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RESEARCH ARTICLE

Sterols regulate endocytic pathways during flg22-induced defense responses in *Arabidopsis*

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ABSTRACT

The plant transmembrane receptor kinase FLAGELLIN SENSING 2 (FLS2) is crucial for innate immunity. Although previous studies have reported FLS2-mediated signal transduction and endocytosis via the clathrin-mediated pathway, whether additional endocytic pathways affect FLS2-mediated defense responses remains unclear. Here, we show that the *Arabidopsis thaliana* sterol-deficient mutant *steroid methyltransferase 1* displays defects in immune responses induced by the flagellin-derived peptide flg22. Variable-angle total internal reflection fluorescence microscopy (VA-TIRFM) coupled with single-particle tracking showed that the spatiotemporal dynamics of FLS2-GFP changed on a millisecond time scale and that the FLS2-GFP dwell time at the plasma membrane increased in cells treated with a sterol-extracting reagent when compared with untreated counterparts. We further demonstrate that flg22-induced FLS2 clustering and endocytosis involves the sterol-associated endocytic pathway, which is distinct from the clathrin-mediated pathway. Moreover, flg22 enhanced the colocalization of FLS2-GFP with the membrane microdomain marker Flot 1-mCherry and FLS2 endocytosis via the sterol-associated pathway. This indicates that plants may respond to pathogen attacks by regulating two different endocytic pathways. Taken together, our results suggest the key role of sterol homeostasis in flg22-induced plant defense responses.

KEY WORDS: Sterols, VA-TIRFM, FLS2, Spatiotemporal dynamics, Endocytosis, Plant immunity

INTRODUCTION

Plants possess a multilayered defense system of innate immunity that confers resistance against many pathogens. Plant cells have a variety of immune receptors, also known as pattern recognition receptors (PRRs), that recognize conserved microbial- or pathogen-associated molecular patterns (PAMPs) (Dodds and Rathjen, 2010; Monaghan and Zipfel, 2012; Felix et al., 1999), such as fungal chitin, and the bacterial factor elongation factor EF-Tu (Kunze et al., 2004), flagellin (Robatzek et al., 2006) and lipopolysaccharides (Mulder et al., 2006; Radutoiu et al., 2007). The *Arabidopsis thaliana* receptor kinase FLAGELLIN SENSING 2 (FLS2) is a well-known PRR that perceives a conserved 22 amino acid domain in the N terminus of flagellin (flg22) (Mulder et al., 2006; Radutoiu et al., 2007). Upon perception of flg22, FLS2 physically associates with another leucine-rich repeat receptor-like kinase (LRR-RLK), BAK1 (BRASSINOSTEROID-INSENSITIVE 1-ASSOCIATED KINASE 1) (Heese et al., 2007; Roux et al., 2011), and is phosphorylated by BIK1 (BOTRYTIS-INDUCED KINASE 1) (Segonzac and Zipfel, 2011). Activation of FLS2 triggers a series of downstream immune responses (Asai et al., 2002; Boudsocq et al., 2010; Henry et al., 2013), including the production of reactive oxygen species (ROS), induction of defense genes, deposition of secondary compounds, such as callose, and accumulation of defense hormones (Grossie et al., 2009; Gómez-Gómez and Boller, 2000; Boudsocq et al., 2010).

Numerous reports support the concept that endocytosis of receptor kinases physically terminates signaling through degradation of receptors, sustains signaling through recycling or relays signals inside the cell through the formation of signaling endosomes (Du et al., 2013). The recycling/signaling and the degradative fates preferentially associate with different endocytic routes (Sigismund et al., 2008). In animal cells, multiple pathways of endocytosis have been identified and classified, including clathrin-dependent and clathrin-independent pathways (Doherty and McMahon, 2009). Similarly, two endocytic pathways have been found in plant cells: the clathrin-mediated and the sterol/raft-associated endocytic pathways (Fan et al., 2015). The clathrin-mediated endocytic pathway (CME) is the major and best-studied endocytic pathway in plant cells (Ortiz-Morea et al., 2016).

The sterol/raft-associated endocytic pathway has also been described and may involve specific membrane microdomain proteins such as flotillin 1 (Flot1) (Li et al., 2012). Membrane microdomains are thought to temporally and spatially organize proteins and lipids into dynamic signaling complexes (Cacas et al., 2012). In animal and yeast cells, sterol-rich domains may function as sorting platforms for proteins that function in signal transduction, pathogen entry, secretion and endocytosis (Simons and Toomre, 2000). Sterol-associated endocytic pathways have been described in plant cells (Men et al., 2008), although the mechanisms of this pathway in plant immunity have yet to be investigated.

Here, we describe a series of investigations into the mechanism of FLS2 endocytosis, to explore the link between sterols and FLS2 dynamics in response to the plant immunity signal flg22. Phenotypic analyses showed that sterols can affect the flg22-induced immune response. Variable-angle total internal reflection fluorescence microscopy (VA-TIRFM) in combination with single-particle tracking (Li et al., 2011) showed that FLS2 is mobile and heterogeneously distributed at the plasma membrane, and that sterols affected this distribution. Furthermore, we demonstrate that the *sterol methyltransferase 1* (*smt1*) mutation does not change the homo-oligomeric state of FLS2 but does affect FLS2 cluster formation. In addition, endocytosis of FLS2 is impaired in *smt1* mutants.

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