nature of callus lines was further assessed by the expression of the GFP gene that was present in the construct and led to visible presence of GFP (Fig. 11A). The LuERA1 expression was estimated in three lines: it was dramatically reduced but not totally decreased (Fig. 11B). This strong decrease was accompanied by an increase of *LuPLR1* gene expression (Fig. 11B) thus confirming the hypothesis of a down regulation of *LuPLR1* activity by the LuERA1 protein.

Transgenic RNAi calli with reduced *LuERA1* expression contained significantly more lignan than the control WT calli. These results are in accord with those of ManuA experiments and support the involvement of LuERA1 in the regulation of lignan biosynthesis in flax.

3. Conclusion

The upregulation of the *LuPLR1* gene expression and the positive impact on lignan biosynthesis observed with a specific inhibitor of the PFT (ManuA), in both flax and Arabidopsis cells systems, suggest the involvement of a protein farnesylation event in the promotion of the lignan synthesis by ABA through the transcriptional activation of the synthesis of PLR enzyme. The PFT may inhibit the *LuPLR1* expression directly or indirectly, with an antagonistic action to ABA. A negative regulation by farnesylation events of the AtABI3 (ABSCISIC ACID INSENSITIVE 3) transcription factor involved in ABA signaling, has been shown in *A. thaliana* [9] and supports the existence of farnesylated proteins implicated in the ABA signaling and in the transcriptional activation of the *LuPLR1* gene in flax.

This hypothesis is reinforced by RNAi experiments in flax targeting the PFT β-subunit encoded by the *LuERA1* gene: the transgenic calli present a reduced LuERA1 expression level and increased lignan accumulation.

This is confirmed by the reversion of the positive effect of flax *LuERA1* mutation on LuPLR1 expression in *A. thaliana*.

Altogether these results strongly suggest a role of the product of the *LuERA1* gene in the ABA-mediated upregulation of lignan biosynthesis in flax cells. This ABA signaling pathway involving ERA1 probably acts through the ABRE box found in the promoter sequence of *LuPLR1*, a key gene for lignan synthesis in flax, as demonstrated by promoter–reporter experiments in flax cells using WT and mutated promoters.

4. Methods

4.1. Plant material

The LuCY1 cell suspension culture of *L. usitatissimum* cv Barbara was established from hypocotyl-derived calli and maintained as described in Ref. [64].

Flax (cv. Barbara) *LuPLR1* promoter fused to the *GUSint* reporter gene transgenic plants were described in Ref. [22]. The seed development stages were defined in Ref. [22].

A. thaliana plants, Columbia (Col-0) wild type and *era1* homozygote mutant line (SALK_116854C) were provided by the SALK Institute. *A. thaliana* cell suspensions were initiated from hypocotyl-derived calli and grown in the dark at 22 °C with gentle shaking at 120 rpm as described by Berger et al. [65]. The cells were inoculated at a 1:5 dilution into fresh medium (4.3 g/l MS basal salts including vitamins (Duchefa), 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/l sucrose pH 5.8 at seven day intervals).

4.2. ABA and ManuA treatments

Cell suspension cultures were submitted to exogenous addition of various concentrations of (±) *cis-trans* ABA or ManuA (Sigma) at the end of the exponential growth phase at day 10. ABA or GA solutions (dissolved in DMSO and diluted with the M2 culture medium) were added to 2.5 ml of 1:5 diluted plant cell culture in six-well sterile culture plates (Greiner) at final concentrations of 10, 50 and 100 μM (for ABA) or 0.5, 1 and 5 μM (for ManuA). Control cells were inoculated with the same volume of DMSO-containing-M2 culture medium. Microplates were maintained on a gyratory shaker at 120 rpm in darkness at 25 °C. Cells were collected after 24 h for RT-sqPCR analysis, 48 h for GUS analysis and 96 h for lignan analysis, separated from the culture medium by centrifugation, washed twice with 1.5 ml distilled sterile water, and the pellet was stored at −80 °C for further use.

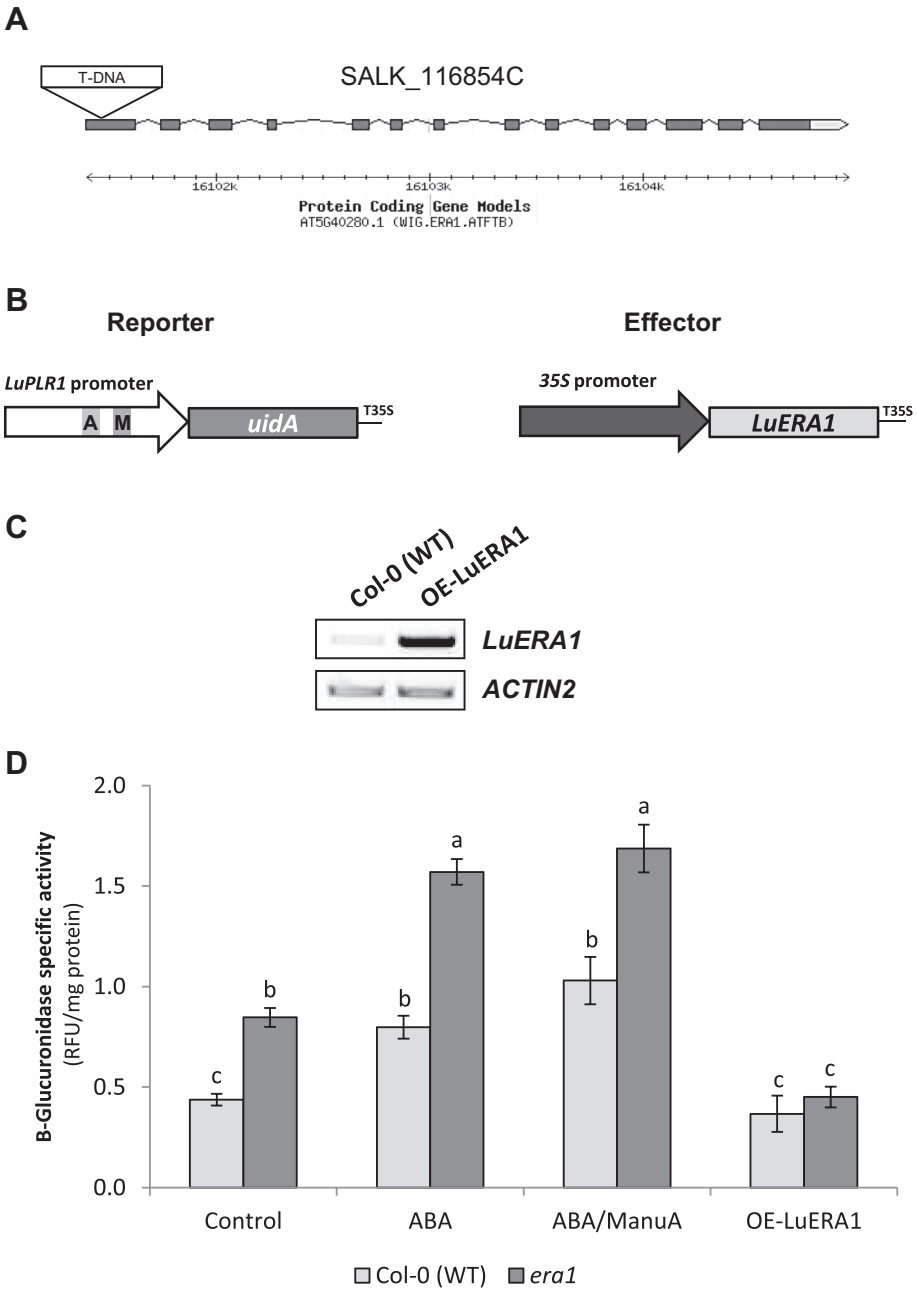


Fig. 9. Transcriptional activity of the *LuPLR1* gene promoter in wild type Col-0 and *era1* mutant *Arabidopsis thaliana* cell cultures. A – Schematic representations of the *AtERA1* mutated gene by T-DNA insertion in the first exon present in the Arabidopsis *era1* mutant (Salk-116854C). B – Schematic representations of the reporter (*β-glucuronidase* reporter gene expression driven by the *LuPLR1* gene promoter) and effectors constructs (*LuERA1* expression driven by the viral *35S* promoter) used for the *Agrobacterium* mediated-transient transformation of the flax cell cultures. (T35S: 35S terminator. A: ABRE Box, M: MYB2 box.). C – RT-sqPCR analysis of *LuERA1* gene expression respectively in non-transformed and *LuERA1* overexpressing wild type (Col0 WT). Total RNAs isolated were subjected to RT-sqPCR analysis *ACTIN2* gene as internal control. Ten microliters of RT-PCR products were loaded on 1% (w:v) agarose gel. This figure is representative of independent experiments performed in the same conditions. D – Effect of 50 μM ABA or ABA + ManuA 5 μM on the transcriptional activity of the *LuPLR1* gene promoter (β-glucuronidase reporter activity) in *Arabidopsis thaliana* cell suspensions (Col-0 (WT only transformed with the promoter–reporter construct) and *era1* mutant) determined 48 h after treatment. OE-LuERA1 are data obtained after transient expression of the effector construct in both lines. CTL is the control without treatment. Data are expressed as the mean of 3 independent experimentations ± standard deviation of the mean and different letters indicate significant differences between conditions ($P < 0.05$).

Capsules containing immature developing flax seeds were submitted to exogenous administration of ManuA and/or (±)-ABA (Sigma) following an experimental process described by Ford et al. [16] with modifications. Briefly, secondary inflorescence stems of wild or transgenic flax plants (stage 2) were harvested and placed in deionized water. Capsules with a remaining peduncle (around 1 cm) were pruned from stems and immediately size sorted. Three fresh capsules for each treatment were placed in one well of a 96-well microplate containing 150 μl of water containing DMSO (control seeds), 150 μl of water containing 20 μM ManuA in DMSO, 150 μl of water containing 100 μM ABA in DMSO or 150 μl of water containing both ManuA (20 μM) and ABA (100 μM) dissolved in DMSO and A. Microplates were maintained at 25 °C under a 16 h photoperiod. After uptake, capsules from each condition were dissected (at 48 h and 96 h post-treatment for glucuronidase activities and SDG contents, respectively), and seed (ca 60 per

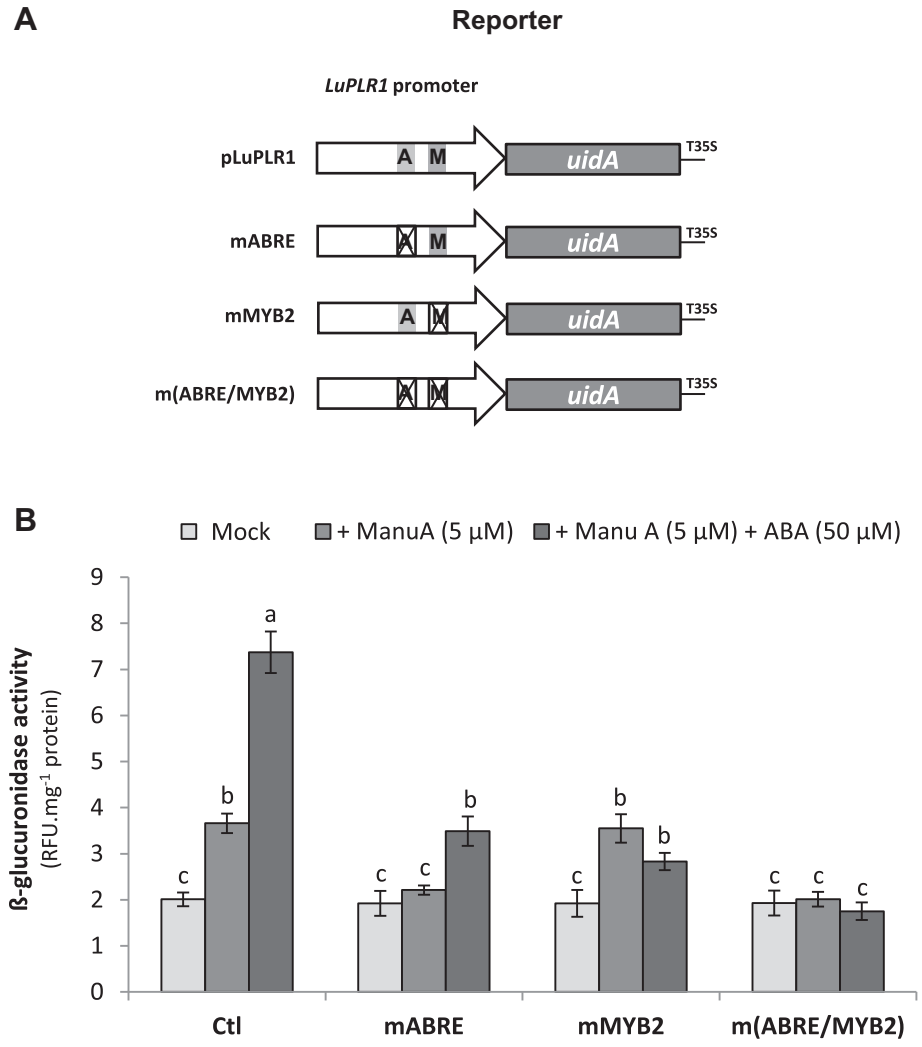


Fig. 10. Effects of mutations in the ABRE and/or MYB2 *cis*-acting elements on the *LuPLR1* gene promoter activity in flax cell suspensions in response to ManuA and ABA treatments. A – Schematic representations of the four constructions *pLuPLR1:GUS* used for the transgenesis of the flax cell cultures containing either the complete promoter or a mutated promoter for one or both of the ABA-response *cis*-acting elements (ABRE et MYB2). Crosses indicate mutations. B – β-Glucuronidase activity in transformed cell suspensions containing the pLuPLR1:GUS with complete or mutated sequences and treated with ManuA 5 µM only or in combination with ABA 50 µM. CTL is the control without treatment. Data are expressed as the mean of 3 independent experimentations ± standard deviation of the mean and different letters indicate significant differences between conditions ($P < 0.05$).

condition) were stored at −80 °C until used for molecular and chemical analysis.

4.3. RNA extraction and RT-sqPCR

Total RNA were extracted from 100 mg of ground frozen tissues using the RNeasy Plant Kit (Qiagen) and the RNase free DNase set (Qiagen) in order to eliminate DNA contamination, as described in Ref. [22]. PCR was performed with specific primers designed from the *LuPLR1* sequence [21] and the sqLuERA-F (CCGATCCTGCCCCTCCG) and sqLuERA-R (TCCCGGACCCACCTGGC) primers for *LuERA1* amplification. To normalize the amount of mRNA in each PCR reaction, a PCR product (632 bp) corresponding to the exon 2 of the *ACTIN2* gene was amplified with the same conditions described previously [22]. cDNA fragments were amplified during 23, 25 and 27 cycles.

4.4. Plasmid constructions

The *LuPLR1* gene constructs used in this study were described in Ref. [24]. The complete cDNA sequence of LuERA1 was amplified using the following set of primers: OE-LuERA1-CyF (5′-CCACATGAACATGGCATGC-3′) and OE-LuERA1-CyR (5′-TTAA-GAATTAGAGAAGAAGTC-3′) for the generation of the over-expression vector. A 517 bp fragment of the LuERA1 gene was amplified using the following set of primers: i-LuERA1-F (5′-ATGAACATGGCGATGCCGACGA-3′) and i-LuERA1-R (5′-GGGGAAATTGATGTGAGGGCTT-3′) for the generation of the LuERA1-RNAi vector. Each PCR fragment was amplified by PCR using the Pfx polymerase (Invitrogen), cloned into the entry vector pENTR/D-TOPO (Invitrogen) and verified by sequencing. Each fragment was then inserted into the pK7FWG2 and pK7GWIWG2-D(II) destination vectors [66] by recombination. The plasmids were then transferred into the disarmed *A. tumefaciens* strain GV3101 (pGV2260) by triparental mating with *E. coli* strain HB101 (pRK2013) as helper.

4.5. Plant transformations

For cells suspension studies the transient expression system described by Berger et al. [65] was used.

For plant RNAi stable *Agrobacterium*-mediated transformation was performed as described by Ref. [22]. Briefly: 5-day-old flax (cv.

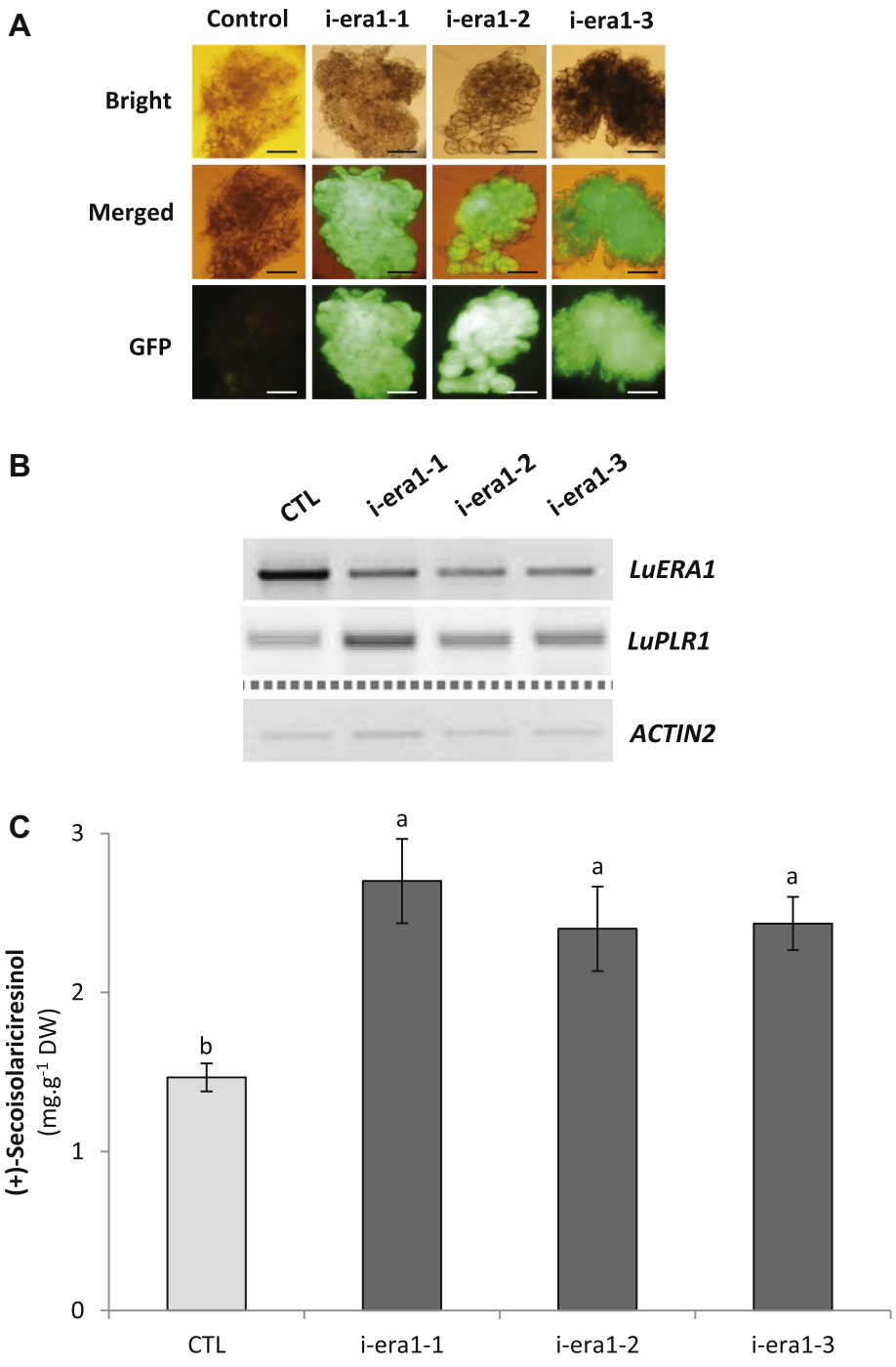


Fig. 11. Effect *LuERA1* RNAi transgenesis on *LuERA1* and *LuPLR1* genes expressions and lignan accumulation in flax calli. A – Wild type (*Linum usitatissimum* cv. Barbara; CTL) and three stably transformed (with *LuERA1-RNAi* construct; i-era1-1 to –3) callus lines expressing *LuERA1-RNAi* construction. Tissues were observed under bright field or fluorescence microscopy (using a 488 nm excitation filter and a 540 nm wavelength pass filter). B – RT-sqPCR analysis of *LuERA1* gene expression in wild type and 3 transgenic lines of callus interfered for the *LuERA1* gene compared to wild type (CTL). Total RNAs isolated were subjected to semi quantitative RT-PCR analysis *ACTIN2* gene as internal control. Ten microliters of RT-PCR products were loaded on 1% (w:v) agarose gel. This figure is representative of three independent experiments performed in the same conditions. C – (+)-Secoisolariciresinol accumulation in stably transformed flax callus in response to *LuERA1* transcript level reduction compared to untransformed callus (CTL). Data are expressed as the mean of 3 independent experimentations ± standard deviation of the mean and different letters indicate significant differences between conditions ($P < 0.05$).

Barbara) seedling hypocotyls were excised and co-cultivated for 2 days with *A. tumefaciens*. Timentin (500 mg l^{-1}; Kalys) was used to remove agrobacteria in the kanamycin-containing (300 mg l^{-1}) selective medium. Putatively transformed hypocotyl-derived calli, actively growing on selective medium, were subcultured every 2 weeks and prepared for bud production by transfer to MS derived medium [67] containing both auxin and cytokinin, maintained at 25 °C under a 16 h photoperiod. After 6 months of subculture on selective medium, the absence of remaining bacteria was checked, and the presence of the transgene in the regenerated *in vitro* grown plantlets was verified by isolating DNA and using PCR to detect the presence of the transgene. Calli were initiated from shoots on M2 medium and cultured in growth room in the dark as described earlier for wild type plants [64]. A parallel transformation was carried out with a construct containing the CaMV35S promoter fused to the *uidA* gene.

4.6. β-Glucuronidase activity

Quantitative assays for GUS activities were determined by measuring 4-methylumbelliferone (4-MU, Sigma) as described [68].

4.7. (+)-Secoisolariciresinol extraction

(+)-Secoisolariciresinol extraction procedure was based on the method derived from Ref. [69] adapted for lignan extraction from cell suspensions. Briefly, 500 mg of lyophilized cells were extracted by stirring with 20 ml of 80% (v/v) of aqueous methanol, for 2 h at 60 °C in a water bath. Following centrifugation, the solid residue was discarded and the supernatant was evaporated to dryness at 40 °C and then resuspended in 1 ml of 0.1 M citrate-phosphate pH4.8 buffer containing 5 unit ml^{-1} of β-glucosidase from almonds (Sigma) for (+)-secoisolariciresinol release during incubation for 4 h at 40 °C. The extract was then centrifuged and the supernatant was filtered (0.45 μm) before HPLC injection. (+)-Secoisolariciresinol was quantified by HPLC on a Varian liquid chromatographic system as described by Renouard et al. [69].

4.8. Fluorescence microscopy

Microscopy was performed using stably transformed flax cells placed in water on a glass slide and covered with a cover slip. An Olympus System TCS SP2 (Mannheim, Germany) was used for imaging GFP signal with an argon laser. Cells were observed under bright field or fluorescence microscopy (using a 488-nm excitation filter and a 515–565 nm wavelength pass filter).

4.9. Modeling

Both subunits were independently modeled using the "@tome V2" metaserver [30] provided by the Centre de Biochimie Structurale (Montpellier, France) and using the multi-template option. Taking advantage of a fully piped tried and tested and straight forward meta-approach of @tome V2 [30], we were able, once a multiple alignment was validated, to generate the expected coordinates by using the Modeller option [31]. For each subunit those appeared reliable since a good confidence score was obtained when further tested with ProSA-web [32]. Those were unified into a complex in a single superimposition of each chain on a high resolution solved complex structure as a template, the structure of the human farnesyltransferase solved by Ref. [36] (pdbcode: 2h6f). As controlled in PISA analysis [70], the complementary residues at the interface were quite well placed resulting in hydrogen bonding network after removing roughly the possible steric clashes by a single GROMACS [71] minimization stage. The catalytic pocket was in suitable conformation for the ligands to be placed into, also by superimposition.

4.10. Alignments

A blast on the RefSeq non-redundant and well-annotated sequence DataBase [72] with a DELTA-BLAST [73] was performed. It was followed by iterated psiblasts [74] to explore larger panel of homologs resulting in 283 sequences of farnesyl and geranyl transferases beta-subunits. The retrieved sequences were aligned within Jalview [75] with a Clustal-w [76] with the default parameters. A tree was then built using the average distance based on the BLOSUM62 matrix [77].

The alignment of the Cryptococcus neoformans farnesyl transferase sequence with the one from LuERA1 was achieved by T-Coffee [78].

4.11. Protein–ligand interaction

The network of the interacting residues within the catalytic site was established and rendered in a PyMOL [79,80] illustration thanks to Ligplot+ [81]. Structure images and superimpositions were realized with PyMOL. The alignment figures were rendered by Jalview [75].

4.12. Statistical analysis of data

All quantitative GUS assays and (+)-secoisolariciresinol quantization by HPLC presented in this study are the mean and the standard deviation of at least three independent replicates. All RT-sqPCR gel images presented here were repeated at least three times using independent replicates. Comparative statistical analyses of groups were performed using Student's *t*-test or one way analysis of variance according to the data.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.06.001.

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