**EIN3 interferes with the sulfur deficiency signaling in *Arabidopsis thaliana* through direct interaction with the SLIM1 transcription factor**

Anna Wawrzyńska and Agnieszka Sirko

*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5A St, 02-106 Warsaw, Poland*

**Corresponding author:**

Anna Wawrzyńska

Institute of Biochemistry & Biophysics PAS

Pawińskiego 5A St, 02-106 Warsaw, Poland

Phone: +48 22 5925749

Fax: +48 22 6584804

e-mail: blaszczyk@ibb.waw.pl

**Abstract**

Sulfur deficiency in plants leads to metabolic reprogramming through changes of gene expression. SLIM1 is so far the only characterized transcription factor associated strictly with sulfur deficiency stress in *Arabidopsis thaliana*. It belongs to the same protein family as EIN3, a major positive switch of ethylene signaling pathway. It binds to the specific *cis* sequence called UPE-box. Here we show that SLIM1 interacts with UPE-box as a homodimer. Interestingly, the same region of the protein is used for heterodimerization with EIN3; however, the heterodimer is not able to recognize UPE-box. Expression of several SLIM1-dependent genes is enhanced in sulfur deficiency grown Arabidopsis *ein3-1* seedlings (with mutated EIN3 protein). This implies a possible regulatory mechanism of ethylene in sulfur metabolism through direct EIN3-SLIM1 interaction.

**Keywords**

Arabidopsis; ethylene signaling; EIN3; sulfur deficiency; SLIM1; transcriptional regulation

**Abbreviations**

aa, amino acids; BD, basic domain; dS, sulfur deficient; EBS, EIN3-binding site; EMSA, electrophoretic mobility shift assay; JA, jasmonic acid; qRT-PCR; quantitative real-time PCR; sS, sulfur sufficient; TEBS, TEIL binding site; wt, wild type; Y2H, yeast two hybrid; Y1H, yeast one hybrid

**1. Introduction**

Sulfur is an essential nutrient required for plant growth and development. Though it was originally not considered a limiting factor, nowadays fertilizers rich in sulfur are used worldwide to increase crop yield and quality. Decades ago it was observed that plants start to suffer from sulfur deficiency paradoxically because of environmental protection and decreased emission of sulfur dioxide to the atmosphere. As the regulation of gene expression in response to sulfur depletion appears an important aspect of plant metabolism it has been the subject of intense research for several years. Thus far only one transcription factor has been described in *Arabidopsis thaliana* that is strictly assigned to the reprogramming of gene transcription owed to sulfur deficiency [1]. Sulfur LIMitation1 (SLIM1/EIL3) is a member of a small plant-specific EIL protein family. There are only six members of this family [2] in Arabidopsis with best described EIN3 and its functional homologs EIL1 and EIL2 dedicated to the regulation of ethylene responsive genes. The EIL family proteins are characterized by five short basic clusters placed mostly in the first half of the protein, acidic N-terminus, and a proline-rich domain in the middle [3]. The high homology of EIL proteins is shared mostly by the first 300 amino acids, whereas the second half of the protein is more divergent. The common N-terminal half present in SLIM1 served as a template to build a model of a primary structure of the DNA-binding domain using the surface plasmon resonance technique [4]. This DNA-binding domain appeared to be unique and marked by a novel fold of five alpha-helices, forming a globular shape. The structure was recently confirmed by the crystallization of EIN3 core binding domains [5].

It was found that the proteins from the Arabidopsis EIL family bind directly to the 28-nt imperfect palindromic sequence found in the promoter of Ethylene-Response-Factor1 (ERF1), an early ethylene-responsive gene [6]. Base substitution experiments revealed that the two palindromic repeats only flank one central EIN3-binding site (EBS). This sequence shares 6-nt core with the TEIL binding site (TEBS), the consensus binding sequence defined for the tobacco homolog of EIN3 [7]. Of note is the observed binding preference between EIN3 from tobacco and Arabidopsis as the former binds to TEBS with considerably more affinity than to the EBSdemonstrating differences between EIL family members towards DNA recognition motif*.* The latest systematic biochemical analysis showed that both the number of EBS and the length of spacing between two EBSscan clearly impact binding affinity of EIN3. The presence of two inverted EBSs allows optimal binding of EIN3, with an ideal interval of 10 bp [5]. Interestingly, SLIM1 that does not take part in ethylene signaling was shown to bind to TEBS, though the interaction is very unstable and only detectable with surface plasmon resonance while electro-mobility shift assay (EMSA) failed to confirm it [4, 6]. As TEBS has a relatively short consensus it occurs highly frequently in the promoter regions of various ethylene-inducible genes and sulfur deficiency-inducible genes but also in other sites of plant genomes. This is not the case for UPE-box, a specific 20-nt binding consensus for SLIM1 consisting of two TEBSs, partially overlapping in opposite orientation to each other [8]. The UPE-box sequence is present only eight times in the Arabidopsis genome, always in the vicinity of the genes described as strongly induced by sulfur deficiency [8]. Interestingly, a potential function for SLIM1 in sensing sulfur deficiency can be suggested as some of those genes belong to a co-regulated gene cluster induced by the cysteine precursor *O*-acetylserine, which is one of the candidates for a sulfur status sensor [9].

To elucidate the molecular bases of the SLIM1 interaction with DNA we investigated the ability of this protein to form SLIM1-SLIM1 homodimers or SLIM1-EIN3 heterodimers. Next, we determined whether these dimers bind to the UPE-box sequence. Finally, we assayed the expression of several SLIM1-dependent genes in the Arabidopsis *ein3-1* mutant seedlings. Our findings contribute to the knowledge on the mechanism responsible for regulating gene expression in response to sulfur starvation and demonstrate the possible involvement of the elements of an ethylene signaling pathway in such regulation.

**2. Material and methods**

*2.1. Gene cloning, plasmid construction, protein purification*

Standard techniques were used for DNA restriction/ligation manipulation and *Escherichia coli* transformation [10]; all enzymes were from Thermo Scientific (Lithuania). Gateway BP and LR recombination reactions were performed as described in the manufacturer protocols using original plasmids (pENTR/D-TOPO, pDEST22, pDEST32, pDEST15) and enzymes (Invitrogen, USA). All plasmids constructions were verified by restriction digests and/or DNA sequencing. Primers used for amplification and appropriate constructs obtained after cloning are listed in Table 1.

For Y2H constructs, the corresponding fragments of *SLIM1* or *EIN3* cDNA were amplified with primers listed in Table 1 and cloned into pENTR/D-TOPO vector. The final bait and prey construct were prepared by recombination into pDEST32 and pDEST22 vectors. The bait plasmid for a Y1H\_Plus screen was described previously [8]. The constructs allowing for simultaneous protein production in yeast cells based on pGADT7-Rec2 (Clontech, Pao Alto, USA) and pGFP-C-FUS [11]. PCR products of full length *SLIM1* or *EIN3* (Table 1) were inserted into the NcoI/XhoI sites of the pGADT7-Rec2 vector, and only the full length *EIN3* was ligated into BamHI/HindIII sites of the pGFP-C-FUS vector.

The constructs for the bacterial protein production used in EMSA were obtained by cloning the PCR fragments of either *SLIM1* or *EIN3* into pENTR/D-TOPO followed by recombination into pDEST15 to form the fusion with N-terminal GST (Table 1). To produce the truncated SLIM1 and EIN3 proteins fused with GST tag the plasmids were introduced to *E. coli* Rosetta DE3 strain (Novagen, USA) and the protein production was induced at *log* phase with 1 mM isopropyl-β-d-thiogalactopyranoside for 3 h. After sonication, the GST-tagged proteins were purified in the native condition on a glutathione sepharose column (GE Healthcare Bio-Sciences AB, Sweden). Next, denaturing polyacrylamide gel electrophoresis was performed to check the quality and quantity of the purified proteins (Fig. 1B).

*2.2. Electrophoretic mobility shift assay (EMSA)*

The binding reaction proceeded in a volume of 15 l which contained 30 to 50 ng of the DNA probe, 200 to 300 ng of the recombinant protein in the buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 40 mM KCl, 1 mM DTT, 5% glycerol). Once the protein was added to the reaction, the mixture was incubated for 30 min at 25 °C and then loaded onto native 6% polyacrylamide gel. Before loading, the gel was pre-run at 12 V cm-1 for 30 min, and electrophoresis was performed in 0.5x Tris-borate, pH 8.3, for 2.5 h. Images were visualized under UV light using a GelDoc XR+ Imaging System (Bio-Rad, USA) after staining the DNA with SYBR Green (Thermo Scientific).

*2.3. Yeast two hybrid (Y2H) and yeast one hybrid plus (Y1H\_Plus) assays*

Manipulation of yeast cells and protein interaction screening were performed according to standard protocols (Clontech Yeast Protocol Handbook, PT3024-1). The Y2H Gold strain of *Saccharomyces cerevisiae* was used for transformation and the protein interactions in Y2H were confirmed for their ability to activate the reporter gene *HIS3* in triplicate (three independently transformed yeast colonies). The Y1H\_Plus assay consisted of the expression of two proteins (SLIM1 and/or EIN3) in yeast cells previously transformed with the pHIS-UPE plasmid (containing either wild-type or mutated, dysfunctional UPE-box). Their ability to bind to the UPE-box sequence manifested as the induction of reporter gene *HIS3* in MaV203 strain was checked.

*2.4. Plant material and growth conditions*

Seeds of Arabidopsis wild type (wt) accession Columbia-0 and homozygous *ein3-1* knock-out line N8052, were obtained from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK). Seeds were surface sterilized before sowing onto Petri dishes with agar-solidified Hoagland’s medium [12] containing 1% sucrose and sealed with parafilm. Two types of media were used in the experiments: either normal sulfur supply (‘sS’; 1 mM sulfate) or sulfur deficient (‘dS’; 10 M sulfate) media. After stratification for three days at 4 °C in the dark, plants were grown under a 12/12 h light/dark cycle at a temperature of 22 °C. Seedlings were harvested from separate Petri dishes always at the same time of day (3 h after light illumination) at three developmental time points: 3, 7 and 11 days. Each sample consisted of 15-20 seedlings pooled together and frozen immediately in liquid nitrogen for RNA preparation.

*2.5. Gene expression analysis*

Total RNA was isolated from seedlings using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) according to protocol [13]. For the quantitative real-time PCR (qRT-PCR) analysis, 2 μg of RNA was reverse-transcribed using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer’s instructions. The synthesized cDNA was diluted 100 times with sterile water and used as a template for qRT-PCR. qRT-PCR was performed in a PikoReal™ Real-Time PCR System (Thermo Scientific). The gene encoding *ACTIN 2* (TAIR: At3g18780) was selected as an internal control to normalize the quantity of total RNA present in each sample. All the primers used for qRT-PCR are listed in Table 2. The primers were designed to amplify a 70–90-bp fragment close to the 3’ end of the gene. The specificity of the forward and reverse primers to the candidate gene was checked using the NCBI-BLAST website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and melting curve analysis following qRT-PCR. The reaction mixture (6 μL) contained 3 μL of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific), 0.6 μM each of forward and reverse primers, and 1 μL of cDNA template. To ensure the reproducibility of the results qRT-PCR was carried out in triplicate (technical repeats). Two independently grown biological repeats were performed on each line and treatment and those were split into three replicates before RNA isolation. Relative gene expression levels were calculated using the delta-delta Ct method [14] as the transcription level under dS stress treatment compared to the transcription level in control conditions (sS) assayed in samples collected simultaneously at the same time and the adequate Arabidopsis line (although the differences in the expression levels between lines and times of treatment were almost negligible between samples from seedlings grown in sS). Statistical differences were analyzed using Student’s t-test.

**3. Results and discussion**

*3.1. Only three basic domains are necessary for SLIM1 interaction with UPE-box*

The EMSA test was used to check the binding ability of SLIM1 to the sequence of UPE-box in vitro. Because of the problems with the production of full-length SLIM1 in *E. coli* cells, it was decided to use truncated versions of the protein, all comprising the N-terminal part where basic domains (BD), necessary for DNA binding, reside (Fig.1A). Studies with the deletion mutants of EIN3 proved that BD I and V were not required for the DNA-binding activity of EIN3 [6]. The exact location of the core of EIN3 DNA binding domains was recently defined as the region of 174 to 306 aa, containing only BD II, III and IV, whereas the presence of the other BDs enhances the DNA-binding capacity [5]. The DNA-binding activity of SLIM1 truncated only to the BD III and IV (aa:162-288) was demonstrated by surface plasmon resonance [4]. We constructed three truncated versions of SLIM1, namely SLIM1-BD (aa: 1-330), SLIM1-N (aa: 1-286) and SLIM1-3BD (aa: 75-286) and produced the proteins in a bacterial system followed by affinity chromatography purification (Fig. 1A,B). Next, their binding to the DNA fragment containing the UPE-box motif was checked. The results for SLIM1-N binding to the wild type and mutated versions of the UPE-box sequence are shown in Fig. 1C. No binding was detected when at least one of the TEBSs present in the UPE-box was mutated underlining the necessity for two functional TEBSs for SLIM1 recognition. As expected, the SLIM1-BD and also shorter SLIM1-3BD, lacking BD I, likewise demonstrated binding ability to the UPE-box sequence (Fig. 1D,E). Our results proved previously published results for the SLIM1 and UPE-box interaction observed in yeast one-hybrid screen [8].

*3.2. SLIM1 forms homodimer while binding to UPE-box*

It was demonstrated for Arabidopsis EIN3, EIL1 and EIL2 that during binding to DNA they form homodimers [6]. EIN3 protein was also shown to form a dimer, even in the absence of DNA [5, 6]. In contrast, the truncated SLIM1 protein (aa: 162-288) produced in a large-scale, cell-free expression system was considered to exist in a monomeric form [4]. The authors suggested the possibility that the dimerization is important for stable binding to a palindromic sequence, though a monomeric protein can putatively still bind to a single TEBS. As none of the experiments were performed with SLIM1’s preferred binding sequence, we decided to check the dimerization abilities of SLIM1 using EMSA and UPE-box interaction with two truncated forms of SLIM1, SLIM1-BD and SLIM1-3BD. The size difference between the proteins allowed us to observe the bands corresponding to the binding of each truncated SLIM1 version to UPE-box and also a faint band of intermediate mobility that appeared when both proteins were combined with the DNA fragment (Fig. 1D). The intermediate band most probably corresponds to a heterodimer of SLIM1-BD and SLIM1-3BD, indicating that SLIM1 binds to the UPE-box as a dimer. To provide additional evidence that SLIM1 has the capacity to form dimers we performed a screen of the interaction using the Y2H system. SLIM1 as a transcriptional regulator has an activating domain, and therefore, to avoid the activation of the reporter gene, only its truncated derivatives, SLIM1-N and SLIM1-BD, fused to the GAL4-BD were used as bait. As prey, the GAL4 activation domain was fused to either full-length SLIM1 or its truncated versions, SLIM1-N or SLIM1-BD. The growth of yeast on selective medium lacking histidine indicated the interaction between the variants of SLIM1 protein confirming homodimerization abilities of SLIM1 even in the absence of UPE-box (Fig. 2A).

*3.3. EIN3 can heterodimerize with SLIM1*

EIN3 is probably not capable of forming heterodimers with EIL1 and EIL2 as assessed by EMSA assays [6]. However, we decided to use the Y2H system for testing the possible SLIM1 and EIN3 interaction. Again, because of problems with activation by the full length EIN3, only partial EIN3-N protein (aa: 1-299) was fused with the GAL4-BD and its interaction with EIN3-N fused with GAL4-AD confirmed its homodimerization abilities (Fig. 2B) [5, 6]. Most importantly; different fragments of SLIM1 also demonstrated their capacity to form heterodimers with EIN3-N (Fig. 2B). SLIM1-3BD was the shortest interacting SLIM1 derivative and therefore the interaction region between the two proteins most likely resides in the 1-299 aa region of EIN3 and the 75-286 aa region of SLIM1. To confirm the heterodimerization *in planta*, in the future studies we plan to analyze the temporal and spatial interaction between both proteins in transgenic Arabidopsis lines using the bimolecular fluorescence complementation (BiFC) technique.

*3.4. EIN3 interaction with SLIM1 prevents its binding to UPE-box*

To assess whether EIN3 can also heterodimerize with SLIM1 during binding of SLIM1 to DNA we performed EMSA using a combination of fragments of SLIM1 and EIN3 proteins (Fig. 1E). To be able to see the size differences between DNA bound to each protein on the gel we used SLIM1-BD and a smaller truncated EIN3 protein, EIN3-IV (aa: 55-276). Interestingly, the results of EMSA showed that truncated EIN3-IV alone is not able to bind to the UPE-boxmotif, despite the presence of the EIN3 optimal DNA binding domain [5] as well as the two parallel TEBSsin the DNA sequence. This is in agreement with the recently published data that EIN3 requires a certain spacing between two inverted EBSsin order to bind to DNA [5]. Even more surprising was the fact that the addition of EIN3-IV to SLIM1-BD prevents binding of the latter to DNA (Fig. 1E). It suggests that SLIM1 forms heterodimers with EIN3 through the same region as the region used for homodimerization and that probably the very same region is necessary for efficient binding to the UPE-box motif or for the dimerization prior to DNA binding in order to form specific conformation. The additional proof of the interference of EIN3 with SLIM1 binding to DNA comes from the experiments using the Y1H\_Plus system. Three plasmids were introduced to yeast cells, enabling simultaneous expression of two proteins and the reporter protein (enabling growth on histidine lacking media) under the control of a UPE-box*-*containing promoter. The growth of yeast on the selective media was observed only when SLIM1 was co-expressed with GFP as additional control protein (Fig. 2C). In an analogous situation when the mutated UPE-box was used almost no yeast growth could be seen. EIN3 was not able to induce the reporter gene either when used with GFP control protein or with EIN3-G (some yeast growth could be visible due to leakiness of reporter gene). Most importantly, when SLIM1 and EIN3-G were co-expressed the yeast was not able to grow on the selective medium. This result indicates that the presence of EIN3 interferes with SLIM1 binding to the UPE-box motif.

It is well known that DNA binding by EIN3 can be regulated by other proteins. For example, JAZ proteins directly interact with EIN3/EIL1 and recruit a corepressor HDA6 to repress the transcriptional activity of EIN3/EIL1 via the action of histone deacetylation [15]. JAZ1 interacts with the fragment of EIN3 (aa: 200-500) that overlaps with its DNA binding domains (aa: 174-306), and therefore it physically interferes with the binding abilities of EIN3. Also, another protein from the jasmonic acid (JA) signaling pathway, MYC2, interacts with EIN3 to attenuate its effect on the transcription of target genes, especially in inhibition of hook formation and disease resistance against necrotrophic pathogens [16]. Conversely, this interaction also represses MYC2 in its ability to inhibit JA-induced expression of wound-responsive genes and herbivory-inducible genes and to attenuate JA-regulated plant defense against generalist herbivores [17].

*3.5. EIN3 negatively regulates the transcription of several SLIM1-dependent genes in sulfur deficiency-grown seedlings*

To examine whether the EIN3 protein indeed has an impact on gene expression in plants suffering from sulfur deficiency the transcript level of several genes was assayed in Arabidopsis *ein3-1* mutant line. For our studies we choose six genes described as being induced during sulfur deficiency and mostly being SLIM1-dependent [1]. In the promoters of three of them, a gene encoding unknown protein (TAIR: At1g12030), *LSU1* (TAIR: At3g49580) and *APR1* (TAIR: At4g04610) (Fig. 3D,E,F) there are UPE-boxes, therefore those genes are directly targeted by SLIM1. Interestingly, despite the presence of this regulatory element, *APR1* has not been listed as a SLIM1-dependent gene [1]. Transcription was monitored in very young seedlings after short (3 days of starvation; germinating seedlings) and prolonged sulfur starvation (11 days of starvation) as the responses to nutrient stress might differ depending on the duration. While at day 3 we expect mainly primary responses, after additional days of inadequate sulfur nutrition we might presume emerging secondary transcriptional and metabolic changes activated by adaptation mechanisms. No phenotypic differences were noticed between wt and *ein3-1* mutant seedlings; only the slight growth retardation as well as other stress-related symptoms such as increased accumulation of anthocyanin was visible already at day 7 in dS conditions (data not shown). In seedlings grown in sS conditions all the assayed genes sustained similar expression levels for 11 days which did not differ significantly between lines. However, transcription of all sulfur deficiency-responsive genes was induced at every tested time of seedling development and the levels increased gradually with the duration of dS treatment (Fig. 3). All six genes clearly showed significantly enhanced expression levels in the *ein3-1* mutant line in comparison to the wt. It is noteworthy that the pattern of induction was slightly different for the genes with UPE-boxin their promoter, especially for the highly inducible genes. While *BGLU28*, *SDI1* and *SULTR1;1* transcript levels did not differ significantly between wt and the *ein3-1* mutant after prolonged starvation (11 days), the accumulation of mRNA of *At1g12030* and *LSU1* was still twice as high in the absence of functional EIN3 protein (Fig. 3A,B,C and D,E respectively). This might suggest that EIN3 interferes with the sulfur deficiency-depended induction and represents a control mechanism of ethylene signaling over the response to nutritional stress. To check whether the ethylene signaling pathway was induced in seedlings grown in dS conditions, we assayed the level of *ERF1* transcript, encoded by a gene under direct EIN3 control [18]. Increasing accumulation of *ERF1* transcript could be observed in wt and the *ein3-1* mutant (Fig. 3G) confirming our previous studies on 2-days sulfur-starved tobacco plants [19]. In the Arabidopsis *ein3-1* mutant *ERF1* induction was to a lesser extent due to a lack of functional EIN3 protein (Fig. 3G). However, its functional homologs, EIL1 and EIL2, might still compensate for EIN3 function [20]. EIN3 and EIL1 are thought to function redundantly in the ethylene signaling pathway, with EIL1 being a minor player [21]. Although the EIN3 protein is generally more effective than EIL1/2 in activating ethylene responses, the latter factors play a pivotal role in regulating a specific subset of reactions to that hormone [22]. In our studies we only followed the interaction between SLIM1 and EIN3; however, it cannot be excluded that EIL1/2 also can form heterodimers with SLIM1 in specific tissues, developmental stages and/or environmental perturbations. The induction of the *ERF1* gene during sulfur deficiency indicates the induction of ethylene signaling resulting in stabilization of the EIN3 protein abundance. Despite this, the transcripts of SLIM1-dependent genes still accumulate in wt seedlings, suggesting that either EIN3 and SLIM1 are no longer forming heterodimers or that the other transcriptional factors are involved in the regulation. It is also possible that other proteins are regulating complex stability, depending on the sulfur status of the plant or tissue. It seems the interplay between EIN3 and SLIM1 is very subtle and certainly awaits detailed studies.

The role of ethylene in the metabolism of various nutrients including sulfur is well described [23, 24] with the examples of direct involvement of EIN3 protein in the regulation of nitrate and iron nutrition [25, 26]. In a recent paper, many sulfur-related genes, including *SULTR1;1*, *LSU1* and *APR1* described here, have been found among genes directly regulated by EIN3 in etiolated seedlings after ethylene exposure during the course of 24 h [18]. In such a short time ethylene induced four distinct waves of transcription, suggesting the presence of EIN3 transcriptional control layers starting a multitude of downstream cascades. The ‘hormone co-regulation’ category was largely over-represented in the list of the genes modulated by EIN3 pointing out its involvement in the ‘cross-talk’ events and the orchestration of other hormone pathways. However, a general conclusion must be drawn carefully, since in the presented experiment whole seedlings were used, while the expression of a given gene might be differentially controlled depending on tissue, developmental stage of the plant and other environmental factors. The possibility of the complex formation between EIN3 and SLIM1 might depend on the abundance, tissue and subcellular localization of both proteins, which makes the situation even more complex. SLIM1 is expressed predominantly in vascular tissues, and neither its transcription level nor protein abundance is modulated by the changes in sulfur conditions [1]; unlike EIN3, whose protein level is constantly controlled by ethylene and carbon status (for review, see: [27]). More research is needed to evaluate the spacing and timing of the heterodimer formation but more importantly to dissect how far the sulfur deficiency response can be attributed to ethylene signaling and whether SLIM1 can be considered part of it. Moreover, the possible occurrence of ‘cross-talk’ between SLIM1 and ethylene receptors was recently suggested [28]. We also should keep in mind that the conditions of sulfur deficiency might be met by plants quite frequently, as these conditions accompany the increased demand for reductive thiol compounds during oxidative stress. Various studies show that elevated oxidative stress enhances ethylene biosynthesis and ethylene-related effects [23]. Glutathione is the major reservoir of non-protein thiols but also the major soluble antioxidant in cells. Its concentration is controlled by a complex homeostatic mechanism with the availability of external sulfur as a prerequisite [23]. Therefore, the interplay between EIN3 and SLIM1 can additionally putatively regulate the transcriptional response to counteract oxidative damage.

Moreover, it remains to be clarified what other factors are influencing EIN3-SLIM1 heterodimer stability/formation and whether EIN3 counteracts SLIM1 transcriptional activity to prevent or to attenuate its effect. In our studies, we observed a much quicker and stronger response to sulfur deprivation in the *ein3-1* mutant which suggests that under adequate sulfur conditions, EIN3 binds SLIM1 to disable its transcriptional activity. Because *SLIM1* is being constantly transcribed irrelevant the sulfur status, it is possible that the interaction serves to adjust SLIM1 abundance, with SLIM1 being degraded by the 26S proteasome together with EIN3. EIN3 protein level is constantly controlled by the recognition of specific E3 ubiquitin ligases EBF1/2 and proteasomal degradation in the absence of ethylene signal (for review, see: [27]). In addition, one can imagine that a sulfur deficiency signal impacts the recognition of EIN3 by EBF1/2 and thus regulates the turnover rate of this transcription factor additionally adjusting the SLIM1 abundance. Either an ethylene or sulfur deficiency signal might prevent the recognition or action of EBF1/2 by posttranslational modification of the targets.

Though awaiting further detailed exploration, our studies clearly underline the involvement of the EIN3 protein in governing the strength and timing of sulfur deficiency responses in Arabidopsis. Since ethylene production and sulfur assimilation pathways share some metabolites [29, 30] it is possible that these two pathways might indeed have in common some sensing or signaling elements; however, the coordination and dissection of specific processes are still unclear. The results presented here have unveiled another regulatory layer directly linking these two pathways. Together with other molecular and cellular details on the ‘cross-talk’ between ethylene signaling and sulfur metabolism the translation of this information into crop plants might provide innovative tools for sustainable agriculture.

**References:**

[1] A. Maruyama-Nakashita, Y. Nakamura, T. Tohge, K. Saito, H. Takahashi, Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism, Plant Cell, 18 (2006) 3235-3251.

[2] H. Guo, J.R. Ecker, The ethylene signaling pathway: new insights, Curr Opin Plant Biol, 7 (2004) 40-49.

[3] Q. Chao, M. Rothenberg, R. Solano, G. Roman, W. Terzaghi, J.R. Ecker, Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins, Cell, 89 (1997) 1133-1144.

[4] K. Yamasaki, T. Kigawa, M. Inoue, T. Yamasaki, T. Yabuki, M. Aoki, E. Seki, T. Matsuda, Y. Tomo, T. Terada, M. Shirouzu, A. Tanaka, M. Seki, K. Shinozaki, S. Yokoyama, Solution structure of the major DNA-binding domain of *Arabidopsis thaliana* ethylene-insensitive3-like3, Journal of Molecular Biology, 348 (2005) 253-264.

[5] J. Song, C. Zhu, X. Zhang, X. Wen, L. Liu, J. Peng, H. Guo, C. Yi, Biochemical and structural insights into the mechanism of DNA recognition by Arabidopsis ETHYLENE INSENSITIVE3, PLoS One, 10 (2015) e0137439.

[6] R. Solano, A. Stepanova, Q. Chao, J.R. Ecker, Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1, Genes Dev, 12 (1998) 3703-3714.

[7] S. Kosugi, Y. Ohashi, Cloning and DNA-binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog, Nucleic Acids Res, 28 (2000) 960-967.

[8] A. Wawrzynska, M. Lewandowska, A. Sirko, *Nicotiana tabacum* EIL2 directly regulates expression of at least one tobacco gene induced by sulphur starvation, J Exp Bot, 61 (2010) 889-900.

[9] H.M. Hubberten, S. Klie, C. Caldana, T. Degenkolbe, L. Willmitzer, R. Hoefgen, Additional role of O-acetylserine as a sulfur status-independent regulator during plant growth, Plant J, 70 (2012) 666-677.

[10] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular cloning: a laboratory manual: 2nd ed., Cold Spring Harbor, New York, 1989.

[11] R.K. Niedenthal, L. Riles, M. Johnston, J.H. Hegemann, Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast, Yeast, 12 (1996) 773-786.

[12] H. Schat, R. Vooijs, E. Kuiper, Identical major gene loci for heavy metaltolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*., Evolution, 50 (1996) 1888-1895.

[13] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal Biochem, 162 (1987) 156-159.

[14] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods, 25 (2001) 402-408.

[15] Z. Zhu, F. An, Y. Feng, P. Li, L. Xue, M. A, Z. Jiang, J.M. Kim, T.K. To, W. Li, X. Zhang, Q. Yu, Z. Dong, W.Q. Chen, M. Seki, J.M. Zhou, H. Guo, Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis, Proc Natl Acad Sci U S A, 108 (2011) 12539-12544.

[16] S. Song, H. Huang, H. Gao, J. Wang, D. Wu, X. Liu, S. Yang, Q. Zhai, C. Li, T. Qi, D. Xie, Interaction between MYC2 and ETHYLENE INSENSITIVE3 modulates antagonism between jasmonate and ethylene signaling in Arabidopsis, Plant Cell, 26 (2014) 263-279.

[17] X. Zhang, Z. Zhu, F. An, D. Hao, P. Li, J. Song, C. Yi, H. Guo, Jasmonate-activated MYC2 represses ETHYLENE INSENSITIVE3 activity to antagonize ethylene-promoted apical hook formation in Arabidopsis, Plant Cell, 26 (2014) 1105-1117.

[18] K.N. Chang, S. Zhong, M.T. Weirauch, G. Hon, M. Pelizzola, H. Li, S.S. Huang, R.J. Schmitz, M.A. Urich, D. Kuo, J.R. Nery, H. Qiao, A. Yang, A. Jamali, H. Chen, T. Ideker, B. Ren, Z. Bar-Joseph, T.R. Hughes, J.R. Ecker, Temporal transcriptional response to ethylene gas drives growth hormone cross-regulation in Arabidopsis, Elife, 2 (2013) e00675.

[19] M. Lewandowska, A. Wawrzynska, G. Moniuszko, J. Lukomska, K. Zientara, M. Piecho, P. Hodurek, I. Zhukov, F. Liszewska, V. Nikiforova, A. Sirko, A contribution to identification of novel regulators of plant response to sulfur deficiency: characteristics of a tobacco gene UP9C, its protein product and the effects of UP9C silencing, Mol Plant, 3 (2010) 347-360.

[20] K. Schellingen, D. Van Der Straeten, T. Remans, J. Vangronsveld, E. Keunen, A. Cuypers, Ethylene signalling is mediating the early cadmium-induced oxidative challenge in *Arabidopsis thaliana*, Plant Sci, 239 (2015) 137-146.

[21] J.M. Alonso, A.N. Stepanova, R. Solano, E. Wisman, S. Ferrari, F.M. Ausubel, J.R. Ecker, Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis, Proc Natl Acad Sci U S A, 100 (2003) 2992-2997.

[22] F. An, Q. Zhao, Y. Ji, W. Li, Z. Jiang, X. Yu, C. Zhang, Y. Han, W. He, Y. Liu, S. Zhang, J.R. Ecker, H. Guo, Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in Arabidopsis, Plant Cell, 22 (2010) 2384-2401.

[23] N. Iqbal, A. Masood, M.I. Khan, M. Asgher, M. Fatma, N.A. Khan, Cross-talk between sulfur assimilation and ethylene signaling in plants, Plant Signal Behav, 8 (2013) e22478.

[24] G. Moniuszko, M. Skoneczny, K. Zientara-Rytter, A. Wawrzynska, D. Glow, S.M. Cristescu, F.J. Harren, A. Sirko, Tobacco LSU-like protein couples sulphur-deficiency response with ethylene signalling pathway, J Exp Bot, 64 (2013) 5173-5182.

[25] G.B. Zhang, H.Y. Yi, J.M. Gong, The Arabidopsis ethylene/jasmonic acid-NRT signaling module coordinates nitrate reallocation and the trade-off between growth and environmental adaptation, Plant Cell, 26 (2014) 3984-3998.

[26] S. Lingam, J. Mohrbacher, T. Brumbarova, T. Potuschak, C. Fink-Straube, E. Blondet, P. Genschik, P. Bauer, Interaction between the bHLH transcription factor FIT and ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between the regulation of iron acquisition and ethylene signaling in Arabidopsis, Plant Cell, 23 (2011) 1815-1829.

[27] A. Wawrzynska, A. Sirko, To control and to be controlled: understanding the Arabidopsis SLIM1 function in sulfur deficiency through comprehensive investigation of the EIL protein family, Front Plant Sci, 5 (2014) 575.

[28] G. Moniuszko, Ethylene signaling pathway is not linear, however its lateral part is responsible for sensing and signaling of sulfur status in plants, Plant Signal Behav, 10 (2015) e1067742.

[29] A. Wawrzynska, G. Moniuszko, A. Sirko, Links between ethylene and sulfur nutrition-a regulatory interplay or just metabolite association?, Front Plant Sci, 6 (2015) 1053.

[30] A. Koprivova, S. Kopriva, Hormonal control of sulfate uptake and assimilation, Plant Mol Biol, 91 (2016) 617-627.

**Figure captions**

**Fig. 1. EMSA results showing DNA-protein interaction.**

(**A**) Scheme of the tested truncated forms of SLIM1 and EIN3 proteins. SLIM1-FL – full length SLIM1 protein with domains characteristic for EIL protein family: ‘acidic’, N-terminal region rich in acidic aa; BD, Basic Domain rich in basic aa; P-rich, region rich in prolines (**B**) Truncated proteins in fusion with N-terminal GST were overproduced in *E. coli* and purified by an affinity column before applied in EMSA. The expected sizes of protein fusions with GST: SLIM1-BD: 63.4 kDa, SLIM1-N: 58.6 kDa, SLIM1-3BD: 50.2 kDa and EIN3-IV: 51.5 kDa. The sizes of the protein ladder (Spectra BR, Thermo Scientific) are on the left. (**C**) Binding of the SLIM1-N protein to UPE-box and various base substitution in UPE-boxas in [8] (UPEbox-1TEBS\_mut: AGA**G**A**A**ATTGAACCTGGACA; UPEbox-2TEBS\_mut: AGATACATT**T**A**C**CCTGGACA; UPEbox: AGATACATTGAACCTGGACA). The unbound DNA probe is present at the bottom of the photo. (**D**) Truncated SLIM1 proteins binding to the UPE-box showing SLIM1 homodimerization. Proteins were incubated with DNA alone or mixed together at similar concentration ratio. Due to longer running time of the gel for better bands separation there is no unbound DNA probe at the bottom (**E**) EIN3-IV prevents SLIM1-BD from binding to UPE-box; no DNA mobility retardation was observed when SLIM1-BD were incubated with EIN3-IV protein at similar concentration ratio (1:1 i.e. 250 ng of each SLIM1-BD and EIN3-IV) while 10 times dilution of EIN3-IV protein had slight impact on SLIM1-BD – UPE-box binding. The unbound DNA probe is present at the bottom of the photo. All EMSA experiments were carried out at least thrice.

**Fig. 2. Interactions of protein-protein and DNA protein in yeasts.**

(**A**) and (**B**) Y2H screen results. The ‘bait’ plasmid is enlisted as first above the yeast colony in the photo, as second the ‘prey’ plasmid. ‘Empty’ stands for negative control of interaction where only pDEST22 vector was used. The growth on the selective SD-LWH media is an indicator of protein-protein interaction whereas the growth on SD-LW is the positive control (selection for transformed plasmids). (**C**) Y1H\_Plus results. Each yeast colony harbors three plasmids listed above as follows: the first one harbors pHIS2.1 with UPE-box sequence (‘UPE’; AGATACATTGAACCTGGACA) or dysfunctional mutated UPE-box sequence (‘UPEm’; AGA**G**A**A**ATT**T**A**C**CCTGGACA); the second one either SLIM1 or EIN3 effector or an empty pGADT7\_Rec2 plasmid (‘GAD’; negative control); and the third one enables the expression of either EIN3 with GFP in C-terminal fusion (‘EIN3-G’) or GFP (negative control of an empty pGFP-C-FUS plasmid). Yeasts were grown for 4 d at 300 C on either control SD-LWU (selection of the transformed plasmids) or selective SD-LWUH medium with 100 mM 3-aminotriazole (3-AT; used to reduce background growth on media lacking histidine). The strong growth indicates induction of reporter gene *HIS* through the presence of an effector able to bind to UPE-boxsequence.

**Fig.3. Relative expression levels of selected genes in seedlings grown on dS media for 3, 7 and 11 days analyzed by qRT-PCR.**

The Arabidopsis gene for *ACT2* was used as an internal control to normalize the expression data. Data are shown as the log2-transformed values of the fold change levels of expression assayed in dS grown wt and *ein3-1* mutant seedlings in comparison with the expression in adequate Arabidopsis line grown for the same time in sS. The data for the seedlings grown in sS conditions are not shown as all the assayed genes sustained similar expression levels throughout the experiment with no statistically important differences between lines. Bars represent mean expression levels and SE from two independent biological split into three technical replicates each containing a pool of 15-20 plants. Asterisks indicate significant differential expression (P < 0.05; Student’s t-test) observed in the *ein3-1* mutant in comparison with wt at a given time point.