Understanding cargo sorting and interactive effects of membrane vesicles in fungal phytopathogens: Current knowledge and research gap

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Abstract

Organisms from all kingdoms of life release membrane vesicles, which are tiny, spherical structures made of a lipid bilayer. These vesicles carry out several functions, such as forming new cell membranes, removing waste products from the cell, and transporting lipids and other substances. The payloads often contained in the vesicles are sorted via the endosomal sorting complex required for transport (ESCRT) pathway stepwise. Furthermore, alterations to this endomembrane system reduces formation of vesicles and produce aberrant endosomal compartments. In pathogenic fungi, studies that have generated mutants with disruption in the ESCRT genes demonstrated negative effects on virulence and growth. Despite these important roles, only a few fungal species have to date been evaluated for the ESCRT pathway. In this review, we comprehensively evaluate recent developments in the ESCRT fungal pathway and its role in plant fungal pathogenesis.

Keywords: Plant pathogens; Fungi; Endocytosis; ESCRT pathway; Extracellular vesicles; Ubiquitination

1. Introduction

Extracellular vesicles (EVs) mediate cell-cell-dependent environmental responses (Anand et al., 2019; Baldrich et al., 2019; Rodrigues et al., 2015). Numerous studies substantiate that isolated fungal pathogen EVs produce phytotoxic effects, among other things, when inoculated on plant tissues such as leaves (Bleackley et al., 2020), suggesting that cargo within these vesicles can be disease-promoting. How this cargo is sorted is currently elusive in plant pathogenic fungi, which limits our understanding of virulence factors transmitted through non-canonical pathways. A repertoire of EV constituents, including nucleic acids, proteins, and secondary metabolites often reflect the pathophysiological state of the cell from whereabout the EVs are secreted. These cargos can be taken up by other cells naked or enclosed in EVs to yield the different EV-mediated physiological states in the acceptor cells (Yáñez-Mó et al., 2015). As EVs carry various bioactive molecules, many of which facilitate cell-cell communication (Anand et al., 2019; Rodrigues et al., 2015), they have also been implicated in plant-fungal interaction and pathogenesis. For instance, EVs of several species of fungi have been shown to traffic virulence factors, such as cell wall degrading enzymes, protein effectors, and toxins with phytotoxic effects on their plant host tissues (Bleackley et al., 2020; Costa et

al., 2021; Regente et al., 2017). However, there is still a lack of understanding about the regulatory mechanisms involved in cargo sorting, packaging and trafficking of the respective vesicles, especially in filamentous fungi.

The membrane trafficking pathway, the ESCRT (endosomal sorting complex required for transport) pathway is utilized by many eukaryotes including fungi. This system is involved in the formation and sorting of endosomal vesicles. In filamentous fungi, the ESCRT pathway plays a role in the biogenesis of filamentous growth and pathogenesis (Sun et al., 2022). Studies have shown that the ESCRT pathway is required for the conventional organization of the fungal cytoskeleton (An et al., 2006; Henne et al., 2013), which is essential for the formation of hyphae. The ESCRT pathway has also been highlighted in the splitting of daughter nuclei during cell division and in the sorting of proteins destined for the plasma membrane (Henne et al., 2013). Additionally, the ESCRT pathway is involved in the formation of endosomal vesicles that are important for nutrient uptake and stress response (Fan et al., 2015; Wang et al., 2020). In pathogenic fungi, the ESCRT pathway also partakes in the secretion of virulence factors and evasion of host immunity (Regente et al., 2017; Martínez-López et al., 2022).

The ESCRT pathway is vital for endocytosis whereby it allows for the packaging of extracellular materials and membrane proteins into EVs for trafficking into the cytoplasm (Fig. 1(A), Ahmed et al., 2019). Endocytosis is characterized by the presence of late endosomes or multivesicular bodies (MVBs), therein harbouring intraluminal vesicles (ILVs) that usually arise from the invagination and budding of the endosomal membrane (Ahmed et al., 2019; Haag et al., 2015). The process of endocytosis is fundamental to various cellular processes ranging from signal transduction to morphogenesis (Schimid et al., 2014). In certain fungi, endocytosis has been demonstrated to contribute during interaction with plants including in the apical growth of hyphae (Bielska et al., 2014; Toshima et al., 2006). In addition to growth and development, ESRCT-based regulation of cellular function was further determined to be crucial for adaptation and response to both external and internal stimuli such as biotic and abiotic stress factors (Mosesso et al., 2019; Rosa et al., 2020). Consequently, many of the basic components of the ESCRT pathway are conserved across eukaryotes, albeit with notable lineage-specific adaptations in some taxa (Leung et al., 2008).

Most of the significant contributions to our understanding of ESCRT derive strongly from studies on model organisms (Herz et al., 2006; Spitzer et al., 2006; Thompson et al., 2005; Vaccari and Bilder, 2005). Adding to these, several studies highlighted the key roles of ESCRT proteins in mammals (Pornillos et al., 2002; Skibinski et al., 2005; Parkinson et al., 2006; Saksena and Emr, 2009; Stuffers et al., 2009; Hurley et al., 2015). The ESRCT pathway was originally discovered in Archaeal cytokinesis (Lindas et al., 2008; Samson et al., 2008) and in fungi, it was first discovered in the model fungus *Saccharomyces cerevisiae* (Katzmann et al., 2001). In this yeast, the ESCRT pathway's constituents were named based on their functions in sorting ubiquitinated membrane proteins into lysosome/vacuole lumens for degradation (Babst et al., 2002a; Katzmann et al., 2001; Xie et al., 2019b). According to several studies, protein complexes such as sec6, play a key role in transporting post-Golgi vesicles to the cell membrane (Hsu et al., 1999; Panepinto et al., 2009). For example, the exocyst complex, which was originally identified in the budding yeast, is a multiprotein complex required for exocytosis (Novick et al., 1980; TerBush et al., 1996; TerBush and Novick, 1995). This complex houses sec6 alongside other sec-related proteins, such as Sec6, Sec8, and Sec15, and several Exo proteins. Of note, the significance of sec6 extends to pathogenesis-related to *Cryptococcus neoformans*, where its suppression reduces the release of virulence factors (e.g., laccase and urease) carried by EVs (Hsu et al., 1999; Panepinto et al., 2009). Therefore, these protein

complexes are not only conserved across different organisms (like the budding yeast and *C. neoformans*) but they influence functions extending beyond vesicle transport and secretion. The ESCRT pathway represents a complex endomembrane system that consists of five complexes, namely ESCRT-0, –I, –II –III and Vps4 (vacuolar protein sorting 4). Together with various accessory proteins, the individual elements of the pathway act in concert to form MVBs during diverse processes including cytokinesis, membrane repair and autophagy (Henne et al., 2013; Hurley et al., 2015; Pla-Martín and Reichert, 2024; Roxrud et al., 2010).

As in other eukaryotes, fungal MVBs are critical for transporting ubiquitinated membrane proteins to the vacuole with the aid of the ESCRT machine, which both recognizes and packages these ubiquitin-modified proteins onto the ILVs contained inside MVBs (An et al., 2006; Henne et al., 2011; Hurley and Hanson, 2010).Studies in yeast also demonstrated that ILVs form when MVB membranes evaginate and undergo fission (Ahmed et al., 2019; Anand et al., 2019), and that impairment in the ESCRT apparatus can lead to reduced formation of ILVs and aberrant endosomal compartments (Raymond et al., 1992; Xie et al., 2019a). In the filamentous ascomycete, *Fusarium graminearum*, such defects can significantly impact cellular processes like deoxynivalenol production, growth and pathogenicity, as well as sexual and asexual reproduction (Xie et al., 2019a). However, most fungal work pertaining to the ESCRT pathway is mostly focused on human pathogenic yeasts including *Cryptococcus neoformans* and *Candida albicans* (Godinho et al., 2014; Hu et al., 2015; Park et al., 2020; Zhang et al., 2016). Details regarding this pathway have been considered in only a few phytopathogens, most notably *Magnaporthe oryzae* and *F. graminearum* (Oh et al., 2012; Xie et al., 2016, 2019a, 2019b; Cheng et al., 2018; Que et al., 2020). However, the elucidation of the core functions of the ESCRT pathway in fungal plant pathogens represents a critical aspect given the functional importance of this pathway. Therefore, this review reflects on the recent ESCRT discoveries as they relate to filamentous fungi and elucidate the significance of released EVs, to what end are the end-products of ESCRT, in fungal biology and fungal-host interactions.

2. Vacuolar protein sorting genes (Vps) facilitate the assembly of vacuoles

Fungal vacuoles are complex cellular organelles involved in several functions including homeostatic regulation and degradative processes among others (Klionsky et al., 1990). During EV biogenesis, fusion of MVBs occurs with vacuoles and autophagosomes and the MVB-endocytosed cargo is delivered and then undergo degradation as means of cellular homeostasis (Fig. 1(D)–Luzio et al., 2003; Luzio et al., 2007, Pla-Martín and Reichert, 2024). Induced autophagy has been shown to result in reduced EV release due to increased fusion of the MVBs to the autophagosomes (Fader et al., 2008). Though little is known about directive mechanisms involved in trafficking of MVBs to either the plasma membrane or the vacuole, these organelles serve an important role in maintaining cytosolic balance.

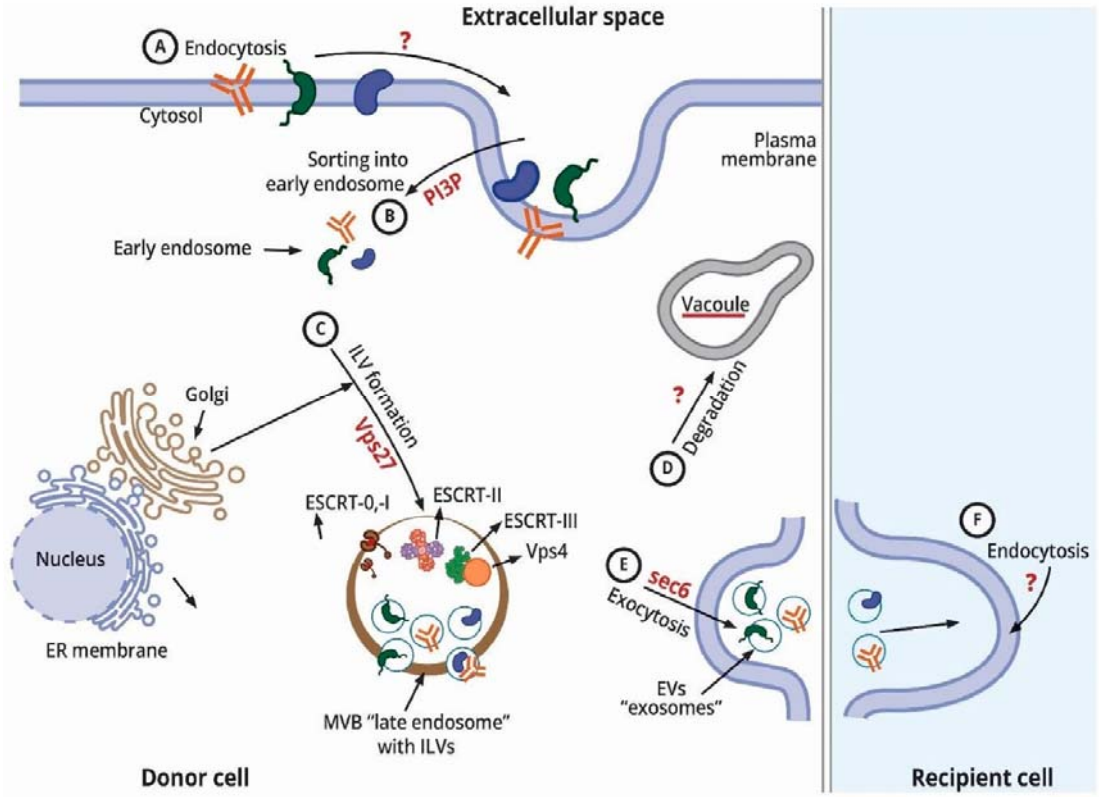

Fig. 1. Schematic representation of the biogenesis, cargo, and secretion of 'exosomes' as well as sentinel genes involved in regulating each process, according to Gurung et al. (2021), with slight modifications. (**A**) extracellular vesicles (EVs) are formed by the invagination of the endocytic membrane and formation of intraluminal vesicles (ILVs) inside the multivesicular bodies (MVBs). No specific gene has been determined as multiple regulatory genes may be involved in this process. (**B, C**) During maturation, the cargoes (nucleic acids, proteins, and lipids) under-go ubiquitination as they are incorporated into ILVs in an ESCRT-dependent fashion, and the maturation of early endosomes gives rise to MVBs. PI3P gene is essential for MVB formation and endocytic trafficking while Vps27 ensures sorting of ubiquitinated proteins and the formation of ILVs within MVBs. (**D**) MVBs may be delivered to the vacuole/lysosome for degradation, or (**E**) fuse with the plasma membrane where the ILVs then release EVs into the extracellular space by donor cells. Regulatory genes involved in determining lysosome/vacuole degradation of late MVBs specifically are not known, while exocytosis of the MVBs to the extracellular space involves sec6 which is associated with vesicle trafficking and pathogenesis (Panepinto et al., 2009) (**F**) The EV cargoes from the donor cell are delivered to recipient cells via endocytosis or direct membrane fusion etc.

Vacuolar proteins are recognized by certain cellular components and are sorted, packaged into transport vesicles, and delivered to the vacuole through a prevacuolar compartment (Hedman et al., 2007). The process involves the assembly of the Vps genes (e.g., Vps 23 or Vps 27) into the five sub-complexes of the pathway, in that respect a stepwise '*modus operandi*,' interact, recognize and sort ubiquitinated cargo (Fig. 2, Mosesso et al., 2019). These proteins are encoded by class E vacuolar protein sorting genes that are structurally classified as type A-F. Among these, the first Vps genes were classified into type A, B and C, with class D, E, and F added later (Hedman et al., 2007). These Vps genes are associated with the formation of the vacuole. For instance, mutants of the class A genes exhibit defects in acidification of the vacuole, class B mutants exhibit small-vacuole like organelles, and thus together with class F

mutants are characterized by large to fragmented small vacuole-like structures (Banta et al., 1988; Hedman et al., 2007).

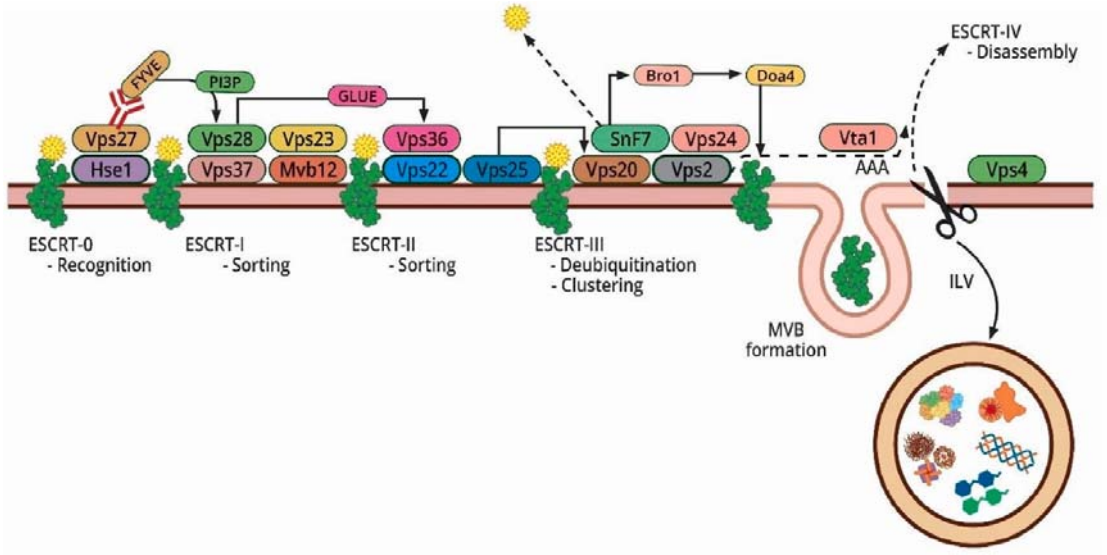

Fig. 2. The ESCRT dependent pathway in *Saccharomyces cerevisiae and* its components. The figure was drawn according Leung et al. (2008) with slight modifications. (**A**) Ubiquitin-modified protein (Ub-protein) triggers the ESCRT-dependent pathway and initiate ESCRT-0 (Hse1 and Vps27) which interact with components of (**B**) ESCRT-I (Vps28, Vps37, Vps23 and Mvb12), (**C**) the ESCRT-I recruits the components of ESCRT-II (Vps22, Vps36 and Vps25). The three subcomplexes work together in transporting Ub-protein and instigate the invagination of the multivesicular body (MVB). (**D**) ESCRT-III components (Vps20, SnF7, Vps2, Vps24) and associated accessory protein (Bro1) recruits deubiquitinating enzyme Doa4 (Degradation of alpha-4) to remove the ubiquitin from protein. (**E**) Vps4 is finally recruited post-deubiquitination and sorting and packaging of protein into the formed MVBs.

Class C Vps is associated with normal assembly of the vacuole as mutants display severely aberrant vacuole formation and sensitivity to osmotic stress in *S. cerevisiae* (Banta et al., 1988). Although their absence has minimal effects in vacuolar biogenesis, and trafficking of endocytosed proteins, class D gene products are limited to the vesicular pathway (Bryant et al., 1998). Class E Vps are important in forming unique class E compartments that house the vacuolar hydrolase carboxypeptidase S (CPS), a transmembrane protein required in the vacuole (Raymond et al., 1992; Shaw et al., 2001; Coonrod and Stevens, 2010). While CPS accumulates in class E compartments, CPS that fails to reach the vacuole lumen remains in the limited membranes of the vacuole (Reggiori et al., 2001; Piper and Katzmann, 2007). In yeast, about 15 components of the class E Vps family, most of which associated with MVB sorting and augmented endosomal compartments, were reported (Katzmann et al., 2001).

In yeasts and mammals, studies have indicated that Vps proteins interact sequentially, resulting in the development of various cellular events such as scission, transport, and the formation of distinct budding membranes (Henne et al., 2011; Hurley and Hanson, 2010; Schuh and Audhya, 2014; Tang et al., 2016). Hence, ESCRT-I and ESCRT-II have been identified as crucial elements in trafficking ubiquitinated cargo to the vacuole. ESCRT- 0 on the other hand, is not as essential in either mammals or yeast for the aforementioned functions, which is in accordance with the dynamic endosomal pathway in plants where ESCRT- 0 is also absent (Henne et al., 2011; Hurley, 2015).

3. Components and assembly of the ESCRT-dependent pathway in fungi

The ESCRT-0 heterodimer is comprised of two subunits, Vps27 and Hse1 (Fig. 2), found in *S. cerevisiae* (Williams and Urbé, 2007). They are orthologous to the human protein Hrs (hepatocyte growth factor regulated tyrosine kinase substrate) and STAM (signal transducing adaptor molecule), respectively (Henne et al., 2011; Mosesso et al., 2019; Raiborg and Stenmark, 2009; Xie et al., 2019a). The ubiquitin-interacting motifs (UIMs) of both subunits comprise an N-terminal VHS (named after the proteins Vps27, Hrs and STAM) domain (Ren and Hurley, 2010). Although both subunits display some structural resemblance, the Vps27/Hrs structure is slightly different in that it encompasses a FYVE (named after the proteins Fab1, YOTB, Vac1 and EEA1) zinc finger domain (Henne et al., 2011; Katzmann et al., 2003). The cysteine-rich FYVE binds to an endosomal lipid, phosphatidylinositol (Ptdlns) 3-phosphate (PI3P), that is crucial for MVB formation and endocytic trafficking and required for the Vps27/Hse1 complex functions and localization (Gillooly et al., 2001; Katzmann et al., 2003; Raiborg et al., 2003; Xie et al., 2016). In *Drosophila melanogaster*, inhibition of PI3P alters the recruitment of Hrs and consequently obstruct MVB formation (Lloyd et al., 2002; Raiborg et al., 2003). Moreover, defects in the Vps27 UIM do not impact vesicle budding and delivery onto the MVB lumen, but hinder sorting of ubiquitinated cargo protein into MVBs (Conibear, 2010; Katzmann et al., 2001, 2002; Piper and Katzmann, 2007).

The ESCRT-I complex is comprised of four subunits, namely Mvb12, Vps23, Vps37 and Vps28 (Fig. 2). However, only Vps23 and Vps28 are present in *F. graminearum* and interact with each other (Xie et al., 2019a). Vps23 and Vps28 also interact with both ESCRT-0 and ESCRT-II at opposite ends of the complexes (Chu et al., 2006; Curtiss et al., 2007; Henne et al., 2011; Katzmann et al., 2001). In mammals, there are multiple isoforms of these ESCRT-I subunits. These may reflect their variations based on tissue-specificity (Henne et al., 2011). The N-terminal ubiquitin E2 variant (UEV) domain of the Vps23 subunit propels to the ESCRT-I stalk, while the UEV binds to the PTAP-like motifs (Pro-Thr/Ser-Ala-Pro) of Vps27, an upstream MVB sorting component, then recruits the ESCRT complex protein to the endosomal membrane (Katzmann et al., 2003; Xie et al., 2019a).

The ESCRT-II protein sub-complex is comprised of three subunits, namely Vps22, Vps36, and Vps25 (Babst et al., 2002b). Through the interaction between Vps25 and Vps20, ESCRT-II recruits downstream ESCRT-III, thus, in turn activating this protein sub-complex (Xie et al., 2019a). The interaction of ESCRT-II with ESCRT-I involves provision of the endosomal localization, the GLUE (GRAM-like ubiquitin-binding in Eap45) domain of Vps36 and Vps28 subunits that bind to ubiquitin PI3P, while Vps25 binds Vps20 and attach to ESCRT-III, thus exhibiting the crucial role of ESCRT-II in activating the formation of ESCRT-III complex (Hanson et al., 2009; Henne et al., 2011; Teo et al., 2006).

The ESCRT-III sub-complex is comprised of four major subunits, Vps20, Snf7/Vps32, Vps24, and Vps2 (Fig. 2). They occur as monomers in the cytosol and cluster rapidly on the endosomal membrane into an active complex (Babst et al., 2002a; Xie et al., 2019b). SnF7 homo-oligomerise post interaction with Vps20 and it is also the most abundant component of ESCRT-III and can self-interact as reported in previous studies (Lin et al., 2005; Teis et al., 2008; Xie et al., 2019b). The major subunits may contain a few accessories or adaptor proteins such as Did2, Bro1, and Vps60, Ahmed et al., 2019). ESCRT-III protein sub-complex is unique from the first three ESCRT complexes because it does not form part of the stable cytoplasmic complex but rather exist in a closed autoinhibited condition in the cytoplasm (Henne et al., 2011; Xie et al., 2019b).

According to Teo et al. (2004), ESCRT–III–dependent membrane modelling is triggered by the interaction between Vps25 and Vps20 from which ESCRT-III forms a complex, as it is recruited to the endosome. Prior to the activation of ESCRT-III, ESCRT-I, and ESCRT-II complexes link through the interactions between Vps23 and Vps22, Vps23 and Vps36, and finally Vps28 and Vps36, wherein the subunits Vps25 and Vps36 of ESCRT-II complex directly interact with ESRCT-III protein complex (Xie et al., 2019b). Despite the differences in the components of some ESCRT complexes, the sequential recruitment and clustering of the ESCRT complexes from interactions among ESCRT protein subunits in *F. graminearum* has exhibited some consistencies comparable to the yeast and mammalian models (Xie et al., 2019b). Therefore, the main interactions of this pathway are well-conserved in eukaryotes (Bowers et al., 2004; Martin-serrano et al., 2003; Teis et al., 2008; Von Schwedler et al., 2003; Xie et al., 2019b), in essence suggesting that Vps23, Vps28, Vps22, Vps25, and Vps36 serve as "connectors" that activate ESCRT–III–mediated membrane remodelling.

Lastly, ESCRT-IV disassembles the ESCRT-III complex when ESCRT-II interacts with Vps20, whereabout SnF7 is recruited (Tang et al., 2016). SnF7 has been demonstrated to be important in processes that lead to fungal pathogenesis including cell wall integrity, endocytosis, vesicle trafficking, as well as growth and differentiation (Cheng et al., 2018). SnF7 then recruits Vps24 and Vps2, thereby completing the assembly of ESCRT-III, shortly after Vps2 engages Vps4 (Obita et al., 2007; Teis et al., 2008; Henne et al., 2011). For stabilization, SnF7 may also recruit adaptor proteins from ESCRT-III i.e., Bro1 and Doa4 DUBs (Luhtala and Odorizzi, 2004; Odorizzi et al., 2003).

For ESCRT-III to disassemble from the membrane, energy is required and that is usually provided by class I AAA (ATPase associated with various cellular activities) ATPase Vps4 (Fig. 1) (Babst et al., 1998). As with SnF7, other ESCRT-III accessory proteins also regulate the interaction between ESCRT-III and Vps4 (i.e., Did2, Vta1 or Vps60) to modulate the functions of Vps4 in several ways, such as facilitating the self-interaction of Vps4, Vps4 interaction with ESCRT-III subunits, or the stimulation of ATP hydrolysis (Ahmed et al., 2019). Furthermore, Vta1 directly interacts with Vps60 of the ESCRT-III complex. A super complex that is stabilized by a flexible Vta1 cap is formed when Vta1 stimulates the ATPase activity of Vps4 (Ahmed et al., 2019).

4. Posttranslational modification of protein cargo – ubiquitination

Ubiquitin is a highly conserved regulatory protein, consisting of 76 amino acid residues (Ahmed et al., 2019; Wang et al., 2012, 2019). Ubiquitination is a process where a ubiquitin is covalently attached to a targeted lysine residue on the cytoplasmic tail of a transmembrane protein (Ahmed et al., 2019; Wang et al., 2012). Monoubiquitination serves as a signal for the lysosomal targeting process, where monoubiquitinated proteins are trafficked to the lysosome (Anand et al., 2019; Raiborg et al., 2003). By contrast, polyubiquitinated proteins are intended for proteolysis (Hicke and Dunn, 2003). Ubiquitination thus appears to serve centrally both in endocytosis and protein turnover (Leung et al., 2008; Mosesso et al., 2019). The process of ubiquitination (and de-ubiquitination, see below) takes place along the endosomal membrane. Ubiquitination involves the activities of three enzymes, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), which are used for the development of iso-peptide bonds between the C-terminal glycine of ubiquitin and the amine of the lysine (K) (e.g., −63 etc.) residue of the target protein (Bhoj and Chen, 2009; Liu and Xue, 2011; Schwihla and Korbei, 2020).

The E3 ligase mostly regulates the specificity of the targeted protein (Oh et al., 2012). So far, only seven lysine residues within the ubiquitin protein are used in the ubiquitination reaction to create ubiquitination chains (i.e., K6, -11, −27, −29, −33, −48, and −63) and may lead to mono-ubiquitinated or poly-ubiquitinated substrates (Schwihla and Korbei, 2020; Wang et al., 2012). The most common polyubiquitin chains are, however, K48- and K63-linked polyubiquitin chains, with K63 reportedly associated with endocytic transportation (Paez Valencia et al., 2016; Rodrigo-Brenni et al., 2010). In yeast, monoubiquitination alone is sufficient to stimulate endocytic internalization of ubiquitinated cargo into MVBs (Kim et al., 2007; Lucero et al., 2000). However, the existence of K63-linked chains also proved to be particularly important during the MVB sorting of CPS (Erpapazoglou et al., 2008; Lauwers et al., 2010; Lucero et al., 2000). Similarly, this phenomenon has been reported in *Arabidopsis* where K63 is the second most abundant polyubiquitin chain after K48 (Dubeaux et al., 2018; Kasai et al., 2011; Leitner et al., 2012; Lu et al., 2011; Martins et al., 2015). Furthermore, defects in the polyubiquitin K63 link chains from *M. oryzae* are alluded to cause substantial alteration on fungal growth and development as well as morphological changes (Oh et al., 2012).

5. Sorting of ubiquitinated-protein cargo into ILVs

Ubiquitination plays major roles in trafficking plasma membrane proteins into the endosomal system (Schwihla and Korbei, 2020). Endosomal ubiquitin modification is critical for ESCRT recognition and sorting of ubiquitin-modified protein cargo into ILVs (Villarroya-Beltri et al., 2014; Williams and Urbé, 2007). This endosomal modification facilitates ubiquitinated proteins to lysosomes through MVBs (Williams and Urbé, 2007). Ubiquitination also serves as essential signal for proteins lacking signal peptides recruited to the ESCRT-dependent pathway during MVB sorting (Agrawal et al., 2010; Piper and Luzio, 2001) Early endosomes, in this respect originate from the *trans*-Golgi network (TGN) as part of the endocytic membrane transport pathway, are major sorting stations that accept molecules from the extracellular environment. On the other hand, MVBs are prelysosomal organelles acting during endocytosis to regulate incoming and outgoing traffic through homotypic and heterotypic fusion events (Huotari and Helenius, 2011; Luzio et al., 2003, 2007). Although, homotypic fusion of MVBs occurs with lysosomes and autophagosomes, heterotypic fusion is different in that it can occur with the plasma membrane. Nonetheless, in both scenarios, fusion results in the delivery of endocytosed cargo. In the case where MVBs fuse with the plasma membrane, ILVs are released into the extracellular space as exosomes, and this has been implicated in cell-cell and *trans*-kingdom communication including plant-fungal interaction (Cai et al., 2018).

The sorting of MVB is important for a series of biological events, including endosomal sorting, organelle biogenesis, and vesicular transportation (Colombo et al., 2014; Yáñez-Mó et al., 2015; Hyka, 2017). Assembly of these molecular cargos takes place inside membrane microdomains of the MVBs and on the plasma membrane (Kuipers et al., 2018). Several ESCRT proteins have been implicated as constituents enriched in EVs, particularly 30–150 nm cup-shaped vesicles (i.e., Tsg101, a protein associated with ESCRT-I, and Alix, an accessory protein associated with ESRCT-III, both of which make up components of the EVs) and are frequently used as biomarkers for them (Anand et al., 2019; Juan and Fürthauer, 2018).

Prior to being sorted into ILVs, protein cargo transverse between membrane-bound protein complexes that recognize and bind to the ubiquitin chains attached to them (Paez Valencia et al., 2016). Equipped with the ubiquitin-interacting domains essential for cargo sorting, the ESCRT complexes −0, –I and –II interact stringently with ubiquitinated cargos and traffic them

to the endosomal MVB pathway (Fig. 3 (B)). These sub-complexes can recognize and retain endosomal ubiquitinated cargos, and transport them into MVBs (Anand et al., 2019; Mosesso et al., 2019). In addition, endocytosis modulators such as epsins (e.g., Epsin15) possess ubiquitin-interacting motifs (UIM) that function in ubiquitin recognition (Mayers and Audhya, 2012). The yeast homologs of epsin, Ent1 and Ent2, are reportedly essential for endocytosis of ubiquitin-dependent α-factor receptor that requires ESCRT-0 Vps27 for sorting (Shih et al., 2000; Raiborg et al., 2003). Finally, membrane scission of the ECRT complex is promoted by the recruitment of the ESCRT-III assembly together with its accessory proteins including Bro1 or Vps60 (Moreno-Gonzalo et al., 2014; Stoorvogel, 2015, Fig. 2).

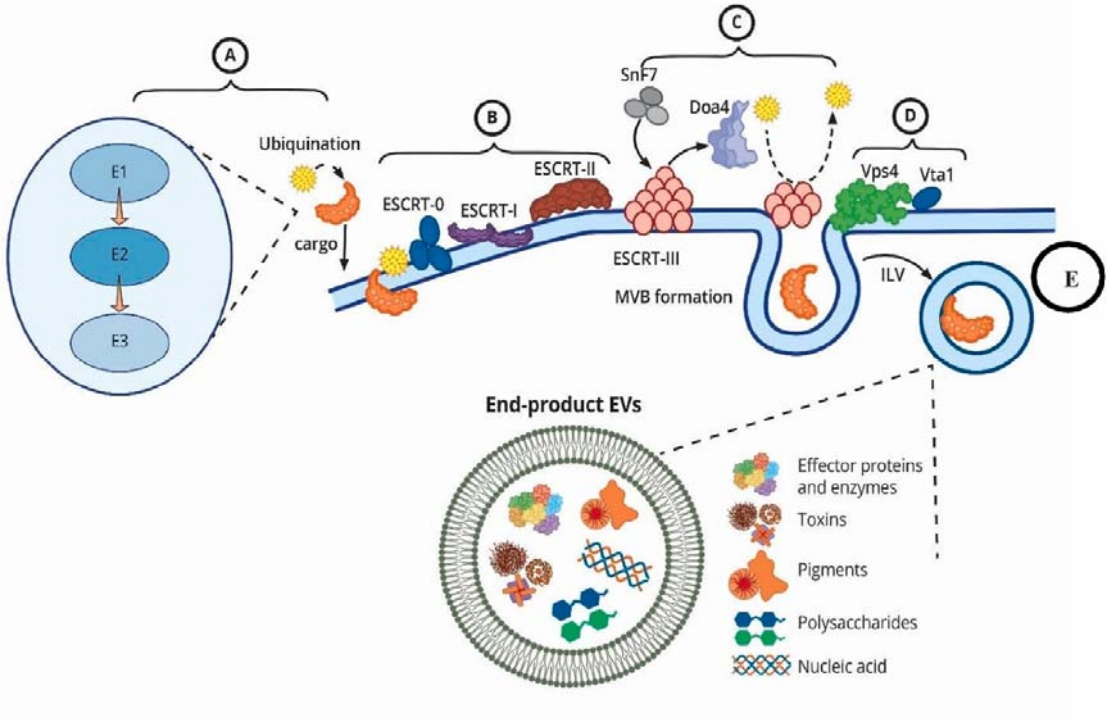

Fig. 3. ESCRT components involved in sorting and trafficking of ubiquitinated cargos and the biogenesis of multivesicular bodies (MVBs) in plant pathogenic fungi. **A.** Cargo lacking signal peptides trigger the process of ubiquitination (E1-ubiquitin-activating enzyme, E2-ubiquitin-conjugating enzyme and E3-ubiquitin ligase). A ubiquitin serving as a signal is then attached to the cargo where it instigates the ESCRT pathway. **B.** The subcomplexes of the pathway (ESCRT-0, ESCRT-I and ESCRT-II) recruit and work in concert to sequentially convey ubiquitin modified cargo. This initial process prompts the evagination of the endomembrane into MVBs. **C.** ESCRT-III employs deubiquitinating enzyme Doa4 (Degradation of alpha-4) to remove the ubiquitin from cargo and recruits ESCRT-IV (Vps4) after sorting and packaging cargo into the formed MVBs. **D.** The Vps4 then dissembles ESCRT-III and promote MVB scission. **E.** End product, EVs containing cargo are released to extracellular space.

6. Deubiquitination: ubiquitin is detached and protein cargo is packaged

The process of ubiquitination can be reversed or eliminated by de-ubiquitination enzymes (DUBs). These enzymes represent a large family of proteases that are responsible for cleaving ubiquitin from proteins (Schwihla and Korbei, 2020). In fungi and mammals, deubiquitination appear to neutralize and maintain the amount of ubiquitinated membrane proteins trafficked for digestion, through recycling cargo back onto the plasma membrane (Schwihla and Korbei, 2020). Deubiquitination also represent a crucial step during the formation of MVBs.

As MVBs form, prior to incorporation of the endocytic cargo into ILVs, DUBs remove their attached ubiquitin (Raiborg et al., 2003). For instance, the ESCRT-III Snf7 attached to Bro1 activate deubiquitination by recruiting Doa4 (degradation of alpha-4) to the endosome before incorporation of ubiquitin modified cargos into MVBs (Amerik et al., 2000b; Katzmann et al., 2001; Odorizzi et al., 2003; Luhtala and Odorizzi, 2004; Wemmer, 2011). However, the deubiquitination process does not preclude certain ubiquitinated proteins from remaining in the ILVs. Research has established that certain EV constituents contained polyubiquitinated non-integral membrane proteins (Buschow et al., 2005). Though this phenomenon is not entirely understood, ubiquitinated proteins in EVs are hypothesized to have escaped deubiquitination and consequently, they might demonstrate microautophagy uptake or the cytoplasmic cargo destined to degradation in lysosomes (Buschow et al., 2005).

DUBs actively link with the ESCRT components (e.g., SnF7-Bro1) to modify and control the status and fate of ubiquitinated cargo (Que et al., 2020). The ESCRT-III sub-complex does not have a ubiquitin recognizing domain, yet it can actively recruit DUBs such as Ubp7 (ubiquitin-specific processing protease 7) and Doa4 that are regulated by ESCRT-III adaptor protein Bro1 in yeasts (Williams and Urbé, 2007; Roxrud et al., 2010; Que et al., 2020). In *S. cerevisiae,* Doa4 significantly contributes to ubiquitin homeostasis and ubiquitin-dependent proteolysis (Amerik et al., 2000b; Que et al., 2020). In addition, Doa4 apparently also recover ubiquitin from ubiquitinated cargo en-route to MVBs (Que et al., 2020). Nevertheless, biological functions of most DUBs are not known in plant pathogenic fungi, especially in filamentous fungi (Wang et al., 2018; Que et al., 2020).

7. Maturation of early endosomes to late endosomes

Late endosomes (LEs), commonly known as multivesicular bodies (MVBs), contain intraluminal vesicles (ILVs) that are released as exosomes into the extracellular space (Huotari and Helenius, 2011). Early endosomes (EEs) serve as preludes for the biogenesis of MVB maturation, and therefore, are perceived as the main sorting station in the endocytic pathway (Helenius et al., 1983; Huotari and Helenius, 2011). On the other hand, MVBs are considered as the second traffic sorting station in the endosomal pathway (Scott et al., 2014). Nonetheless, the structural complexity of the EEs is composed of tubular and vacuolar domains enriched in Rab GTPase proteins (e.g., Rab4, Rab5 and Rab11) as well as retromers and ArF1/COPI (ADP-ribosylation factor 1/coat protein 1) (Aniento et al., 1996; Huotari and Helenius, 2011; Sparvoli et al., 2020). A key component of the endosomal maturation involves the conversion of the small Rabs GTPases i.e., Rab5 and Rab7 (Vps21 and Ypt7 in yeast), which have been extensively investigated in endosomal maturation accounting to the switch between motile EEs to static MVBs (Rink et al., 2005; Segev, 2011). In essence, switching of the two Rab proteins imply that EEs containing Rab5 in their domain are transmuted into MVBs containing Rab7 in their domains, meaning that, Rab7 effectors stand-in for Rab5 effectors and result in MVBs (Abenza et al., 2012; Zerial and McBride, 2001). The switch between Rab5 and Rab7 (RabB and RabS in *A. nidulans*, respectively), is mediated by complexes of homotypic-fusion-and-protein-sorting (HOPS) and class-C-core vacuole/endosome-tethering (CORVET) by prompting EEs' fusion with MVBs, and successively vacuoles (Balderhaar and Ungermann, 2013).

The HOPS and CORVET complexes share similar core subunits (i.e., Vps11, Vps16, Vps18 and Vps33) but also have distinct units including Vps39 and Vps41 found in HOPS only and Vps3 and Vps8 found only in CORVET (Nickerson et al., 2009; Peplowska et al., 2007). During endosomal maturation, CORVET may convert into HOPS through an interchange

between other distinct complex-subunits as they bind to Rab proteins and act as molecular tethers that assemble the acceptor and donor membranes (Markgraf et al., 2009; Ostrowicz et al., 2010; Peplowska et al., 2007). Moreover, mutations of genes from both the HOPS and CORVET complexes lead to deformed Vps genes and impaired vacuoles (Raymond et al., 1992; Rieder and Emr, 1997). The identity of whether the endosomal membrane domain either becomes a vacuole or an MVB is determined by the lipid contents of the PI3P (phosphatidylinositol (Ptdlns) 3-phosphate) as it is the main effector invigorating EE maturation (Penalva et al., 2014). Paralogues of Rab5, RabA, and RabB in *A. nidulans* play unique roles in that RabB facilitates the enrichment of PI3P by recruiting Vps34 PI-3-kinase to EE. MVB biogenesis is initiated through the ESCRT machinery by the PtdIns3P effector Vps27 (Penalva et al., 2014).

The maturation of EEs to MVBs in the filamentous fungi *A. nidulans* is critical for the physiology of the pathogen. It is not surprising that the deletion of the genes encoding proteins involved in this process leads to severe impairment of MVBs (Abenza et al., 2010; Calcagno-Pizarelli et al., 2011). For instance, as Vps33 is crucial for the maturation of EEs into MVBs and survival of the fungus, its deletion resulted in impaired fungal growth without conidia (Abenza et al., 2010, 2012; López-Berges et al., 2017). Moreover, several other studies have shown that this impairment can be restored through the deactivation of the Slt pathway. In *A. nidulans*, this pathway involves the zinc finger transcription factors sltA/sltB whereabout it mediates cation stress tolerance and detoxification (Calcagno-Pizarelli et al., 2011; Mellado et al., 2015, 2016). sltB can reportedly recover phenotypes and therefore sltB mutants have been shown to rescue severe growth defect of the ESCRT null mutants blocked in endosomal maturation (Calcagno-Pizarelli et al., 2011). The morphology of vacoules is extensively affected by mutants of the COVERT and HOPs complex subunits. For example, in *A. nidulans* defects of rabB (encoding Vps21p in yeast) leads to reduced vacuole size. Similar findings were reported in yeast class B vacoular phenotype that has also been observed in ESCRT mutants impeding the MVB pathway (Abenza et al., 2012; Calcagno- Pizarelli et al., 2011). Moreover, deletion of HOPS encoding gene Vps41 and Vps39 also resulted in formation of various small vacuoles (Abenza et al., 2012; López-Berges et al., 2017). Thus, maturation of EEs through the COVERT and HOPS complexes is key for *A. nidulans* to thrive (López-Berges et al., 2017).

8. ESCRT-dependent components in plant fungal pathogenesis

At the molecular level, components of the ESCRT pathways are well understood in only a few model phytopathogenic fungi, including *F. graminearum*, *M. oryzae* and *Ustilago maydis*. Of these, the two ascomycetes (*F. graminearum* and *M. oryzae*) are respectively responsible for Fusarium head blight of barley/wheat and rice blast disease (Sun et al., 2022; Xie et al., 2019a). The basidiomycetous species *U. maydis* is the causal agent of smut on maize and teosinte (Pérez- Rodríguez et al., 2021). Overall, these studies showed that the basic assembly and sequential recruitment of ESCRT components is likely consistent with that of the yeast model. This is consistent with the fact that the ESCRT pathway is conserved with only some components apparently non-essential in certain lineages (Leung et al., 2008). Also, some ESCRT components are critical for growth, stress response reproduction system, while others affect pathogenesis.

Several gene knockout studies have been performed to determine the impact of ESCRT-associated genes in these pathogens. A recent study by Xie et al. (2016) was the first to report on ESCRT-0 subunit Vps27 functions in *F. graminearum*. The authors revealed that Vps27 is

crucial for the pathogen's development, conidiation and virulence. They also showed that ESCRT proteins –I, –II and –III are essential for endocytic delivery into the vacuole, therefore aiding in the production of mycotoxins (Xie et al. (2019b)). In *U. maydis*, the removal of ESCRT-III Did2 caused defects on hyphal growth as well as impaired conveyance of early and late endosomes (Haag et al., 2017). In *M. oryzae*, deubiquitination enzyme Doa4 is essential for deubiquitination to ensure pathogenicity and infection-related morphogenesis (Wang et al., 2018; Que et al., 2020).

The above studies on phytopathogenic fungi are consistent with those conducted on human pathogenic fungi. For example, in *C. neoformans,* Doa4 mutants exhibited reduced pathogenicity and phenotypic defects (Amerik et al., 2000a; Fang et al., 2012; Que et al., 2020). In *C. albicans*, deletion mutants of ESCRT-III SnF7 is associated with acute impairment on hyphal germination (Yang et al., 2020). Additionally, DUBs such as Ubp14, Ubp8 and Ubp4 have been shown to play critical roles in conidiation, growth and, most importantly, pathogenicity (Wang et al., 2018; Que et al., 2020; Yang et al., 2020).

9. End products of the ESCRT-pathway contribute to virulence

In recent years, EVs have gained prominence in the field of biology due to their ubiquitous nature in organisms and their composition, thus making them appealing for better understanding plant-microbe interactions (Kalluri and LeBleu, 2020). Indeed, EVs have been found to be secreted across a spectrum of microbes including plant pathogenic fungi (e.g., *Penicillium digitatum*), bacteria (e.g., *Xanthomonas campestris pv., campestris)* and protozoans (e.g., *Trypanosoma cruzi*) (Chatterjee and Das, 1967; Coelho and Casadevall, 2019; Costa et al., 2021; Raiborg et al., 2003; Rybak and Robatzek, 2019; Sidhu et al., 2008; Torrecilhas et al., 2009). Presently, EVs are better characterized in human bacterial pathogens than any other microbes whereby they contribute to trafficking and delivery of effector proteins, which induce host immune response (Rybak and Robatzek, 2019).

Studies on fungal EVs, especially those of plant pathogens, are gradually gaining momentum. Recently, a study by Costa et al. (2021) has demonstrated that EVs are used by filamentous fungal pathogens to export the phytotoxic compound, tryptoquialanines A, during plant infection and consequently cause tissue damage on citrus seeds. This is consistent with several other studies showing that EVs derived from fungal pathogens exert toxic effects on plant tissues (e.g., Silva et al., 2014; Bleackley et al., 2020). In other words, EVs may trigger a type of immune response in plants, to what end leading to hypersensitivity and subsequent discoloured areas on the plant leaf. This suggests the potential use of pathogen-derived EVs as plant improvement agents by serving as biological biomarkers.

10. EVs and plant-host interactions – the fungal pathogen viewpoint

The first report of EVs in a plant fungal pathogen came from the powdery mildew fungus *Blumeria graminis* (Hippe, 1985; Hippe-Sanwald et al., 1992). However, their earliest detailed characterization originated from research conducted around the 2007, beginning with work on *C. neoformans* and expanding to other systems (Rodrigues et al., 2007a, Rodrigues et al., 2007b; Rodrigues et al., 2008a; Samuel et al., 2015; Zhao et al., 2019). Nonetheless, the mechanism behind the production of EVs from the fungal cell wall is not well understood, although several mechanisms have been suggested (e.g., via passage channels, turgor pressure, cell-wall degrading enzymes, and viscoelasticity of the cell wall) (Brown et al., 2015; Kuipers et al., 2018). For instance, a recent study on *S. cerevisiae* showed that EVs may contain

enzymes and cell wall-related proteins that possibly drive the transition of EVs across the fungal cell wall (Zhao et al., 2019).

Whilst a shortfall of protocols and specific biomarkers for EV isolation continues to be a challenge in the field of EV biology, particularly for filamentous fungi, numerous studies on fungal EV production remain biased towards human fungal yeast pathogens (Albuquerque et al., 2008; Bielska et al., 2018; Gehrmann et al., 2011; Ikeda et al., 2018; Lavrin et al., 2020; Leone et al., 2018; Peres da Silva et al., 2019; Vallejo et al., 2011; Vallejo et al., 2012; Vargas et al., 2015). Consequently, very little is known about the release of EVs in plant pathogenic fungi, although a large number of studies have confirmed their presence in these organisms (Silva et al., 2014; Bitencourt et al., 2018; Liu et al., 2018; de Paula et al., 2019; Souza et al., 2019; Bleackley et al., 2020; Brauer et al., 2020; Rizzo et al., 2020; Costa et al., 2021; Garcia-Ceron et al., 2021). However, the production of EVs in fungi is considered unconventional because they mostly transport proteins lacking signal peptides (Samuel et al., 2015). For example, *B. graminis* secrete avirulence proteins (i.e., AVRa10 and AVRk1) that lack a signal peptide (Ridout et al., 2006; Samuel et al., 2015). Mammalian studies have, however, reported the delivery of proteins with or without signal peptides by EVs, suggesting highly complex and unique mechanisms of cargo sorting and loading into these entities (Samuel et al., 2015).

Fungal virulence is associated with the release of EVs that are involved in modulating acceptor cells. Thus, EVs are required for fungal pathogenesis due to their ability to modulate receipt cells to promote virulence (Joffe et al., 2016; Rodrigues et al., 2007a, Rodrigues et al., 2007b; Vargas et al., 2015). In addition to their phytotoxic activity as mentioned previously, *F. oxysporum f. sp. vasifectum* EVs were found to comprise of virulent polyketide synthases and proteases etc. (Garcia-Ceron et al., 2021). Also, EVs in *C. neoformans* were found to contain virulence factors such as glucuronoxylomannan (GXM) and glucosylceramide (Rodrigues et al., 2007a, Rodrigues et al., 2007b; Rodrigues et al., 2008a).

Several studies on filamentous plant pathogens (fungi and oomycetes) revealed that the ESCRT pathway is utilized to export virulence-associated molecules via vesicles (Giraldo et al., 2013; Wang et al., 2017). The citrus fungal pathogen, *Penicillium digitatum*, is reported to release EVs of myriad cargos containing mycotoxins and alkaloids (tryptoquialanine A) during infection (Costa et al., 2021). Moreover, *B. cinerea* EVs have been reported to carry sRNAs that promote pathogen infection by silencing the plant immunity genes, suggesting that EVs mediate the delivery of fungal virulent factors into host plants (Rodrigues et al., 2008b; Weiberg et al., 2013; Wang et al., 2016, 2018). Recently, clathrin-mediated endocytosis (CME), which operates upstream of the ESCRT pathway, was shown to be crucial in the internalization of fungal sRNA-containing EVs using *B. cinerea* as a model (He et al., 2023). Not long ago, two separate research groups demonstrated that cytoplasmic effectors released by the fungus *M. oryzae* and oomycete *Phytophthora infestans*, both filamentous pathogens of plants, enter their host cells via CME (Oliveira-Garcia et al., 2023; Wang et al., 2023), suggesting they can be released within EVs and play a role in modulating host immune responses. EVs from human fungal pathogens including *Histoplasma capsulatum* and *C. parapsilosis* have also been shown to contain effector proteins that may play a role in host immune responses (Albuquerque et al., 2008; Gil-Bona et al., 2015; Vargas et al., 2015). Taken together, these findings suggest CME and the ESCRT system, though distinct processes, are functionally linked i.e., while CME is responsible for the initial cargo internalization, ESCRT takes over at the endosomal level, concerned with sorting and loading cargo into EVs. This molecular collaboration ensures that intracellular trafficking and its cellular outcomes are carried out in an efficient manner.

11. EVs in fungal biofilm biology

Membrane vesicles play a significant role in the formation and maintenance of fungal biofilms (Fig. 4) (Zarnowski et al., 2018, 2022). Biofilms are complex structures composed of communities of sessile cells embedded in a self-produced extracellular matrix (ECM) (Harding et al., 2009). Formation of biofilms generally involves a succession of phases commencing with adherence of cells to surfaces, followed by the growth of cells which are covered by the ECM, leading up to maturation and dispersal of cells (Chandra et al., 2001; Harding et al., 2009). Nonetheless, the exact mechanisms by which membrane vesicles contribute to biofilm formation are not fully understood, but several mechanisms have been proposed.

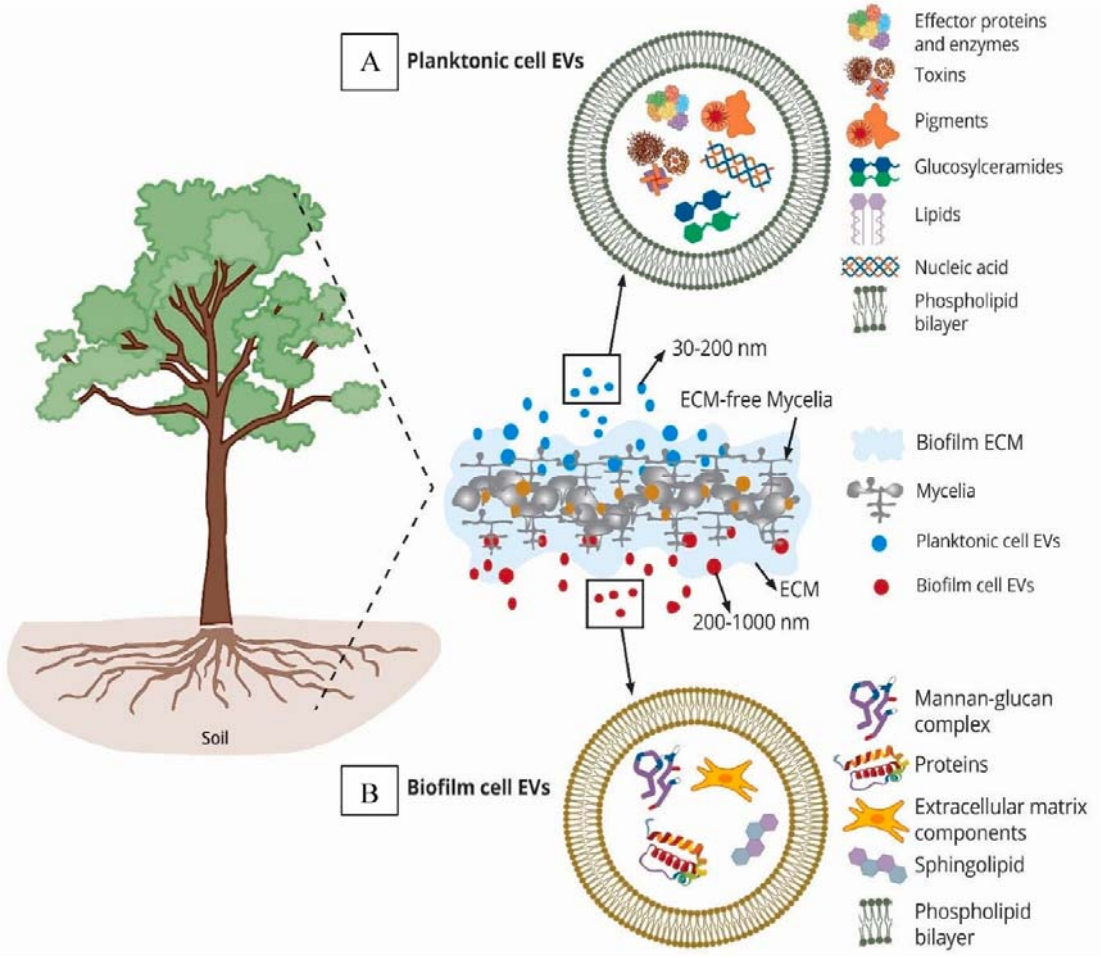

Fig. 4. Phytopathogenic fungi and their interactive membrane vesicles. **A.** Planktonic-derived EVs promoting cell-cell communication among other functions. **B.** Biofilm-derived extracellular vesicle(EVs) (200–1000 nm) trafficking potentially drug-tolerant components and mostly extracellular matrix components.

One proposed mechanism is that membrane vesicles are involved in the secretion of ECM components (Fig. 4), such as polysaccharides and proteins, which are necessary for the formation of the biofilm structure (Di Martino, 2018; Harding et al., 2009). For instance, in *C. albicans*, EVs have been shown to be a major source of matrix material, and delivering up to 45% of the matrix-associated proteins (Zarnowski et al., 2018). Such vesicle cargos included ESCRT subunits such as Hse1 and Vps27, and cultures devoid of these ESCRT genes and

others (i.e., Vps4, Snf7 etc.) showed decreased vesicle production, which resulted in biofilms with increased susceptibility to antifungal agents (Zarnowski et al., 2018). This means that defects in EV production potentially compromise the structural integrity of the biofilm and led to the biofilm matrix inability to sequester antifungals (Zarnowski et al., 2018). As elegantly demonstrated through a 'vesicle add back' protocol, which entail feeding WT biofilm vesicles to a vesicle-defective mutant, EVs are indeed crucial in carrying essential matrix components (Zarnowski et al., 2018). Vesicles are also known to transport enzymes that are involved in the degradation of host tissues, which can create a suitable environment for biofilm formation. In *F. oxysporum f. sp.vasinfectum* enzymatic metabolites trafficked by membrane vesicles have been reported to cause tissue damage when injected into leaves of either cotton plants or *Nicotiana benthamiana* (Bleackley et al., 2020).

Another proposed mechanism is that membrane vesicles are involved in intercellular communication within the biofilm (Fig. 4). Therefore, vesicles play a pivotal role in ensuring sharing of community resources such as the biofilm matrix material and maintenance of the biofilm. Moreover, studies have demonstrated that vesicles can transfer signaling molecules, such as lipopeptides and small RNAs, between cells, which can coordinate the behavior of the cells within the biofilm (Leone et al., 2018). Additionally, membrane vesicles are also thought to play a role in the development of antifungal resistance. Vesicles have been found to sequester drugs and protect the biofilm cells from their effects, and also to detoxify drugs, breaking them down before they reach the cells (Zarnowski et al., 2018, 2022). Components of the ESCRT pathway (i.e., Vps28, Vps22, SnF7etc.) displayed major roles in membrane vesicle production with effects in their functional cargo, thus it can be concluded that some of these genes are crucial in EV biogenesis.

Although the role of EVs in biofilm biology is beginning to be understood in yeast species, there are some indications that the EVs are also essential in biofilms of filamentous plant fungi (Motaung et al., 2023). For instance, vesicles obtained from biofilms and planktonic cells of *Fusarium circinatum*, an important fungal pathogen of pine trees, have been observed to increase ECM production within the fungus (Motaung et al., 2023), somewhat reminiscent with Zarnowski's findings in their 2018 paper (Zarnowski et al., 2018). Considering that biofilms of this fungus also exhibit resistance to antifungals (Ratsoma et al., 2024), this hints that EVs, through their ability to enhance ECM, could potentially be a mechanism behind the antifungal resistance seen in this particular plant pathogen. Recent studies demonstrated that the structural complexity of biofilms (i.e., high cell density, ECM etc.) led to the production of EVs which can enhance drug resistance and are unique to planktonic (free-living) cells (Bielska and Ma, 2019, Honorato et al., 2021; Zarnowski et al., 2022). The cargo of biofilm EVs in *C. albicans* ferry compounds including cell-wall degrading enzymes as well as mannan and glucan (Garcia-Ceron et al., 2021; Mitchell et al., 2015; Zarnowski et al., 2014). Compatible with the assembly of the ECM, the delivery mannan-glucan complex by EVs is critical for drug resistance (Mitchell et al., 2015; Zarnowski et al., 2014).

ESCRT-I genes including Hse1 and Vps27 were found enclosed in secreted EV cargo of *C. albicans*. These ESCRT-I containing EVs are reported to restore the biofilm matrix architecture and quantities of the key mannan–glucan components, an indication that they may function in biofilm EV production and promotion of matrix biogenesis (Zarnowski et al., 2018). EVs are also believed to control morphogenesis in *C. albicans* as their presence hinders the process of biofilm formation and dimorphic transition (Honorato et al., 2021). For instance, EVs containing RNA are assumed to actively take part in the dimorphic transition of *Pichia fermentans* (Leone et al., 2018). Overall, membrane vesicles are involved in multiple aspects

of biofilm formation, including the secretion of matrix components, intercellular communication, and drug resistance effects in their functional cargo, thus it can be concluded that some of these genes are crucial in EV biogenesis.

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12. Future Directions

Since the discovery of EVs, there has been a growing interest in the mechanisms involved in the sorting and packaging of their cargo (proteins, nucleic acids, and so on). The components of ESCRT pathway function together to deliver proteins into EVs. ESCRTs are thus crucial in plant pathogenic fungi as they contribute to their pathogenesis, developmental and growth phenotypes. We hypothesize that plant pathogens use the ESCRT pathway to sort and package protein lacking signal peptides, and that ubiquitin modification is necessary for fungal pathogenesis. The exact role of this pathway requires further studies as proteins with signal peptides are also reported in EVs.

Proteins of the ESCRT-dependent pathway have been implicated in the virulence of EVs. Thus, a few studies conducted in plant pathogens have highlighted the importance of ESCRT proteins, especially in the sorting of virulent factors into EVs. We suggest that the core functions of the ESCRT pathway genes need to be investigated further in plant pathogenic fungi, particularly since it has been demonstrated that ESCRT protein dysfunction causes aberrant endosomal compartments and reduced ILV formation. The much-needed drive contributed by genome sequencing enables the intelligible characterization of this system in

fungi with completely sequenced genomes. Through the characterization and detection of particular cargos sorted by the ESCRTs, we can gain further insights into possible mechanisms involved in the pathogenesis of plant pathogen fungi. The ESCRT pathway is involved in the formation of MVBs, which contain ILVs that can be released as EVs ('exosomes') that are loaded with bioactive molecules during heterotypic fusion.

Devising prospective strategies to inhibit components of this pathway may yield improved agricultural products for pathogen control. However, it is ideal that we first investigate and understand how this pathway works. Intercellular contact, pathogenicity, and cellular homeostasis all depend on EVs. As a result, their release can serve as a barometer of disease, stress, or health. The information gained on the mechanisms involved in the pathogenicity effects of plant pathogenic EVs can serve as a stepping stone toward improving our ability to manage their damaging effects on agricultural crops and forests. EVs should thus be explored as a delivery platform for agricultural products such as either active ingredients of fertilizers, antimicrobial or plant priming agents to facilitate and promote improved plant health.

Credit authorship contribution statement

Manchela F. Ratsoma: Conceptualization, methodology, investigation, analysis, writing-original draft, writing review and editing. Quentin C Santana: Methodology, investigation, analysis, writing review and editing. Brenda D Wingfield: Conceptualization, methodology, investigation, analysis, writing review and editing. Emma T Steenkamp: Conceptualization, methodology, investigation, analysis, writing review and editing Thabiso E Motaung: Conceptualization, Methodology, writing review and editing, resources, supervision and funding acquisition.

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Declaration of competing interest

None.

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