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A component of the Sec61 ER protein transporting pore is required for plant susceptibility to powdery mildew

Wen-Jing Zhang†, Susanne Hanisch†, Mark Kwaaitaal, Carsten Pedersen and Hans Thordal-Christensen*

Section for Plant and Environmental Sciences, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark

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INTRODUCTION

Many filamentous plant pathogenic fungi and oomycetes rely on placing a feeding structure, a so-called haustorium inside host cells in order to exploit host resources and to transfer effector proteins to the host cytosol. By unknown mechanisms, these pathogens trigger the host cells to generate an extrahaustorial membrane (EHM), which allows the host cells to stay alive despite the severe haustorial invasions (Cao et al., 2012). In between the haustorium and the EHM, a sealed compartment, called the extrahaustorial matrix (EHMs) is present. Many of these pathogens, such as powdery mildew fungi, have genetically lost certain general life-sustaining processes during their evolution (Spanu et al., 2010). This prevents them from living on dead biological material, making them strict biotrophs. In the meantime, they secrete hundreds of effectors from the haustorium, mediated by signal peptides (SPs) and default secretion. Many of these effectors are transferred to the host cytosol, where they play important roles in pathogenicity by assisting in nutrient acquisition, suppression of defense and membrane trafficking. However, the mechanisms behind effector delivery are largely unknown. This paper provides a model for and new insights into a putative transfer mechanism of effectors into the plant cell. We show that silencing of the barley Sec61β transcript results in decreased susceptibility to the powdery mildew fungus. HvSec61β is a component of both the endoplasmic reticulum (ER) translocon and retrotranslocon pores, the latter being part of the ER-associated protein degradation machinery. We provide support for a model suggesting that the retrotranslocon function of HvSec61β is required for successful powdery mildew fungal infection. HvSec61β-GFP and a luminal ER marker were co-localized to the ER, which was found to be in close proximity to the EHM around the haustorial body, but not the haustorial fingers. This differential EHM proximity suggests that the ER, including HvSec61β, may be actively recruited by the haustorium, potentially to provide efficient effector transfer to the cytosol. Effector transport across this EHM-ER interface may occur by a vesicle-mediated process, while the Sec61 retrotranslocon pore potentially provides an escape route for these proteins to reach the cytosol.

Birotrophic pathogens, like the powdery mildew fungi, require living plant cells for their growth and reproduction. During infection, a specialized structure called the haustorium is formed by the fungus. The haustorium is surrounded by a plant cell-derived extrahaustorial membrane (EHM). Over the EHM, the fungus obtains nutrients from and secretes effector proteins into the plant cell. In the plant cell these effectors interfere with cellular processes such as pathogen defense and membrane trafficking. However, the mechanisms behind effector delivery are largely unknown. This paper provides a model for and new insights into a putative transfer mechanism of effectors into the plant cell. We show that silencing of the barley Sec61β transcript results in decreased susceptibility to the powdery mildew fungus. HvSec61β is a component of both the endoplasmic reticulum (ER) translocon and retrotranslocon pores, the latter being part of the ER-associated protein degradation machinery. We provide support for a model suggesting that the retrotranslocon function of HvSec61β is required for successful powdery mildew fungal infection. HvSec61β-GFP and a luminal ER marker were co-localized to the ER, which was found to be in close proximity to the EHM around the haustorial body, but not the haustorial fingers. This differential EHM proximity suggests that the ER, including HvSec61β, may be actively recruited by the haustorium, potentially to provide efficient effector transfer to the cytosol. Effector transport across this EHM-ER interface may occur by a vesicle-mediated process, while the Sec61 retrotranslocon pore potentially provides an escape route for these proteins to reach the cytosol.

Keywords: powdery mildew, haustorium, extrahaustorial membrane (EHM), endoplasmic reticulum-associated degradation (ERAD), Sec61 complex, susceptibility factor

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The Sec61 pore can translocate proteins bidirectionally, and it is primarily known as the translocon pore, mediating the process of SP-dependent protein translocation into the ER. The Sec61 pore is a doughnut-shaped heterotrimeric complex, consisting of the subunits, Sec61α, Sec61β, and Sec61γ. SP and Sec61-dependent translocation into the ER can occur either co- or post-translationally (Zimmermann et al., 2011). The ERAD pathway has in several cases been shown to be recruited by opportunistic pathogens for transfer of polypeptides into the host cell cytosol. For example, cholera toxin, Shiga toxin, and Pseudomonas aeruginosa exotoxin enter the cytosol through retrotranslocon pores, but escape from ubiquitination and proteasome-mediated degradation (Rochgut et al., 2002; Blank, 2006). Retrotranslocation of cholera toxin occurs through the Sec61 retrotranslocon pore, and depletion of the Sec61 complex prevented the retrotranslocation of this toxin into the cytosol (Schmitz et al., 2000; Teter et al., 2002).

Here we aimed at studying the role of the Sec61 pore in plant susceptibility to the powdery mildew fungus. Barley (Hordeum vulgare) has two Sec61α, two Sec61β, and one Sec61γ protein (Deng et al., 2007; Mayer et al., 2012), and to unravel the role of the pore, we made use of the fact that the Sec61β component is essential for retrotranslocon activity for various substrates, but less important for translocon activity under non-stressed conditions (Finke et al., 1996; Van den Berg et al., 2004; Liao and Carpen-
RESULTS

HvSec61βa is a potential susceptibility factor for the barley powdery mildew fungus

In barley two Sec61 genes have been identified, which are named HvSec61βa and HvSec61βb. Interestingly, the HvSec61βa transcript accumulates in leaves after attack by Bgh (Dash et al., 2012). Therefore, we selected to analyze the role of HvSec61βa in the barley-Bgh interaction, and performed single cell TIGS of this gene. A 35S-promoter-driven RNAi construct, covering the full-length coding region of this gene, was generated and transiently transformed together with a GUS reporter gene construct into barley epidermal cells (Douchkov et al., 2005). After 2 days, the leaves were inoculated with Bgh and transformed cells were stained for GUS activity 3 days thereafter. Infection success of Bgh was evaluated microscopically by scoring the total number of GUS-stained cells and the number of GUS-stained cells containing one or more haustoria. Subsequently, the data were normalized to the empty vector control. The RNAi construct of HvSec61βa resulted in more than 40% reduction in susceptibility to Bgh (Figure 1A). As a positive control, the relative susceptibility of cells transformed with an Mlo RNAi construct was included (Douchkov et al., 2005). These cells were 70% less susceptible than the control cells. In order to confirm that the RNAi construct in fact results in silencing of HvSec61βa, we co-transformed barley epidermal cells with a 35S promoter-driven HvSec61βa-GFP fusion construct. Five days after transformation, together with a reference construct for cytosolic mCherry expression, confocal imaging revealed that the RNAi construct prevented appearance of GFP signal, while it did not affect the signal from mCherry in the same cell (Figure 1B). The reduced HvSec61βa-GFP signal indicated that the HvSec61βa RNAi silencing construct indeed induced degradation of HvSec61βa encoding mRNA and likely as well impaired endogenous HvSec61βa transcript and protein accumulation. Thus, the observed increased resistance of HvSec61βa-silenced cells indicates a potential role of HvSec61βa as a susceptibility factor for efficient Bgh infection.

In order to analyze whether the reduced susceptibility could be due to reduced viability of the cells in which HvSec61βa was silenced, a second experiment was performed. Co-transformation was performed with an anthocyanin biosynthesis gene activation construct, pBC17, causing the transformed cells to accumulate the red anthocyanin pigment as long as they stay alive (Schweizer et al., 2000). Two days after transformation, the leaves were inoculated with a high density of Bgh conidia (~200 per mm²). Similar numbers of anthocyanin accumulating cells were detected in HvSec61βa-silenced and non-silenced cells after Bgh infection (Figure 1C). Therefore, this result confirmed that the HvSec61βa RNAi construct did not affect the viability of the barley cells after inoculation.

HvSec61βa localization in uninfected and infected barley cells

Next we aimed to subcellularly localize HvSec61βa to search for clues for the powdery mildew-related function of this protein. Sec61β is a small ~8 kDa protein with a single transmembrane domain, and GFP-tagging has previously been used for its localization (Rolls et al., 1999; Voeltz et al., 2006). Therefore, we co-expressed our 35S promoter-driven HvSec61βa-GFP fusion construct together with a 35S promoter-driven SP-mCherry-HDEL construct (Nelson et al., 2007) in infected and uninfected barley epidermal cells. The SP targets mCherry to the ER and the ER retrieval motif HDEL (His-Asp-Glu-Leu) at the C-terminus retains it in the lumen of the ER (Gimeno et al., 1997). Confocal images of epidermal single cells expressing HvSec61βa-GFP and SP-mCherry-HDEL were recorded 48 h after particle bombardment (Figure 2). Intense GFP signal was observed in the ER cortical network throughout the cells expressing GFP-tagged HvSec61βa-GFP but not the fluorescence signal from cytosolic mCherry 5 days after transformation. Micrographs show maximum intensity projections. (C) Number of pBC17-transformed cells accumulating anthocyanin, reflecting cell viability, after co-bombardment with either an empty vector control or the HvSec61βa-RNAi construct on similarly sized pieces of leaf. Two days after bombardment, leaves were inoculated with Bgh, and the number of anthocyanin-accumulating cells was scored 3 days later (n = 4).
FIGURE 2 | HvSec61βa co-localizes with an ER luminal marker (A). Maximum intensity projection of a z-series of confocal images of a barley epidermal cell expressing HvSec61βa-GFP reveals the ER localization of HvSec61βa-GFP with the typical distribution within the reticular ER network. (B) In the same epidermal cell, the 35S promoter-driven SP-mCherry-HDEL construct is expressed and labels the ER. (C) The merged image shows that the HvSec61βa-GFP and SP-mCherry-HDEL signals largely overlap. Scale bar, 20 μm.

HvSec61βa (Figure 2A). In addition, the HvSec61βa-GFP signal largely colocalized with mCherry signal from the luminal ER marker (Figures 2B,C). The colocalisation is near perfect in the tubular parts of the ER, while the cisternal parts have relatively more mCherry signal. This likely reflects that HvSec61βa-GFP is membrane bound, and that the soluble mCherry luminal marker dominates the more voluminous cisternal ER. In conclusion, our observations indicate that HvSec61βa is localized to all parts of the ER.

Since we confirmed the ER localization of HvSec61βa-GFP in barley and have observed increased resistance after silencing this gene, we were interested in knowing how the ER changes its location after pathogen attack. It is often described that infected host cells re-localize organelles and specific proteins, which results in their accumulation at the pathogen attack site (Takenoto et al., 2003; Koh et al., 2005; Caillaud et al., 2012). We used the 35S promoter-driven SP-mCherry-HDEL construct to study the localization of the ER after attack by Bgh. Confocal imaging of an infected barley cell revealed that the mCherry ER-luminal marker was located around the body of the Bgh haustorium. Meanwhile, this ER marker was most often not present around the haustorial fingers (Figures 3A,B). In a 3D projection (Figure 3C) of the mCherry fluorescent signal, this distinction between the haustorial body and fingers is clearly visible. These observations revealed that the ER network is in close proximity to the EHM around the haustorial body.

Similar to the mCherry ER-luminal marker (Figure 3), the HvSec61βa-GFP signal was present in the ER network around the Bgh haustorial body as well (Figure 4). Contiguous accumulation of HvSec61βa-GFP was detected around the nucleus, which was
observed close to the haustorium, supporting the re-localization of this organelle upon pathogen attack (Figures 4A–E), as previously described (Schmidz, 2002). As for the ER-luminal marker, HvSec61βa-GFP confirmed that the ER and EHM are in close proximity around the haustorial body. In summary, these confocal microscopy results suggest that the HvSec61βa-GFP-labeled ER is differentially recruited to the proximity of the EHM around the haustorial body, but not around the fingers.

**DISCUSSION**

The fact that silencing of HvSec61βa causes the barley cells to become resistant to powdery mildew suggests that HvSec61βa either is a negative regulator of defense or a susceptibility factor required for disease. Sec61β is, as described above, associated with protein-transmitting pores in the ER. While it has been barely studied in plants, yeast data suggest that one of its activities is to be part of a post-translational translocon complex, but that this role is not essential under non-stressed conditions (Finke et al., 1996). Furthermore, Sec61β has also been associated with protein retrotranslocation from the ER (Kawaguchi and Ng, 2007; Nakatsuku and Brodsky, 2008; Willer et al., 2008), and the question is, which of these activities is important in barley cells attacked by Bgh.

Silencing of HvSec61βa would result in inhibition of secretion if this protein is generally required for co- or post-translational translocation.}

![Figure 4](https://www.frontiersin.org/article/10.3389/fpls.2013.00127/gi001)

**FIGURE 4** | HvSec61βa-GFP localizes around the Bgh haustorial body. Confocal image of an epidermal cell, transformed with the HvSec61βa-GFP construct, taken 48 h after Bgh inoculation. (A–C) Three different focal planes from an image series of an infected cell with a haustorium. HvSec61βa-GFP localizes to the ER around the nucleus (arrow head, A) and surrounding the haustorium in an ER-like tubular pattern (asterisk, A). (C–E) GFP fluorescence (C), bright field (BF) (D), and merged image (E) show HvSec61βa-GFP localization at the surface of the haustorial body. HvSec61βa-GFP labels the tubular ER network, which is further illustrated in the 3D projection (F) (Image Surfer 1.2). Scale bar, 10 μm.
protein translocation into the ER. This can hardly explain our phenotype, as inhibition of secretion in barley results in increased susceptibility to Bgh (Ostertag et al., 2013). A more likely explanation might be found in a specific HvSec61β-fuction in post-translational translocation. This could involve the so-called "unfolded protein response" (UPR), which results from ER stress due to accumulation of unfolded proteins. During UPR, ER chaperones and components of the ERAD system are up-regulated to prevent the cell from undergoing programmed cell death (Travers et al., 2000). Similarly, ER stress induced by, e.g., tunicamycin (an N-glycosylation inhibitor) increases transcript levels of genes encoding proteins of the ER-QC machinery and the secretory pathway (Martines and Christen, 2001; Hutten and Strasser, 2012). A more likely explanation might be found in a specific HvSec61β-function in post-translational translocation. This could involve the so-called "unfolded protein response" (UPR), which results from ER stress due to accumulation of unfolded proteins. During UPR, ER chaperones and components of the ERAD system are up-regulated to prevent the cell from undergoing programmed cell death (Travers et al., 2000). Similarly, ER stress induced by, e.g., tunicamycin (an N-glycosylation inhibitor) increases transcript levels of genes encoding proteins of the ER-QC machinery and the secretory pathway (Martines and Christen, 2001; Hutten and Strasser, 2012). An important chaperone that counter acts UPR is the ER-luminal protein, BiP, which is taken up post-translationally through the translocon complex in a Sec61β-dependent manner (Finke et al., 1996). Therefore, a model could be that HvSec61β silencing causes ER-deprivation of BiP, in turn resulting in UPR as well as increased resistance. An Arabidopsis BiP knock-out line has previously been suggested to be prone for UPR. However, in disagreement with the model, the BiP knock-out line had reduced resistance (Wang et al., 2005). This may indirectly suggest that reduced BiP import into the ER is not the cause of the Sec61β phenotype we observe, while Bgh resistance increases in this situation. We therefore favor a function for Sec61β in protein retrotranslocation in the interaction with the powdery mildew fungus.

In the meantime, we had an indication of active recruitment of ER by the fungus, supporting that HvSec61β functions as a susceptibility component. We observed a close association of the ER, labeled by HvSec61β-GFP, and the Bgh haustorial body. The ER has also in other cases been found to be closely associated with haustoria (Koh et al., 2005; Micali et al., 2011). However, only Blumeria haustoria differentiate in two parts and provide a chance to distinguish variations in ER association. Interestingly, there is little ER association with the haustorial fingers, which could suggest that the ER proximity to the haustorial body is not due to ER being present wherever there is cytosol. Therefore, it is possible that the fungus controls the ER-haustorium association. Vogele et al. (2009) proposed that effector proteins are transferred to the cytosol via the ER. Effectors need to cross a membrane in order to reach the host cytosol, and the ER retrotranslocon pore offers an escape route for this. The resistance phenotype seen after HvSec61β silencing is in agreement with a model, where this protein is necessary for pore function. As illustrated in Figure 5, we suggest that vesicle trafficking transfers the effectors from the EHM to the ER in order for them subsequently to employ the retrotranslocon to enter the cytosol. While we consider the model in Figure 5 to describe the most likely mode of action of Sec61β in plant powdery mildew interactions, other scenarios are possible. An unexpected function has for instance been described for Drosophila Sec61β, which is

![FIGURE 5 Schematic model for a possible Sec61-dependent route of effector release into the host cytosol. Effectors are hypothesized to be transferred from the extrahaustorial matrix to the cytosol through Sec61 retrotranslocon pores in the ER. Trafficking from the matrix to the ER is envisaged to take place in vesicles dependent or independent of Golgi. Adapted from Vogele et al. (2009).](image-url)
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