P-proteins in *Arabidopsis* are heteromeric structures involved in rapid sieve tube sealing

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INTRODUCTION

The complex phloem system of higher plants distributes photosynthates and signaling molecules throughout the plant body. The conducting sieve tubes that accomplish this long-distance transport comprise sieve elements connected end-to-end with intervening perforated sieve plates to promote efficient mass flow. Angular sieve elements are characterized by abundant structural phloem proteins (P-proteins; Cronshaw and Esau, 1967), the identity and function of which has been debated for decades.

P-proteins of the fascicular phloem are today known to be encoded by members of the widespread *sieve element occlusion* (*SEO*) gene family (Pélassier et al., 2008; Rüping et al., 2010; Noll et al., 2007, 2009; Bucsenez et al., 2011). This raises the interesting possibility that P-proteins of the SEO gene family, and tobacco SEO proteins were shown to be directly involved in sieve tube sealing thus preventing the loss of photosynthate. Analysis of the two *Arabidopsis* SEO proteins (*AtSEOa* and *AtSEOb*) indicated that the corresponding P-protein subunits do not act in a redundant manner. However, there are still pending questions regarding the interaction properties and specific functions of *AtSEOa* and *AtSEOb* as well as the general function of structural P-proteins in *Arabidopsis*. In this study, we characterized the *Arabidopsis* P-proteins in more detail. We used *in planta* bimolecular fluorescence complementation assays to confirm the predicted heteromeric interactions between *AtSEOa* and *AtSEOb*. *Arabidopsis* mutants depleted for one or both A*SEO proteins lacked the typical P-protein structures normally found in sieve elements, underlining the identity of A*SEO proteins as P-proteins and furthermore providing the means to determine the role of *Arabidopsis* P-proteins in sieve tube sealing. We therefore developed an assay based on phloem exudation. Mutants with reduced A*SEO expression levels lost twice as much photosynthate following injury as comparable wild-type plants, confirming that *Arabidopsis* P-proteins are indeed involved in sieve tube sealing.

Keywords: sieve element occlusion, phloem, translocation, wound sealing, exudation, forisome

Structural phloem proteins (P-proteins) are characteristic components of the sieve elements in all dicotyledonous and many monocotyledonous angiosperms. Tobacco P-proteins were recently confirmed to be encoded by the widespread *sieve element occlusion* (*SEO*) gene family, and tobacco SEO proteins were shown to be directly involved in sieve tube sealing thus preventing the loss of photosynthate. Analysis of the two *Arabidopsis* SEO proteins (*AtSEOa* and *AtSEOb*) indicated that the corresponding P-protein subunits do not act in a redundant manner. However, there are still pending questions regarding the interaction properties and specific functions of *AtSEOa* and *AtSEOb* as well as the general function of structural P-proteins in *Arabidopsis*. In this study, we characterized the *Arabidopsis* P-proteins in more detail. We used *in planta* bimolecular fluorescence complementation assays to confirm the predicted heteromeric interactions between *AtSEOa* and *AtSEOb*. *Arabidopsis* mutants depleted for one or both A*SEO proteins lacked the typical P-protein structures normally found in sieve elements, underlining the identity of A*SEO proteins as P-proteins and furthermore providing the means to determine the role of *Arabidopsis* P-proteins in sieve tube sealing. We therefore developed an assay based on phloem exudation. Mutants with reduced A*SEO expression levels lost twice as much photosynthate following injury as comparable wild-type plants, confirming that *Arabidopsis* P-proteins are indeed involved in sieve tube sealing.
pseudogenes) in Arabidopsis to 26 (including seven pseudogenes) in soybean (Rüping et al., 2010; Ernst et al., 2011) and the conserved sequences and expression profiles suggest potential functional redundancy among the corresponding proteins (Lynch and Conery, 2000). This issue was recently addressed in Arabidopsis, where transfer DNA (T-DNA) insertion mutants lacking either AtSEOa or AtSEOb did not accumulate the typical P-protein filaments in sieve elements, although the remaining AtSEO gene was expressed normally (Anstead et al., 2012). This was confirmed by microscopy in the same mutant lines complemented with a green fluorescent protein (GFP)-tagged version of the missing AtSEO protein. These data indicate that AtSEOs and AtSEOb are likely to be structural plasmid filament proteins that are jointly required for filament formation. However, the fundamental ability to form heteromers could not be confirmed.

We therefore set out to characterize the role of SEO proteins in Arabidopsis in more detail. The anticipated heteromeric interaction between AtSEOs and AtSEOa was confirmed by bimolecular fluorescence complementation (BiFC) studies in planta. Furthermore, the generation and analysis of mutants with limited expression of both AtSEO genes confirmed the hypothesis that AtSEOb and AtSEOb are Arabidopsis P-proteins because the typical P-protein structures could accordingly not be detected in these plants by transmission electron microscopy (TEM). The P-protein-depleted plants also allowed us to examine the functional role of structural P-proteins in Arabidopsis. We found that the mutants lost twice as much photosynthetic as comparable wild-type plants following injury, providing the first direct evidence that P-proteins are required for rapid plasmolysis wound sealing in the model plant Arabidopsis.

MATERIALS AND METHODS

Arabidopsis T-DNA INSERTION MUTANTS

Seeds of T-DNA insertion mutants for the genes AtSEOb (At3g01680) and AtSEOa (At3g01670) were obtained from the European Arabidopsis Stock Centre (NASC). Homozygous plants were identified from the lines SALK_146614C (AtSEOb) and SALK_3_14780 (AtSEOa), which were used for further analysis. All mutants are in a Columbia (Col-0) background.

PLANT GROWTH CONDITIONS

Plants were typically grown in phytochambers at 23/18°C, 40% relative humidity and under long-dark conditions (16/8-h light/dark period). To increase the leaf size for examination, the corresponding lines were cultivated under short-day conditions (8/16-h light/dark period).

AGROINFILTRATION OF Nicotiana benthamiana

The interaction characteristics of AtSEOb and AtSEOa were analyzed by BiFC in a plant background (Bracha-Drori et al., 2004; Walter et al., 2004). The coding sequences of both genes were amplified with and without translational stop codons using primer combinations 5'-AGA TCAGAG AGA TGG CCC GAC GCT TTC TCA ATT-3' and 5'-ACA TCTGAG TTA CTC AAG GCA GCA TGG GTC-3' or 5'-AGA TCAGAG AGA TGG CCC GAC GCT TTC TCA ATT-3' and 5'-ACA TCTGAG TTA CTC AAG GCA GCA TGG GTC-3' (AtSEOb) and 5'-AGA TCAGAG AGA TGG CCC GAC GCT TTC TCA ATT-3' and 5'-ACA TCTGAG TTA CTC AAG GCA GCA TGG GTC-3' (AtSEOa) and 5'-AGA TCAGAG AGA TGG CCC GAC GCT TTC TCA ATT-3' and 5'-ACA TCTGAG TTA CTC AAG GCA GCA TGG GTC-3' (AtSEOa) and 5'-AGA TCAGAG AGA TGG CCC GAC GCT TTC TCA ATT-3' and 5'-ACA TCTGAG TTA CTC AAG GCA GCA TGG GTC-3' (AtSEOa). The recombinant entry vectors were used to insert the AtSEO sequences into Gateway-compatible pBatTL vectors, mediated by LR Clonase (Invitrogen). The pBatTL vectors contained reporter sequences encoding split variants of the monomeric red fluorescent protein mRFP1-Q66T (Jach et al., 2006) resulting in eight different constructs: pBatTLAtSEObΔmRFP, pBatTLAtSEOaΔmRFP, pBatTLNlmRFP, pBatTLAtSEOa, and pBatTLAtSEOaΔmRFP. A monomeric version of Emerald (mEmerald) was used as a control by converting the phenylalanine residue at position 223 to arginine by polymerase chain reaction (PCR) mutagenesis using primers 5'-AGA ACCATGGGT AAA GGA GAA G-3' and 5'-AGA ACCATGGGT ATT GCC TAT ATG TTT TTC TGG TTC TC-3' corresponding lines were cultivated under short-day conditions (8/16-h light/dark) or long-day conditions (16/8-h light/dark) using primer combinations 5'-ACA TCTGAG TTA CTC AAG GCA GCA TGG GTC-3' and 5'-ACA TCTGAG TTA CTC AAG GCA GCA TGG GTC-3'. The pGJ2628 vector was used as the template (kindly provided by Dr. Guido Jach). The mEmerald fragment was digested with NcoI and inserted into vector pENTR4, which was again used to insert the gene into the corresponding pBatTL vectors resulting in constructs pBatTLmEmeraldNlRFP and pBatTLmEmeraldCmRFP.

For transient expression in N. benthamiana epidermal cells, all BiFC constructs were introduced into Agrobacterium tumefaciens strain GV3101:pMP90. Leaves from 4-week-old plants were infiltrated simultaneously with A. tumefaciens containing the pCH32 helper plasmid encoding Tomato bushy stunt virus RNA silencing repressor p19 (Voigt et al., 2004). Infiltrated leaf discs were punched out after 3 days and analyzed by confocal laser scanning microscopy (CLSM).

CLSM IMAGING

N. benthamiana leaf discs were analyzed by CLSM using a Leica TCS SP5X microscope with excitation/emission wavelengths of 543/570–630 nm for the detection of mRFP and 488/500–580 nm for the detection of mEmerald.

GENERATION OF ASEO KNOCKOUT/KNOCKDOWN PLANTS

To obtain Arabidopsis plants with reduced levels of both AtSEO proteins, RNAi vectors producing AtSEOs or AtSEOa hairpin (hp) RNAs were generated and used to transform the homozygous T-DNA insertion mutants (ΔAtSEOb or ΔAtSEOa) described above. To ensure strong transgene expression in sieve elements, the Cauliflower mosaic virus (CaMV) 35S promoter from the RNAi vector pHANNIBAL (Wesley et al., 2001; kindly provided by CSIRO Plant Industry) was removed by digestion with SacI and EcoRI (including Klenow treatment) or SacI/XbaI, and replaced with 997-bp promoter fragments designated PASexoA and PASexoB, amplified from Arabidopsis Col-0 genomic DNA. The PASexoA/SexoB/EcoRI primer combination was 5'-AGA CCCGGG GTC GAGGGGCGCGTC ACC GGG AGA TCA TTC-3' and 5'-AGA CCCGGG ATT GCC GAG GTT GGT AG-3' to generate PASexoA of pHANNIBAL. The PASexoA/SexoB/EcoRI primer combination was 5'-AGA CCCGGG GTC GAGGGGCGCGTC ACC GGG AGA TCA TTC-3' and 5'-AGA CCCGGG ATT GCC GAG GTT GGT AG-3' to generate PASexoB of pHANNIBAL.

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Each case, the Norf site was introduced for a subsequent cloning step. 400-bp fragments with different restriction sites were then amplified from complementary DNA (cDNA) for AtSEOa (primer combinations 5′-GAATTC CCC GAT CCG CCG AGC-3′/5′-AGA TCG CCG CCG AGC-3′) and AtSEOb (primer combinations 5′-AAGCTT TGG TGC ACA GGG ACC 3′/5′-AATCTAGCT CTG TGC ACA GGG ACC 3′) and AtSEOa and AtSEOb (primer combinations 5′-GAATTC TGG TGC ACA GGG ACC 3′/5′-AAGCTT TGG TGC ACA GGG ACC 3′) and AtSEOa and AtSEOb (primer combinations 5′-AATCTAGCT CTG TGC ACA GGG ACC 3′/5′-AAGCTT TGG TGC ACA GGG ACC 3′). The gene fragments were inserted into the EcorRI/Kpn1 and BamHI/XbaI sites (AtSEOa) or Kpn1 and HindIII/XbaI sites (AtSEOb) of the modified pHANNIBAL vectors to obtain pAtSEOaHPAtSEOaHPHANNIBAL and pAtSEObHPAtSEObHPHANNIBAL. The hprRNA cassettes were isolated by digestion with Norf and, after Klenow treatment, inserted into the Smal sites of binary plant transformation vectors pBIN19 (Bevan, 1984) or plab12.1 (Post et al., 2012), resulting in the final constructs pPAtSEOaHPAtSEOa and pLPAtSEObHPAtSEOb.

STABLE PLANT TRANSFORMATION

All binary vectors used for stable plant transformation were introduced into A. tumefaciens strain LBA4404 by electroporation. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998).

REVERSE TRANSCRIPTASE PCR

AtSEO gene expression in the mutant lines was analyzed by isolating total RNA from young leaves using the NucleoSpin RNA Plant Kit (Macherey-Nagel) followed by reverse transcriptase (RT). Superscript II (Invitrogen) was used for reverse transcription, followed by RNA digestion (RNaseH, New England Biolabs). Intron-spanning gene fragments were amplified using primer combinations 5′-TGA TGT CAC A TC ACT TCT CTC CG-3′/5′-TGA TGT CAC A TC ACT TCT CTC CG-3′. The gene fragments were amplified as an internal control using primers 5′-TGA TGT CAC A TC ACT TCT CTC CG-3′/5′-TGA TGT CAC A TC ACT TCT CTC CG-3′ (AtSEOa) and 5′-ATG GAC ATC AAG GAG AAG CTC TC-3′/5′-ATG GAC ATC AAG GAG AAG CTC TC-3′ (AtSEOb). The Arabidopsis actin gene ACT2 (accession number U41998) was amplified as an internal control using primers 5′-CCT CAT ACT CAT GCG CCT TGC AG-3′ and 5′-GTA GAG GAC ATC AAG GAG AAC TCC TCA-3′.

TEM

The ultrastructure of the Arabidopsis sieve element was analyzed by cutting small stem segments, bisecting them longitudinally, fixing the explants in 2% (vol/vol) glutaraldehyde and 3.5% (wt/vol) sucrose for 3 h and incubating them in 1% (vol/vol) osmium tetroxide for 2 h. Samples were dehydrated in ethanol and embedded in LR White (Sigma). Ultrathin sections were stained for 30 min in 2% (wt/vol) uranyl acetate and 3 min in 4% (wt/vol) lead citrate. The sections were examined with a Zeiss EM900 microscope fitted with an SIS Morada digital camera.

EXUODATION ANALYSIS

Arabidopsis plants (wild-type, n = 38; ΔAtSEOa, n = 44; ΔAtSEOb, n = 60; ΔAtSEOa/ΔAtSEOb lines, n = 30; II, n = 52; III, n = 54; ΔAtSEOa/ΔAtSEOb lines, n = 30; II, n = 54; III, n = 54) were grown under short-day conditions for 9 weeks. Fifteen mature leaves were cut from every plant included in the analysis with a razor blade, jointly placed in one vial [containing 3 ml 1 mM MES (2-(N-morpholino)ethanesulonic acid), pH 7] and exuded for 10 min. Exudation experiments were carried out consecutively for 2 days between zeitgeber times 2 and 8 with plants of all lines used in an alternating manner. After exudation, samples were frozen in liquid nitrogen and lyophilized. Freeze-dried samples of two plants were combined and dissolved in 120 μL distilled water, and 50 μL of the solution was then used to determine the quantity of D-glucose and sucrose using the sucrose/D-glucose/D-fructose kit (Roche) as described by Ernst et al. (2012). To determine the total sucrose content of total leaves, these were ground under liquid nitrogen, and the extraction and quantification of sugars was carried out using the sucrose/D-glucose/D-fructose kit (Roche) according to the manufacturer’s instructions.

RESULTS

ΔAtSEOa and ΔAtSEOb form heteromers in a plant background

ΔAtSEOa and ΔAtSEOb were assumed to be non-redundant proteins that undergo heteromeric interactions to form P-protein structures because the analysis of AtSEO complementation lines by microscopy showed typical filaments only when both proteins were expressed (Anstead et al., 2012). However, these data could not be confirmed in yeast two-hybrid experiments, which showed that both ΔAtSEOa and ΔAtSEOb could readily form homodimers but not heterodimers. To resolve this apparent discrepancy and to check whether heteromeric interaction might occur in higher-order structures, we analyzed the interaction behavior of ΔAtSEOa and ΔAtSEOb in a plant background using BiFC. To reduce any potential impact of the reporter fragments, we fused both split variants of the monomeric red fluorescent protein mRFP-Q66T (Iach et al., 2006) to the N- and C-terminus of ΔAtSEOa and ΔAtSEOb, respectively, resulting in eight different constructs (pBatTLAtSEOa/BpNmRFP, pBatTLAtSEOa/BpCmRFP, pBatTLNmRFP:AtSEOb, and pBatTLNmRFP:AtSEOa/Δ). These were introduced into N. benthamiana leaves by agroinfiltration using all possible heteromeric combinations along with appropriate controls (Figure 1).

The infiltration of MES buffer alone did not provoke any defense reactions that might cause red fluorescence in the epidermal cells (Figure 1A). Furthermore, to exclude reporter fluorescence caused by the non-specific interaction of the mRFP split fragments, we fused these to the coding sequence of the non-interacting protein mEmerald. Leaves infiltrated with these control constructs showed green fluorescence confirming that the mEmerald fusions were efficiently expressed (upper part of Figure 1B), whereas no mRFP fluorescence could be detected (lower part of Figure 1B). Homomorphic interaction events could be detected for both ΔAtSEOa and ΔAtSEOb in all tested combinations. ΔAtSEOa homomeric interactions always resulted in rather large fluorescent complexes (Figure 1C), showing the combination ΔAtSEOa/BpNmRFP + ΔAtSEOa/BpCmRFP as an example), but ΔAtSEOb homomeric interaction often yielded cytoplasmic fluorescence, although some combinations also yielded complexes (Figure 1D, showing the combination ΔAtSEOb/BpNmRFP + ΔAtSEOb/BpCmRFP as an example). Most interestingly, the in planta
AtSEO proteins seal sieve plates

AtSEOa:NmRFP
AtSEOb
(Figures 1E,F).
Depending on the tag position and often, restricted to large protein complexes (Frontiers in Plant Science, showing the combination AtSEOa:CmRFP as an example). These experiments confirmed that AtSEOa and AtSEOb can indeed interact in a heteromeric manner.

**AISEO KNOCKOUT/KNOCKDOWN PLANTS LACKING STRUCTURAL P-PROTEINS SHOW NO DISTINCT MORPHOLOGICAL PHENOTYPE**

AtSEOa and AtSEOb are thought to be non-redundant proteins and single knockout mutants lacking either AtSEOa or AtSEOb are known to lack the typical P-protein filaments (Amstead et al., 2012). However, because the precise functions of AtSEOa and AtSEOb in P-protein formation are still unknown, we next set out to generate plants completely lacking AtSEO proteins for further analysis. Therefore, the T-DNA insertion mutants ΔAtSEOa and ΔAtSEOb were supertransformed with hpRNA cassettes specific for the remaining functional AtSEO gene, resulting in knockout/knockdown plant lines designated ΔAtSEOa ΔAtSEOb and ΔAtSEOa ΔAtSEOb. The efficiency of the knockout/knockdown combination was analyzed at the mes-senger RNA (mRNA) level by RT-PCR using intron-spanning primers. Figure 2A shows the expression profiles of AtSEOa and AtSEOb in the young leaves of wild-type Arabidopsis plants, T-DNA insertion mutants and the RNAi lines, confirming the knockout of AtSEOa and AtSEOb in ΔAtSEOa and the efficient silencing of the non-interrupted remaining AtSEO gene in ΔAtSEOa ΔAtSEOb and ΔAtSEOa ΔAtSEOb. Minimal residual expression of the silenced AtSEO genes was detected in the knockout/knockdown lines (Figure 2A) and we were unable to confirm silencing directly at the protein level because no antibodies are available for the specific detection of AtSEOa or AtSEOb.

Next, we used TEM to investigate the structural properties of sieve elements. Therefore, stem sections from 5-week-old plants (wild-type, ΔAtSEOa, ΔAtSEOb, ΔAtSEOa ΔAtSEOb, and ΔAtSEOa ΔAtSEOb) were prepared as described above. Sieve elements of Arabidopsis wild-type plants were always characterized by the abundance of P-protein filaments (Figure 2B). Because of the wounding caused by explant preparation, P-proteins were usually detected in the dispersed state with filaments detached from their parietal positions and clearly occluding the sieve pores (Figure 2E). In agreement with previous studies, we found that the T-DNA insertion mutants ΔAtSEOa and ΔAtSEOb (only impaired in expression of one single AtSEO gene) were also unable to form typical P-protein structures, confirming that both AtSEOa and AtSEOb are needed to form the filaments (Froelich et al., 2011; Amstead et al., 2012). The knockout/knockdown lines with only residual AtSEO gene expression (ΔAtSEOa ΔAtSEOb and ΔAtSEOa ΔAtSEOb) also completely lacked P-protein-like structures in the sieve elements (Figures 2C,D) and no fil-aments accumulated at the sieve plates occluding sieve pores (Figures 2E,G). Sieve element morphology was not affected by the lack of structural P-proteins.

Plants of all lines were grown under standard conditions for several weeks and none of the mutants showed any obvi-ous phenotype differing from wild-type plants. The availability of these plants therefore enabled us to analyze the long-discussed functional role of Arabidopsis P-proteins in sieve tube sealing.
Arabidopsis plants lacking P-proteins show enhanced bleeding following injury

Although P-proteins are known to disperse from the cell periphery and accumulate on downstream sieve plates after injury, their direct role in sieve tube sealing has long been subject to debate. The first direct evidence for their capacity to prevent phloem translocation was provided recently when tobacco mutants lacking P-proteins were tested in exudation assays, showing that the depleted plants lost on average nine times more photoassimilate than corresponding wild-type plants (Ernst et al., 2012). However, the function of P-proteins in *Arabidopsis* has been questioned based on the microscopic analysis of plants expressing AtSEO-yellow fluorescent protein (YFP) fusion constructs (Froelich et al., 2011). To address this discrepancy, we developed an exudation assay to compare the loss of photoassimilate in wild-type Arabidopsis plants and P-protein-depleted mutants following injury.

We therefore grew wild-type Arabidopsis plants, single-knockout mutants ΔAtSEOa and ΔAtSEOb and corresponding knockout/knockdown plants (ΔAtSEOa/ΔAtSEOb, lines I, II, and III). To increase the average leaf size, all plants were cultivated under short-day conditions and again no significant morphological phenotype was visible when exudation experiments were carried out at the age of 9 weeks (Figure 3). In *Arabidopsis*, most of the sugar is translocated as sucrose (Haritatos et al., 2000). Therefore, the amount of sucrose lost after injury should mirror the efficiency of sieve tube sealing.

An experimental overview of the exudation assay is provided in Figure 4A. We removed 15 mature leaves from each assayed plant and pooled the exudates in one vial. We limited exudation to 10 min, thus measuring the rapid sealing activity of P-proteins while avoiding the onset of callose deposition, which promotes the long-term sealing of sieve pores (van Bel, 2003; Xie et al., 2011). After exudation, the samples were lyophilized and the freeze-dried contents from two vials were redissolved and combined, therefore representing the total exudates of 30 leaves derived from two individual plants of the same line. We then determined the D-glucose concentration of these final samples before and after the enzymatic hydrolysis of sucrose using the sucrose/D-glucose/D-fructose
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FIGURE 3 | Arabidopsis plants used for exudation analysis. Arabidopsis wild-type (A), single-knockout ΔAtSEOA (B) and ΔAtSEOB (C) and knockout/knockdown plants ΔAtSEOA/ΔAtSEOB, lines I–III (D,E,F), and ΔAtSEOB/ΔAtSEOA, lines I–III (G,H,I), did not show any obvious morphological phenotype prior to the exudation assay. All plants were grown under short-day conditions to increase total leaf size.

kit (Roche), and sucrose concentrations were deduced from the difference between the D-glucose concentrations before and after enzymatic inversion (Figures 4A,B). Remarkably, all the mutant lines tested in the exudation experiments showed a similar enhanced loss of photoassimilate compared to wild-type plants (Figure 4C). Mutant plants lacking one AtSEO gene and knockout/knockdown plants impaired in expression of both AtSEO genes lost on average twice as much sucrose as wild-type plants. To exclude the possibility that the differences in sucrose content might reflect the presence of more sucrose in the leaves of the mutant plants, we prepared total leaf extracts from all plant lines included in the assay and determined the sucrose concentrations as described above. These measurements confirmed that the sucrose contents in leaves of mutants were not generally increased (Figure 4D) and that the enhanced loss of transport sugars should therefore be a direct consequence of diminished P-protein contents. Our experiments therefore show that the lack of P-protein reduces the wound-sealing capacity of Arabidopsis plants. Furthermore, comparable exudation rates of the single-knockout and knockout/knockdown mutants confirm that both AtSEOA and AtSEOB are required to form functional P-protein structures in Arabidopsis sieve elements.
FIGURE 4 | Analysis of exudation rates in wild-type Arabidopsis plants, single AtSEO knockout mutants and AtSEO knockout/knockdown mutants. (A) Schematic overview of the exudation studies. (B) The sucrose content of exudates from wild-type plants (WT) and mutants impaired in the expression of either one (ΔAtSEOa and ΔAtSEOb) or both AtSEO genes (ΔAtSEOa/ΔAtSEOb; ΔAtSEOb/ΔAtSEOa, lines I–III, respectively) was calculated from the difference in D-glucose concentration before (light blue parts of bars) and after (total bars) the enzymatic conversion of sucrose. (C) Summary of the exudation assays. The mean value of sucrose exuded from wild-type plants was set as 1. The results represent means ± SD; nWT = 19, nΔAtSEOa = 22, nΔAtSEOb = 14, nΔAtSEOa/ΔAtSEOb line I = 15, nΔAtSEOa/ΔAtSEOb line II = 26, nΔAtSEOa/ΔAtSEOb line III = 27, *P ≤ 0.001, Mann–Whitney test. (D) Total leaf controls: total sugars were extracted from ground tissue and sucrose concentrations were determined as described above. The results represent means ± SD; nWT = 8, nΔAtSEOa = nΔAtSEOb = nΔAtSEOa/ΔAtSEOb I–III = 4.


DISCUSSION

NEW INSIGHTS INTO ASSEO INTERACTIONS

The analysis of ASSEO complementation lines clearly indicated that both ASSEOa and ASSEOB are required to form the typical P-protein structures in sieve elements, because filaments could only be detected when both proteins were present (Anstead et al., 2012). This would almost certainly require some form of heteromeric interaction between the subunits, although heterodimerization events have not been detected in previous yeast two-hybrid experiments. We therefore carried out interaction studies based on BiFC in a plant background. The infiltration of homogeneous combinations led to the formation of rather large complexes of ASSEOa protein, whereas homomeric interaction events for ASSEOB often resulted in cytoplasmic fluorescence but also yielded similar complexes. Our experiments therefore confirmed the homomeric interaction potential of both ASSEOa and ASSEOB (Anstead et al., 2012) and established that the interactions also occur in planta. The combinatorial expression of ASSEOa and ASSEOB fusions finally confirmed the predicted ability of the proteins to interact in a heteromeric manner. These combinations yielded complexes as well as evenly distributed cytoplasmic fluorescence. Because heteromers could be demonstrated in plants but not in the yeast two-hybrid system, it is possible that the interaction between ASSEOa and ASSEOB involves higher-order structures, perhaps requiring the initial assembly of homogeneous multimers, although this will need to be investigated in future experiments.

ASSEOa and ASSEOB are both required to form typical P-protein filaments in Arabidopsis, indicating that the proteins are not functionally redundant, although their functions may overlap (Anstead et al., 2012). Our investigation of heteromeric interactions revealed different reporter signals for each protein. ASSEOa formed complexes, whereas ASSEOB formed complexes but was also detected as uniform cytoplasmic fluorescence. Such distinct behavior may provide evidence that ASSEOa and ASSEOB have different roles in the formation of P-proteins, but the data should not be over-interpreted because the interaction was investigated in a non-phloem-cell background under the control of a non-native promoter. The structures observed during the interaction experiments were also clearly influenced by the position and/or size of the fused split mRFP fragments.

Analogous investigations focusing on tobacco SEO proteins also demonstrated a strong potential for interaction (Ernst et al., 2012; Jekat et al., 2012). Further comparative analysis of the SEO proteins from different species could provide insight into the detailed mode of SEO interaction and polymerization, and the relevant motifs that allow SEO proteins to form multimers. However, Arabidopsis is currently the only angiosperm known to possess just two expressed SEO genes, whereas, e.g., 19 SEO genes (including SEO-F genes) are expressed in Glycine max (Rüping et al., 2010), suggesting there may be species-dependent functional differences among these proteins.

ASSEO PROTEINS ARE INVOLVED IN SIEVE TUBE SEALING

The involvement of P-proteins in rapid sieve tube sealing in response to phloem wounding is a matter of debate. Phloem explants normally contain P-proteins in the wounding state with dispersed filaments accumulating at sieve plates and clearly occluding pores, which is why the structural components were proposed to be involved in sieve tube sealing (Cronshaw, 1981; Will and van Bel, 2006; Furch et al., 2010). The first direct evidence that P-proteins can indeed affect translocation was provided by the analysis of tobacco mutants depleted for SEO proteins, which lost on average nine times more photosynthate than wild-type plants upon injury (Ernst et al., 2012). However, microscopic analysis of ASSEO-YFP fusion proteins expressed in Arabidopsis sieve elements revealed slow movement of the resulting protein plugs through sieve plates, leading to the hypothesis that ASSEO proteins might not be able to affect transport through sieve tubes (Fivelich et al., 2011). We therefore developed an Arabidopsis exudation assay to measure the direct loss of photosynthate after wounding.

The generation of ASSEO knockout/knockdown mutants lacking P-protein filaments in the sieve elements (but otherwise showing no significant morphological differences to wild-type plants) allowed us to investigate the function of Arabidopsis P-proteins following injury. Our results clearly showed that Arabidopsis P-proteins are required for sieve tube sealing because mutants lacking P-protein structures lost significantly greater amounts of transport sugars after wounding than corresponding wild-type plants. Furthermore, our experiments provided a potential explanation for the inconsistent results from earlier studies: our BiFC experiments showed that the interaction properties of ASSEOa and ASSEOB are influenced by the fused reporter fragments, as also reported for the assembly properties of N/SEO proteins fused to YFP (Ernst et al., 2012). The recombinant ASSEO fusion proteins monitored in sieve elements passing the sieve pores were therefore likely to be constrained in their interaction properties, which might have led to a reduced sealing capacity.

Although the Arabidopsis exudation assay demonstrated the significant impact of ASSEO proteins on phloem translocation, the loss of photosynthate in P-protein-depleted Arabidopsis mutants was far less than previously reported in tobacco. This may reflect the different sieve tube conductivity of the two species and its impact on exudation (Thompson and Wologiak, 2008) but it is also possible that the impact of P-proteins in sieve tube sealing or their general sealing efficiency varies among different species. Sieve element and sieve plate anatomy (e.g., the number and size of pores) differ widely between species (Mulendore et al., 2010) and the response to injury may consequently involve differing mechanisms. Therefore, the detailed interplay between P-proteins, callose and potential additional sieve element components (such as sieve element plastids) in phloem wound sealing will certainly be the topic of interesting future studies.

ACKNOWLEDGMENTS

We thank Christiane Fischer, Claudia Hansen, Heike Hinte, and Raphael Sonner (Fraunhofer Institute for Molecular Biology and Applied Ecology) for technical assistance. We also thank Florentin Schmidt, Marion Rehners, and Stefan Mester for assisting in the plant exudation assays, and Till Mattat for help with transmission electron microscopy.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 March 2013; accepted: 10 June 2013; published online: 03 July 2013.

Clarenhov Jekat SR, Ernst AM, van Bel LA, Zielonka S, Torreyen RM, Noll GA and Pürrer D (2013) P-protein in Arabidop- sis is an homo-birefringent structure involved in rapid sieve tube sealing. Front. Plant Sci. 4:225. doi: 10.3389/fpls.2013.00225

This work was submitted to Frontiers in Plant Science, a specialty of Frontiers in Plant Science.

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