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LOL2* and *LOL5* loci control latex production by laticifer cells in *Euphorbia lathyris

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SUMMARY

- Laticifers are specialized plant cells capable of indefinite elongation that ramify extensively and are responsible for latex biosynthesis and accumulation. However, it remains largely unknown the mechanisms underlying laticifer cell differentiation, growth and production of latex.

- In a search for mutants showing enhanced accumulation of latex we identified two *LOT OF LATEX* (*LOL)* loci in *Euphorbia lathyris*. *lol2* and *lol5* mutants show enhanced production of latex contained within laticifer cells. The recessive *lol2* mutant carries increased biosynthesis of the plant hormone jasmonoyl-isoleucine (JA-Ile) and therefore establishes a genetic link between JA signaling and latex production in laticifers. Instead, heighten production of latex in *lol5* plants obeys to enhanced proliferation of laticifer cells.

- Phylogenetic analysis of laticifer-expressed genes in *E. lathyris* and in two other latex-bearing species, *E. corallioides* and *E. palustris*, allowed the identification of canonical JA responsive

elements present in the gene promoter regions of laticifer marker genes. Moreover, we identified that the hormone JA functions not as a morphogen for laticifer differentiation but as a trigger for the fill out of laticifers with latex and the associated triterpenoids.

- The identification of *LOL* loci represents a further step towards the understanding of mechanisms controlling latexproduction in laticifer cells.

Keywords: *Euphorbia lathyris*; latex; laticifer cells; *lol* mutants; triterpenoids.

INTRODUCTION

Tracheophytes are vascular plants characterized by the presence of two prominent tubing systems: the tracheal-appearing xylem and the vessels of the phloem. These two transport systems have been widely studied. An additional tubing system based on living cell(s) which occurs throughout the Plantae, yet not as extensively conserved as the xylem and phloem, is that conformed by laticifer cells. Laticifers are specialized cells (or row of cells) that synthesize and accumulate latex (Fahn, 1990). Latex contained within laticifers is highly variable in chemical composition and contains a great variety of macromolecules (Konno, 2011).

Latex is produced in more than 12,000 plant species belonging to twenty different plant families that grow in a great variety of ecosystems (Metcalfe, 1967; Lewinsohn, 1991). Early histochemical studies of latex-bearing plants revealed the existence of two different types of laticifer cells, each with a distinct ontogeny and cellular morphology: articulated and non-articulated laticifers (Esau, 1965; Fahn, 1990; Mahlberg, 1993). Articulated laticifers arise from the cambium meristem at different phases of plant growth. Adjacent cells, derived by trans-differentiation from the cambium, undergo the partial or complete perforation of the cell walls at their distal ends. Upon anastomosis, connected cells give rise to a somewhat elongated cell representing the summation of a number of cells connected through the highly perforated cell walls (Nessler and Mahlberg, 1979; Nessler and Mahlberg, 1981). Hao and Wo (2000) reported that exogenous applications of jasmonic acid (JA) and linoleic acid to the surface of young stems in epicormic shoots stimulated the formation of articulated laticifer cells, therefore providing the first evidence pointing towards a hormonal regulation for the differentiation of articulated laticifers. Examples of articulated laticifers are those of rubber-producing plants such as the tree *Hevea brassilensis* or the tubers of dandelion plants (*Taraxacum* spp.). On the contrary, non-articulated laticifers are single cells that grow by elongation and do not form a middle lamella with adjacent cells. Non-articulated laticifers grow intrusively between other cells, a process requiring disruption of cell wall connections within the surrounding mesophyll cells (Mahlberg, 1963; Castelblanque et al., 2016). Therefore, the non-articulated

laticifer constitutes an exceptional example of a non-static pectin-free plant cell type that drifts into adjacent tissues. Non-articulated laticifers, like those present in petroleum plant *Euphorbia lathyris* (Castelblanque et al., 2016) or in other Euphorbiaceas (Mahlberg, 1961; Mahlberg and Sabharwal, 1968) develop from cells that are present in the embryo (i.e., laticifer initials). As the embryo grows into a mature plant, the laticifer initials elongate from their tips and undergo karyokinesis without forming cell plates; therefore, non-articulated laticifer is a multinucleate coenocyte. Due to continued growth by elongation non-articulated laticifers become large (e.g., tens of centimeters long), and constitute the lengthiest cell type described in plants. Despite the widespread distribution in the plant kingdom, laticifers have not received much attention in recent years even though they are the cell factories where rubber or opium is produced in *H. brasilensis* and *Papaver somniferum,* respectively. From an ecological point of view, laticifers have been touted for years as a defense against insect herbivory (Dussourd and Eisner, 1987) where the pressurized flow of latex may function as a form of "squirt gun" defense (Becerra and Venable, 1990). This, in addition to the potential antibiotic effects of the secondary metabolites stored in the latex (Agrawal and Konno, 2009; Huber et al., 2016), serves decreasing the performance of insect herbivores and benefits plant vegetative and reproductive fitness under insect attack. However, the conclusion of a defensive role of laticifers is mostly based on observations of how purified latex affects the growth and performance of herbivores, but precise genetic data on the importance of laticifers for host defense remain scarce.

Recently, a survey for mutants in *E. lathyris* defective in laticifers, and therefore deficient in the production of latex, allowed the identification of the *pil* (*poor in latex*) series of mutants (Castelblanque et al., 2016). The fact that in *pil* mutant plants normal growth and fitness remains unaltered, at least when grow under control conditions, revealed that laticifers are not essential for plant development. This thus reinforces the contention on the importance of laticifers for special ecophysiological adaptations of latex-bearing plants to specific natural environments. In any case, there remains a paucity of information regarding the mechanisms of cellular differentiation and how non-articulated laticifer growth and activity is integrated within plastic developmental programs. The identification of the signals from the plant influencing non-articulated laticifer growth and activity, and the understanding of the genetic basis of laticifer cell differentiation and metabolic reconfiguration, will contribute to a better understanding of the biology of this paradigmatic type of cell.

Here, we document on the identification and characterization of two *LOT OF LATEX* (*lol*) mutants showing enhanced production of latex: *lol5* and *lol2.* Moreover, we present molecular evidences indicating that the plant hormone jasmonic acid (JA) regulates the metabolic activity of laticifer cells and latex formation in *E. lathyris.* In particular, the characterization of *lol2* plants, which carry elevated endogenous levels of JA-Ile, served establishing *in vivo* that the JA pathway and the activity of laticifer cells are genetically linked. Furthermore, in *E. lathyris* JA does not seem to act as a morphogen but instead as a hormone controlling latex production in laticifer cells.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Euphorbia lathyris wild-type plants and *pil* mutants used here have been described previously (Castelblanque et al., 2016). *Euphorbia corallioides* and *Euphorbia palustris* wild-type plants were obtained from Jelitto Seeds (Germany). Plants were grown in growth chamber (19-23 ºC temperature, 85% relative humidity, 120-150 µmol m-2 s-1 fluorescent illumination, 16-hr light photoperiod).

RNAseq Analysis

Total RNA was extracted from latex of *E. lathyris, E. corallioides* and *E. palustris* and from leaves of *E. lathyris*. RNA extraction was done using several phenol-chloroform-isoamyl alcohol extractions, lithium chloride precipitation and treatment with DNA-free DNase Treatment and Removal Kit (Invitrogen). Pyrosequencing was performed on a Roche Genome Sequencer FLX instrument (454 Life Science Roche, Lifesequencing SL) as previously described (Castelblanque et al., 2016).

Gene Expression Analysis

Selectedgenes (Table S1) were analyzed for gene expression by RT-qPCR. Total RNA was extracted as described above. RNA quality was assessed with a 2100 Bioanalyzer (Agilent Technologies). For reverse transcription, RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) was used. Quantitative PCR (qPCR) was performed using an ABI 7500 Fast Real-Time sequence detection system and Power SYBR Green PCR Master Mix (Applied Biosystems). Histone H3 was chosen as the reference gene. Three biological replicates from three independent experiments were used for qPCR analysis. The $2^{\Delta\Delta Ct}$ method was used for relative gene expression analysis. Primer sequences (Table S2) were checked in the NCBI database and in our transcriptome database using a BLAST search to ensure the detection of a single gene. Prior to qPCR, a standard curve for each gene was performed, obtaining efficiencies between 92 and 105% with R2 values higher than 0.92.

Promoter Regions, Gene Structure and Promoter Regulatory Elements

Genomic DNA was extracted from leaves using the CTAB method (Doyle and Doyle, 1990) and treated with RNase (Fermentas). Promoter regions of *E. lathyris EH, E. lathyris MLP, E. lathyris SQE, E. palustris EH* and *E. corallioides EH* genes were identified using the Genome Walker Universal Kit (Clontech) with gene specific primers (GSP) (Table S4). The gene full length was amplified from genomic DNA by PCR with the Phusion Hot Start II DNA Polymerase (Thermo Scientific) using specific primers (Table S3). Gene structure (exon and introns regions) was established by alignment of the sequences of cloned genomic amplification products and cDNAs. The regulatory elements in the promoters were identified with the PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

MeJA Plant Treatments

Methyl jasmonate (Sigma-Aldrich) was dissolved in ethanol, prepared in water to a final concentration of 50, 100 and 200 µM and supplemented with 0.02% Silwet L-77 (Phytotechnology Laboratories). 6 weeks old plants were sprayed with either water (mock) or MeJA three times on alternate days and sample were

collected three day after the last treatment. For gene expression analysis, leaf samples were collected 6 and 12 hours after a single treatment. For triterpene quantification and laticifer staining, leaf samples were collected 7 days after treatment. Three independent treatments were performed using three biological replicates for each experiment.

Gene Construct *pEH::lathyris-GUS*, Arabidopsis Transgenic Lines and MeJA Treatment

The promoter sequence of the *E. lathyris EH* gene was amplified from genomic DNA by PCR with Phusion Hot Start II DNA Polymerase (Thermo Scientific) using specific primers carrying restriction endonuclease sites: the forward primer with the EcoRI site 5'-TAGTCGACGAATTCACCCTTACCCGTTAAGAATCAA and the reverse primer with the XbaI site 5'-TAGTCGACTCTAGACATATTATCTCTCTGCTCTGTTT (restriction sites with underlined letters). The amplified fragment (1712 bp upstream start codon) was digested with EcoRI and XbaI endonucleases. The standard binary vector pTF102 (Frame et al., 2002), kindly provided by Dr Kan Wang (Iowa State University), was used to generate the *pEH::lathyris-GUS* gene construct. This vector contained a β-glucuronidase (*uidA, GUS*) gene with intron as the reporter gene and a *bar* gene as the selectable marker gene (conferring resistance to glufosinate), with the CaMV 35S promoter driving both the *bar* and the *GUS* genes. The vector was digested by EcoRI and XbaI to remove the CaMV 35S promoter in the *GUS* gene and ligated with the digested promoter fragment using the T4 ligase (Takara), attaining the *EH* promoter fused to the coding sequence of the *GUS* intron gene. The obtained construct was verified by sequencing and promoter was confirmed to be in frame with the coding sequence. Arabidopis transgenic lines containing the gene construct were generated in the Col-0 background, using the floral dipping method (Clough and Bent, 1998) and the strain C58 of *Agrobacterium tumefaciens*. T1 lines were obtained by selection with commercial glufosinate and resistant lines were used for MeJA treatment. MeJA was prepared as described above at a final concentration of 50 μM and was applied by spray to Arabidopsis transgenic lines carrying *pEH::lathyris-GUS* construct. Rosette leaves were collected before and 24h after the MeJA treatment and incubated overnight at 37°C in GUS staining buffer, containing the X-Glc substrate, as previously described (Jefferson et al., 1987). A total of 12 independent T1 lines were analyzed.

Triterpene Quantification

Isoprenoids were extracted from leaves with heptane and quantified by GC-MS analysis as previously described (Castelblanque et al., 2016).

Laticifer Staining and Laticifer Index

Laticifers were visualized in entire plants by whole-mount staining with Sudan Black B and laticifer index (LI) was calculated by measuring the total length (in mm) of the Sudan Black B-stainedlaticifer cells in a microscopic field area (in mm2), as previously described (Castelblanque et al., 2016).

Mutagenesis, Mutant Identification and Genetic Analysis

E. lathyris wild-type seeds were mutagenized by gamma ray at a dose of 300 Gy at the IAEA Laboratories in Seibersdorf (Vienna, Austria). The M2 population (a total of 3000 M2 lines) was screened for latex production by pricking leaves and lines showing higher latex oozing than the wild-type plants were selected. These mutants were coined "*lot of latex*" (*lol*). They were selfed and phenotype was corroborated in the M3 and M4 generations. To clean up unrelated mutations the mutants were backcrossed twice with the parental line and segregation of phenotypes in the F2 generation was analyzed with the X^2 test for goodness of fit (Table S4). For complementation analysis, each *lol* mutant was crossed with each of the other *lol* mutants and appearance of the *lol* phenotype in F1 plants recorded.

Analysis of JA Sensitivity

For the root growth inhibition assay, seeds from wild-type plants and *lol* mutants were surfaced-sterilized and sown in MS medium (Duchefa Biochemie) for germination. When radicle emerged, seedlings were transferred individually to culture tubes containing 0, 1, 5 and 10 μM MeJA in MS medium. Tubes were incubated in a growth chamber and two weeks after, root length in the seedlings was measured. For anthocyanin content, samples from the same seedlings were collected in liquid nitrogen. Extraction of anthocyanins was performed in acidic methanol and quantification was performed by a spectrophotometric method as previously described (Ramirez et al., 2010). 40 seedlings for each treatment were analyzed. Analysis of variance (ANOVA) was used to test the significance of the results. The experiment was repeated three times obtaining similar results.

Analysis of JA Levels

Plant hormone JA-Ile was quantified by a liquid chromatography system (UPLC, Waters) connected to a triple quadrupole mass spectrometer (TQD, Waters) using as standard JA-Ile-d6. The extraction and quantification was performed asdescribed in Sánchez-Bel et al., 2018. Three biological replicates were used for each genotype, being each biological replicate a pool of three plants.

Accession Numbers

Nucleotide sequence data for the genes described here are available from the GenBank database under the following accession numbers: *E. lathyris* EH gene, promoter region and complete CDS (JX088585), *E. lathyris* MLP gene, promoter region and complete CDS (JX133160), *E. lathyris* SQE gene, promoter region and complete CDS (MG755201), *E. palustris* EH gene, promoter region and complete CDS (MG755202), *E. corallioides* EH gene, promoter region and complete CDS (MG755203), MLP (JQ694158), SQE (JQ694153), EH (JQ694156), PEI (JQ694161), PE (JQ694160), DHDDS (JQ694157), LOX2 (MG755200), AOS (MG755199), OPR3 (MG755198), JAR1 (MG755197), COI1 (MG755196), MYC2 (MG755195), JAZ1 (MG755194), JAZ2 (MG755193), JAZ3 (MG755192), JAZ8 (MG755191), JAZ9 (MG755190), JAZ10 (MG755189), JAZ12 (MG755188) and H3 (JQ966276).

RESULTS

Laticifer-associated gene expression

Previous RNAseq analysis of the latex oozing from leaves of *Euphorbia lathyris* plants allowed identification of genes related to laticifer cell activity and identity (Castelblanque et al., 2016). Here we selected some of these laticifer-enriched genes, including a gene encoding an epoxide hydrolase-like enzyme (EH), a squalene epoxidase (SQE) and a major-latex protein (MLP) and performed comparative gene expression analysis, by quantitative PCR (RT-qPCR), in full expanded leaves from wild-type plants and the laticifer-deficient *poor in latex 1* (*pil1*) mutant. Results indicate these genes are down-regulated in the *pil1* mutant (Fig. 1a). *EH* expression was nearly undetectable in the mutant, and *MLP* and *SQE* expression, although still detectable, was severely repressed in *pil1* plants. The expression of *PEI*, encoding a pectinase inhibitor, and whose mRNA was previously shown to be absent in latex (Castelblanque et al.,2016), was used as a control. In marked contrast, *PEI* expression showed no variation between wild-type and *pil1* tissue samples (Fig. 1a).

In an attempt to identify regulatory elements in the promoter regions of the *EH*, *MLP* and *SQE* genes that could give us a clue to what signal mediates expression of these genes in laticifer cells, genomic DNA from *E. lathyris* was isolated and DNA fragments corresponding to these three genes were isolated by using a PCR walking approach. DNA fragments were sequenced, aligned to cDNA sequences, and 5 ´promoter regions, introns, exons and 3´ UTRs sequences were identified for each of the three genes under consideration (Fig. 1b). A search for the presence of common *cis* regulatory elements in their 5 ´promoter regions revealed the conspicuous presence of the canonical jasmonate (JA) responsive element CGTCA (Rouster et al., 1997) and the JA-related MYC binding motif CACGTG (Chini et al., 2007; Dombrecht et al., 2007; Lorenzo et al., 2004;) in the three genes (Fig. 1b). For the latter motif, single nucleotide polymorphic variants were considered in our search, as single nucleotide variants still showed high affinity to MYC proteins (Godoy et al., 2011) (Fig. S1). The isolated 1638 bp promoter region of the *EH* gene showed 2 and 4 copies, respectively, of each of these responsive elements. The 2415 bp promoter region of *MLP* contained 2 copies of the CGTCA element and 3 copies for the CACGTG motif. The 1450 bp isolated promoter region of *SQE* contained 1 copy of the CGTCA and 3 copies of CACGTG elements. Therefore, these results suggest that the expression of these laticifer-marker genes might be regulated by JA.

Promoter shadowing of *EH*

The presence and possible evolutionarily conservations in promoter regions of responsive elements mediating laticifer gene expression was also addressed by phylogenomic shadowing. This method assumes that during evolution noncoding DNA sequences of orthologous genes diverged except for regions containing functionally important *cis*-motifs mediating gene expression (Boffelli et al., 2003). Therefore, we compared the promoters of *EH* genes from three distant but related plant species: *E. lathyris*, *E. corallioides* and *E. palustris* in search for conserved orthologous regions. These three species have diverged along evolution (Barres et al., 2011), and despite gross anatomical differences (Fig. 2a), *E. corallioides*and *E. palustris* have retained the characteristic distribution pattern and organization of the laticiferous system that was previously documented for *E. lathyris* (Castelblanque et al., 2016). Whole-mount histochemical staining employing Sudan Black B, which stains for lipids (including triterpenoids)

(Jensen, 1962), of intact leaves of *E. corallioides* and *E. palustris* revealed the presence of main laticifers that distributed parallel along the midrib of the leaf. By repeated bifurcations from their tips these main laticifers gave rise to secondary laticifers which resumed cell elongation at right or near-right angles. The derived secondary laticifers repeat this cell growth process allowing expansion of the laticifer network in every direction of the leaf blade (Fig. 2b).

RNAseq of latex samples derived from *E. corallioides* and *E. palustris*, and subsequent search for sequences homologous to *EH* gene from *E. lathyris*, allowed identification of sequences encoding EH from these two plant species and showed 84.8 % and 85.4 % similarity to *E. lathyris* EH, respectively (Fig. S2). Similarity between *E. corallioides* and E. *palustris EH* was even higher, reaching 98.1 % (Fig. S2). This high conservation was also noted when the genomic DNA sequences corresponding to *EH* genes from these two species were compared to that of *E. lathyris*. While exon organization was similar between the three species, the length of the first intron was much shorter in *E. lathyris* (Fig. 2c). Comparative sequence analysis of the promoter regions of these three *EH* genes did not revealed conservation of any region that may be indicative of a laticifer specific sequence motif. However, as was revealed above for *E. lathyris EH*, the promoter regions of the *E. corallioides* and *E. palustris EH* genes also carried the conspicuous presence of a number of CGTCA and CACGTG *cis* conserved elements (Fig. 2c and Fig. S1). Therefore, the conserved abundance of these motifs further suggests that JA might be important for expression of *EH* in laticifer cells.

JA mediates *EH* gene expression and activates triterpenoid metabolism in *E. lathyris*

The previous observations prompted us to search if JA could promote expression of the *EH* laticifer marker gene in *E. lathyris*. Spray application of 50 µM MeJA to *E. lathyris* plants promoted a rapid (6h) enhancement of EH transcript accumulation as revealed by RT-qPCR (Fig. 3a). The effect of JA on *EH* gene expression was transient and at 12 h the level of EH transcript accumulation resumed their normal value. Next, we generated stable transgenic Arabidopsis lines carrying a gene construct with the 5 ´promoter region of the *E. lathyris EH* gene fused to the β-glucuronidase gene (*pEH-lathyris::GUS*). Expression of GUS activity in leaves of untreated transgenic Arabidopsis plants was only marginal and poorly detected (Fig. 3b). However, the sole application by spray of 50 µM MeJA promoted transcriptional activation of *pEH-lathyris::GUS* as revealed by the corresponding intense GUS staining throughout the rosette leaves (Fig. 3b). These results thus indicated a JA-mediated *EH* transcriptional activation.

We next wondered if JA could similarly promote the metabolic activity of laticifer cells in *E. lathyris*. Thus a comparative analysis of triterpenoid content in leaves revealed that spray application of MeJA to plants was able to induce a three-fold increase in triterpene accumulation (Fig. 3c). This increased accumulation was similarly achieved at 50, 100 and 200 µM MeJA, indicating that the JA-mediated effect on de novo biosynthesis of these compounds has reached its maximum with 50 µM MeJA. The triterpenoid metabolic footprint of laticifer cells in *E. lathyris* is represented by four major isoprenoids species (i.e., cycloartenol (CYC), lanosterol (LAN), butyrospermol (BUT), and 24-methylene cycloartanol (24M)) (Castelblanque et al., 2016). The relative content of each of these four major

laticifer-bearing compounds in mock- and JA-treated plants was analyzed by GC-MS (Fig. S3). Upon quantification (Fig. 3d), results revealed that MeJA-mediated promotion of isoprenoid biosynthesis occurred without substantial alteration in the relative content of each of these four end products. This indicates that MeJA-mediated effect on enhancing isoprenoid metabolism might operate at an early step of the pathway: e.g., by promoting accumulation of squalene, the common precursor of the four major isoprenoids that accumulate in laticifer cells. To better characterize the effect of MeJA on the metabolic activity of laticifers, we performed comparative whole-mount histochemical staining with Sudan Black B. This allowed visualizing laticifer cells and their distribution along the leaf lamina (Fig. 3e). Results revealed that MeJA-mediated increase in isoprenoids (Fig. 3c) occurs specifically in laticifer cells, which became intensively stained when compared to mocked plants.This drastic difference in staining reflected the JA-mediated triterpenoid fill-out effect of laticifers (Fig. 3e). The determination of the laticifer index (LI; Castelblanque et al., 2016), either in young or full expanded leaves from mock- and JA-treated plants (Fig. 3f) revealed no statistically significant differences in LI. This suggests that MeJA appears to have no effect on laticifer differentiation or growth. The small variation of LI value observed in JA-treated plants, despite not being statistically significant, may be attributable to the ease in identifying the intensely stained laticifers under the microscope from MeJA-treated plants, when compared to mocked-treated plants.

Identification of *E. lathyris* mutants showing enhanced latex content

In view of the positive effect that JA has on enhancing triterpenoid accumulation in laticifers, we next approached the identification of *E. lathyris* mutants showing increased accumulation of latex in order to identify locus regulating latex production in laticifer cells. We screened for mutants with enhanced latex production by pricking leaves of a M2 mutagenized population of *E. lathyris* and selecting those plants showing enhanced latex oozing from the pricked site when compared to the parental line. Six candidate mutants were initially identified and selfed. These mutants were coined "*lot of latex*" (*lol*). For two of these mutants, i.e., *lol2* and *lol5* (Fig. 4a), we re-confirmed the phenotype in the M3 and M4 generations and these two mutants were selected for further studies. Backcrossing with parental plants and segregation analysis of the F2 progenies indicated that the two selected *lol* mutants manifested as Mendelian recessive genes (Table S4). Also, reciprocal crosses between *lol2* and *lol5* plants and characterization of F1 plants revealed a wild-type phenotype, thereby indicating that *lol2* and *lol5* are not allelic.

Characterization of the *lol2* and *lol5* mutants

The two *lol* mutants were found affected in distinct aspects of laticifer activity. In *lol2* plants, inspectionof whole-mount preparations of primary leaves indicated that laticifer cell differentiation and growth appeared normal, with a pattern of distribution along the leaf similar to that of wild-type plants (Fig. 4b) and *lol2* plants showed LI values similar to wild-type plants (Fig. 4c). This suggested that enhanced production of latex in *lol2* appeared not due to an increase in the number of laticifer cells. However, laticifers from *lol2* plants became heavily stained with Sudan Black B when compared to wild-type laticifers (Fig. 4b), being this indicative of a higher accumulation of triterpenoids within the laticifers. In

fact, *lol2* plants doubled triterpenoid content compared to wild-type plants (Fig. 4d). Interestingly, triterpenes enhancement in *lol2* plants was accompanied by an alteration in the relative content of the four major triterpenoid species present in the latex of wild-type plants (Fig. 4e). 24-methylene cycloartanol (24M) showed enhanced accumulation in *lol2* mutant compared to wild-type plants (Fig. 4e). This enhancement in 24M appears to occur at the expenses of lanosterol (LAN), and to a minor extend also of butyrospermol (BUT), whose accumulations were reduced in the mutant (Fig. 4e). On the other hand, RT-qPCR analysis of laticifer-specific genes, such as *EH*, *PE, DHDDS* (Castelblanque et al., 2016) revealed the expression of these genes not to be substantially altered in *lol2* plants (Fig. 4f), congruent with *lol2* mutants showing unaltered LI value (Fig. 4c). Therefore, enhanced production of latex in *lol2* plants appeared due to enhanced metabolic activity of laticifer cells.

For *lol5*, the enhanced production of latex was accompanied with a denser network of laticifers cells expanding along the leaf lamina (Fig. 4b) which was reflected into a higher LI value (Fig. 4c). This enhanced density of laticifers concurred with increased expression of the laticifer marker genes analyzed (Fig. 4f). However, at variance with *lol2* plants, the increased accumulation of triterpenes in *lol5* plants (Fig. 4d) was not accompanied by an alteration in the relative content of the four major triterpene constituents present in latex (Fig. 4e), and neither laticifer cells became more heavily stained with Sudan Black. Therefore, the enhanced production of latex in *lol5* plants appeared to be the result of an increase in the number of laticifer cells and not to a higher metabolic activity of the laticifers, thus indicating that LOL5 controls laticifer cell growth.

Laticifer arrangement in the *lol2* mutant.

The enhanced accumulation of triterpenes in the *lol2* mutant allowed a clearer visualization of the intact laticifer network in whole-mount preparations. In the primary leaf, the laticifer system is initially established with 4-5 elongated laticifers which lay proximal to the central vasculature of the midrib (Fig. 5a,b). These founder laticifers of the leaf run parallel to each other, and extend from the petiole to the tip of the elongating leaf. Concurring with the process of leaf expansion, these central laticifers initiate repeated episodes of bifurcation from their growing tips and give rise to secondary laticifers. These secondary laticifers penetrate the surrounding tissues of the leaf by intrusive growth and continue with the bifurcation and elongation process, finally conforming the complex tubular laticifer system (Fig. 5b). This cellular network, which engulfs the entire leaf lamina, is kept interconnected through the characteristic Y and H joining points (Fig. 5c). As a result, myriads of tubular laticifer cell structures form a complex web within and along the entire leaf, resembling the blood circulatory system present in metazoans. The laticifer network, despite showing numerous superpositions at different planes, never anastomoses and each laticifer cell is kept independent from each other (Fig. 5c). Interestingly, laticifers abound proximal to the leaf epidermal layer and are easily dragged along with this tissue layer when taking epidermal peels from the leaf (Fig. 5d). In their close association with the epidermis, the laticifers frequently appear proximal to stomata (Fig. 5d). Indeed, in many cases, the laticifer is in such proximity with guard cells that even the hole of the open stomata appears internally occluded by the laticifer (Fig. 5e,f). The meaning of this cellular strategy, and whether these two cell types establish any type of

communication or physical contact is unknown. The lack of an apparent alteration in the abundance, distribution and organization pattern of laticifers in the *lol2* mutant in comparison to that previously described in wild-type plants (Castelblanque et al., 2016) indicated that enhanced accumulation of triterpenoids in laticifers appeared to have no effect in the differentiation and growth of this type of cell.

lol2 shows enhanced sensitivity to JA

The higher accumulation of triterpenoids in *lol2* plants and the observation of a JA-promoted enhanced triterpenoid biosynthesis in wild-type plants prompted us to search if the *lol2* mutant might be affected in JA signaling. We studied *lol2* plants in comparison to wild-type plants for altered responses to JA using the widely applied root growth inhibition assay (Staswick et al., 1992). *lol5* plants were also included in these studies. In the absence of JA, primary root length of *lol2* seedlings was shorter than that of wild type and *lol5* plants (Fig. 6a), and in the presence of 1, 5 and 10 μM MeJA, root growth reduction in *lol2* progressively increased. Reduction in root growth, relative to control seedlings without MeJA, was much more conspicuous in the *lol2* mutant which showed significant differences when compared to root growth reduction attained in wild-type and *lol5* seedlings (Fig. 6b). Induced accumulation of anthocyanins in leaves is another characteristic response of plants to JA treatments (Tamari et al., 1995). Therefore, we measured anthocyanin accumulation in seedlings after similar MeJA treatments. Results revealed that *lol2* seedlings accumulated more anthocyanins at any of the three doses of MeJA than those of wild-type and *lol5* (Fig. 6c); however, the difference between genotypes was already established at 1 μM MeJA. Therefore, *lol2* seedlings responded with increased sensitivity to the hormone in comparison to wild-type and *lol5* seedlings. Moreover, *lol2* plants were still able to respond to MeJA with further increases in triterpenoid content (Fig. 6d), indicating that triterpenoid biosynthesis, despite being hyperactivated in *lol2* still has not reached its maximum of activity. Interestingly, whole-mount comparative analysis of laticifer cells of *lol2* mutant after application of JA revealed the presence of appendages in laticifer cells which appeared formed de novo only in JA-treated plants (Fig. 6e). These appendages appear to function as laticifer-connected deposits where the excess of triterpenoids induced by JA appears to be stored. In the case of *lol5* plants, this mutant responded to JA with a further enhancement in the accumulation of triterpenoids over its basal levels (Fig. 6d), an enhancement that was of a magnitude similar to that attained in wild-type plants.

Therefore, *lol2* plants appeared not to show defectsin JA signal transduction. In fact, the expression of genes encoding cardinal constituent of the signaling pathway (Fig. S4), including COI1, MYC2 and seven different JAZs (i.e. JAZ1,-2,-3,-8,-9,-10, and -12), whose sequences were retrieved from our RNAseq gene bank and annotated on the basis of highest sequence homology with Arabidopsis orthologs (Figures S5, S6 and S7), was not compromised in *lol2* plant; neither could we find polymorphism in cDNA sequences derived from *lol2* mRNAs. Only could we note that expression of *JAZ1*, *-2*, *-3*, *-8*, *-10*, and *-12* appear to be partially up-regulated in the mutant. The expression of these latter genes was markedly triggered in *lol2* and in wild-type plants soon (i.e., 6h) after application of MeJA (Fig. S4), thus reinforcing the idea that JA perception is not compromised in the mutant.

lol2 plants accumulate higher levels of JA

We next hypothesized that the linked hypersensitivity to JA and enhanced accumulation of triterpenoids observed in *lol2* plants could be the consequence of the mutant having increased endogenous levels of the hormone. If so, it was likely that JA biosynthesis could be up-regulated in *lol2* plants. To test this hypothesis, we first identified in our RNAseq gene bank of *E. lathyris* genes homologous to those encoding key enzymes of the JA biosynthesis pathway (Figures S8, S9, S10 and S11) and then checked their expression level in the mutant in comparison to wild-type plants and *lol5*. RT-qPCR analysis of mRNAs for 4 key enzymes involved in production of JA (i.e. the plastid-localized lipoxygenase (LOX2) and allene oxide synthase (AOS), the peroxisone-localized oxophytodienoate reductase 3 (OPR3) and the JA-amido synthase (JAR1)) (Fig. 6f) revealed that *LOX2* and *AOS* were highly expressed in *lol2* plants in comparison to wild-type and *lol5* plants. *OPR3* and *JAR1* expression suffered not such up-regulation. Therefore, early steps of the JA biosynthesis would appear hyper-activated in *lol2* plants thus providing higher amounts of precursor to presumably potentiate JA-Ileaccumulation. If so, then higher levels of JA-Ile would be expected in *lol2* plants. Results (Fig. 6g) revealed that *lol2* plants showed a 100-fold increase in JA-Ile over the normal levels observed in wild-type plants and also in *lol5* plants. Therefore, *lol2* plants carry sustained higher accumulation of JA-Ile which in turn potentiates isoprenoid biosynthesis in laticifer cells. Moreover, the alteration in the relative content of the four major triterpenoid species present in the latex of *lol2* plants (Fig. 4e) contrasts with a lack alteration in the relative content observed in wild-type plants when treated with MeJA (Fig. 3d). This difference might suggest that the *lol2* mutation may be exerting a pleiotropic effect affecting not only JA biosynthesis, a situation previously documented for other mutants affected in JA signaling in Arabidopsis (e.g., *cev1* (Ellis et al., 2002), *fou2* (Bonaventure et al., 2007). In summary, *LOL2* represents a locus that negatively regulates JA-Ile biosynthesis and that ultimately limits excessive accumulation of triterpenoids in laticifers.

DISCUSSION

Latex constitutes the cytoplasmic content of laticifer cells (Hagel et al., 2008), a specialized plant cell type historically identified for the first time by de Bary in 1884. An advance in the understanding of laticifer cell biology derived from the recently identified *pil* (*poor in latex*) mutants in *E. lathyris* which allowed identification of loci regulating laticifer cell growth and activity: i.e., *PIL1* and *PIL6* regulate laticifer elongation while *PIL2* and *PIL3* regulate laticifer cell bifurcation from the cell tip. Overall, the identification of *pil* mutants revealed for the first time that laticifer represent a specialized cellular adaptation of latex-bearing plants to fulfill specific ecophysiological role(s) since plant development and morphogenesis remain unaltered in all *pil* mutants. However, identification of additional genes regulating laticifer growth and activity, and understanding how developmental and hormonal cues influence laticifer organization and activity within the plant is still needed for a full comprehension of the biology of the laticiferous system.

In this study, through the sequence analysis of the 5´promoter regions of genes abundantly expressed in laticifer cells (i.e. MLP, SQE, and EH), and by the comparison of promoter regions of a laticifer marker gene (i.e., *EH*) in three distantly related plant species (i.e., *E. lathyris*, *E. corallioides* and *E. palustris),*

we identified the common abundance and evolutionarily conserved presence of canonical JA responsive elements CGTCA (Rouster et al., 1997) and CACGTG (Chini et al., 2007; Dombrecht et al., 2007) in the promoter regions of these genes. This led us to hypothesize that specific aspect of laticifers might be regulated by the plant hormone JA. In fact, expression of the laticifer marker gene *EH* was transiently activated in *E. lathyris* plants by the external application of MeJA, indicating a JA-mediated transcriptional reprograming in laticifer cells. Furthermore, the 5´promoter region of the *E. lathyris EH* gene, when fused to the GUS reporter and introduced in stable transgenic Arabidopsis plants, revealed a remarkable transcriptional activation promoted by the sole application of MeJA to transgenic plants. These observations thus points laticifer cell as a target for JA action and prompted us to search whether laticifer cell activity and metabolism might be regulated by the plant hormone JA. In this regard, we observed that JA when applied exogenously to *E. lathyris* plants promoted enhanced accumulation of cycloartenol, lanosterol, butyrospermol, and 24-methylene cycloartanol which constitute the metabolic footprint of latex contained in laticifer cells of *E. lathyris*. The effect of JA in promoting triterpenoid biosynthesis provoked a fill out effect of triterpenoids in laticifer cells which facilitated the identification of these cells in whole-mount preparations of intact leaves of *E. lathyris*. JA-mediated triterpenoid biosynthesis and concurrent massive accumulation of these compounds within laticifer cells have no effect on laticifer network organization and neither showed any toxicity to these cells. These observations thus suggest that the JA pathway positively regulates triterpenoid metabolism in laticifer cells. Moreover, the hormone appears not to function as morphogen controlling non-articulated laticifer differentiation and growth since the pattern of laticifer distribution and the index of laticifer cells (LI) appears not to change upon JA treatment. This lack of morphogenetic effect of JA on laticifer cells contrast with the effect of JA in *Hevea brasiliensis* (latex tree) reported by Hao and Wu (2000) who concluded that JA promoted the differentiation of articulated laticifers, a distinct type of laticifer cells. At variance to the non-articulated laticifers generated in the embryo, like those of *E. lathyris*, articulated laticifers originate from the cambium and show different cellular behavior, growth strategies and organization pattern. Therefore, in theparticular case of *H. brasiliensis* JA might function as a morphogen acting on cambial cell activity to promote their differentiation into articulated laticifers, an effect that nevertheless was possibly influenced by the fact that wounding was necessarily used to introduce the hormone into the tree.

In the present work we continued our search for additional locus regulating laticifer cell activity in *E. lathyris* and identified the *lol* mutants, which in contrast to *pil* mutants, accumulate more latex. The characterization of the recessive *lol5* mutant revealed that these plants carry a denser laticifer network, suggesting that LOL5 negatively regulates laticifer growth. We speculate that this effect could be exerted by influencing the timing and/or the abundance of laticifer bifurcation events from the cell tips; a process likely coordinated by interplaying with PIL2 and PIL3, which ultimately may shape laticifer density in the plant. On the other hand, the identification of the recessive *lol2* mutant unveiled a locus critical for laticifer triterpenoid metabolism but not for laticifer cell growth. The enhanced and exclusive over-accumulation of triterpenoids in laticifer cells observed in *lol2* plants further indicates that laticifer cell growth is not influenced by the net accumulation of latex. This reconciles with the observation that JA-mediated enhanced accumulation of triterpenoids in laticifers of wild-type plants neither affect laticifer cell growth. Also, this conforms to previous observation that in the *pil10* mutant of *E. lathyris*, which is

blocked in triterpenoid metabolism in laticifer cells and produces no latex, the differentiation and growth of laticifer cells are not affected (Castelblanque et al., 2016). Therefore the identified *LOL2* locus may represent a metabolic switch controlling excess production of triterpenoid and latex accumulation in differentiated laticifer cells. A series of observations in *lol2* mutant, including (1) its hypersensitivity to JA, (2) its enhanced expression of genes required for the first committed steps of JA biosynthesis (e.i., *LOX2* and *AOS*) and (3) its 100-fold increase in the basal accumulation of JA-Ile, lead us to conclude that *LOL2* is critical in the control of JA biosynthesis in the plant. Positive effect of JA on latex production in *H. brasiliensis* (Hao and Wu, 2000; Laosombut et al., 2016) and the effect of latex harvesting procedures have on genes of JA pathway and responsive genes were previously reported (Pirrello et al., 2014; Laosombut et al., 2016). Also, the metabolic pathways that originate from squalene, a common precursor for the synthesis of triterpenes, saponins and sterols on different plant species has been reported to be affected by the action of JA (Suzuki et al., 2005; Mangas et al., 2006; James et al., 2013). Also, in the concertedtranscriptional regulation of terpenoids biosynthetic genes JA has been shown to play a crucial role (Pauwels et al., 2009; De Geyter et al., 2012; Mertens et al., 2016a), which for triterpene saponin biosynthesis in *Medicago truncatula* is mediated through the bHLH transcrition factors TSAR1 and TSAR2 (Mertens et al., 2016b). Likewise, JA application to opium poppy (*Papaver somniferum*) promotes accumulation of alkaloids in latex produced in poppy capsules, a process mediated through the JA-responsive AP2/ERF-domain type ORCA factors and MYC factors (Memelink et al., 2001; De Geyter et al., 2012). Therefore, the identification of the *E. lathyris lol2* mutant offer genetic evidence linking JA to the metabolism of laticifer cells and production of latex, a correlation so far established through pharmacological experiments. Since JA is a critical hormone mediating plant adaptation to environmental changes, including biotic stress and resistance to insect attack, the readiness and fine-tuning of laticifer cell activity to fluctuations in the environment may thus become controlled by JA. Our findings thus represents a step forward on how the general metabolism of JA can influence specific aspects of the plant life style by exerting cellular specificity in its mode of action; in this particular case by specifically influencing the metabolic rate of laticifer cells.

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AUTHOR CONTRIBUTION

P.V., L.C., B.B. and C.M. designed the research; B.B., C.M., M.O., and L.C. performed the experiments; P.V. wrote the article.

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SUPPORTING INFORMATION

The following Supporting Information is available for this article.

Figure S1. MYC binding motifs in promoter regions.

Figure S2. Similarity between EH genes.

Figure S3. Comparative triterpenoid content of *E. lathyris* wild-type mock and MeJA treated plants

Figure S4. Gene expression of selected JA signaling pathway genes in *E. lathyris*.

Figure S5. Alignments of *E. lathyris* MYC2 protein with selected orthologs.

Figure S6. Alignments of *E. lathyris* COI1 protein with selected orthologs.

Figure S7. Comparison of JAZ proteins *of E. lathyris* with the *A. thaliana* orthologs.

Figure S8. Alignments of *E. lathyris* LOX2 protein with selected orthologs.

Figure S9. Alignments of *E. lathyris* AOS protein with selected orthologs.

Figure S10. Alignments of *E. lathyris* OPR3 protein with selected orthologs.

Figure S11. Alignments of *E. lathyris* JAR1 protein with selected orthologs.

Table S1. Selected genes for transcriptomic analysis in *E. lathyris.*

Table S2. Primer sequences used in this study for RT-qPCR analyses in *E. lathyris.*

Table S3. Primer sequences used in this study for *E. lathyris*, *E. corallioides* and *E. palustris*.

Table S4. Segregation analysis in *lol* mutants of *E. lathyris.*

FIGURE LEGENDS

Figure 1. Analysis of *E. lathyris* laticifer marker genes. (a) Expression of the *MLP*, *SQE*, *EH* and *PEI*, genes in wild-type and *pil1* plants. Relative expression was assayed by RT-qPCR. Expression was normalized to the constitutive Histone *H3* gene, then to expression attained in wild-type plants. Data represent means ± SD (n = 3 biological replicates). Asterisk above the bars indicates statistically significant differences with respect to reference values from wild-type plants using an ANOVA with a $P < 0.05$ level of significance. (b) Gene structure of the *MLP*, *SQE* and *EH* genes showing the JA-related and MYC-binding motifs found in the promoter regions of these genes using the PlantCARE tool.

Figure 2. Phylogenomic shadowing of promoter regions of *EH* genes. (a) *E. corallioides* and *E. palustris* are latex-bearing plant species related to *E. lathyris*. (b) Distribution pattern of laticifer cells in *E. corallioides* and *E. palustris* leaves as revealed by whole-mount staining with Sudan Black B. The final image was processed from multiple images using Panorama Maker 3.0 (c) Gene structure of the *EH* gene from *E. corallioides, E. palustris* and *E. lathyris* showing the JA-related and MYC-binding motifs found in the promoter regions of using the PlantCARE tool.

Figure 3. JA promotes *EH* gene expression and triterpenoid metabolism in *E. lathyris*. (a) Expression of the *EH* gene after jasmonate treatment. Plants were sprayed with water (mock) or 50 μM MeJA. Relative expression was assayed by RT-qPCR. Expression was normalized to the constitutive Histone *H3* gene, then to expression attained in wild-type plants. Data represent means ± SD (n = 3 biological replicates). Asterisk above the bars indicates statistically significant differences with respect to reference values from mocked plants using an ANOVA with a $P < 0.05$ level of significance. (b) Transcriptional activation of the *GUS* gene driven by the *E. lathyris EH* promoter in transgenic Arabidopsis plants 24h after a 50 μM MeJA treatment. (c) Triterpenoid content in leaves, quantified by CG-MS analysis, 7 days after treatment with different concentrations of MeJA. Data represent mean ± SD (n = 9 independent plants). Letters above the bars indicate different homogeneous groups with statistically significant differences using an ANOVA with a $P < 0.05$ level of significance. (d) Relative content of each of the four major *E. lathyris* latex triterpenes: LAN, lanosterol; BUT, butyrospermol; CYC, cycloartenol; 24M, 24-methylenecycloartanol. Triterpeneswere quantified by CG-MS analysis, 7 days after treatment with different concentrations of MeJA. Data represent mean ± SD, (n = 9 independent plants). (e) Whole-mount staining with Sudan Black B of mock and MeJA treated leaves, showing the laticiferous system (above) and magnification of a leaf sector showing details of stained laticifer cells (below). (f) Laticifer index (LI) recorded in leaves located at the same position in mock and MeJA-treated plants. Bars represent means ± SD (n = 5 independent plants). ANOVA with a $P < 0.05$ level of significance was performed and no statistically significant differences were found.

Figure 4. Characterization of *E. lathyris lot of latex* (*lol)* mutants. (a) Comparative oozing of latex upon pricking of leaves from wild-type, *lol2* and *lol5* plants. (b) Whole-mount Sudan Black B staining of leaves and close-up of a sector of the leaf blade, showing the laticifer network in the indicated genetic backgrounds. (c) Laticifer index. Letters above the bars indicate different homogeneous groups with statistically significant differences using an ANOVA with a $P < 0.05$ level of significance. (d) Triterpenoid content in leaves, quantified by CG-MS analysis, of wild-type, *lol2* and *lol5* plants. Data represent mean ± SD, n = 9 independent plants. (e) Relative content of each of the four major *E. lathyris* latex triterpenes: LAN, lanosterol; BUT, butyrospermol; CYC, cycloartenol; 24M, 24-methylenecycloartanol. Triterpenes were quantified by CG-MS analysis in wild-type, *lol2* and *lol5* plants. Data represent mean ± SD (n = 9 independent plants). (f) Expression of the laticifer marker genes *EH*, *PE* and *DHDDS* in wild-type, *lol2* and *lol5* plants. Relative expression was assayed by RT-qPCR. Expression

was normalized to the constitutive Histone *H3* gene, then to expression attained in wild-type plants. Data represent means ± SD (n = 3 biological replicates).

Figure 5. Laticiferarrangement in the *E. lathyris lol2* mutant. (a) Whole-mount staining with Sudan Black B of a primary leaf close to the apical meristem showing predominant distribution of laticifer cells close to the midrib. The final image was processed from multiple images using Panorama Maker 3.0 (b) Details showing the laticifer network along the leaf blade of a primary at different magnifications. (c) Detail of laticifer cells showing the characteristic Y and H bifurcations. (d) Epidermal peels of the leaf showing the proximity of the laticifers to the stomata. The different intensity in the laticifer staining is attributed to the different content in triterpenoids. (e,f) Serial sectioning of epidermal peels showing laticifer cells in close proximity to the stomatal pore. The purple arrows show consecutive images at different focal positions from the same fixed epidermal peel.

Figure 6. Responses of wild-type, *E. lathyris lol2* and *lol5* seedlings to JA. (a) Effect of 1 μM MeJA on root growth. (b) Root lenght of wild-type, *lol2* and *lol5* seedlings grown in 1, 5 and 10 μM MeJA expressed as percentage of the root length measured in control seedlings grown without MeJA. Data represent mean ± SD (n = 40 seedlings). Letters above the bars indicate different homogeneous groups with statistically significant differences using an ANOVA with a $P < 0.05$ level of significance. (c) Anthocyanin content of wild-type, *lol2* and *lol5* seedlings grown in 1, 5 and 10 μM MeJA expressed as arbitrary units $((A_{530}-0.25A_{657})/g)$. Bars represent means ± SD (n = 40 seedlings). (d) Triterpenoid content in leaves from wild-type, *lol2* and *lol5* plants after MeJA treatment. Plants were sprayed with water (mock) or 50 μM MeJA and7 days after treatment triterpenes were quantified by CG-MS analysis. Data represent mean ± SD (n = 9 independent plants). Asterisks indicate statistical differences compared with its respective genotype as referred to its mock control ($P<0.05$) analyzed using a Student's t-test. (e) Whole-mount Sudan Black B staining of *lol2* leaves treated or not treated with MeJA. Laticifer appendages appearing in MeJA-treated leaves are indicated by white arrows. On the right, close-up of two sectors of the MeJA treated leaf where laticifer appendages appeared (red arrows). (f) Expression of the JA biosynthesis genes *LOX2*, *AOS*, *OPR3* and *JAR1* in wild-type, *lol2* and *lol5* plants. Relative expression was assayed by RT-qPCR. Expression was normalized to the constitutive Histone *H3* gene, then to expression attained in wild-type plants. Data represent means ± SD (n = 3 biological replicates). (g) Quantification of JA-Ile in wild-type, *lol2* and *lol5* plants. Data represent means ± SD (n = 3 biological replicates).