SOBIR1/EVR prevents precocious initiation of fiber differentiation during wood development through a mechanism involving BP and ERECTA

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In plants, secondary growth results in radial expansion of stems and roots, generating large amounts of biomass in the form of wood. Using genome-wide association studies (GWAS)-guided reverse genetics in *Arabidopsis thaliana*, we discovered *SOBIR1/EVR*, previously known to control plant immunoresponses and abscission, as a regulator of secondary growth. We present anatomical, genetic, and molecular evidence indicating that SOBIR1/EVR prevents the precocious differentiation of xylem fiber, a key cell type for wood development. SOBIR1/EVR acts through a mechanism that involves BREVIPEDICELLUS (BP) and ERECTA (ER), 2 proteins previously known to regulate xylem fiber development. We demonstrate that BP binds *SOBIR1/EVR* promoter and that *SOBIR1/EVR* expression is enhanced in *bp* mutants, suggesting a direct, negative regulation of BP over *SOBIR1/EVR* expression. We show that SOBIR1/EVR physically interacts with ER and that defects caused by the *sobir1/evr* mutation are aggravated by mutating *ER*, indicating that SOBIR1/EVR and ERECTA act together in the control of the precocious formation of xylem fiber development.

meristem | cambium | xylem | wood development

The development of multicellular organisms is determined by the integration and coordination of multiple growth programs. In plants, the secondary growth (radial expansion) of stems, hypocotyls, and roots is a central developmental process that provides the mechanical support and stability that plants need to expand and sustain new organs. Secondary growth is mediated by the activity of a specialized meristem termed vascular cambium. The cambium arises postembryonically in the shape of a cylinder that surrounds the growth axes of stems, hypocotyls, and roots (1). The cambium differentiates, exclusively, new vascular tissues: the water and solutes conducting tissue (xylem/wood) toward the inner part of the plant and the assimilates conducting tissue (phloem/bast) toward the external part of the plant (2). The radial accumulation of secondary vascular tissues is what ultimately results in secondary growth. Xylem cell production is higher than phloem cell production, and, as a result, secondary xylem represents the largest proportion of the secondary vascular tissues (3).

Secondary xylem is composed of 3 cell types: vessels, fibers, and parenchyma. Vessels and fibers undergo programmed cell death during their development and are the major components of wood in trees: vessels being specialized in water and solute transport and fibers on sustaining the plant body. Secondary xylem differentiation in *Arabidopsis* hypocotyl resembles that of tree stems (4) and occurs in 2 clearly distinguishable phases: phase I, in which parenchyma is formed and only vessels mature, and phase II (also known as expansion phase [5]), in which no parenchyma forms and, in addition to vessels, large amounts of fibers develop (4). Understanding the transition from phase I to phase II is key to understanding the regulation of secondary growth. Such transition coincides with flowering time (6) and is positively regulated by gibberellins (GAs) (5) and the KNOTTED1-like HOMEOBOX (KNOX) transcription factor BREVIPEDICELLUS (BP) (7). BP positively regulates the expression of the SECONDARY WALL THICKENING PROMOTING FACTOR/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN (NST/SND) NST1 and NST3/SND1 transcription factors, 2 master regulators of xylem fibers differentiation, in a GA-dependent manner (7–10). Indeed, BP has recently been demonstrated to be limited in function by the DELLA proteins through physical interaction (11). The leucine-rich repeat receptor-like kinases (LRR-RLKs) ERECTA (ER) and ER-LIKE 1 (ERL1) redundantly prevent the excessive radial growth of the hypocotyl by controlling the amount of fiber production during phase II of xylem expansion, presumably in a BP-dependent manner (12).

The identification of new genetic regulators of secondary growth has traditionally been hampered by (i) the difficulties entailed by the use of classical genetics in long life-cycle plants such as trees and (ii) the fact that phenotyping secondary growth necessarily needs sectioning and imaging when using genetically amenable model organisms such as *Arabidopsis thaliana*, thus making large-scale forward genetics extremely challenging. Here, we have substantially overcome such difficulties by using genome-wide association studies (GWAS)-guided reverse genetics in *A. thaliana* as gene discovery strategy. With such an approach, we identified the LRR-RLK EVERSHED (EVR) also named SUPPRESSOR OF BIR-1 (SOBIR1), previously associated with immunoresponses (13) and abscission (14), as a regulator of secondary growth. Our results demonstrate that SOBIR1/EVR prevents the

Significance

Secondary growth (thickening of stems and roots) provides the mechanical support that plants need to grow and sustain their structures, and results in wood formation. Although wood accounts for the largest proportion of terrestrial biomass, the identification through classical genetics of key elements that control secondary growth is hampered by the challenge of phenotyping traits associated with inner tissues. We have taken advantage of natural genetic variation to identify SOBIR1/EVR, a gene previously known for its involvement in plant immunity and abscission, as a regulator of the process, and demonstrate that it prevents precocious formation of xylem fiber cells through a mechanism that involves the activity of previously discovered master regulators of xylem fiber development.

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precocious differentiation of xylem fibers during secondary growth. We show evidence for a regulatory mechanism in which BP down-regulates the expression of *SOBIR1/EVR* by binding to its promoter and SOBIR1/EVR interacts with ER to exert its regulatory activity.

Results

Identification of *SOBIR1/EVR* as a Secondary Growth Regulator. The aim of this project was to identify new regulators of secondary growth. To that end, we carried out a GWAS-guided reverse genetics approach. Natural variation data for running GWAS was generated by screening secondary growth in 166 *A. thaliana* accessions. Because secondary xylem is the tissue that proliferates the most during secondary growth, we focused on quantifying such tissue for our analyses as readout (an example is provided in *SI Appendix*, Fig. S1*A*). A preliminary time-lapse experiment of in vitro-grown Col-0 plants (*SI Appendix*, Fig. S1 *B–M*) confirmed our previous observation that extensive secondary xylem is found at 21 d after germination (DAG) in the root–hypocotyl junction, the anatomical position where secondary growth is first detected in *Arabidopsis* (15) (*SI Appendix*, Fig. S1 *L* and *M*). Thus, we measured secondary xylem area at 21 DAG in our panel of 166 accessions. Our results revealed broad natural variation for this trait across accessions, reflected in a smooth distribution of the trait in which we found a maximum fold change of 20.3 between the accessions developing the most and the least secondary xylem (Fig. 1*A* and Dataset S1). Quantitative data associated with this natural variation was used to carry out GWAS mapping by using the Web-based tool GWAPP (16). We used a mixed-model algorithm and SNP data from the 250K SNP chip (17, 18). To exclude false positives, we used a 5% false discovery rate threshold calculated through the Bonferroni testing correction. Our analyses identified 1 locus, located at chromosome 2 (Fig. 1 *B* and *C*), which significantly associated with natural variation of secondary growth.

Based on (i) the fact that the identified locus contained a number of significant SNPs that were distributed across several genes and (ii) the linkage between causal and noncausal genomic sites (18, 19), we selected for subsequent analyses a genomic window within the identified locus that contained 12 genes: from position 13520325 (*AT2G31800*) to 13559767 (*AT2G31890*). From those genes, *AT2G31820*, *AT2G31850*, and *AT2G31880* were retained for further experimentation, as their expression levels are reported to significantly decrease (*AT2G31820*) or increase (*AT2G31850* and *AT2G31880*) in (pro)cambial cells compared with adjacent tissues, according to published transcriptomic data (1, 20, 21). In addition, we searched for genes within the locus containing SNPs, the presence of which is preferentially found in accessions with enhanced or decreased capacity for secondary growth. Hence, we compiled all of the individual SNPs within the locus while, in parallel, we ranked all of the accessions that we used in our GWAS according to their ability to develop secondary xylem, based on our phenotypic data (Fig. 1*A* and Dataset S1). To that end, we used a normalization system in which the accession displaying the lowest secondary xylem area received a value of 0 and the accession displaying the highest secondary xylem area received a value of 1 (*SI Appendix*, Fig. S2*A*). We then ran a script that we specifically developed to detect the bias of SNPs (*SI Appendix*, Files S1, S2, and S3) for specific groups of accessions, and we identified a subset of SNPs that was particularly present in the accessions ranked with values ≥0.8 and a second subset that was

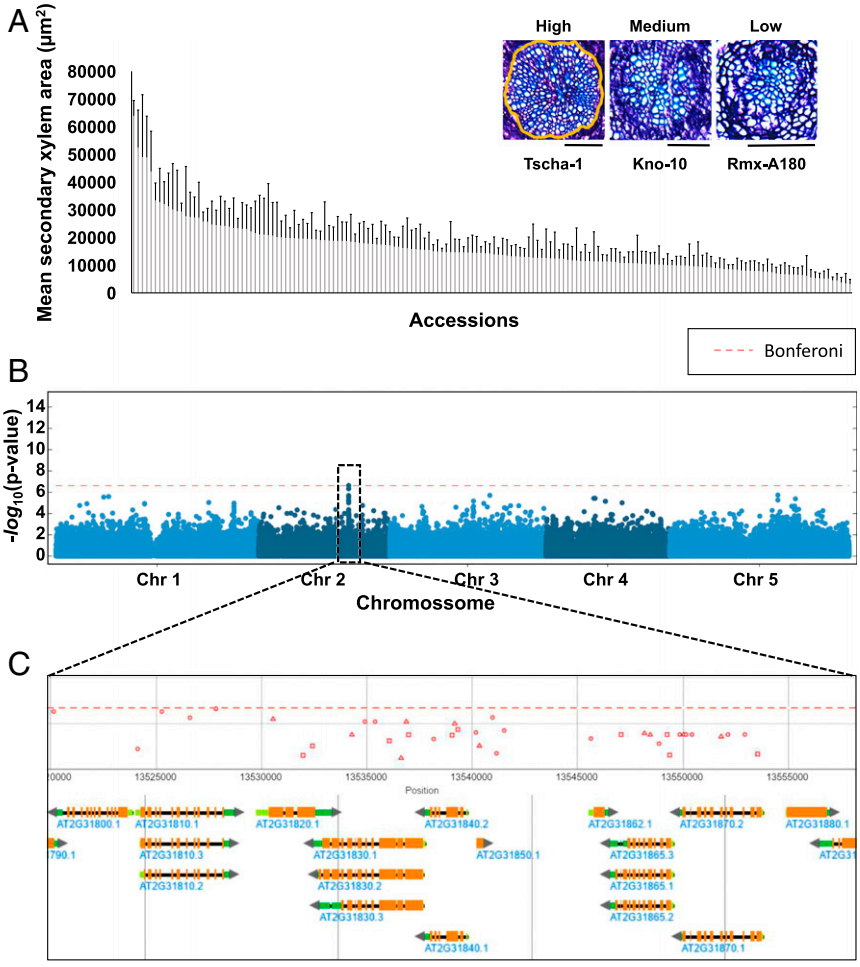

Fig. 1. Natural variation of secondary xylem in 166 *A. thaliana* accessions and GWAS identify a locus significantly associated to secondary growth regulation. (*A*) Secondary xylem area distribution in the hypocotyl of 166 *Arabidopsis* natural accessions at 21 DAG. The average of 3 to 5 individuals per accession is shown (gray bars; error bars marked in black). Representative accessions showing high, medium, or low secondary xylem area phenotypes are shown. (Scale bars, 100 µm.) Orange line on Tscha-1 illustrates the measured secondary xylem area. (*B*) Manhattan plot for the GWAS on hypocotyl mean secondary xylem area at 21 DAG depicting genome-wide SNP associations using GWAPP (16). The 5 *Arabidopsis* chromosomes are shown. The *x*-axis represents genomic positions within chromosomes, and the *y*-axis depicts −log10(*P* value) significance of the association. The dashed red line represents the nominal 0.05 significance threshold after Bonferroni false discovery rate correction. Black box indicates the significantly associated locus detected within chromosome 2. (*C*) Detail of the genomic region contained within the significantly associated locus.

particularly present in accessions with a value ≤0.2 (*SI Appendix*, Fig. S2*A* and Dataset S2). In other words, with our script, we identified SNPs that are more commonly found in accessions within the top 20% or the bottom 20% in our ranking of accessions. We subtracted all of the SNPs that were present in both categories of accessions and identified 8 SNPs that were present exclusively in the accessions belonging to the lowermost 20% in our ranking. No SNP was found to be exclusively present in the top 20% of accessions (*SI Appendix*, Fig. S2*B*). Among the 8 selected SNPs, 1 is intergenic, 1 is intronic (intragenic), and 6 are exonic, among which 3 are silent mutations and 3 are missense, nonsynonymous mutations (*SI Appendix*, Fig. S2*C*). The reported regulated expression of *AT2G31820*, *AT2G31850*, and *AT2G31880* in cambial cells (1, 20, 21), together with the presence of missense mutations in genes *AT2G31810*, *AT2G31830*, and *AT2G31880*, and the intragenic SNP in *AT2G31860*, were the basis for the selection of genes for subsequent analyses (*SI Appendix*, Fig. S2*C*). Mutants for all selected genes (*AT2G31810*, *AT2G31820*, *AT2G31830*, *AT2G31850*, *AT2G31860*, and *AT2G31880*) were obtained and phenotyped for their secondary growth capacities. Our results revealed that loss-of-function mutants on *AT2G31880* were impaired in secondary growth (*SI Appendix*, Fig. S3). Mutants for the rest of the selected genes displayed no phenotype compared with WT. *AT2G31880* encodes SUPRESSOR OF BIR1/EVERSHED (SOBIR1/EVR), a leucine-rich repeat receptor-like kinase that has been shown to participate in the regulation of immune responses (13) and floral organ abscission (14) in *Arabidopsis*. Our studies revealed that both the *evr-1* and *evr-2* mutant lines (14) displayed the same phenotype: significant reduction of secondary xylem area, resulting in a concomitant significant reduction of the overall secondary growth in the root–hypocotyl junction (Fig. 2 *A–D*, *M*, and *N* and *SI Appendix*, Fig. S4 *A* and *B*).

SOBIR1/EVR Regulates Secondary Growth by Preventing Precocious Xylem Fiber Formation. We next aimed at understanding the precise role of *SOBIR1/EVR* in secondary growth. Given that both *evr-1* and *evr-2* displayed the same phenotype, we focused on the *evr-2* mutant for subsequent analyses.

As expected for a gene involved in secondary growth, *SOBIR1/EVR* expression was reduced in *wox4* (2), a mutant impaired in secondary growth, and slightly enhanced in *mol1*, a mutant in which secondary growth is slightly enhanced (1) (*SI Appendix*, Fig. S5*A*). To determine the origin of the significant reduction of secondary growth in *evr* mutants, we compared the xylem developmental phases between *evr-2* and WT. Transition between the 2 xylem developmental phases at the *Arabidopsis* hypocotyl is coordinated with flowering (5–7). Thus, we collected hypocotyl samples of WT and *evr-2* at developmental stages before, during, and after the transition from phase I to well-advanced phase II, that is, before bolting, at bolting, when the first flower fully opened, and at adult stage (when stems were 20 cm in height), respectively. Xylem parenchyma, which is not lignified, is developed only in phase I, and fibers, which are strongly lignified, develop only in phase II (4). Therefore, to diagnose the developmental stages, we characterized all samples for lignin deposition (Fig. 2 *E–L*). We observed that transition from phase I to phase II occurred at earlier developmental stages in *evr-2* hypocotyls than in WT. Indeed, whereas, in *evr-2*, fibers consistently appeared between bolting and when the first flower fully opened (Fig. 2*J*), they could not be detected in WT plants at such developmental stages (Fig. 2*I*). This implies that fibers formed later in WT development than in *evr-2*. We reasoned that, in such a scenario, the phase I-derived xylem area should be smaller in *evr-2* than in WT. Indeed, we observed that phase I xylem area was significantly decreased in *evr-2* in comparison with WT (Fig. 2*O*). However, the phase II-derived xylem area did not significantly change in the mutant when compared with WT (Fig. 2*P*).

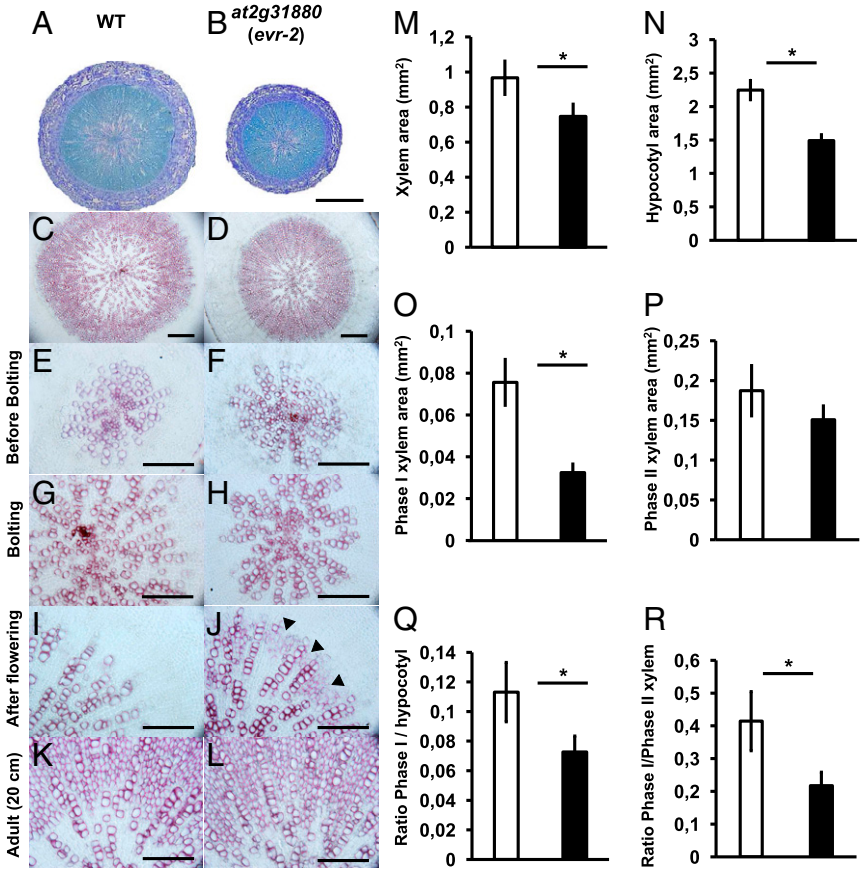

Fig. 2. Anatomical characterization of *evr-2* mutants. (*A–D*, *M*, and *N*) Representative micrographs of toluidine blue (*A* and *B*) and phloroglucinol lignin staining (*C* and *D*) of hypocotyl transverse sections from adult (20 cm in height) plants; WT (L*er*) and *evr-2* mutants. Both the xylem and whole hypocotyl area are significantly decreased in *evr-2* in comparison with WT (L*er*) (*M* and *N*). (*E–L*) Representative micrographs of hypocotyl transverse sections stained for lignin with phloroglucinol from WT (L*er*) plants and *evr-2* mutants before bolting (*E* and *F*), at bolting (*G* and *H*), after flowering (*I* and *J*), and at adult (20 cm in height) stages (*K* and *L*). Arrowheads in *J* depict appearance of first xylem fibers in *evr-2*. Note that, at this developmental stage, fibers are not detected in wild type (*I*). (Scale bars: *A* and *B*, 500 μm; *C* and *D*, 200 μm; *E* and *L*, 100 μm.) (*O* and *P*) Mean area of xylem in phase I (*O*) or in phase II (*P*) stage of development in hypocotyl sections of adult plants. (*Q* and *R*) Ratio between phase I xylem area and whole hypocotyl area (*Q*) or phase I and phase II xylem areas (*R*). Asterisks indicate significantly different values determined by Welch's *t*-test [$P < 0.01$; significance *P* values are 6.798e-06 (*M*), 1.179e-07 (*N*), 0.0004662 (*O*), 0.07709 (*P*), 0.007013 (*Q*), and 0.005186 (*R*)]. White bars, WT-L*er*; Black bars, *evr-2*. Each experiment was performed twice, with $n \geq 8$ per genotype per experimental round. Error bars indicate SD.

Hence, the differences in secondary xylem development and, as a result, the overall hypocotyl secondary growth between *evr-2* and WT are caused by the observed decrease in phase I-derived xylem development. This is reflected in a significant reduction of the ratios between phase I and whole hypocotyl and between phase I and phase II xylem areas in *evr-2* mutants (Fig. 2 *Q* and *R*). In summary, our results indicate that SOBIR1/EVR prevents the precocious xylem fiber formation (i.e., entry into phase II) in the *Arabidopsis* hypocotyl, therefore regulating the relative amounts of phase I- and phase II-derived secondary xylem tissue and the overall hypocotyl secondary growth.

Transcriptomic Data Support the Association of *SOBIR1/EVR* to Secondary Growth. In further support of SOBIR1 playing a role in the molecular regulation of secondary growth, we used ATTED (22), a plant coexpression database, to identify the most common *SOBIR1/EVR* coexpressors and found that, among the first 300, 161 (53.66%) are up-regulated in at least 1 of the previously published secondary growth transcriptomes (1, 2, 21, 23, 24) (Dataset S3). In addition, we found that *SOBIR1/EVR* itself was up-regulated in previously reported secondary growth-related transcriptomes (1, 21). In addition, we performed RNA-seq to compare the transcriptome of WT hypocotyls with that of *evr-2* and identified 305 genes up-regulated in *evr-2* in comparison with WT, among which 84 had been shown to be up-regulated during secondary growth in previous transcriptomic analyses (1, 2, 21, 23, 24) (Dataset S4 A and B). Among the 84 genes, we found *CPK28*, a gene encoding a calcium-dependent protein kinase. Interestingly, *cpk28* mutants display increased secondary growth and lignification (25). Moreover, we also found genes coding proteins involved in the metabolism and signal transduction of hormones previously shown to regulate secondary growth, such as auxin, jasmonic acid, ethylene, and abscisic acid (26–28), and genes coding proteins involved in the regulation of cell wall metabolism, lignin biosynthesis, programmed cell death, cellular proliferation, and lateral organ formation. All such processes are related to xylogenesis. The up-regulation of genes within these categories is consistent with the precocious formation of xylem fibers in *evr* mutants in comparison with WT that we observed anatomically. In summary, our own as well as previously reported secondary growth-related transcriptomic data support the involvement of *SOBIR1/EVR* in the molecular regulation of the process.

***SOBIR1/EVR* Expresses in Cambium and Developing Xylem Cells during Phase I to Phase II Transition of Xylem Development.** To understand the possible mechanism by which SOBIR1/EVR prevents the precocious entrance into phase II of secondary xylem development, we initially investigated *SOBIR1/EVR* transcript accumulation pattern during secondary growth through mRNA in situ hybridization in WT (L*er*) hypocotyl sections. *SOBIR1/EVR* transcript accumulation was not detectable during the first phases of growth at 7 DAG, an early stage of phase I in which only few secondary xylem cells have been formed (*SI Appendix*, Fig. S5 *B–D*). Similarly, *SOBIR1/EVR* transcript accumulation was still not detectable at 14 DAG, when xylem development is still in phase I (*SI Appendix*, Fig. S5 *E–G*). These results suggest that *SOBIR1/EVR* is not involved in phase I of xylem expansion. Remarkably, *SOBIR1/EVR* transcript strongly accumulated in the cambial zone and in developing xylem during the transition from phase I to phase II (Fig. 3 *E* and *F*), precisely during the developmental stage in which we showed that the first xylem fibers appear (Fig. 3*D*). *SOBIR1/EVR* transcript accumulation seemed to be detectable but minor before (phase I) and after (phase II) transition. Our results are consistent with *SOBIR1/EVR* being a regulator of the initiation of fiber formation.

SOBIR1/EVR Activity Is Integrated within the BP-Mediated Mechanism Regulating Xylem Fiber Development. Fiber formation during secondary xylem development depends on the activity of the transcription factor BREVIPEDICELLUS (BP) (7). BP belongs to the KNOX family of proteins and has been shown to promote

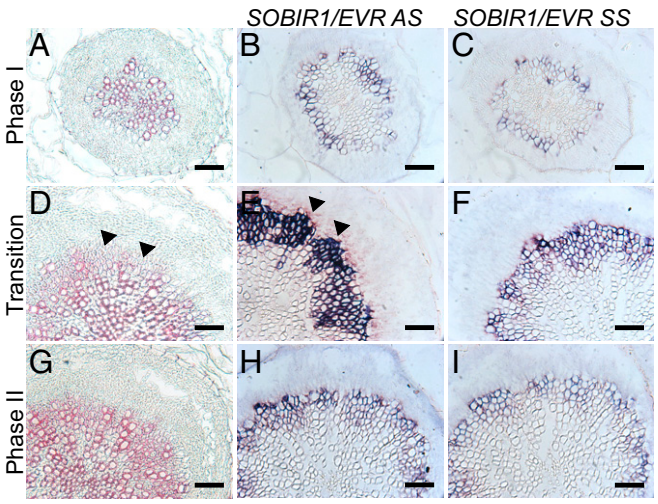

Fig. 3. *SOBIR1/EVR* transcript accumulation increases during specific stages of secondary xylem development. (*A*, *D*, and *G*) Micrographs of phloroglucinol-stained hypocotyl sections of *Arabidopsis* representing key stages during transition from phase I to phase II of secondary xylem development: (*A*) phase I, (*D*) transition from phase I to phase II, (*G*) phase II. (*B*, *C*, *E*, *F*, *H*, and *I*) In situ hybridization highlighting mRNA signals of *SOBIR1/EVR* expression during phase I (*B* and *C*), phase I to phase II transition (*E* and *F*), and phase II (*H* and *I*). (*B*, *E*, and *H*) Antisense (AS) probe. (*C*, *F*, and *I*) Sense (SS) probe. Experiment repeated twice with $n = 5$ in each experimental round.

fiber formation by positively regulating the transcription levels of *NST1* and *NST3* (7, 12). Our gene expression analyses in the hypocotyl across relevant developmental stages for fiber formation revealed that *BP* mRNA abundance is undistinguishable between *evr-2* and WT in any of these developmental stages (*SI Appendix*, Fig. S6*A*), whereas *SOBIR1/EVR* mRNA is more abundant in the *bp-1* mutant in comparison with WT at all examined stages, suggesting that BP is upstream of *SOBIR1/EVR* and negatively regulates its expression (*SI Appendix*, Fig. S6*B*). To determine whether BP exerts a direct control on the expression of *SOBIR1/EVR* by binding to its promoter, we performed chromatin immunoprecipitation analyses (ChIP). In silico studies of the *SOBIR1/EVR* promoter identified GTAACATA, the reverse complementary sequence of a previously identified binding site for the BP-related tobacco KNOX protein NTH15 (29), in position −794 and a partial site (TATGT) in position −223 (Fig. 4*A*). We selected both as potential AtBP-binding sites and designed primers to specifically amplify the regions containing each of these sites (amplicons named as P1 and P2; Fig. 4*A*). We performed qRT-PCR with such primers on DNA obtained from a ChIP assay on *pBP*::*BP-CFP* plants (30) by using anti-GFP antibodies. We included the promoters of *AT1G77530* and *AT4G35160*, 2 previously reported BP targets (11), as control in our experiment. Our results clearly demonstrated that BP binds the promoter of *SOBIR1/EVR* (Fig. 4*B*) in the 2 studied regions.

As BP has been shown to positively regulate the expression of *NST1* and *NST3*, we reasoned that, if SOBIR1/EVR is in the BP regulatory pathway of fiber formation, *SOBIR1/EVR* expression is down-regulated directly by BP, and SOBIR1/EVR's function is preventing the precocious formation of fibers, then SOBIR1/EVR may act upstream of NST1 and NST3. We observed that the expression of *NST3* and (to a lesser extent) *NST1* is higher in *evr-2* at all tested developmental stages (*SI Appendix*, Fig. S6 *C* and *D*), suggesting that SOBIR1/EVR may negatively regulate the expression of such genes. Moreover, in silico analyses of our RNA-seq data revealed that NST3 explains a part of the up-regulated transcriptome in *evr-2* hypocotyls when compared with WT (*SI Appendix*, Fig. S7).

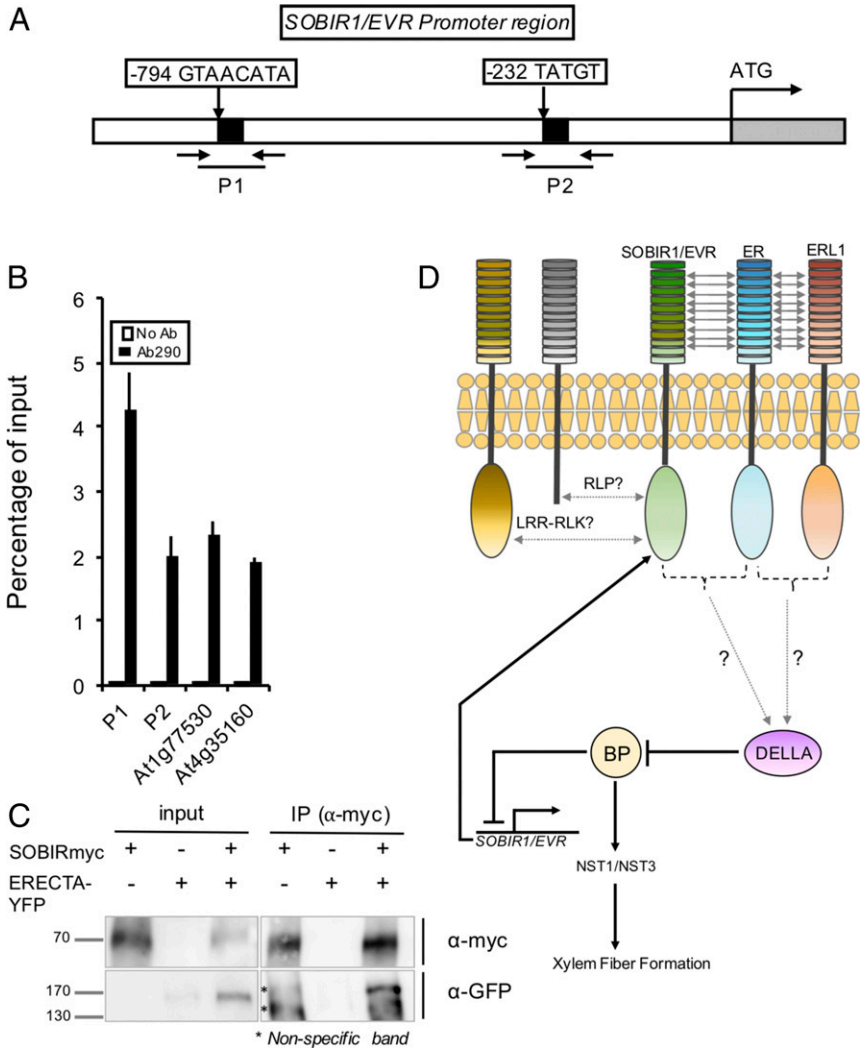

Fig. 4. SOBIR1/EVR acts through a mechanism that involves BP control of *SOBIR1/EVR* expression and SOBIR1/EVR interaction with ERECTA. (*A*) Identification through in silico analyses of BP-binding sites in the *SOBIR1/EVR* promoter. Putative binding sites identified at positions −794 and −232 within the promoter. (*B*) q-RT-PCR of *SOBIR1/EVR* on chromatin-immunoprecipitated BP-CFP lines demonstrating direct interaction of BP on *SOBIR1/EVR* promoter. No signal was detected when no antibody (AB) was used. Previously described BP targets were used as control. (*C*) Coimmunoprecipitation assay showing the interaction between SOBIR1/EVR and ERECTA in leaves of *N. benthamiana*. SOBIR1/EVR-Myc and ERECTA-GFP were expressed alone or together in leaves of *N. benthamiana* and were detected by immunoblotting with anti-MYC or anti-GFP antibodies. (*D*) Proposed mechanism for xylem fiber formation (*Discussion*).

SOBIR1/EVR and ERECTA Act Together in the Control of Xylem Fiber Development. Apart from SOBIR1/EVR, other LRR-RLKs have been shown to repress fiber differentiation: ERECTA (ER) and ERECTA-LIKE1 (ERL1), which act redundantly (12). Given that *evr-1* and *evr-2* mutants are in the L*er* background, which carries a loss of ER function, we studied the possible genetic interactions between SOBIR1/EVR and the ER and ERL pathway by examining the phenotypes of *sobir1/evr* mutants in the presence of active *ER* alleles. Thus, we initially obtained a *sobir1/evr* T-DNA mutant with strong reduction in *SOBIR1* expression: *salk_031580* (Col-0), from now on *sobir1-13* (*SI Appendix*, Fig. S8 *A–D*), and characterized its secondary growth. In *sobir1-13*, there is a slight, not significant, decrease in overall secondary xylem development in the hypocotyl, but no differences in xylem progression relative to WT Col-0 (*SI Appendix*, Fig. S8*E*). We then generated a double-mutant *sobir1-13 er* in the Col-0 background by crossing *er-124* (31) with *sobir1-13*, both in Col-0 (*SI Appendix*, Fig. S8 *F* and *G*). As is the case in *evr-2* mutants in comparison with L*er*, the double-mutant *er-124 sobir1-13* in Col-0 background developed significantly less overall secondary xylem than WT Col-0 plants, whereas, remarkably, the *er-124* single mutant developed similar amounts of secondary xylem in comparison with WT (*SI Appendix*, Fig. S8 *E*, *H*, and *I*). The overall hypocotyl secondary growth and the parenchyma/fibers ratio were also reduced in *er-124 sobir1-13* in comparison with WT Col-0, implying, again, that the double mutant in Col-0 displays the same tendency that is observed in *evr-2* mutants when compared with WT (precocious initiation of phase II leading to less secondary xylem development and, therefore, overall secondary growth). However, the reduction in the overall hypocotyl secondary growth and in the parenchyma/fibers ratio was not statistically significant, suggesting that the effect of the combined mutations, despite showing the same tendency in Col-0 as in Landsberg-1 (La-1; from which L*er* was generated), is not as strong in Col-0 as it is in La-1. We ascribe this disparity to the fact that the *er-124 sobir1-13* (Col-0) mutant is not a null mutant. Indeed, *SOBIR1/EVR* still displayed detectable mRNA levels in the *sobir1-13* mutant in comparison with WT (*SI Appendix*, Fig. S8*D*).

Having revealed a genetic interaction of *SOBIR1/EVR* and *ER* in the control of fiber formation, we next aimed at discerning whether SOBIR1/EVR and ERECTA physically interact at the protein level. To that end, we performed a coimmunoprecipitation assay after transiently expressing c-myc-SOBIR1/EVR (32) and GFP-ERECTA in *Nicotiana benthamiana* leaves. GFP-ERECTA coimmunoprecipitated when c-myc-SOBIR1/EVR was pulled down from leaf extracts (Fig. 4*C* and *SI Appendix*, Fig. S9). These results demonstrate that the 2 proteins interact.

Discussion

The aim of this work was to identify new regulators of secondary growth. To that end, we used GWAS-guided reverse genetics. In this way, we identified *SOBIR1/EVR* as regulator of xylem fiber differentiation that prevents the precocious initiation of their development. In addition, we have shown that the activity of SOBIR1/EVR occurs through a mechanism involving BP (regulating

SOBIR1/EVR expression) and ERECTA (physically interacting with SOBIR1/EVR).

We suggest a mechanistic model that integrates ours and previous findings (Fig. 4*D*). Previous works showed that both BP and the ER-ERL1 module regulate fiber formation and that the 2 pathways converge in the control of the activity of the NST1 and NST3 transcription factors (7, 12). The fact that the *er erl1* double mutant (but not the single *er* or *erl1* mutants) displays precocious initiation of xylem fibers implies a genetic interaction between *ER* and *ERL1* in the control of xylem fiber developmental timing (12). In a similar manner, our results point out that a double mutation *sobir1/evr er* (but not a single mutation *sobir1/evr*) also leads to precocious xylem fiber development in Col-0 and that SOBIR1/EVR physically interacts with ER. In this way, our findings and previous evidence (12) indicate that ERECTA does not maintain xylem fiber formation on-check alone, but through interaction with other LRR-RLKS (so far either ERL1 [12] or SOBIR1). The regulation of fiber formation by ER and ERL1 was proposed to be BP-dependent and to involve down-regulation of GA signaling (12, 33). Recently, DELLA proteins were shown to limit the BP activity through physical interaction (11). Considering such reports and that the *sobir1/evr er* and *er erl1* phenotypes are very similar, we suggest that the interaction of ER with SOBIR1/EVR or with ERL1 leads to the same or very similar signaling outcomes. We propose that the interaction of ER with SOBIR1/EVR (and, possibly, that of ER with ERL1) results in the down-regulation of GA signaling, presumably implying DELLA accumulation. In such a scenario, the DELLA-mediated limitation of BP activity would reduce the *NST1* and *NST3* expression and, as a result, fiber formation. At the same time, the system would be regulated by the BP-mediated down-regulation of *SOBIR1/EVR* expression that we have shown (*SI Appendix*, Fig. S6*B* and Fig. 4 *A* and *B*), which would lead to a reduction of SOBIR1/EVR protein abundance; consequently, less DELLA would accumulate, resulting in enhanced BP activity (Fig. 4*D*). In agreement with this mechanistic model, in *sobir1/evr* mutants, we found enhanced GA signaling (reflected in enhanced *GA20 OXYDASE1*, *GA3 OXYDASE1*, and *SCL3* expression; *SI Appendix*, Fig. S10). Such GA signaling enhancement matches with a potential decrease in DELLA accumulation in *sobir1/evr* mutants that would result in increased BP activity, leading to the enhanced *NST1* and *NST3* expression and the precocious initiation of fiber formation we have observed in *sobir1/evr* mutants (*SI Appendix*, Fig. S6 *C* and *D*).

SOBIR1/EVR was first described as a protein involved in plant immune responses that acts as a positive regulator of programmed cell death (PCD) upon pathogenic attacks (13). It was also shown as a controller of precocious floral organ abscission, a process that is also regulated by BP and in which PCD also plays an important role (14, 34). Interestingly, (i) SOBIR1/EVR also prevents precocious formation of fibers and (ii) PCD is a crucial step in xylem vessels and fibers development (35), so perhaps the involvement of SOBIR1/EVR in PCD might be a (or the only) link between all 3 processes, and the linkage with BP as well as the prevention of precocious initiation of "a" process in which PCD is crucial might even represent a further link between abscission and fiber formation.

The general molecular function of SOBIR1/EVR is to act as a scaffold or adaptor that associates with other LRR-RLKs or, more commonly, with receptor-like proteins (RLPs), in which case it provides them with a kinase domain that can be activated upon ligand–RLP interaction, generating bimolecular equivalents to RLKs (36). It remains to be studied whether, apart from interacting with ERECTA, SOBIR1/EVR also interacts with one (or more) RLPs or other LRR-RLKs during secondary xylem formation (Fig. 4*D*)—in other words, whether SOBIR1/EVR acts exclusively by interacting with ERECTA or whether other SOBIR1/EVR interactors that could play a role in the prevention of the precocious initiation of fiber formation exist.

Materials and Methods

Plant material is listed in Dataset S5. All plants were grown in LD conditions (16 h light/8 h dark). All phenotyping was performed by using toluidine blue or phloroglucinol-stained micrographs as described previously (1, 11). GWAS was performed by using the GWAPP application (16). Expression analyses were performed as described previously (1). Further details on experimental procedures are described in *SI Appendix, SI Materials and Methods*.

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