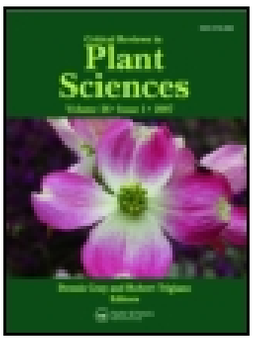

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Flotillins, Erlins, and HIRs: From Animal Base Camp to Plant New Horizons

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ABSTRACT

Plant stomatin/prohibitin/flotillin/HflK/C (SPFH) proteins are represented by prohibitins, flotillins, stomatins, erlins, and hypersensitive induced reaction proteins (HIRs). The purpose of this review is to summarize the current state of knowledge regarding plant flotillins and HIRs and to assign putative functions of plant flotillins and erlins based on the known functions of their mammalian homologs. Similar to human flotillins, plant flotillins are localized in membrane microdomains, and involved in endocytosis, and interact with receptor kinases. HIRs play an important role in plant immunity by promoting the hypersensitive response and binding to leucine-rich repeat proteins. In this way, they participate in resistance to bacterial or fungal pathogens. We further focused on flotillins, HIRs, and erlins in *Arabidopsis thaliana* and, using public databases, described them in terms of the following: 1) their transcription throughout plant ontogeny and under various environmental conditions; 2) the presence of conserved domains or characteristic motifs in their amino acid sequences; and 3) their potential interactions with other proteins. Based on these data, we hypothesize about their additional functions and properties.

KEYWORDS

Arabidopsis thaliana; erlin; flotillin; HIR; membrane microdomains; SPFH domain

I. Introduction

Cellular membranes provide many indispensable functions and serve as a crucial interface for communication, signaling, or transport. The membranes are functionally compartmentalized into distinguishable areas of various size—i.e., macro, micro, and nanodomains (Sekeres *et al.*, 2015; Zarsky *et al.*, 2009). The lateral discontinuum of the lipid composition of membranes was originally reported for sphingolipid-enriched areas preexisting within Golgi complex membranes that are sorted preferentially to the apical rather than basolateral plasma membrane in epithelial cells (Van Meer *et al.*, 1987). A subsequent association of sphingolipid-enriched membrane areas with glycophosphatidylinositol (GPI)-anchored proteins containing a sorting signal to the apical plasma membrane was observed (Brown and Rose, 1992). Finally, the term "lipid rafts" was proposed as the membrane trafficking principle resulting in different lipid-protein compositions of apical versus basolateral plasma membrane as well as signaling platform recruitment and clustering of proteins involved in membrane signaling (Simons and Ikonen, 1997). However, this has not yet been unambiguously confirmed (Kraft, 2013), and the term is inappropriate for plant membranes, for which the terms micro or nanodomains are to be used instead (Tapken and Murphy, 2015).

The stomatin/prohibitin/flotillin/HflK/C (SPFH) domain (also known as prohibitin homology (PHB) domain or Band_7 domain) protein superfamily comprises several types of proteins with different functions that are found in most evolutionary lineages (Rivera-Milla *et al.*, 2006). According to their cellular localization and biological function, the SPFH proteins are distinguished in several subfamilies. Metazoan SPFH proteins include flotillin/reggie (Schulte *et al.*, 1995), stomatin (Stewart *et al.*, 1993), prohibitin (Nuell *et al.*, 1991), erlin (Browman *et al.*, 2006), and podocin (Boute *et al.*, 2000). The bacterial membrane proteins HflK and HflC (Rivera-Milla *et al.*, 2006), and vacuolin of *Dictyostelium* (Rauchenberger *et al.*, 1997) are also SPFH protein superfamily members. Most of these proteins form microdomains in cell membranes. Besides, they are also enriched in detergent-resistant membrane (DRM) fraction (Browman *et al.*, 2007).

Plant SPFH proteins comprise prohibitins, flotillins, stomatins, erlins, and the plant-unique hypersensitive

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induced reaction proteins (HIRs) (Di *et al.*, 2010). SPFH protein homolog genes were identified in many plant species, including most common plant models such as *Arabidopsis thaliana, Chlamydomonas reinhardtii, Medicago truncatula, Oryza sativa, Physcomitrella patens, Populus tremula*, and *Sorghum bicolor* (Di *et al.*, 2010).

In this review, we attempt to summarize the knowledge acquired for HIRs, erlins, and flotillins. Because very little information is available for plant erlins and flotillins, we hypothesize about their putative functions based on current findings for their mammalian homologs, which play substantial roles in several essential processes—mainly signaling events. Plant stomatins and prohibitins, however, are not included in this review because they were recently reviewed by Gehl and Sweetlove (2014).

II. Flotillins—a wide range of functions

Flotillins were first discovered in goldfish (*Carassius auratus*) retinal ganglion cells (Schulte *et al.*, 1995) and originally named "reggie" because of their induced expression during optic nerve regeneration (Schulte *et al.*, 1997). Independent of the reggie discovery, a DRM protein found in caveolae membranes in mouse fibroblast tissue culture was denoted flotillin (Bickel *et al.*, 1997). The protein was homologous to a previously described human epidermal surface antigen (Bickel *et al.*, 1997; Schroeder *et al.*, 1994).

The *Arabidopsis thaliana* genome contains three coding regions for homologs of flotillin (At5g25250, At5g25260, and At5g64870). The first one was designated AtFlot1 (Borner *et al.*, 2005) or AtFLOT1A (Jarsch *et al.*, 2014), and the second was designated AtFLOT1B (Jarsch *et al.*, 2014). For the purpose of this review, we will refer them as follows: AtFlot1 (At5g25250), AtFlot2 (At5g25260), and AtFlot3 (At5g64870). *AtFlot1* and *AtFlot2* transcriptions predominate in leaves and shoots, whereas *AtFlot3* is mostly transcribed in flower parts and siliques (Figure 3).

A. The SPFH domain of flotillins is necessary for their proper membrane localization

The interaction of flotillins with membranes is provided through the SPFH domain in animal cells. The entire SPFH domain is pivotal for the localization of human Flotillin-2 to the plasma membrane and to endosomes in HeLa cells (Langhorst *et al.*, 2008). Similarly, the Flotillin-2 SPFH domain itself is sufficient for plasma membrane trafficking in Vero cells (Morrow *et al.*, 2002), whereas the truncated SPFH domain of Flotillin-2 lacking the N-terminal 1–40 aa (a hydrophobic stretch with acylation sites) does not localize to the plasma membrane in N2a cells (Solis *et al.*, 2007). Pronounced localization in Golgi cisternae has been observed for the truncated version of Flotillin-2 consisting of the N-terminal 1–30 aa stretch (containing palmitoylation and myristoylation sites) fused to the flotillin domain and lacking most of the SPFH domain (Langhorst *et al.*, 2008). Interestingly, whereas Flotillin-2 trafficking is impaired following treatment with Brefeldin A (BFA) and the protein was retained in accumulated Golgi vesicles (Langhorst *et al.*, 2008), Flotillin-1 localization is insensitive to BFA treatment. Thus, Flotillin-1 cellular trafficking is Golgi-independent (Morrow *et al.*, 2002).

The N-terminus of the SPFH domain is necessary for anchoring flotillins to membranes with palmitoyl and myristolyl residues in animal cells; truncated versions of both flotillins containing only the SPFH domain localize properly to the plasma membrane, whereas those lacking the SPFH domains accumulate in soluble fractions (Langhorst *et al.*, 2008; Morrow *et al.*, 2002; Neumann-Giesen *et al.*, 2004). Intriguingly, no palmitoylation or myristoylation sites were predicted in the N-terminus of any of the three *A. thaliana* flotillin homologs using public databases (Figure 1), and *A. thaliana* flotillins are thus considered to interact with membranes in a manner that differs from animal flotillins.

B. Flotillins are present in membrane microdomains and in detergent-resistant membranes

Flotillins are found in many animal cell types and lines (Volonté *et al.*, 1999; Zhao *et al.*, 2011). Regardless of rare findings in mitochondria in human (Ogura *et al.*, 2014) and murine cells (Zhang *et al.*, 2008), and in nuclei in human cell lines (Santamaria *et al.*, 2005), flotillins were most frequently associated with membrane microdomains (Baumann *et al.*, 2000; Dermine *et al.*, 2001; Frick *et al.*, 2007; Glebov *et al.*, 2006; Langhorst *et al.*, 2007, 2008; Neumann-Giesen *et al.*, 2007; Pust *et al.*, 2010; Riento *et al.*, 2009; Slaughter *et al.*, 2003; Solis *et al.*, 2007; Stuermer *et al.*, 2001). These are membrane areas that can be distinguished by physical properties and an enrichment of sterols, sphingolipids, saturated phospholipids, and GPI-anchored proteins from the surrounding membrane. DRM comprises a nonsolubilized membrane fraction that is extracted with mild, cold nonionic detergents (Brown and London, 1997; Brown and Rose, 1992); DRMs have traditionally been considered membrane microdomain counterparts. The association with DRMs is provided by hydrophobic stretches present at the N-termini of human flotillins (Liu *et al.*, 2005). Membrane microdomains, which have been reasonably classified by some authors as nanodomains with distinct lipid and protein composition, and DRMs have also been reported in plants

(Jarsch *et al.*, 2014; Mongrand *et al.*, 2004; Tapken and Murphy, 2015). The idea of microdomains being directly defined by areas of DRM has recently been overcome (Heerklotz, 2002; Lichtenberg *et al.*, 2005; Tanner *et al.*, 2011; Tapken and Murphy, 2015), but some authors still do not clearly distinguish between membrane microdomains and DRMs (Cacas *et al.*, 2016; Ishikawa *et al.*, 2015). This interconnection may seem intuitive because many proteins are present in both the DRM fractions (usually detected *in vitro*, e.g., on Western blots), and the membrane microdomains (observed as clusters or dots of tagged proteins using microscopic techniques), although neither of the two implies the existence of the other.

The plant flotillins AtFlot1, *Picea meyeri* PmFlot1, and rice OsFlot were enriched in the plasma membrane DRM fraction prepared from *A. thaliana* calli, spruce pollen tubes, and rice cells. Concomitant alteration of the sphingolipid composition of the DRM fraction and OsFlot contents in this DRM fraction was observed in rice overexpressing BI-1 (Ishikawa *et al.*, 2015). AtFlot1 and AtFlot2 fused to green fluorescent protein (GFP) or yellow fluorescent protein (YFP) were observed in plasma membrane clustered in dynamic punctate structures corresponding to membrane microdomains in leaf (Jarsch *et al.*, 2014) and root (Hao *et al.*, 2014; Li *et al.*, 2011, 2012) epidermal cells. Similar localization and punctate structure formation within plasma membrane in root epidermal cells were also observed for flotillin homologs of *M. truncatula* MtFLOT2 and MtFLOT4 (Haney and Long, 2010; Haney *et al.*, 2011).

Plant membrane microdomains can be distinguished according to their localization pattern, e.g., polar, equatorial, and punctate domains. Fluorescent-labeled flotillins, similar to, e.g., remorins, cellulose synthase, or plasma membrane intrinsic proteins, form discrete foci or punctate microdomains in the plasma membrane (Konrad and Ott, 2015).

C. Flotillin microdomains provide specific types of endocytosis

In addition to their plasma membrane localization, flotillins have been observed extensively in endosomes and

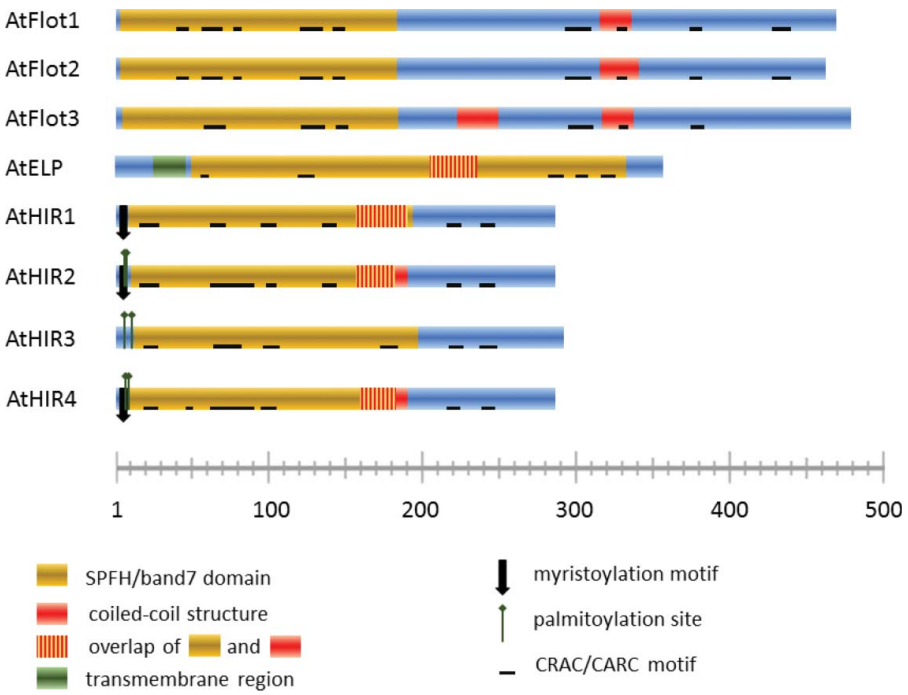

Figure 1. Structure of *Arabidopsis thaliana* SPFH proteins. Putative conserved SPFH (= band 7) domains (yellow), coiled-coil stretches (red), transmembrane stretches (green), N-myristoylation motifs (black downward arrows), and palmitoylation sites (green upward arrows) were identified using the NCBI conserved domain searching tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Max-Planck Institute for Developmental Biology Bioinformatics Toolkit (http://toolkit.tuebingen.mpg.de/pcoils, http://toolkit.tuebingen.mpg.de/marcoil), prediction of transmembrane helices in proteins using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), NBA-Palm—prediction of palmitoylation site (http://nbapalm.biocuckoo.org), CSS-Palm—prediction of palmitoylation site (http://csspalm.biocuckoo.org), PlantsP—plant-specific myristoylation predictor (http://plantsp.genomics.purdue.edu/myrist.html), NTM—the MYR predictor (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm), and Myristoylator (http://web.expasy.org/myristoylator/). CRAC, CARC, and CRAC/CARC-like motifs (black horizontal segments) were searched manually using the ScanProsite tool (http://prosite.expasy.org/scanprosite/). The ruler indicates the protein length as the number of amino acids.

other vesicular compartments. The plasma membrane/endosome distribution ratio is different in various cell types or at different developmental stages (Dermine *et al.*, 2001; Liu *et al.*, 2005). Human flotillins are strongly endocytosed upon stimulation with epidermal growth factor (Babuke and Tikkanen, 2007; Neumann-Giesen *et al.*, 2007; Riento *et al.*, 2009), shiga toxin (Pust *et al.*, 2010), ricin (Pust *et al.*, 2010), or cholera toxin (Ait-Slimane *et al.*, 2009; Glebov *et al.*, 2006; Stuermer *et al.*, 2001).

Human Flotillin-1 is found in plasma membrane curvatures and invaginations, as well as endosomes that are distinct from clathrin-coated vesicles, with no overlap between fluorescentl-labeled flotillin and transferrin, a marker of clathrin-mediated endocytosis (Glebov *et al.*, 2006; Langhorst *et al.*, 2008). Moreover, the rate of transferrin endocytosis was not affected in Flotillin-2-knockdown cells (Ait-Slimane *et al.*, 2009), and Flotillin-1-positive vesicles displayed different dynamics from clathrin-coated vesicles (Glebov *et al.*, 2006). Unlike clathrin-coated vesicles, flotillin microdomain endosome budding is not dependent on dynamin in HeLa cells (Glebov *et al.*, 2006). However, endocytosis of sphingolipid-binding domain peptide, a sphingolipid tracer, GPI-GFP and CD59, a GPI protein, was mediated by Flotillin-2 and dependent on dynamin (Ait-Slimane *et al.*, 2009; Zhang *et al.*, 2009a). Thus, flotillins are crucial for the endocytosis of GPI-anchored proteins.

AtFlot1 was predominantly observed on the plasma membrane from which vesicles budded and co-localized with FM4-64-labeled endosomes in *A. thaliana*. AtFlot1-labeled endosomes differed from clathrin-coated endosomes in terms of size and mobility. The diffusion coefficient of AtFlot1 endosomes was not affected by tyrphostin A23, an inhibitor of clathrin-mediated endocytosis, but was decreased by the application of latrunculin B, an inhibitor of actin polymerization; oryzalin, an inhibitor of tubulin polymerization; and methyl-β-cyclodextrin, a sterol-depleting agent (Li *et al.*, 2012). Flotillin microdomains thus define a clathrin-independent endocytic pathway in both mammalian and *A. thaliana* cells. However, the extent to which one or the other pathway is utilized for the endocytosis of a specific cargo depends on external conditions in *A. thaliana* root cells. In the case of the plasma membrane aquaporin PIP2;1 and NADPH oxidase RbohD, both were predominantly endocytosed via a clathrin-dependent pathway under control conditions, with only a minor contribution of AtFlot1-positive endosomes. This proportion was dramatically increased under salt stress (Hao *et al.*, 2014; Li *et al.*, 2011). Similarly, the brassinosteroid receptor BRI1 is endocytosed in a mostly clathrin-dependent manner when only endogenous brassinosteroids are available. External application of epibrassinolide substantially increases AtFlot1-dependent endocytosis of BRI1 (Wang *et al.*, 2015). Based on these findings, for the same cargo proteins, the clathrin pathway may function as a constitutive endocytic mechanism, whereas flotillin microdomain-based endocytosis is induced by stress or in response to signaling events. This pathway may either serve as a simple contributor to the overall endocytic capacity, or it may direct the endocytosed cargo via an alternative trajectory (e.g., vacuolar degradation versus recycling).

Flotillin pits resemble another type of endocytic structure—caveolae, which are membrane invaginations characterized by the presence of proteins called caveolins (Glenney and Soppet, 1992). The caveolin structure and interaction with the membrane is very similar to those of flotillin, although the domain that interacts with the plasma membrane is located in the N-terminus of flotillins but in the C-terminus of caveolins (Bender *et al.*, 2002; Stuermer, 2010). An interaction of flotillins and caveolins in the formation of endocytic structures was observed in A498 cells (Volonté *et al.*, 1999) and adipocytes (Baumann *et al.*, 2000), and downregulated expression of Flotillin-1 caused a decrease in the concentration of Caveolin-1 in intestinal epithelial cells (Vassilieva *et al.*, 2009). A functional link was found in skeletal muscle cells during insulin-induced glucose transporter type 4 (GLUT-4) trafficking to the plasma membrane. In this process, the stimulated insulin receptor is first endocytosed through caveolae and caveolin-3, and then, is re-localized to GLUT-4-containing Flotillin-1 vesicles, where it provokes Flotillin-1/GLUT-4 vesicle recruitment to the plasma membrane (Fecchi *et al.*, 2006). By contrast, there is no co-localization between caveolins and flotillins in HeLa cells (Frick *et al.*, 2007; Glebov *et al.*, 2006), in which different caveolin and flotillin microdomain dynamics were observed (Frick *et al.*, 2007). Similarly, in human kidney epithelial cells, Flotillin-2 and Caveolin-1 define distinct non-co-localizing microdomains (Roitbak *et al.*, 2005). In DRM prepared from HEK293 cells, Flotillin-1 and Caveolin-1 are found in distinct sub-fractions (Mellgren, 2008). No co-localization was found between endocytosed CD59, a GPI-anchored protein, and caveolin (Ait-Slimane *et al.*, 2009).

Taken together, these findings suggest that there are several populations of flotillin endocytic structures, some of which mediate GPI-anchored protein endocytosis whereas others may contribute to the endocytosis of different cargos delivered by complexes of flotillins and caveolins.

However, the genes for caveolins have not been discovered in plants (Echarri and Del Pozo, 2012; Samaj *et al.*, 2004). Thus, it is tempting to hypothesize that based on structural and functional similarities, plant flotillins could potentially adopt or encompass some

processes that are mediated by caveolins in animal cells. The significance of plant flotillins in membrane transport is supported by the interaction of AtFlot2 and AtFlot3 with several proteins involved in vesicular trafficking and endocytosis, including ESCRT proteins, exocyst and SNARE subunits, and Rab-GTPase (Table 1).

D. Flotillins and sterols

Because sterols are important for the proper membrane microdomain constitution of some plant and yeast proteins (Grossmann *et al.*, 2007; Malínská *et al.*, 2003; Raffaele *et al.*, 2009), it has been questioned whether or not the amount of sterols present within membranes can affect flotillin microdomain properties. The most abundant sterol in animal cells, cholesterol (Espenshade and Hughes, 2007), is recognized by proteins through their cholesterol recognition/interaction amino acid consensus motifs, i.e., CRAC, with the following amino acid sequence: L/V-X_{1-5}-Y-X_{1-5}-K/R, where X = any amino acid (Li and Papadopoulos, 1998). The inverted motifs, K/R-X_{1-5}-Y -X_{1-5}-L/V or CARC, and the modified K/R-X_{1-5}-F-X_{1-5}-L/V or CARC-like were also found to bind cholesterol (Baier *et al.*, 2011). Mammalian Flotillin-2 was predicted to contain two putative CRAC motifs in its amino acid sequence, both of which reside in the SPFH domain (Roitbak *et al.*, 2005). Both murine flotillins were found to interact directly with Niemann-Pick 1-like 1 (NPC1L1) protein, which cycles between the plasma membrane and the endocytic recycling compartment to mediate cholesterol uptake. The complex of flotillins and NPC1L1 forms microdomains at plasma membrane co-localizing with cholesterol-rich foci stained with filipin. Moreover, the complex dissociates in the absence of cholesterol (Ge *et al.*, 2011). In Flotillin-knockdown cells, NPC1L1 and cholesterol are also predominantly present at the plasma membrane, and overall cholesterol uptake is decreased in comparison to cells expressing flotillins at normal levels (Ge *et al.*, 2011). Similarly, in *Aspergillus nidulans* strains with flotillin ortholog depletion, the localization pattern of sterol-rich domains (stained with filipin) differs from that of wild-type strains, although sterol-rich domains do not co-localize with flotillin ortholog microdomains (Takeshita *et al.*, 2012). Flotillin may thus affect sterol uptake/trafficking/localization both directly and indirectly in different cell types or evolutionary lineages.

Proper sterol composition and trafficking to membranes is also important for endocytic processes in plant (Sekeres *et al.*, 2015). The sterol content of DRMs is necessary for proper functioning of membrane microdomain-localized NADPH oxidase in *P. meyeri* (Liu *et al.*, 2009). Decreased sterol content in AtFlot1-knockdown lines (Li *et al.*, 2012) leads to a decrease in the structural order of the membrane (measured as generalized polarization) in these lines (Zhao *et al.*, 2015). Moreover, the depletion of sterol using methyl-β-cyclodextrin results in a decrease in the lateral mobility of AtFlot1 microdomains (Li *et al.*, 2012), which is consistent with the effect observed in N2a cells (Langhorst *et al.*, 2007). Although the effect of sterol depletion may be rather pleiotropic as a result of overall changes in membrane properties, flotillin microdomain dynamics appear to be particularly sensitive to the sterol content of the membrane. The most pronounced decrease in the diffusion coefficient was observed for AtFlot1 (80-fold decrease in the mode value) compared with some other plasma membrane microdomain or punctate-forming proteins such as clathrin (5-fold decrease), RbohD (28-fold decrease), or PIP2;1 (20-fold decrease) in response to the same methyl-β-cyclodextrin treatment (Hao *et al.*, 2014; Li *et al.*, 2011, 2012). The function of flotillins is thus affected by the presence or the amount of sterols, but flotillins also influence sterol uptake and its levels in the cell. *A. thaliana* possesses two sequence homologs of NPC1L1 (At1g42470 and At4g38350), but they do not appear to be involved in sterol metabolism or trafficking (Feldman *et al.*, 2015).

The sequestration of sterols from the plasma membrane leads to changes in the membrane order and fluidity following exposure to oomycetal elicitins such as cryptogein, cactorein, or parasiticein, which are produced by different species of *Phytophthora* (Mikes *et al.*, 1998; Vauthrin *et al.*, 1999). The transcription of all *A. thaliana* flotillin isoforms is highly induced in response to treatment with *Phytophthora* and *Hyaloperonospora* (Figure 2), which are common plant pathogens that encode numerous elicitins or elicitin-like proteins (Chen *et al.*, 2014). Given that *AtFlot1*-knockdown plants contain less sterols in plasma membranes (Li *et al.*, 2012), it is possible that AtFlots binds to sterols and thus prevents them from being trapped and removed from the plasma membrane by elicitins.

Because CRAC/CARC motifs have not yet been demonstrated to recognize sterols other than cholesterol, neither have they been investigated in plants. Thus, it is difficult to predict whether plant flotillin homologs can bind to sterols. In plant cells, phytosterols predominate over cholesterol, which is present in rather minute amounts. The CRAC/CARC motif recognizes the A or B ring of the sterane structure and iso-octyl chain of cholesterol (Fantini and Barrantes, 2013). The A and B cycles are common to cholesterol and the main phytosterols (i.e., β-sitosterol, campesterol, stigmasterol, and brassicasterol), whereas they differ from one another in terms of side chains (iso-octyl in cholesterol). However, phytosterols have been confirmed to bind with a relatively high efficiency to two cholesterol-binding proteins,

Table 1. Proteins that interact with flotillins or HIRs of *Arabidopsis thaliana*.

AGI	Name	Function/type	SPFH	Source
AT1G21240	WAK3	Signaling/receptor-like kinase	F1, F2, F3	a
AT2G42290	LRR protein kinase	Signaling/receptor-like kinase	F2, F3	a
AT2G20850	SRF1	Signaling/receptor-like kinase	F3	a
AT2G17290	CPK6	Signaling/calcium-dependent kinase	F3	a
AT3G48260	WNK3	Signaling/MAPKKK	F2	a
AT3G48040	ROP10	Rop GTPase	F1, F2, F3	a
AT5G42980	TRX3	Signaling/thioredoxin	F1, F2, F3	a
AT1G45145	TRX5	Signaling/thioredoxin	F2	a
AT2G26180	IQD6	Calcium binding	F1, F2, F3	a
AT4G37445	Unknown	Calcium binding	F2, F3	a
AT1G03950	VPS2.3	Protein sorting/ESCRT	F2	a
AT3G10640	VPS60.1	Protein sorting/ESCRT	F2, F3	a
AT1G54090	EXO70D2	Vesicular transport/exocyst subunit	F3	a
AT3G03800	SYP131	Vesicular transport/SNARE subunit	F3	a
AT4G35860	GB2	Rab GTPase	F3	a
AT1G17280	UBC34	Ubiquitination/conjugation enzyme	F2, F3	a
AT3G60820	PBF1	Proteasome subunit	F2, F3	a
AT4G38690	PLC-like	Phospholipid metabolism	F2, F3	a
AT3G08510	PLC2	Phospholipid metabolism	F3	a
AT4G21540	SPHK1	Sphingosine metabolism	F3	a
AT4G04850	CPA2	Monovalent cation:proton antiporter	F2	a
AT3G12180	Cornichon	Potassium/sodium transport	F1, F2, F3	a
AT5G22290	NAC089	Transcription factor	F2, F3	a
AT5G28290	NEK3	Cell-cycle regulator	F3	a
AT4G05370	BCS1	AAA ATPase	F2, F3	a
AT1G14700	PAP3	Purple acid phosphatase	F2	a
AT3G54260	TBL36	Trichome birefringence-like	F2, F3	a
AT3G01500	Carbonic anhydrase 1	Carbonic anhydrase	F2	a
AT1G34760	GRF11	14-3-3 protein	F3	a
AT1G19570	DHAR1	Dehydroascorbate reductase	F3	a
AT2G24940	MAPR2	Progesterone binding protein	F3	a
AT4G27610	Unknown	Unknown	F2	a
AT3G03210	Unknown	Unknown	F2	a
AT4G26090	RPS2	NB-Leucine-rich repeat protein	H1, H2	b, c
AT3G58140	Phe-tRNA synthetase	Phe-tRNA synthetase	H1, H2, H3, H4	b
AT5G35750	AHK2	Histidine kinase/cytokinin receptor	H1, H4	c
AT3G01670	SEOR2	Sieve element occlusion related	H3	c

Protein that interact with *Arabidopsis thaliana* SPFH proteins retrieved from protein-interaction databases. F1 = AtFlot1, F2 = AtFlot2, F3 = AtFlot3, H1 = AtHIR1, H2 = AtHIR2, H3 = AtHIR3, H4 = AtHIR4; a = Associomics (https://associomics.dpb.carnegiescience.edu/Associomics/Home.html), b = String (http://string-db.org/), c = Anap (http://gmdd.shgmo.org/Computational-Biology/ANAP/ANAP_V1.1/). Only interactions obtained experimentally (pull-down assay or Y2H screen) were considered. Concerning Associomics, only interactions of at least two positive screen results were assumed.

oxytocin receptor and cholecystokinin (type B) receptor (Gimpl *et al.*, 1997), of which the latter contains the CRAC motif essential for binding to cholesterol (Desai and Miller, 2012). In plants, only a limited number of proteins have been experimentally demonstrated to bind sterols. A sitosterol-binding protein, ORP3, contains an oxysterol-binding region (Saravanan *et al.*, 2009) with four putative CRAC/CARC/CARC-like motifs. Moreover, one of these motifs is contained within the conserved KPFNPLLGETF region that is shared among several oxysterol-binding proteins from *A. thaliana* and rice (Umate, 2011). Similarly, a stigmasterol- and phosphatidylethanolamine-binding protein, ROSY1, contains three putative CRAC/CARC/CARC-like motifs within its MD2 lipid-binding domain (Dalal *et al.*, 2016). Taken together, it is possible that CRAC/CARC motifs can recognize at least some phytosterols; therefore, we investigated the presence of putative CRAC/CARC motifs in *A. thaliana* flotillins, HIRs, and erlins (Figure 1). Several motifs were predicted for each protein within but also outside the SPFH domain.

E. Protein-protein interactions—flotillin mode of action

Because flotillins do not dispose of an activity like enzymes, transporters, and molecular motors, among others, their function likely consists of affecting other proteins via protein-protein interactions. First, mammalian flotillins interact with one another to form homotetramers (Neumann-Giesen *et al.*, 2004; Solis *et al.*, 2007) and heterotetramers (Solis *et al.*, 2007). Oligomerization is provided by coiled-coil structures outside of the SPFH domain within so-called flotillin domains in the C-terminal parts of the proteins (Solis *et al.*, 2007). The tetramers are quite resistant toward denaturation; they remain

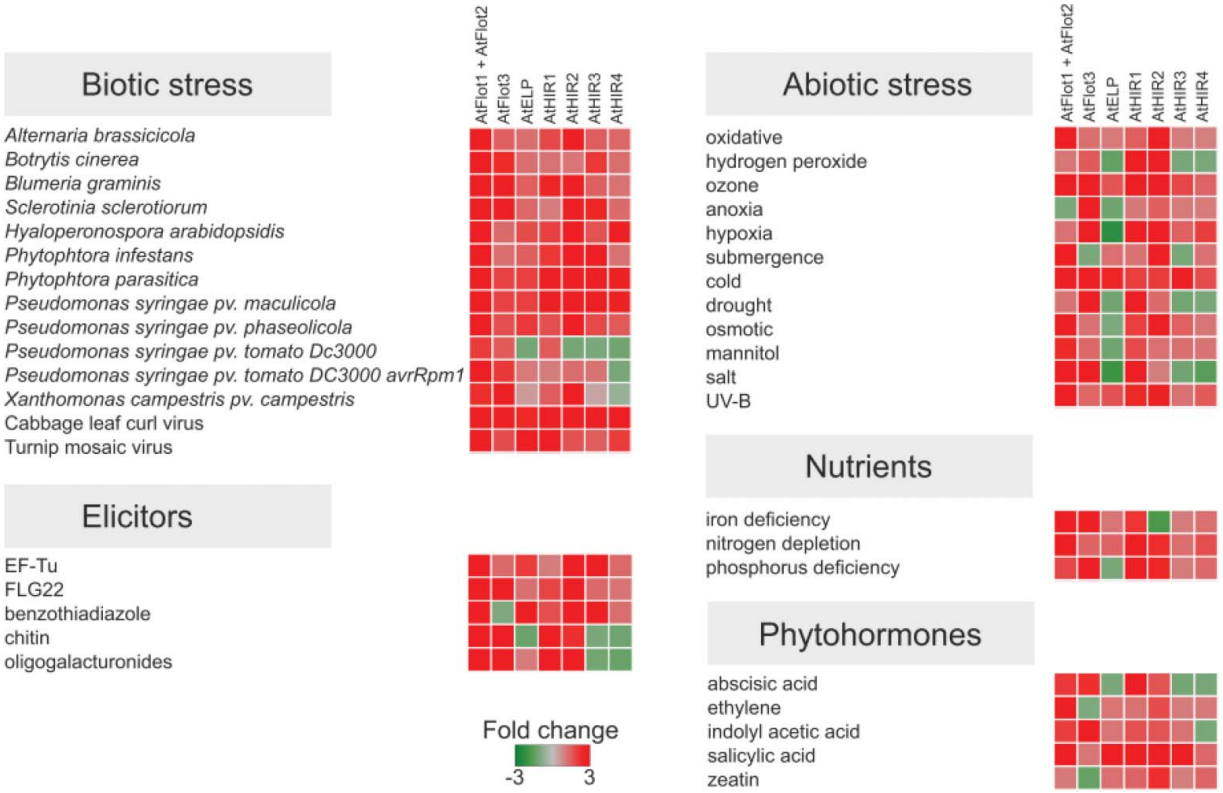

Figure 2. *Arabidopsis thaliana* SPFH gene transcription is affected by many factors. Affymetrix 22 K microarray data were retrieved from Genevestigator.

stable in 8 M urea (Solis *et al.*, 2007). The effects of mutual flotillin interactions on their localization within the plasma membrane have been established: when only one of the isoforms is overexpressed, it is evenly distributed throughout the plasma membrane, whereas under a similar expression level of both flotillins these two are predominantly concentrated and co-localize in plasma membrane microdomains (Frick *et al.*, 2007). Flotillin-2 affects Flotillin-1 stability by preventing its 26S proteasome degradation, while knockdown of Flotillin-1 had little or no effect on the Flotillin-2 concentration (Pust *et al.*, 2010; Solis *et al.*, 2007). However, endocytosis of the Flotillin-1/Flotillin-2 complex was impaired in Flotillin-1-knockout cells, demonstrating the importance of flotillin heterooligomerization for endocytosis (Babuke *et al.*, 2009). Given that there are three isoforms of flotillin homologs in the genome of *A. thaliana*, the situation in plant cells may be more complex.

Another relationship with 26S proteasome protein degradation was demonstrated via human Flotillin-1 binding to antisecretory factor (AF) protein, which displays antisecretory and anti-inflammatory features (Bjorck *et al.*, 2000; Davidson and Hickey, 2004) and has been shown to interact with the 26S proteasome (Lange *et al.*, 1999). AF is 42% identical to *A. thaliana* 26S proteasome non-ATPase regulatory subunit 4, which is also known as multiubiquitin chain binding protein 1 (At4g38630). Moreover, AtFlot2 and AtFlot3 were demonstrated to bind two proteins involved in ubiquitination and proteasome degradation (Table 1). Thus, plant flotillin activity may be controlled by ubiquitination or, moreover, flotillins themselves may be involved in regulating the degradation of other proteins.

F. Flotillins interact with kinases

One prominent group of proteins that interact with mammalian flotillins are tyrosine kinases such as epidermal growth factor receptor (EGFR) (Amaddii *et al.*, 2012), a transmembrane receptor kinase (Ullrich and Schlessinger, 1990), and Src family kinases (Neumann-Giesen *et al.*, 2007). Src proteins are nonreceptor kinases that are involved in many cellular processes, among which signal transduction is of the most substantial one (Parsons and Parsons, 2004; Sirvent *et al.*, 2015). The distribution of EGFR within the plasma membrane changes in response to stimulation with its ligand, EGF, forming clusters, and EGFR is enriched in the DRM (Roepstorff *et al.*, 2002). This clustering is impaired when Flotillin-1 is knocked down (Amaddii *et al.*, 2012). Upon stimulation with EGF, both flotillins co-precipitate with EGFR, even when EGFR kinase activity is inhibited (Amaddii *et al.*, 2012; Riento *et al.*, 2009), and are endocytosed together with EGFR from the plasma membrane

(Amaddii *et al.*, 2012; Neumann-Giesen *et al.*, 2007). This re-localization consists of the phosphorylation of Flotillin-2 (Neumann-Giesen *et al.*, 2007) but is independent of Flotillin-1 (Amaddii *et al.*, 2012). However, in Flotillin-1-knockout cells, the phosphorylation of EGFR itself is reduced (Amaddii *et al.*, 2012). Thus, flotillins mediate EGFR function and endocytosis, whereas EGFR does not affect flotillins by phosphorylating them.

Both human flotillins are phosphorylated on several Tyr residues within the SPFH domain by Fyn (Riento *et al.*, 2009) and Src (Neumann-Giesen *et al.*, 2007; Riento *et al.*, 2009) kinases belonging to Src family kinases. The phosphorylation of a specific Tyr of Flotillin-2 is necessary for endocytosis of the Flotillin-based complex (Neumann-Giesen *et al.*, 2007; Riento *et al.*, 2009). Src, Fyn, and Lyn kinases co-precipitate with flotillins (Kato *et al.*, 2006; Liu *et al.*, 2005; Neumann-Giesen *et al.*, 2007), and this interaction depends on the kinase activity of these Src family kinases (Neumann-Giesen *et al.*, 2007) and is mediated by Tyr residues of flotillins (Liu *et al.*, 2005).

Src-phosphorylated Flotillin-1 co-precipitates with the succinate dehydrogenase iron-sulfur subunit in mitochondria, and this interaction is impaired when Src kinase activity is inhibited (Ogura *et al.*, 2014). Thus, the phosphorylation of flotillins may be required for flotillins to bind to other proteins.

Although plants lack Tyr kinases, numerous dual specificity kinases have been described in *A. thaliana* that have structural similarities to animal Tyr kinases including Src, Lyn, and Fyn (Rudrabhatla *et al.*, 2006). Taken together with the observation that the proportion of phosphotyrosine among all phosphorylated amino acids in *A. thaliana* is similar to that in humans (Sugiyama *et al.*, 2008), it would be interesting to investigate whether the function of *A. thaliana* flotillins is also modulated by phosphorylation.

In plants, a pattern similar to the interaction of mammalian flotillin and EGFR induced by EGF stimulation has been described for *M. truncatula* MtFLOT4 and Lysin motif receptor-like kinase 3 (LYK3), a receptor of nodulation factor (NF) in root hairs. After stimulation of LYK3 and MtFLOT4 with NF, both proteins re-localize to the apex of the root hair and cluster and co-localize in the resulting microdomains, whereas without stimulation, they are both present in puncta that are evenly distributed throughout the plasma membrane without significant co-localization. The amount of MtFLOT4 in the plasma membrane of root hairs decreased in LYK3 kinase-inactive plants (Haney *et al.*, 2011). The LYK3 homolog in *A. thaliana* is Chitin elicitor receptor kinase 1 (At3g21630). Moreover, *AtFlot* transcription is upregulated by chitin (Figure 2). All AtFlot isoforms also bind to receptor-like kinases that can also play a role in signal transduction (Table 1).

An important type of plant kinase is wall-associated kinases (WAKs). The extracellular domain of WAK shares sequence similarity with EGFR (He *et al.*, 1996), and AtWAK3 binds to all the three isoforms of AtFlots (Table 1). Thus, it is possible that plant flotillins may be involved in cell responses to extracellular signals in a manner similar to mammalian flotillins. AtWAK1 and AtWAK2 have been reported to bind oligogalacturonides—products of cell-wall pectin cleavage caused by pathogens—and thus AtWAKs may mediate the defense response (Brutus *et al.*, 2010; Kohorn *et al.*, 2009). WAKs and WAK-likes have also been reported to be involved in mineral processing, especially heavy-metal uptake and responses (Hou *et al.*, 2005; Sivaguru *et al.*, 2003). Plant flotillin involvement in these WAK-mediated processes is supported by the upregulation of all *AtFlot* by oligogalacturonides and in response to iron and nitrogen deficiency (Figure 2).

G. Flotillin interactions with the cytoskeleton and extracellular matrix

In mammalian cells, flotillins co-localize with F-actin attached to the plasma membrane (Langhorst *et al.*, 2007; Liu *et al.*, 2005). This interaction is achieved via a multivalent adaptor protein, CAP/ponsin, which binds directly to both flotillins (Baumann *et al.*, 2000; Liu *et al.*, 2005) in their SPFH domain (Langhorst *et al.*, 2007) and actin (Liu *et al.*, 2005). Blocking actin polymerization with cytochalasin D does not affect the number or organization of flotillin domains (Langhorst *et al.*, 2007; Liu *et al.*, 2005), but their lateral mobility increases or decreases in response to disruption or enhanced polymerization of actin filaments, respectively. No such effect has been observed for microtubules in animal cells (Langhorst *et al.*, 2007).

The opposite situation is observed for the AtFlot1 diffusion coefficient, which decreases when both actin and tubulin polymerization are disrupted (Li *et al.*, 2012). Moreover, AtFlot1 vesicles co-localize with the myosin-binding protein MyoB1 (Peremyslov *et al.*, 2013). Given that movement along actin filaments is realized by myosin motor proteins (Vale, 2003), these findings suggest a possible functional linkage of plant flotillins and actin.

In animal cells, F-actin also interacts with flotillin associated with cadherin in adherens cell junctions (Guillaume *et al.*, 2013), and both flotillins directly interact with catenin, another protein in the cell junction complex (Kurrle *et al.*, 2013). Flotillins are vital for the stability and integrity of these junctions, and Flotillin-knockdown cells display aberrant junctions (Guillaume *et al.*, 2013; Kurrle *et al.*, 2013; Solis *et al.*, 2012).

Although cadherins are not present in plants (Hulpiau and van Roy, 2009), antibodies against animal cadherins and catenins display significant cross-immunoreactivity in corn, binding mainly to membrane structures (Baluska *et al.*, 1999). Catenins belong to a family of armadillo repeat (ARM)-containing proteins that are also found in plants (Coates, 2003). In *A. thaliana*, 108 ARM-containing proteins have been predicted, most of which are ubiquitin ligases (Mudgil *et al.*, 2004) associated with the plasma membrane (Vogelmann *et al.*, 2014).

As mentioned above, all three isoforms of AtFlots bind WAK3, which belongs to a family of plant kinases that mediate communication between the extracellular matrix and the cell (Wagner and Kohorn, 2001). Plant flotillins may—similarly to mammalian ones—participate in plasma membrane to cell wall communication with respect to structure formation and signaling.

H. The interactome and transcription profiles of Arabidopsis thaliana *flotillins* may indicate their additional function

Because the functions of animal flotillins consist mainly of interactions with other proteins, knowledge of plant Flotillin-interacting partners may indicate their possible roles. We analyzed the amino acid sequences of AtFlots (as well as other *A. thaliana* SPFH proteins) for coiled-coil motifs using web prediction tools. All three AtFlots contain putative coiled-coil motifs (Figure 1), suggesting a potential association with other proteins via these motifs. For three AtFlots, several interacting proteins are present in the Associomics Membrane-based Interactome Database (MIND) based on split-ubiquitin Y2H assays, thus assembling data on the interactions of membrane-bound proteins. As previously described, AtFlots interact with several receptor-like kinases. In addition, other proteins involved in cell signaling or regulation, such as Mitogen-activated protein kinase kinase kinase (MPKKK), calcium-binding proteins, or thioredoxins, have been found to bind AtFlots (Table 1).

Three proteins that interact with *A. thaliana* flotillins participate in phospholipid or sphingolipid metabolism. Because both sphingolipids and saturated phospholipids are necessary for membrane microdomain formation and composition, these interactions suggest possible roles of AtFlots in the actual microdomain establishment in terms of Flotillin-mediated modulation of proper membrane lipid composition.

As mentioned above, both animal and plant flotillins participate in clathrin-independent endocytosis. Therefore, it is not surprising that proteins involved in protein sorting (ESCRT) or vesicular transport (exocyst and SNARE subunits) interact with AtFlots.

The transcription of all *AtFlot* isoforms is highly induced under salt stress (Figure 2). AtFlot2 binds to CPA2, a monovalent cation transporter, and all three flotillin isoforms interact with cornichon, a protein of unknown function in the *A. thaliana*, an ortholog of which interacts with the potassium/sodium transporter in rice (Rosas-Santiago *et al.*, 2015). Thus, AtFlots may take part in monovalent cation uptake.

AtFlot1 co-localizes with PIP2;1 aquaporin within membrane microdomains. In response to NaCl treatment, PIP2;1 is endocytosed from the plasma membrane via a clathrin-independent pathway, i.e., a pathway that is likely mediated by AtFlot1 (Li *et al.*, 2011). Together with the considerable induction of *AtFlot*s transcription by osmotic or water stress, this observation may indicate the potential involvement of AtFlot1 in the regulation of water uptake. A NADPH oxidase, RbohD, which is known to mediate plant responses to pathogens (Pogany *et al.*, 2009; Torres *et al.*, 2002) or salt stress (Xie *et al.*, 2011) through the production of reactive oxygen species (ROS), has also been found to co-localize with AtFlot1 (Hao *et al.*, 2014). In addition, in *P. meyeri*, pollen tube PmFlot1 was associated with the same DRM fraction as NADPH oxidase (Liu *et al.*, 2009). Because ROS signaling accompanies many plant stress reactions, including hypoxia or anoxia (Pucciariello *et al.*, 2012), and single *AtFlot* isoform transcription is upregulated by oxidative stress or hypoxia and anoxia, a contribution of flotillin to this cellular event may also be possible.

III. Erlins—important players in endoplasmic reticulum signaling

Erlins were discovered in human hematopoietic cell lines (Browman *et al.*, 2006). To date, they have been further characterized only in humans and *Caenorhabditis elegans* (Hoegg *et al.*, 2012). Only one sequence of an erlin homolog—erlin-like protein (AtELP, At2g03510)—was identified in *A. thaliana* (Di *et al.*, 2010; Gehl and Sweetlove, 2014). Its transcription throughout plant development and in organs is presented in Figure 3. It is generally expressed throughout all development stages and plant organs, with maximal expression potential in siliques and the lowest values in anthers, pollen, and vegetative parts of the inflorescence. *AtELP* transcription is upregulated in response to pathogens, namely *Phytophthora parasitica*, some strains of *Pseudomonas syringae*, and viruses. Among abiotic stresses and phytohormones, cold and salicylic acid (SA) have the most prominent effects.

A. Erlins form multimeric complexes in the endoplasmic reticulum membrane

Human erlins (Erlin-1 and Erlin-2) are localized to the endoplasmic reticulum (ER) and are highly enriched in

the DRM (Browman *et al.*, 2006). A transmembrane stretch is present in front of the SPFH domains of human erlins, and the N-terminus is localized in the cytoplasm whereas most of the molecule is inside the ER lumen (Pednekar *et al.*, 2011).

Two coiled-coil stretches are present at the C-terminus of human erlins. The coiled-coil motifs mediate the formation of erlin homo and heterooligomers (Hoegg *et al.*, 2009), among which heterotrimers are the most abundant (Pednekar *et al.*, 2011). Oligomers are further organized into an approximately 2-MDa complex consisting of ca 50 monomers with an Erlin-1/Erlin-2 ratio of 1:2 (Pearce *et al.*, 2009). This structure is assembled through an "association domain" that is present in erlin molecules beyond the coiled-coil motifs (Pednekar *et al.*, 2011). The 2-MDa complex is shaped like an open ring (Pednekar *et al.*, 2011), and is stable even during sterol depletion (Hoegg *et al.*, 2009).

AtELP also contains a putative transmembrane stretch as well as a coiled-coil motif (Figure 1). These predictions suggest possible AtELP membrane localization and an ability to bind to the same or other molecules, i.e., to form homooligomers or protein complexes.

B. Erlin supercomplexes are involved in inositol trisphosphate receptor degradation

Human erlins are involved in the ER-associated degradation (ERAD) pathway, a process during which some ER proteins (typically misfolded or undergoing rapid turnover) are polyubiquitinated and consequently degraded by the 26S proteasome (Vembar and Brodsky, 2008). Erlin-2 has been found to interact with several proteins involved in ERAD as well as with the inositol trisphosphate receptor (IP3R), and Erlin-2 knockdown cells display inhibition of the polyubiquitination of some ERAD substrates including IP3R (Pearce *et al.*, 2007). Mammalian IP3R is localized in the ER membrane and releases calcium ions into the cytoplasm following IP3 stimulation (Foskett *et al.*, 2007; Taylor *et al.*, 2004). The rapid increase in the calcium concentration is known to act as a second messenger in many signalization cascades. After stimulation and calcium release, IP3R is promptly degraded via ERAD (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999). Because the 2-MDa erlin complex binds to IP3R and the extent of this interaction increases after IP3 stimulation (Pearce *et al.*, 2007, 2009; Wang *et al.*, 2009), this complex plays a key role in the signal transduction induced by IP3. The interaction takes place before the beginning of IP3R ubiquitination (Pearce *et al.*, 2007). Monomeric erlins do not bind IP3R (Pearce *et al.*, 2009). Upon suppression of erlin expression, IP3R is ubiquitinated to a substantially lower extent (Pearce *et al.*, 2007; Sanchez-Quiles *et al.*, 2010), IP3 signaling is not attenuated, the ER is stressed and the cell can be damaged (Sanchez-Quiles *et al.*, 2010).

Regarding the shape of the 2-MDa erlin complex, it has been hypothesized that the complex acts as a retrotranslocon, a structure that can extrude stimulated IP3R or other proteins from the ER membrane after their interaction or that can draw an ubiquitin ligase that recognizes IP3R and thus mediates their association (Pearce *et al.*, 2009). Binding of the 2-MDa complex and RNF170, an activated IP3R-specific ubiquitin ligase, has been established (Lu *et al.*, 2011).

IP3-induced release of calcium ions into the cytoplasm is also an important signaling event in plant cells (Alexandre and Lassalles, 1992; Malho *et al.*, 1998). Vacuolar (Allen and Sanders, 1994; Brosnan and Sanders, 1993) and microsomal fraction compartments (Dasgupta *et al.*, 1997; Muir and Sanders, 1997; Scanlon *et al.*, 1996) were found to release Ca^{2+} in response to IP3 stimulation in several plant species, suggesting that a putative plant IP3R is present in these membrane compartments. Nevertheless, a plant protein homolog of metazoan IP3R has not been discovered to date (Krinke *et al.*, 2007; Taylor *et al.*, 2004). A binding site for IP3 (but not a specific protein) was found in ER membranes (Martinec *et al.*, 2000) in *Chenopodium rubrum*. Several tens of IP3-binding proteins have recently been identified in the ER membrane of *Oryza sativa* (Nie *et al.*, 2014).

IV. Hypersensitive induced reaction proteins—modulators of plant immunity

HIRs are plant-specific members of the SPFH protein superfamily. They were originally discovered in *Zea mays* (Nadimpalli *et al.*, 2000) and were found highly homologous to a tobacco NG1 cDNA product that causes a spontaneous hypersensitive response (HR) when overexpressed in tobacco leaves (Karrer *et al.*, 1998). HIR homologs have been further identified in barley (Rostoks *et al.*, 2003), rice (Chen *et al.*, 2007; Malakshah *et al.*, 2007), tomato (Jung and Hwang, 2007), pepper (Jung *et al.*, 2008), papaya (Porter *et al.*, 2008), wheat (Liu *et al.*, 2013; Yu *et al.*, 2008; Zhang *et al.*, 2009b, 2011), *A. thaliana* (Di *et al.*, 2010), apple (Zhou *et al.*, 2012), and soybean (Koellhoffer *et al.*, 2015; Xiang *et al.*, 2015). In the Haptophyte algae *Emiliania huxleyi* and *Tisochrysis lutea*, proteins related to *A. thaliana* HIRs in terms of sequence similarity and molecular mass have been identified (Rose *et al.*, 2014; Shi *et al.*, 2015). HIRs of *A. thaliana* are denoted herein according to Qi *et al.* (2011) as follows: AtHIR1 (At1g69840), AtHIR2 (At3g01290), AtHIR3 (At5g51570), and AtHIR4 (At5g62740). The expression of single isoforms differs during plant development and in various organs (Figure 3). *AtHIR1* is expressed

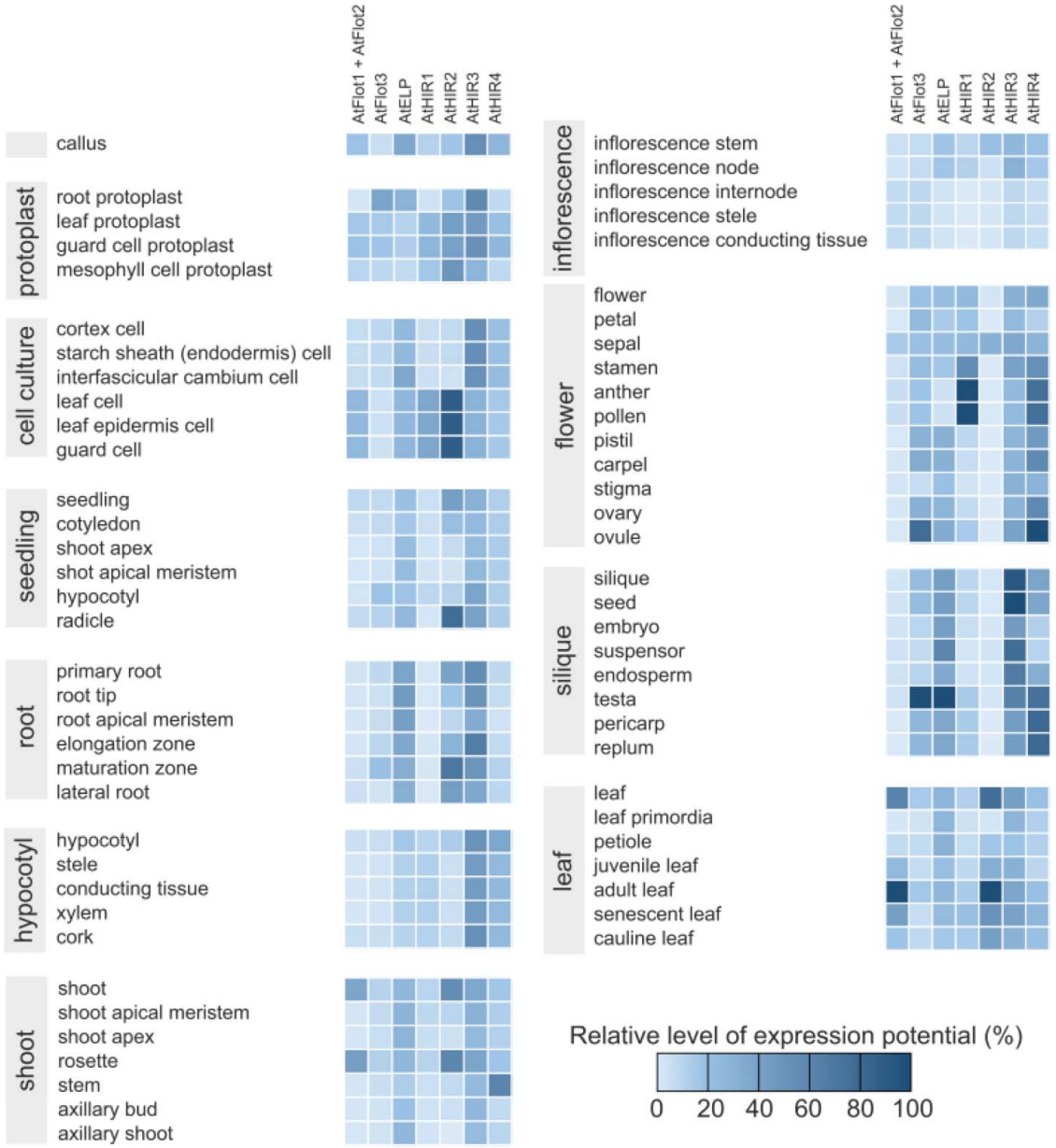

Figure 3. *Arabidopsis thaliana* SPFH genes are expressed differentially in various tissues throughout individual development. Affymetrix 22 K microarray data were retrieved from Genevestigator.

in leaves and some flower parts but is not expressed in root tissues. *AtHIR2* expression is highest in leaves and roots and almost absent in flowers and siliques. *AtHIR3* and *AtHIR4* are, to a certain extent, expressed during all developmental stages and in all organs, with the highest values exhibited in siliques and seeds or gametangia, gametophytes, and seed parts, respectively.

A. HIRs are localized on the plasma membrane

HIR proteins were found on the plasma membrane in rice (Chen *et al.*, 2007; Ishikawa *et al.*, 2015; Malakshah *et al.*, 2007), *A. thaliana* (Qi *et al.*, 2011), and pepper (Choi *et al.*, 2011), and on the tonoplast in rice (Zhou *et al.*, 2010) or the endosomes in pepper (Choi *et al.*, 2013). In wheat, TaHIR1 and TaHIR3 localize on the plasma membrane (Duan *et al.*, 2013), whereas TaHIR2 are present in the cell interior (Zhang *et al.*, 2011).

In pepper, the association of CaHIR1 with the plasma membrane is provided by the SPFH domain (Choi *et al.*, 2011). Zhou *et al.* (2010) and Xiang *et al.* (2015) predicted putative myristoylation sites and transmembrane stretches in the N-terminus for several isoforms of HIRs in soybean, rice, maize, wheat, and barley. However, our search using web prediction tools did not reveal any transmembrane stretches in the amino acid sequences of

any of the four HIR isoforms of *A. thaliana*, whereas N-terminal putative myristoylation and/or palmitoylation sites were identified for all of them (Figure 1). HIR-related protein was found to be a major membrane microdomain protein in *E. huxleyi* (Rose *et al.*, 2014). AtHIR1, AtHIR2, and AtHIR3, as well as OsHIR1, OsHIR3, and OsHIR5, were found in DRMs (Ishikawa *et al.*, 2015; Minami *et al.*, 2009). The association of AtHIRs with the DRM is not altered following sterol depletion induced by methyl-β-cyclodextrin (Kierszniowska *et al.*, 2009).

B. HIRs are upregulated in response to pathogen attack

Plant immunity consists mainly of two types of defense mechanisms corresponding to two types of molecules that are produced by pathogens. The so-called pathogen (or microbe)-associated molecular patterns (PAMPs or MAMPs) such as flagellin, elongation factor thermo unstable (EF-Tu), peptidoglycan, chitin, lipopolysaccharide, and glucan of pathogen cell walls, are generally conserved across pathogen taxa (Chisholm *et al.*, 2006) and play a role as elicitors. PAMPs are recognized by plant pathogen recognition receptors (PRRs) localized at cell surfaces (Jones and Dangl, 2006). The action initiated by PRRs results in the activation of innate immune response termed PAMP-triggered immunity (PTI) (Dangl and Jones, 2001).

To overcome PTI, pathogens have evolved another type of molecule that disrupts early steps in the PRR-induced response (He *et al.*, 2006). These so-called effectors or plant proteins damaged by these effectors are recognized by resistance proteins (R proteins) in plants (Chisholm *et al.*, 2006). Effectors are race or strain-specific, and their presence or absence in a pathogen determines its (a)virulence. An effector that is recognized by host R protein is called an avirulence protein because it provokes a supposed effector-triggered immunity (ETI) response in the host plant (Dangl and Jones, 2001). ETI comprises the production of ROS, reactive nitrogen oxide intermediates, and increased levels of the defense phytohormone jasmonic acid (JA) (Coll *et al.*, 2011), eventually leading to HR. SA signaling and the subsequent upregulation of pathogenesis-related proteins (Stintzi *et al.*, 1993) is involved in both the ETI and the PTI pathways (Janda and Ruelland, 2015).

HIR protein expression is generally increased during bacterial (Chen *et al.*, 2007; Jung and Hwang, 2007; Qi *et al.*, 2011; Zhou *et al.*, 2010) or fungal (or oomycetes) infection (Chen *et al.*, 2012; Liu *et al.*, 2013; Porter *et al.*, 2008; Xiang *et al.*, 2015; Yu *et al.*, 2008, 2013; Zhang *et al.*, 2009b, 2011; Zhou *et al.*, 2012). *HIR* upregulation is usually induced to a much greater extent by avirulent pathogens strains during incompatible interactions than by virulent strains during compatible interactions; however, both virulent and avirulent strains cause an increase in *HIR* expression (Chen *et al.*, 2007, 2012; Jung and Hwang, 2007; Liu *et al.*, 2013; Yu *et al.*, 2008, 2013; Zhang *et al.*, 2009b, 2011; Zhou *et al.*, 2010).

Genevestigator microarray transcription data (Figure 2) also show the upregulation of at least some HIR isoforms in response to several pathogen types including fungi (*Alternaria brassicae, Botrytis cinerea, Blumeria graminis*, and *Sclerotinia sclerotiorum*), oomycetes (*Hyaloperonospora arabidopsidis* and *Phytophthora infestans*), bacteria (*P. syringae* pv. *maculicola* and *phaseolicola, Xanthomonas campestris* pv. *campestris*), and several viruses. Interestingly, the effect of both virulent and avirulent strains of *P. syringae* pv. tomato on the transcription of AtHIR1, AtHIR2, and AtHIR3 (Qi *et al.*, 2011) is rather insignificant. In *E. huxleyi* viral infection causes changes in the size distribution of membrane microdomains defined by HIR-related protein (Rose *et al.*, 2014). Possible involvement of HIRs or HIR-related proteins in response to viral infection is consistent with highly upregulated AtHIR transcription in response to cabbage leaf curl virus or turnip mosaic virus infection (Figure 2).

HIR transcription is also induced by elicitors. *AtHIR1, AtHIR2*, and *AtHIR3* transcription is upregulated by flg22 peptide (Qi *et al.*, 2011), chitin, and EF-Tu (Figure 2). *CaHIR1* transcription is increased by infiltration of the purified Filamentous hemagglutinin-like protein (Fha1) of *Xanthomonas campestris* pv. *vesicatoria* into pepper leaves (Choi *et al.*, 2013). *CaHIR1* transcription is also increased in pepper leaves overexpressing INF1 elicitin of *Phytophthora capsici* (Feng *et al.*, 2014). Both Fha1 and INF1 elicit necrosis and HR in pepper leaves (Feng *et al.*, 2014; Choi *et al.*, 2013).

The effect of stressors and phytohormones on *HIR* expression seems to be specific for species and isoform. *OsHIR1* expression is induced by salt stress (100 mM NaCl) in rice (Malakshah *et al.*, 2007), whereas salt treatment (200 mM NaCl), cold stress (4°C), and osmotic stress (20% PEG6000) in wheat decrease the transcription of *TaHIR1* and *TaHIR3* (Duan *et al.*, 2013). *TaHIR1* and *TaHIR3* transcription are also reduced by ethylene (100 μM) and ABA (100 μM), with only a minor effect of MeJA (100 μM) or SA (2 mM) application (Duan *et al.*, 2013). This phenomenon is in contrast to the situation in pepper, in which SA (5 mM), MeJA (100 μM), ABA (100 μM), and ethylene (5 ppm) application increased *CaHIR1* transcription (Jung and Hwang, 2007). In soybean, hydrogen peroxide treatment (100 μM) repressed the transcription of *GmHIR1,*

GmHIR3, and *GmHIR4* (Xiang *et al.*, 2015). *AtHIR* transcription is upregulated by SA, a phytohormone that is involved in the plant response to pathogens, and zeatin. The effects of oxidative and osmotic stress, ozone, hypoxia UV-B, and cold seem to be similar for all *AtHIR* isoforms, whereas hydrogen peroxide, drought, salt, and ABA have opposite impacts on different isoforms, i.e., they increase or decrease the transcription of AtHIR1 and AtHIR2, or AtHIR3, and AtHIR4, respectively (Figure 2).

C. Overexpression of HIRs promotes the hypersensitive response

The HR regulates cell death at the site of infection, resulting in the restriction of pathogen growth and spreading. It typically occurs during incompatible interactions between plants and pathogens, and is manifested by plasma membrane depolarization and potassium ion efflux into the intercellular space (Atkinson *et al.*, 1990), an oxidative burst (Torres *et al.*, 2006), and callose deposition (Cuypers and Hahlbrock, 1988). These processes are mediated by SA, ABA, JA, and ethylene signaling (Fujita *et al.*, 2006). In pepper overexpressing *CaHIR1*, increased callose deposition and electrolyte leakage, and reduced SA content are observed in the absence of a pathogen (Choi *et al.*, 2011). Interestingly, when *CaHIR1* is expressed in *A. thaliana*, the plants exhibit elevated SA and hydrogen peroxide contents, a higher potassium ion concentration in the intercellular space, and constitutive upregulation of pathogenesis-related proteins (Jung and Hwang, 2007). *OsHIR1* expression in *A. thaliana* results in the spontaneous formation of lesions and a constitutive increase in *PR* expression (Zhou *et al.*, 2010). Barley lesion mimic mutants, i.e., mutant lines exhibiting constitutive necrosis formation, were found to constitutively overexpress *HvHIR1, HvHIR2, HvHIR3*, and *HvHIR4* (Rostoks *et al.*, 2003; Wright *et al.*, 2013). These lesion mimic mutants are more resistant to virulent biotrophic *Puccinia hordei*, but they have been reported to have an increased susceptibility to necrotrophic *Pyrenophora teres* f. sp. *teres* (Wright *et al.*, 2013). This observation suggests that HIR proteins may play a role in the crosstalk between SA and JA. SA is generally responsible for defense responses to biotrophs, whereas JA is involved in defense responses to necrotrophs (Glazebrook, 2005). Ectopic expression of *OsHIR1* and *CaHIR1* and overexpression of *AtHIR1* and *AtHIR2* lead to increased resistance to virulent *P. syringae* pv. tomato DC3000 (Jung and Hwang, 2007; Qi *et al.*, 2011; Zhou *et al.*, 2010). T-DNA-knockout lines of *AtHIR2* and *AtHIR3* consistently exhibit reduced resistance to avirulent *P. syringae* pv. tomato AvrRpt2 (Choi *et al.*, 2011). Similarly, virus-silenced (VIGS) *TaHIR1*- and *TaHIR3*-knockdown leaves have a less intense HR, better pathogen performance and an overall decrease in *PR* gene transcription compared with the control after infection with the avirulent strain of *Puccinia striiformis f. sp. tritici*. In contrast, infection of VIGS-*CaHIR1*-pepper leaves by both virulent and avirulent strains of *Xanthomonas campestris* pv. *vesicatoria* leads to reduced callose deposition and electrolyte leakage but an increased level of SA and the transcription of PR genes, which together result in improved pathogen resistance (Choi *et al.*, 2011).

In rice cells overexpressing BI-1, a cell death suppressor that increases plant tolerance to several stresses and signalization of which involves ROS formation, the association of OsHIR1, OsHIR3, and OsHIR5 as well as OsFlot with the DRM is markedly reduced, although their transcription level is not affected. Accordingly, knockdown or KO mutants of *OsHIR3* (or *OsFlot*) exposed to oxidative stress (induced by menadione or SA) exhibit reduced cell death (Ishikawa *et al.*, 2015). Oxidative stress generally increases *AtHIR* transcription (Figure 2), and because the oxidative burst is one of first steps in the HR, it is possible that the action of HIR occurs through ROS production and that proper functioning of HIRs may be dependent on their localization in the membrane microdomains.

D. HIRs physically and functionally interact with leucine-rich repeat proteins

Similar to mammalian flotillins and erlins, all isoforms of AtHIRs form homo and heterooligomers *in vitro*. All possible pairwise combinations of single isoforms have been detected (Qi *et al.*, 2011). Oligomerization may lead to the clustering of HIR molecules, giving rise to the protein scaffolds of microdomains. Binding of AtHIRs to other protein molecules may occur in coiled-coil regions within AtHIR molecules. Using web predictors, coiled-coil motifs were identified in amino acid sequences of all AtHIR isoforms with the exception of AtHIR3 (Figure 1)

HIRs have been reported to physically and functionally interact with leucine-rich repeat (LRR) superfamily proteins. In rice, an interaction of OsHIR1 with OsLRR1 was found in a Y2H screen. Furthermore, a Y2H screen showed that OsHIR1 can bind to AtLRR1 (At5g21090, an OsLRR1 homolog) and OsLRR1 can bind to AtHIR1 (Zhou *et al.*, 2009). Overexpression of *OsLRR1* upregulates *OsHIR1* expression and increases the localization of OsHIR1 on the plasma membrane (Zhou *et al.*, 2010). Similarly, CaLRR1 interacts with CaHIR1 in plasma membrane microdomains in pepper leaves and *CaLRR1* overexpression results in increased *CaHIR1* transcription (Choi *et al.*, 2011). Binding to CaHIR1 is provided by the

LRR domain of CaLRR1 (Jung and Hwang, 2007). Ectopic expression of *OsLRR1* in *A. thaliana* or overexpression of *CaLRR1* in pepper lead to the upregulation of PR genes and increase resistance of leaves to the pathogens *Xanthomonas campestris* pv. *vesicatoria* and *P. syringae* pv. tomato DC3000, respectively (Choi *et al.*, 2011; Zhou *et al.*, 2009, 2010). By contrast, *CaLRR1* is downregulated in response to *CaHIR1* overexpression. In VIGS-*CaHIR1* leaves, the level of *CaLRR1* transcription together with the SA content is increased to a much greater extent during incompatible interactions, which result in increased pathogen resistance (Choi *et al.*, 2011). Thus, LRR proteins stimulate the increased HIR protein level in rice and pepper, while HIR negatively regulates LRR expression in pepper. LRR seems to attenuate the function of HIR in the development of necrosis because the co-overexpression of *CaHIR1* and *CaLRR1* results in a less intense HR compared with *CaHIR1* overexpressing leaves during treatment with benzothiadiazole, an HR elicitor (Jung and Hwang, 2007).

AtHIR1 and AtHIR2 were found to bind RPS2 (At4g26090) (Qi *et al.*, 2011). RPS2 belongs to the nucleotide-binding-LRR subclass of LRR proteins (Bent *et al.*, 1994). It is a resistance protein that binds to RIN4 protein (At3g25070), a target of the bacterial effector AvrRpt2. When RIN4 is absent, RPS2 promotes HR (Axtell and Staskawicz, 2003; Mackey *et al.*, 2003). Thus, AtHIR1 and AtHIR2 play an important role in ETI.

E. HIRs are parts of protein complexes

As mentioned above, AtHIR1 and AtHIR2 were found to be components of a RPS2-based protein complex that also contained RIN4; a receptor-like kinase (At4g08850); aquaporin PIP1;2 (At2g45960); phototropin 1 and 2 (At3g45780 and At5g58140); patellin-1 (At1g72150)—a phosphoinositide-binding carrier protein; epithiospecifier modifier 1 (At3g14210), which mediates isothiocyanate production during glucosinolate hydrolysis; and heavy metal ATPase 3 (At4g30120) (Qi and Katagiri, 2009). Because these proteins were co-purified together with RPS2, it is possible that at least some of the proteins within this protein complex interact with AtHIR1 or AtHIR2. The transcription of both *AtHIR1* and *AtHIR2* increases during drought and salt stress, whereas the other two isoforms are slightly downregulated under these conditions (Figure 2). Together with the mentioned co-purification of both isoforms in a complex with PIP1;2, these findings suggest a possible effect of AtHIR1 and AtHIR2 on water uptake or transport. Similarly, within the HIR-related protein-based microdomains of *E. huxleyi*, several co-occurring proteins have been identified, including porin, the H^+-PPase pump, the mitochondrial import receptor subunit, heat shock protein, nitrate transporters, actin, histone, and many chloroplast proteins (Rose *et al.*, 2014).

Phospholipase D δ (PLDδ, At4g35790) has been reported to pull-down AtHIR1 together with clathrin heavy chain (At3g08530/At3g11130), heat shock protein 70 (At1g56410/At3g09440/At3g12580/At5g02490/At5g02500), ATP synthase subunits α (AtMg01190) and β (At5g08670/At5g08680/At5g08690), actin 7 (At5g09810), and β-tubulin (At4g20890/At5g23860/At5g44340/At5g62690/At5g62700) (Ho *et al.*, 2009). PLDδ is involved in several signaling processes, including drought, cold and frost, salt and water stress, oxidative and UV-B-induced damage, and biotic stress, particularly during the penetration of fungi into plants (Bargmann *et al.*, 2009; Katagiri *et al.*, 2001; Kawamura and Uemura, 2003; Li *et al.*, 2004; Pinosa *et al.*, 2013; Wang, 2005), and this interaction may suggest a broader-spectrum function of AtHIR1. This suggestion is supported by the altered *AtHIR* transcription in response to UV-B, cold, drought, salt, and oxidative stress (Figure 2). AtPLD participates in the maintenance of root elongation during phosphate deficiency (Li *et al.*, 2006a) by recycling phosphate via phospholipid cleavage, which is then replaced with galactolipids in membranes (Cruz-Ramirez *et al.*, 2006; Li *et al.*, 2006a, b). The transcription of all four AtHIR isoforms increases in response to phosphate deficiency (Figure 2), suggesting the involvement of AtHIR in this type of adaptation to low nutrient conditions.

The AtHIR4 content in DRM is reduced and its content in the detergent-soluble membrane fraction is increased after treatment with cytochalasin D, an actin polymerization inhibitor (Szymanski *et al.*, 2015). Because AtHIR1 was found in the AtPLDδ interactome together with actin 7 (Ho *et al.*, 2009), it is possible that AtPLDδ functions as a bonding bridge between AtHIRs and actin filaments. This interaction may determine the AtHIR microdomain pattern and its possible rearrangement during stress signaling. AtHIRs may be either directed to microdomains via oriented transport along actin filaments and/or retained at certain positions by actin bundles in cortical cytoplasm adjacent to the plasma membrane (Szymanski *et al.*, 2015). AtHIRs have been found to bind to AtAHK2, a cytokinin receptor, and Sieve element occlusion related protein 2 that participates in P-protein formation (Table 1). Considering that *AtHIR2* is upregulated by zeatin (Figure 2), these results suggest the potential involvement of AtHIR in cytokinin signaling.

V. Conclusions

Mammalian flotillins have recently been proven to play roles in many important processes, including membrane microdomain organization, endocytosis, signal transduction, cholesterol uptake, or intercellular communication.

Human erlins are involved in the modulation of IP3 signaling and ER-associated protein degradation. In plants, less is known about flotillins, and there is a complete lack of information for erlin.

However, plant flotillins exhibit features similar to human ones in terms of their occurrence in membrane microdomains, participation in clathrin-independent endocytosis or interactions with receptor kinases. Experimentally obtained data for the binding of *A. thaliana* proteins to flotillins and for the expression of AtFlot suggest the presence of additional functions such as cell signaling, pathogen responses, water and/or ion uptake control, vesicular transport, and protein trafficking. AtFlot1 has been shown to be involved in sterol uptake via the plasma membrane, and AtFlot2 and AtFlot3 have been observed to bind phospholipases and sphingosine kinase. AtFlots may thus affect three lipid components that are all established to increase membrane order and are pivotal for the membrane microdomain identity. Thus, flotillins may be crucial for determining these microdomains.

In most cases, HIR proteins present on the plasma membrane have been shown to promote the hypersensitive response after bacterial or fungal infection, thus playing an important role in plant resistance to pathogens. HIRs have been confirmed to interact with LRRs in pepper, rice, and *A. thaliana*. The ratio of HIR to LRR expression affects the plant response to infection. *A. thaliana* HIRs are part of protein complexes containing phospholipase D, cytoskeletal proteins, and ATPases, among others. The transcription data support the possible involvement of AtHIRs (apart from the response to pathogen attack) in some abiotic stress reactions, such as cold, UV-B, hyperosmotic stress, nutrient deficiency, and both oxidative stress and hypo/anoxia, as well as in hormone signaling. Moreover, two AtHIR isoforms bind to the cytokinin receptor.

VI. Future perspectives

To better understand the functions of plant flotillin, the first step would be experimental *in planta* verification of the putative protein interactions of AtFlots (Table 1) using fluorescence and confocal microscopy techniques such as Förster resonance energy transfer (FRET), fluorescence lifetime imaging (FLIM) or bimolecular fluorescence complementation (BiFC). Because these techniques are based on different principles, each is faced by specific limitations. FRET and FLIM allow the detection of direct interactions because they are sufficiently effective for identifying a distance between fluorophores that does not exceed 10 nm. To obtain relevant results, the concentration of fluorescent-labeled interaction partners must be high enough to permit clear detection. However, assuming that AtFlots are present at the plasma membrane in spatially limited microdomains, expression levels and molecule contents that are too high could lead to "crowding" of the molecules within these areas, resulting in false-positive FRET. False-positive as well as false-negative FRET results may also be obtained when the expression of the donor and acceptor-fused proteins is unbalanced. Thus, great care must be taken in terms of the gene expression of the protein partners (Xing *et al.*, 2016). Cloning both partners fused to fluorophores within one expression cassette or under one promoter in multicistronic vectors containing the sequence for 2A self-cleaving peptide may aid in preparing transformants with a suitable expression ratio.

BiFC is prone to exhibiting false-positive results because of irreversible self-assembly of the fluorophore employed, which is why it is vital to apply the appropriate negative controls and rather low expression levels by using, e.g., native promoters. However, BiFC may enable the detection of transient and weak interactions because they are stabilized through the re-assembled fluorophore. It can also be applied to visualize indirect interactions between proteins (Xing *et al.*, 2016). Ratiometric BiFC (rBiFC) involving internal control of the transformation and expression efficiency and thus allowing proportional quantification should be used preferentially (Grefen and Blatt, 2012). When applying BiFC to membrane-anchored proteins (i.e., flotillins and HIRs), steric constraints that impede split fluorophore reassembly may occur because the anchored proteins may not freely rotate and the split fluorophore fragments may not be able to assume the appropriate position for reassembly. This phenomenon can be, to a certain extent, overcome by using sufficiently long linkers.

Both FRET/FLIM- and BiFC-positive results should be verified by co-immunoprecipitation assays. In addition to the widely used western blot detection, single-molecule pull-down assays using protein bait immobilized on a microscopic slide and total internal reflection fluorescence imaging (TIRF) were also recently introduced in plant science, allowing the determination of not only the interaction itself but also its dynamics and the stoichiometry of the proteins that form the complexes (Husbands *et al.*, 2016).

The dynamics of these interactions (e.g., dissociation or formation of the protein complexes) under various conditions can be further addressed using particle analysis tools such as fluorescence cross-correlation spectroscopy (FCCS), which analyzes the fluctuation of fluorescent particles in a small defined confocal volume and thus provides information about the particle dynamics or concentration on a single-molecule level within microsecond-to-second

timescales. Its application is, however, limited to particles with a diffusion coefficient of no less than 0.1 $\mu m^2\ s^{-1}$. Thus, it is biased toward faster moving molecules when used to measure lateral mobility due to photobleaching of the slow and methodological "invisibility" of immobile molecules lingering within the confocal volume. In fact, this phenomenon might concern at least some AtFlots because the AtFlot1 diffusion coefficient has been approximately hundredths of $\mu m^2\ s^{-1}$ under different conditions, as determined by TIRF acquisition and subsequent tracking of fluorescent foci (Li *et al.*, 2012). In the case of stable molecules and complexes, raster image cross-correlation spectroscopy (RICCS) permits the analysis of particle diffusion coefficients as low as 0.001 $\mu m^2\ s^{-1}$ (Digman *et al.*, 2005). The correlation spectroscopy methods require low (typically nanomolar) concentrations of fluorophores, supporting the use of the native promoters of the analyzed proteins, which would also avoid possible artifacts caused by overexpression. Both methods have been previously applied in analyses of plant membrane proteins (Lankova *et al.*, 2016; Li *et al.*, 2013).

Because many proteins are known to form membrane microdomains, the question is whether some of these microdomains overlap with SPFH protein-based ones. Electron microscopy and super-resolution imaging techniques could be a great asset to more precisely describe the organization of plant flotillin and HIR microdomains. For example, the stimulated emission depletion technique can reveal a substantially smaller size of potato remorin microdomains compared with the same microdomain size obtained using "standard" confocal microscopy (Demir *et al.*, 2013).

The previously reported involvement of AtFlot1 microdomains in endocytosis requires further exploration. One strategy is to focus on differences between flotillin and the well-described clathrin-mediated endocytosis in terms of their cargo specificity or their dependence on cytoskeleton or phosphorylation (which is known to be important for flotillin endocytosis in mammalian cells). The latter two may be addressed using specific inhibitors and cytoskeletal drugs.

Because membrane microdomains are characterized by their distinct lipid composition, it would be very interesting to assess the ability of plant SPHF proteins to bind these lipids. Because we revealed several CRAC/CARC motifs within each *A. thaliana* SPFH protein sequence, detection of phytosterol binding to these proteins should be explored. Another important group of membrane lipids that seems to be associated with plant flotillins are sphingolipids. AtFlot3 binds to sphingosine kinase, and concomitant alteration of glucosylceramide and rice flotillin and HIR content is detected in the DRM. Binding assays using lipid strips and purified proteins can be used to determine whether plant SPFH proteins really bind to these membrane lipids. Surface plasmon resonance would be the next step to better characterize the binding process. Moreover, in the case of cholesterol, it would be possible to explore whether the CRAC/CARC motifs serve to bind to phytosterols in plants because these motifs have, to date, only been reported to bind cholesterol in animal cells.

One of the properties in which defined membrane microdomains may differ is the lipid order. An interesting question emerges concerning whether this difference is a prerequisite for plant SPFH protein localization or *vice versa*. Using cells with different expression levels of plant SPFH proteins (i.e., loss-of-function mutants, ami-RNA lines, and (inducible) overexpressors, among others) and simultaneous monitoring of local fluctuations in the membrane lipid order using fluorescent dyes that exhibit different emission spectra when incorporated into a more ordered or disordered membrane environment (e.g., Laurdan, di-4-ANEPPDHQ) is an effective tool for ascertaining the relationship between flotillins and HIRs and the establishment of areas of different lipid orders in membranes.

Ubiquitination is a substantial pathway that controls specific protein activity. The prominent role of the mammalian erlin complex in ubiquitination and degradation of activated IP3R could be a clue for discovering the identity of plant IP3R, which has not yet been found, although its activity, which is similar to that in animals, has been well reported in plants. A thorough analysis of the ubiquitinated proteome and its differences in wild type compared with KO *AtELP* might identify the protein targets of AtELP-mediated ubiquitination activity, which might include a putative IP3R. However, this approach assumes the same activity of mammalian erlin and its plant homolog toward mammalian IP3R and its plant counterpart. This assumption may not reflect the real situation because plant IP3R is not likely to be a sequence homolog of mammalian IP3R. AtFlot2 and AtFlot3 also bind to proteins involved in ubiquitination and proteasome degradation. An investigation of protein targets (using the same approach applied for AtELP) and their function can also suggest the possible involvement of AtFlots in these functions. Moreover, ubiquitination also serves as a signal that results in protein internalization, as reported for the brassinosteroid receptor and the iron transporter (Barberon *et al.*, 2011; Martins *et al.*, 2015). An analysis of the ubiquitinated proteome obtained from immunopurified AtFlot-positive endosomes may thus be a possible way to identify cargo proteins that have been internalized via AtFlot-dependent endocytosis.

Abbreviations

ABA	abscisic acid
AF	antisecretory factor
AHK2	histidine kinase 2
ARM	armadillo repeat
AtELP	*Arabidopsis thaliana* erlin-like protein
BFA	brefeldin A
CARC	inverted CRAC
CRAC	cholesterol recognition/interaction amino acid consensus sequence
DRM	detergent-resistant membranes
EF-Tu	elongation factor thermo unstable
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated degradation
ESCRT	endosomal sorting complexes required for transport
ETI	effector-triggered immunity
Fha1	filamentous hemagglutinin-like protein 1
FM4-64	N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide
GFP	green fluorescent protein
GLUT4	glucose transporter type 4
GPI	glycophosphatidylinositol
HR	hypersensitive response
HIR	hypersensitive induced reaction protein
IP3	inositol 1,4,5-trisphosphate
IP3R	inositol 1,4,5-trisphosphate receptor
JA	jasmonic acid
LRR	leucine-rich repeat
LYK3	lysin motif receptor-like kinase 3
MAMP	microbe-associated molecular pattern
MeJA	methyl jasmonate
MyoB1	myosin-binding protein 1
NPC1L1	Niemann-Pick C 1-like1
PAMP	pathogen-associated molecular pattern
PHB	prohibitin homology
PLDδ	phospholipase D δ
PIP2;1	plasma membrane intrinsic protein 2;1
PIP1;2	plasma membrane intrinsic protein 1;2
PRR	pathogen recognition receptor
PR	pathogenesis-related
PTI	PAMP-triggered immunity
RbohD	respiratory burst oxidase D
RIN4	resistance to *Pseudomonas syringae* pv. *maculicola* 1-interacting protein 4
ROS	reactive oxygen species
RPS2	resistance to *Pseudomonas syringae* protein 2
SA	salicylic acid
SNARE	soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor
SPFH	stomatin/prohibitin/flotillin/HflK/C
VIGS	virus-silenced gene silencing
WAK	wall-associated kinase
Y2H	yeast two-hybrid screening
YFP	yellow fluorescent protein

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Declaration of interest

The authors declare that there are no conflicts of interest.

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