Chapter 9
Molecular Mechanisms Regulating Root Hair Tip Growth: A Comparison with Pollen Tubes

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Abstract The developmental program of roots is constantly modified according to environmental signals and often includes an elevation in the density of root hairs, which increases the root's absorptive surface in an attempt to meet the ion and water demands of the plant. Root hairs emerge from certain epidermal cells and this depends on a complex genetic cascade. Once this has determined root hair cell fate, local wall loosening and turgor pressure initiate a bulge in the cell wall. The transition from root hair initiation to actual tip growth begins with the accumulation of secretory vesicles at the apical part of the bulge. A complex interplay between ion oscillations, cytoskeleton architecture, vesicle trafficking, cell wall metabolism and hormonal and environmental signals allows the root hair to maintain growth at the tip. This review summarizes the current knowledge on the core components regulating root hair tip growth, critically identifies challenges for future research and points to commonalities and differences with the current knowledge on pollen tube tip growth.

Keywords Arabidopsis • Calcium • Cell wall • Cytoskeleton • Elongation • Pollen tube • Root hair • ROPs • ROS • Tip growth

Abbreviations

ACA	autoinhibited Ca^{2+}-ATPase
ACT	ACTIN
ADF	actin-depolymerizing factor

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G. Obermeyer, J. Feijó (eds.), *Pollen Tip Growth*,
DOI 10.1007/978-3-319-56645-0_9

AFs	actin filaments
AHA	*Arabidopsis* H^+-ATPase
AIP1	AKT1 INTERACTING PROTEIN PHOSPHATASE 1
ANX	ANXUR
ARK1	ARMADILLO REPEAT-CONTAINING KINESIN 1
ARP2/3 complex	actin-related protein 2/3 complex
ATPase	adenosine triphosphatase
ATSFH1	ARABIDOPSIS THALIANA SHORT ROOT HAIR 1
AtSTP6	ARABIDOPSIS THALIANA SUGAR TRANSPORTER 6
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
CA	constitutively active
$[Ca^{2+}_{cyt}]$	cytoplasmic calcium concentration
$[Ca^{2+}_{ER}]$	endoplasmic reticulum calcium concentration
$[Ca^{2+}_{ext}]$	extracellular calcium concentration
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CAP1	$[CA^{2+}]$CYT-ASSOCIATED PROTEIN KINASE 1
CBLs	calcineurin B-like proteins
CBM3a	carbohydrate-binding module 3a
CDPK	calcium-dependent protein kinase
CESA	CELLULOSE SYNTHASE
CIPKs	CBL-interacting protein kinases
$[Cl^-_{cyt}]$	chloride concentration cytoplasmic
CMLs	calmodulin-like proteins
CMTs	cortical microtubules
CNGC	cyclic nucleotide-gated channel
cNMP	cyclic nucleotide
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPK3	CALCIUM-DEPENDENT PROTEIN KINASE 3
CRIB	Cdc42- and Rac-interactive binding
CrRLK1Ls	*Catharanthus roseus* RLK1-like kinases
CSC	cellulose synthase complex
CSLD	CELLULOSE SYNTHASE-LIKE D
CytB	cytochalasin B
DACC	depolarization-activated calcium channel
DCB	2,6-dichlorobenzonitrile
DN	dominant negative
DPI	diphenyleneiodonium
ECA1	ER-type Ca^{2+}-ATPase
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EMTs	endoplasmic microtubules
EXP7	EXPANSIN7
F-actin	filamentous actin
FER	FERONIA
FH1 domain	formin homology 1 domain

FH2 domain	formin homology 2 domain
FRET	Förster resonance energy transfer
G-actin	globular actin
GAE6	UDP-D-GLUCURONATE 4-EPIMERASE 6
GAP	GTPase-activating protein
GDI	guanosine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GLR	glutamate receptor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HACC	hyperpolarization-activated calcium channel
HAK5	HIGH-AFFINITY K^+ TRANSPORTER 5
HEK	human embryonic kidney
HG	homogalacturonan
IP_3	inositol trisphosphate
LatB	latrunculin B
LePT1	*Lycopersicon esculentum* phosphate transporter 1
LRX1	LEUCINE-RICH REPEAT/EXTENSIN 1
MAPK/MPK	MITOGEN-ACTIVATED PROTEIN KINASE
MCA1/2	MID1-COMPLEMENTING ACTIVITY 1/2
MICU	mitochondrial Ca^{2+} uniporter
MOR1	MICROTUBULE ORGANIZATION 1
MRH2	MORPHOGENESIS OF ROOT HAIR 2
MSL2/3	MSCS-LIKE 2/3
MT	microtubules
MyoB1/2	myosin-binding proteins 1/2
NHX1-4	SODIUM HYDROGEN EXCHANGER 1-4
NOX	NADPH oxidase
ORF	open reading frame
OXI1	OXIDATIVE SIGNAL-INDUCIBLE1
PH	pleckstrin homology
pH_{cyt}	cytoplasmic pH
pH_{ext}	extracellular pH
PIN2	PIN-FORMED 2
PI-4Kβ1	PHOSPHATIDYLINOSITOL 4-OH KINASE β1
PI-4P	phosphatidylinositol 4-phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-biphosphate
Plus(+) end	barbed actin filament end
PM	plasma membrane
PME	pectin methylesterase
PMEI	pectin methylesterase inhibitor
prf1	profilin 1
PRONE	plant-specific ROP nucleotide exchanger
PT	pollen tube

qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RabA4b	RAB GTPASE HOMOLOGUE A4B
RBOH	RESPIRATORY BURST OXIDASE HOMOLOGUE
RH	root hair
RHD2	ROOT HAIR DEFECTIVE 2
RHM1	RHAMNOSE BIOSYNTHESIS 1
RHS	root hair specific
RHS8	ROOT HAIR SPECIFIC 8
RIC	ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN
RIP	ROP INTERACTIVE PARTNER
RLK	receptor-like kinase
RNAi	RNA interference
ROPs	Rho-like GTPases from plants
ROS	reactive oxygen species
SCN1	SUPERCENTIPEDE1
SIMK	STRESS-INDUCED MAPK
SLAH3	SLAC1 HOMOLOGUE 3
SOD	superoxide dismutase
TAIR	The Arabidopsis Information Resource
TCH2	TOUCH2
T-DNA	transfer-DNA
TPC1	TWO-PORE CHANNEL 1
UER1	UDP-4-KETO-6-DEOXY-D-GLUCOSE-3,5-EPIMERASE-4-REDUCTASE 1
VGD1	VANGUARD1
VLN	VILLIN
WER	WEREWOLF
XEH	XYLOGLUCAN ENDOHYDROLASE
XTH	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE
XUT1	XYLOGLUCAN-SPECIFIC GALACTURONOSYLTRANSFERASE 1
XXT	XYLOGLUCAN XYLOSYLTRANSFERASE
XyG	xyloglucan
YC3.6	Yellow Cameleon 3.6 (cytosolic calcium sensor)
YFP	yellow fluorescent protein

9.1 Introduction

Two types of cell elongation, named diffuse and tip growth, are found in higher plants. Tip growth is a highly specific and conserved developmental process that governs both root hair (RH) and pollen tube (PT) growth. Following pollination,

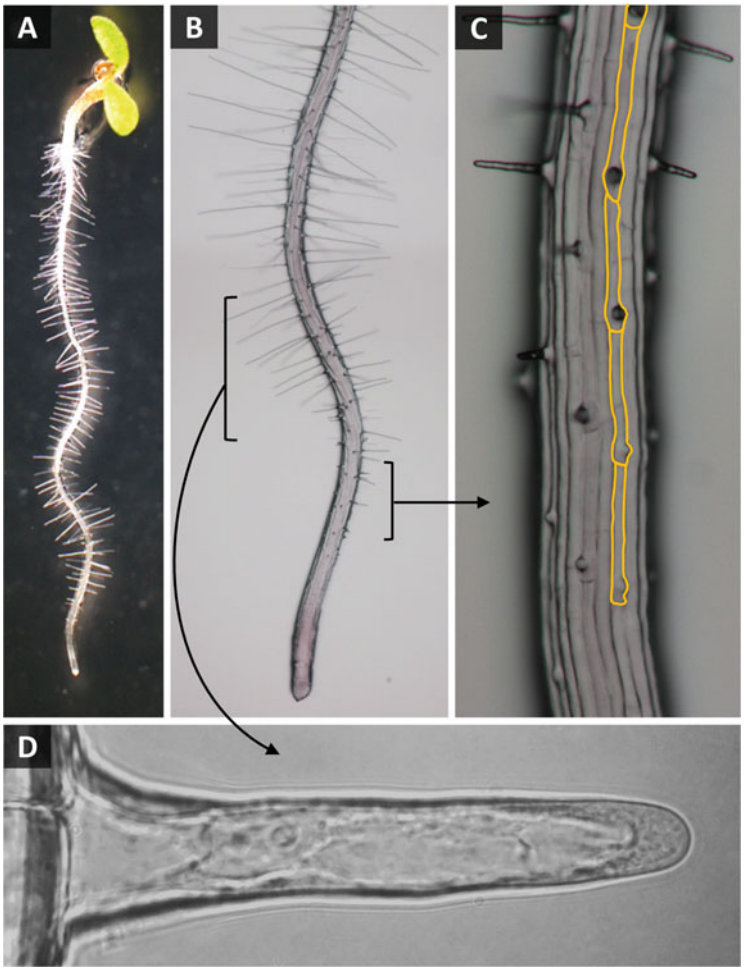

Fig. 9.1 Root hair growth pattern and morphology in *Arabidopsis*. (**a**) 5-day-old *Arabidopsis* seedling with root hairs decorating the primary root. (**b**) Close-up of the root hair growth zone showing the zone of root hair bulge emergence and the fast tip growth zone. (**c**) Close-up of the bulge formation zone, showing emerging root hairs at the basal side of single epidermal trichoblast cells (*orange* = outline of root hair cell file). (**d**) Bright-field microscopy image of a single tip-growing root hair

PTs emerge from the pollen grains and transport the male gametophytes towards the ovule to facilitate double fertilization. RHs on the other hand arise from the root's epidermis, where they drastically increase the root's absorption/contact area (Fig. 9.1). As such, tip growth lies at the basis of the plant's ability to reproduce, acquire nutrients and water, anchor to the soil and interact with soil microbiota. RHs are often exploited to study tip growth since many mutations exist, they are easy to visualize and especially since their presence is not a requirement for plant survival.

The root epidermis is typically built up by RH (trichoblasts) and non-RH cells (atrichoblasts). Cell fate specification and initiation site determination are the processes that regulate whether and where RH bulges are formed on epidermal cells. A genetic cascade involving intra- and intercellular position-dependent signalling and complex feedback loops determines cellular fate, and local wall weakening coupled to turgor pressure initiates a highly localized bulge (reviewed in Balcerowicz et al. 2015). Next, fast tip growth is initiated. Unlike diffuse cell elongation where growth occurs over the whole cell's surface, tip growth is restricted to the very apex of the tubular growing structure. The frequency and amplitude of PT and RH growth depends on extracellular conditions and a highly organized interplay between gene transcription, protein turnover and modification, the cytoskeleton, the cell wall, ion and reactive oxygen species (ROS) gradients and membrane-localized import and export proteins. At first sight, the process by which PTs and RHs grow appears to be highly similar. More so, comparing the RH and PT transcriptome revealed the existence of a common apical cell growth signature (Becker et al. 2014). However, several decades of research has shown that even though some central aspects are shared, multiple regulatory components differ between PT and RH development. Here, we review ROP GTPases, ROS, calcium and pH gradients, the actin and microtubule (MT) cytoskeleton and the cell wall, main molecular determinants controlling both PT and RH tip growth. We will not focus on the hormonal and genetic (mainly *root hair specific-like 4; RSL4*) regulation of these molecular players, but we will identify commonalities, differences and potential targets for future research based on the current knowledge available at the time of writing.

9.2 ROP GTPases as Master Regulatory Switches

9.2.1 ROPs Control Tip Growth

Plant Rho-like GTPases (ROPs) play a key role in the regulation of various developmental and cellular processes with emphasis on polarized growth of RHs and PTs. These small GTPases cycle between an inactive (GDP-bound) and an active (GTP-bound) state and thus act as molecular switches 'turning on and off' a wide range of signalling pathways (Fig. 9.2). ROP activity oscillates with the same frequency as tip growth oscillations (Monshausen et al. 2007; Hong et al. 2015) and depends on the action of several regulatory proteins. GTP-bound ROPs localize to the plasma membrane (PM) at the apex of elongating RHs and PTs (Kost et al. 1999; Molendijk et al. 2001; Jones et al. 2002), allowing them to relay extracellular signals from PM-associated receptors such as receptor-like kinases (RLKs; Nibau and Cheung 2011). ROPs interact with diverse effectors in order to mediate actin dynamics, MT organization, vesicle trafficking (reviewed in Nibau et al. 2006; Yalovsky et al. 2008; Yang 2008), ROS production (Carol et al. 2005) and the formation of a tip-focused Ca^{2+} gradient (Li et al. 1999).

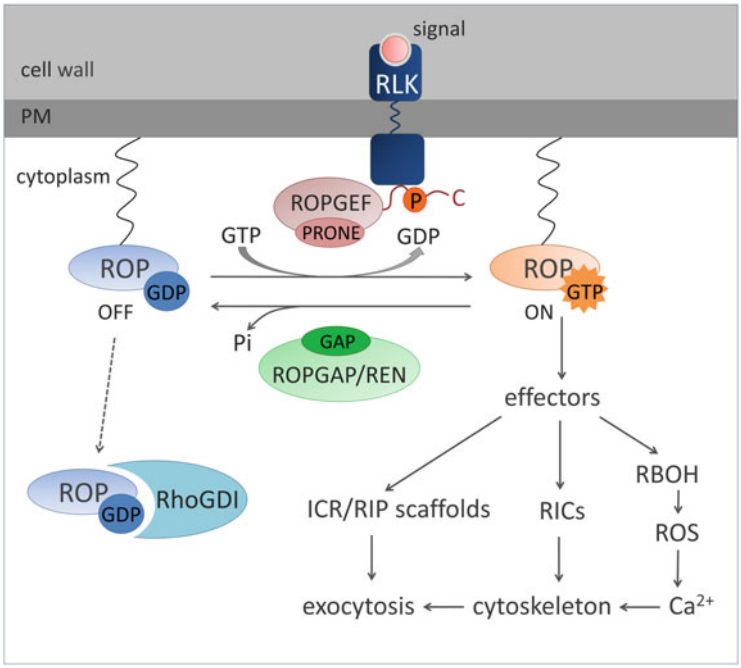

Fig. 9.2 Graphical representation of the ROP-GTPase signalling cascade during root hair growth. All abbreviations are referred to in Sect. 9.2

The *Arabidopsis* genome encodes 11 ROPs from which ROP1, 2, 4 and 6 have been implicated in tip growth of RHs. In general, expression of constitutively active (CA) forms of ROPs leads to non-polarized RH growth, whereas overexpression of dominant negative (DN) variants results in reduced RH elongation (Molendijk et al. 2001; Tao et al. 2002; Jones et al. 2002, 2007; Carol et al. 2005). Furthermore, ROP1, ROP3 and ROP5 have been shown to regulate PT growth and perturbation of their expression, and activity causes similar effects to those observed in RHs (Kost et al. 1999; Fu et al. 2001; Chen et al. 2003; Gu et al. 2003). Interestingly, transcription of a common set of ROP-related genes defines both RH and PT development in *Arabidopsis* (Becker et al. 2014).

9.2.2 ROP-Associated Proteins Regulate ROP-GTPase Activity

Activity of ROPs is mainly regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Fig. 9.2; reviewed by Yang 2002; Nagawa et al. 2010).

9.2.2.1 Guanine Nucleotide Exchange Factors

GEFs are PRONE-domain-containing proteins which activate ROPs at the apical PM by catalysing the exchange of GDP to GTP (Fig. 9.2; Berken et al. 2005). In *Arabidopsis*, 3 of 14 members of the GEF family are implicated in RH development. The RH-specific ROPGEF4/RHS11 is involved in the RH elongation stage since loss of function causes a short RH phenotype (Won et al. 2009; Huang et al. 2013a), while knocking out ROPGEF10 mainly results in a reduced RH number. The fact that ROPGEF10 is expressed in all root epidermal cells (Won et al. 2009) and that no changes in the expression of RH-specific genes were found in *gef10* plants indicates that ROPGEF10 rather plays a role during RH initiation. Truncation and domain-swapping experiments revealed that the distinct functions of ROPGEF4 and ROPGEF10 result from their noncatalytic domains (Huang et al. 2013a). On the other hand, both ROPGEFs are interacting partners for RLK FERONIA (FER) and seem to participate in a common pathway for ROS production which involves activation of ROP2 and ROP6 (see Sect. 9.3; Huang et al. 2013a). Shin et al. (2010) reported that overexpression of the third member, ROPGEF11/phytochrome-interacting ROPGEF1 (PIRF1), affects RH polarity and RH density. Furthermore, growing PTs, similarly to RHs, express seven members of the ROPGEF family and also require the action of ROP activators (Gu et al. 2006; Zhang and McCormick 2007). It has been demonstrated that the GFP-tagged RopGEF1, RopGEF8, RopGEF9, RopGEF12 and RopGEF14 localize to the PM of the PT apex and that overexpression of these GEFs causes loss of polar PT growth with the most severe phenotype seen for RopGEF1 (Gu et al. 2006). Notably, overexpression of RopGEF12 leads to dramatic changes in PT morphology only if RopGEF12 is C-terminally truncated, suggesting that the C-terminal region is auto-inhibitory to GEF activity (Zhang and McCormick 2007). According to more recent findings, the C-terminus of RopGEF1 is directly phosphorylated by the pollen RLK pollen receptor-like kinase 2 (PRK2) (Chang et al. 2013). The authors proposed a model in which PRK2 activates RopGEF1 through releasing its auto-inhibition by phosphorylation, which in turn leads to the activation of ROP1. In addition, interactions with ROPGEFs have also been reported for the RLKs from the *Catharanthus roseus* receptor-like kinase 1-like (CrRLK1L) subfamily (Duan et al. 2010).

9.2.2.2 GTPase-Activating Proteins

Activated ROPs are ‘switched off’ by GTPase-activating proteins (GAPs) which accelerate GTP hydrolysis. In *Arabidopsis*, nine genes have been found to encode for proteins with a GAP catalytic domain (Wu et al. 2000; Hwang et al. 2008). Among these, six members, termed ROPGAP1–ROPGAP6, contain a CRIB motif that is required for binding to ROPs and acts as a positive regulator of GAP activity (Wu et al. 2000). So far nothing is known about the role of ROPGAPs in RH growth. However, according to the *Arabidopsis* eFP Browser (Winter et al. 2007),

ROPGAP1 and ROPGAP6 are specifically expressed in RH cell files. ROPGAP1 has been shown previously to regulate ROP1 activity in PTs since coexpression of this GAP restores polarized PT growth in ROP1-overexpressing plants (Hwang et al. 2010). Consistently, NtRhoGAP1, a tobacco homologue of ROPGAP1, suppresses a phenotype caused by NtRAC5 (a tobacco ROP) overexpression (Klahre and Kost 2006). ROPGAP6 is annotated as a pseudogene although it has an intact ORF and a normal structure. In addition, the ROPGAP6 protein lacks part of a conserved GAP motif (Kost 2010). Hence, it remains unclear whether ROPGAP6 encodes a functional ROPGAP. Furthermore, Hwang et al. (2008) have identified novel structurally distinct *Arabidopsis* ROPGAPs, termed REN1-3, which contain a conserved GAP motif, an N-terminal pleckstrin homology (PH) domain and C-terminal coiled-coil regions. It is likely that a PH domain regulates catalytic activity and/or localization of RENs through binding to phospholipids, as it was shown for non-plant GAPs (Harlan et al. 1994; Lemmon 2008). To date, REN1 is the only PH-type ROPGAP characterized in detail. Knocking out REN1 leads to formation of balloon-shaped PTs indicating that REN1 plays a major role in controlling PT growth polarity. The authors suggested a self-organizing mechanism in which REN1 associates with exocytic vesicles via the coiled-cold part and is targeted to the apical PM where it globally inhibits ROP1 activity. Vesicle trafficking is stimulated by ROP1 signalling which creates a negative feedback loop and prevents excessive ROP activation. It would be interesting to verify whether a similar mechanism involving REN1 exists in RHs.

9.2.2.3 Guanine Nucleotide Dissociation Inhibitors

Spatial control of ROP signalling also depends on RhoGDIs, small cytoplasmic proteins characterized by the presence of a C-terminal immunoglobulin-like domain (IG-like) and a regulatory arm (RA). The IG-like domain is responsible for transferring inactive ROPs from the PM to the cytoplasm, followed by the formation of ROP/RhoGDI heterodimers (Klahre et al. 2006; Kost 2010). The RA sequesters ROPs in their inactive state by preventing GDP dissociation and blocking interactions with regulators and effectors (DerMardirossian and Bokoch 2005). The *Arabidopsis* RhoGDI family consists of three members (RhoGDI1, 2a and 2b) that share high similarity with mammalian RhoGDIs (Bischoff et al. 2000). RhoGDI1/SUPERCENTIPEDE1 (SCN1) is involved in RH growth through promoting the tip-focused production of ROS by ROOT HAIR DEFECTIVE 2 (RHD2)/RBOHC (see Sect. 9.3; Carol et al. 2005). Yeast two-hybrid assay and FRET analysis revealed that RhoGDI1 interacts with ROP2, ROP4 and ROP6 (Bischoff et al. 2000; Wu et al. 2013). Further, it has been demonstrated that the phosphorylation of RhoGDI1 by calcium-dependent protein kinase 3 (CPK3) has an effect on the binding ability to ROPs (Wu et al. 2013). RhoGDI1 might regulate subcellular localizations of ROP2 since ROP2::YFP was ectopically localized at the trichoblast surface in *scn1* (Carol et al. 2005). Similarly, depolarized localization of ROPs has also been observed in *gdi2a-RNAi* PTs (Hwang et al. 2010). Very recently,

Feng et al. (2016) reported that all three RhoGDIs play redundant roles in sustaining cellular homeostasis during PT growth. In addition, RhoGDI2a and RhoGDI2b are mainly expressed in the male gametophyte suggesting that PTs have higher demands for GDI-mediated ROP signalling in comparison to RHs (Feng et al. 2016).

9.2.3 ROP Effector Proteins

In addition to ROP regulators, several ROP effector proteins such as ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEINS (RICs), INTERACTORS OF CONSTITUTIVE ACTIVE ROPS (ICRs)/ROP INTERACTIVE PARTNERS (RIPs), receptor-like cytoplasmic kinases and cysteine-rich receptor kinase have been identified (reviewed in Nagawa et al. 2010).

9.2.3.1 ROP-Interactive CRIB Motif-Containing Proteins (RICs)

The *Arabidopsis* genome encodes 11 RICs, which interact with GTP-bound ROPs (Wu et al. 2001). To date, most of our knowledge about the involvement of RICs in tip growth comes from studies on PTs, where ROPs recruit several RICs to coordinate PT growth. Overexpression of RIC1, RIC2, RIC5, RIC6 and RIC7 inhibits, while overexpression of RIC10 promotes elongation of tobacco PTs (Wu et al. 2001). Moreover, elevated expression of RIC3 and RIC4 leads to non-polar PT growth and causes formation of bulbous tips. Phenotypes of RIC3 and RIC4 overexpression lines are associated with antagonist actions of these two effectors on F-actin dynamics, and co-overexpression of both RICs restores proper tip growth of PTs (Gu et al. 2005). RIC4 promotes actin assembly which is required for vesicle accumulation at the tube apex, whereas RIC3 mediates actin disassembly via activation of Ca^{2+} signalling which induces exocytosis in the growing tip. Importantly, both RICs are downstream components of ROP1 signalling demonstrating that a single ROP can act through activation of antagonist pathways (Gu et al. 2005; Lee et al. 2008). According to a recent report, regulation of F-actin dynamics in PTs also depends on the action of RIC1 (Zhou et al. 2015b). In vitro studies revealed that RIC1 has an ability to sever and cap F-actin in the presence of Ca^{2+}. In addition, RIC1 localizes to the apical PM of PTs and its distribution oscillates together with growth oscillations. Noteworthy, previous studies have shown that ROP6 activates RIC1 in order to promote katanin-mediated MT severing in leaf pavement cells (Fu et al. 2005, 2009; Lin et al. 2013) and to inhibit PIN2 internalization through stabilization of AFs in roots (Lin et al. 2012). Thus, the action of RIC1 on the cytoskeleton seems to differ between tissues or cell types. It would be interesting to determine whether RICs also contribute to RH growth by regulating the actin and/or MT cytoskeleton (Jones et al. 2006; Cole and Fowler 2006).

9.2.3.2 Interactors of Constitutive Active ROPS (ICRs)/ROP Interactive Partners (RIPs)

Activated ROPs also interact with a novel class of plant-specific effector proteins known as ICRs/RIPs. All five members of the *Arabidopsis* ICR/RIP family are characterized by the presence of a highly conserved QWRKAA motif in their C-terminal region which is required for binding to ROPs (Li et al. 2008). Until now, the role of these effectors in RH growth remains unknown. ICR1/RIP1, the most extensively studied member of the group, has been identified from two independent yeast two-hybrid screens using CA forms of ROPs as bait (Lavy et al. 2007; Li et al. 2008). GFP-tagged ICR1/RIP1 localizes to the apical cortex of growing PTs and its distribution depends on the activity of ROP1. At the same time, overexpression of ICR1/RIP1 enhances recruitment of GFP-ROP1 to the PM what suggests that ICR1/RIC1 might participate in a positive feedback loop to ensure polar localization of ROP1 to the PM (Li et al. 2008). Furthermore, Lavy et al. (2007) have demonstrated that ICR1/RIP1 form complexes with the exocyst complex subunit SEC3A and that these complexes can interact with ROPs in vivo. This indicates that ICR1/RIP1 acts as a scaffold mediating interactions between different proteins. More recently, ICR1/RIP1 has also been shown to control polar localization of PIN auxin transporters in roots and embryos (Hazak et al. 2010). Thus, ICR1/RIP1 forms a link between auxin, ROPs and exocytosis. In addition, RIP3, which localizes to MTs, has been found to interact with active ROPs and the plant-specific kinesin-13A, thereby linking ROPs with the MT cytoskeleton (Mucha et al. 2010).

9.3 ROS as Diverse Signalling Molecules

9.3.1 ROS Production at the Tip

It is well established that RHs and PTs require production and accumulation of ROS at the tip in order to maintain polarized growth (Fig. 9.3). This process involves the activity of membrane-bound NADPH oxidases (NOX) which catalyse the reduction of molecular oxygen to the superoxide anion ($O_2^{\bullet -}$), a form of ROS (Sagi and Fluhr 2001). Monshausen et al. (2007) have demonstrated that *Arabidopsis* RHs exhibit oscillating increases in NOX-derived extracellular ROS levels which follow peaks in growth rate by approximately 7 s. *Arabidopsis* NOX, also named RBOH for respiratory burst oxidase homologues, are encoded by 10 genes (*RBOHA-RBOHJ*) (Torres and Dangl 2005). Generation of ROS in RHs is linked to RBOHC/RHD2 since mutations in *RHD2* greatly reduce ROS levels and arrest RH development at the bulge formation stage. A similar effect on RH growth can be observed after treatment of wild-type roots with diphenyliodonium chloride (DPI), a NOX inhibitor (Foreman et al. 2003). Two other members, RBOHH and RBOHJ, share

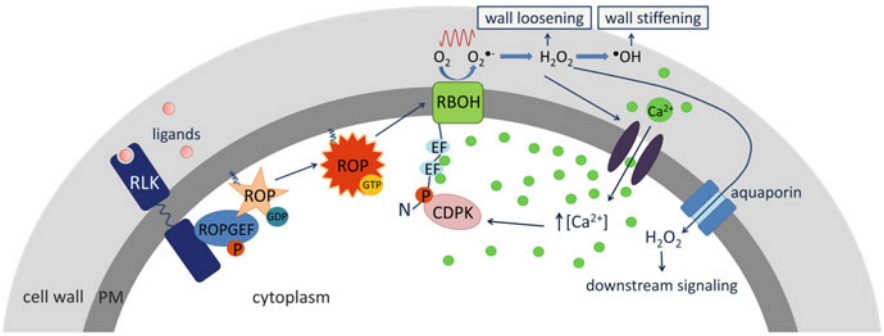

Fig. 9.3 Simplified model showing interactions between ROS and other key regulators at the tip of growing root hairs. All abbreviations are referred to in Sect. 9.3

81% amino acid identity and display partial redundancy in the regulation of PT growth. In vitro and in vivo studies have shown that *rbohH rbohJ* double mutants produce short and bursting PTs and exhibit reduced male fertility (Boisson-Dernier et al. 2013; Kaya et al. 2014). In *Nicotiana*, transfection of PTs with antisense NtNOX nucleotides downregulates the NtNOX level, decreases ROS formation and consequently inhibits tube growth (Potocký et al. 2007).

9.3.2 ROS Interacts with Calcium Signalling

Several lines of evidence support the hypothesis that NOX-derived ROS are involved in the activation of Ca^{2+} channels and Ca^{2+} flux into the cytoplasm at the tips of RHs and PTs (Fig. 9.3; reviewed in Wudick and Feijó 2014). First, *rhd2* plants lack the tip-focused Ca^{2+} gradient in RHs, and exogenous application of ROS increases the level of cytosolic Ca^{2+} and partially restores RH growth (Foreman et al. 2003). Further, knocking out *RBOHH* and *RBOHJ* disrupts Ca^{2+} homeostasis in PTs although the effect is less severe than that caused in the *rhd2* mutant. PTs of *rbohH rbohJ* retain the ability to form the tip-focused Ca^{2+} gradient, but this gradient is unsteady over time (Boisson-Dernier et al. 2013). In addition, it has been demonstrated that ROS activates Ca^{2+} channels in the PM of guard cells in response to abscisic acid (ABA; Pei et al. 2000). Furthermore, all *Arabidopsis* NOX proteins contain two putative EF-hand motifs in their N-terminal cytosolic part that are helix-loop-helix Ca^{2+}-binding domains (Keller et al. 1998; Torres et al. 1998). Takeda et al. (2008) have reported the existence of a positive feedback loop between Ca^{2+} and ROS which results from binding of Ca^{2+} to EF-hand motifs within RHD2 and the Ca^{2+}-dependent phosphorylation of RHD2. The authors demonstrated that point mutations in the EF-hands affect RH growth and that these mutations in both EF-hands and phosphorylation sites disrupt ROS formation in vitro. Ca^{2+}-stimulated ROS production has also been confirmed for other NOX proteins including pollen-

specific RBOHH and RBOHJ (Ogasawara et al. 2008; Kimura et al. 2012; Kaya et al. 2014).

9.3.3 ROS Regulates Cell Wall Properties

Given the fact that RHs of *rhd2-1* burst when switching to tip growth, RHD2-generated ROS are proposed to stabilize the cell wall at the expanding tip. In this context ROS might act in a complementary manner with extracellular pH since increasing the growth medium pH to ≥6.0 rescues the *rhd2-1* phenotype (Monshausen et al. 2007). Several studies indeed have shown that ROS affects cell wall properties although different forms of ROS may play opposite roles (Fig. 9.3). Extracellular superoxide produced by NOX can give rise to hydrogen peroxide (H_2O_2), a more stable type of ROS, either spontaneously or in the presence of superoxide dismutase (SOD; Halliwell and Gutteridge 1999). It has been shown that H_2O_2 causes cell wall stiffening by increasing cross-linking of wall polymers (Hohl et al. 1995; Schopfer 1996). Moreover, H_2O_2 can readily diffuse across the PM through aquaporins (Bienert et al. 2007; Hooijmaijers et al. 2012). Interestingly, a ROS gradient exists in the apical RH cytoplasm (Foreman et al. 2003; Bai et al. 2014b). However, no direct evidence exists for aquaporin-mediated transport of H_2O_2 at the tip of growing RHs. Most interestingly however, Di Giorgio et al. (2016) recently showed that the two aquaporins *NOD26-LIKE INTRINSIC PROTEIN 4;1/2 (NIP4;1/2)* are specifically expressed in PTs and that NIP4;1 can transport H_2O_2. Could aquaporins govern transmembrane H_2O_2 transport in tip-growing cells? In the presence of transition metals such as copper or iron, H_2O_2 can further be converted to hydroxyl radicals (•OH), the most reactive type of ROS (Fry 1998; Halliwell and Gutteridge 1999), which, in contrast, are involved in cell wall loosening through breakdown of wall polysaccharides (Fry 1998; Schopfer 2001; Liszkay et al. 2004). Thus, the proper balance between ROS species may be crucial for regulation of tip growth oscillations.

9.3.4 ROS and Kinase Signalling

Apart from the above-mentioned functions, ROS might also be involved in tip growth by participating in signal transduction pathways. Work by Rentel et al. (2004) has demonstrated that expression of *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*), which encodes a serine/threonine protein kinase, is induced in response to H_2O_2 administration. In addition, H_2O_2 has an ability to stimulate OXI1 kinase activity in vivo. Phenotypic analysis revealed that loss-of-function mutation in *OXI1* results in a slightly reduced RH length. Furthermore, OXI1 has been found to work upstream of mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 since in the *oxi1* mutant both MAPKs showed reduced activation after treatment with

H_2O_2 and cellulase. Hence, it is likely that ROS may act as signalling molecules regulating activity of MAPKs by the intermediate of OXI1. The role of MAPK in RH growth has already been reported in *Medicago sativa* (Šamaj et al. 2002). SIMK, the homologue of MPK6, localizes to RH tips, and overexpression of its gain-of-function form has a stimulatory effect on RH growth. Besides, more recently, MPK3 and MPK6 have been implicated in funicular guidance of PTs (Guan et al. 2014).

ROS are downstream components of *Catharanthus roseus* receptor-like kinase (CrRLK1L) subfamily signalling pathways (Fig. 9.3). CrRLK1Ls are major regulators of cell expansion in response to extracellular signals and are likely to act as cell wall integrity sensors. In a current model, ligand-bound CrRLK1Ls interact with ROPGEFs in order to activate ROP GTPases. ROPs, in turn, stimulate NOX-dependent ROS production leading to the activation of Ca^{2+} channels and changes in cell wall properties (reviewed by Nibau and Cheung 2011; Wolf and Höfte 2014; Nissen et al. 2016). In RHs, ROS formation is controlled by PM-localized CrRLK1L FERONIA (FER) and *fer* mutants show RH defects similar to those seen in *rhd2* plants (Duan et al. 2010). The FER signalling pathway in RHs involves ROPGEF1, ROPGEF4, ROPGEF10 and ROP2 and ROP6 (Duan et al. 2010; Huang et al. 2013a). Interestingly, FER is also expressed in the female gametophyte where it induces ROS-mediated PT rupture (Escobar-Restrepo et al. 2007; Duan et al. 2010, 2014). Furthermore, ERULUS/$[Ca^{2+}]_{cyt}$-ASSOCIATED PROTEIN KINASE 1 (CAP1), which unlike other CrRLK1Ls is localized at the tonoplast, also contributes to ROS-mediated RH growth since abnormal RH growth in the *cap-1* mutant coincides with the absence of a ROS gradient in the hair tips (Haruta et al. 2014; Bai et al. 2014a, b). The ROS gradient in *cap-1* can be restored in NH_4^+-free growth medium suggesting a possible interplay between ROS and ammonium (Bai et al. 2014b). Besides, RHD2 activity also depends on the action of ROPGDI1/SCN1, a ROP regulatory protein. In *scn1* mutants RH bulges are formed at ectopic positions on the trichoblast, which results from delocalized RHD2 accumulation and ROS production (Carol et al. 2005). Proper distribution of RHD2 relies on actin microfilaments because functional loss of *ACTIN2* and treatment with microfilament-disrupting drug cytochalasin D (CytD) both lead to RHD2 accumulation in cytoplasmic clumps (Takeda et al. 2008). The fact that actin microfilament dynamics in RHs are regulated by ROP2, which is a target of SCN1, suggests that SCN1 might spatially control RHD2 localization via this ROP (Jones et al. 2002; Takeda et al. 2008). Similarly to RHs, ROS production in PTs is also controlled by CrRLK1L members. Pollen-specific ANXUR1 (ANX1) and ANXUR2 (ANX2) act redundantly to maintain PT growth within female tissue since PTs of *anx1 anx2* double mutant burst after germinating in vitro and fail to reach the ovules in vivo (Boisson-Dernier et al. 2009). Given the fact that the phenotype of *anx1 anx2* resembles that of *rbohH rbohJ* and that overexpression of *ANX*s and knockout of *RBOHH* and *RBOHJ* enhances exocytosis, it is proposed that ANXs regulate RBOHs to synchronize growth rate with cell wall exocytosis (Boisson-Dernier et al. 2009; Kaya et al. 2014; Lassig et al. 2014; Nissen et al. 2016).

9.4 Ion Oscillations Integrate Extra- and Intracellular Signalling

9.4.1 Calcium

9.4.1.1 Introduction

Calcium ions function as important second messenger in eukaryotic cells. In plants, Ca^{2+} is of crucial importance for signal transduction in processes such as long-distance propagation of electrical signals (reviewed in Steinhorst and Kudla 2014), the response to salt stress (Choi et al. 2014), regulation of exocytosis (Zorec and Tester 1992; Battey et al. 1999) and actin cytoskeleton dynamics (Battey and Blackbourn 1993; Chen et al. 2002; Braun et al. 2004), cell wall remodelling (Holdaway-clarke et al. 1997; Rounds et al. 2011), phosphoinositide signalling (Franklin-Tong 1999), stomatal aperture (Allen et al. 2001; Evans et al. 2001), mechanosensing (Monshausen et al. 2009) and gravitropism (Plieth and Trewavas 2002; Toyota et al. 2007).

Given the low diffusion constant of Ca^{2+} in the cytoplasm (10^{-7} cm^{-2} s^{-1}; Thomas 1982), local Ca^{2+} concentration maxima can be maintained for a prolonged period of time. Ca^{2+} concentration peaks are often keys for local regulation of specific signalling pathways in the cell. For instance, in living systems, integration of biotic and abiotic stimulus perception often occurs through Ca^{2+} signalling by local elevation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$; Hetherington and Brownlee 2004; Trewavas 1999).The existence of Ca^{2+} maxima relies on a well-coordinated release and sequestration from and to inter- and intracellular Ca^{2+} stores, through a collection of Ca^{2+} transporters. Such elevations in $[Ca^{2+}]_{cyt}$ can consist of a single transient peak, but often exhibit a more complex wave-like pattern. Highly specific differences in the temporal and spatial nature of these Ca^{2+} dynamics are often referred to as 'Ca^{2+} signatures' (Dodd et al. 2010). The characteristics (duration, frequency and amplitude) of these signatures determine downstream signal perception and propagation through a number of Ca^{2+}-sensing and Ca^{2+}-relaying proteins. Final perception of the Ca^{2+} signal steers multiple developmental pathways, often related to plant morphogenesis, including RH and PT development.

A central role for Ca^{2+} signalling in tip-growing cells was established over 20 years ago. For instance, both RHs and PTs rely on optimal extracellular Ca^{2+} concentrations ($[Ca^{2+}]_{ext}$) for successful tip growth. Generally, a lack of Ca^{2+} ions results in decreased RH growth in *Arabidopsis* (Schiefelbein et al. 1992). Also for PTs, a lack or excess of Ca^{2+} in the growth medium results in abnormal, stunted tubes (Boavida and McCormick 2007). Recently, advancements in spatial and temporal visualization of in vivo Ca^{2+} dynamics have further contributed to unraveling the complexity of Ca^{2+} signalling in RH and PT development. Whereas both cell types grow by the process of tip growth, some remarkable differences between PT and RH exist in the Ca^{2+}-related machinery. More so, while our

understanding has greatly improved, it is clear that the current knowledge is only scratching the surface, and many discoveries are yet to be made.

9.4.1.2 An Oscillating Tip-Focused Calcium Gradient Controls RH and PT Growth

Growing RHs and PTs exhibit a clear zone at their extreme apex. The clear zone delineates the region to which all tip growth-related machinery is concentrated. Numerous studies have now shown that the clear zone coincides with a tip-focused cytosolic Ca^{2+} gradient in both RHs (Fig. 9.4) and PTs of multiple species

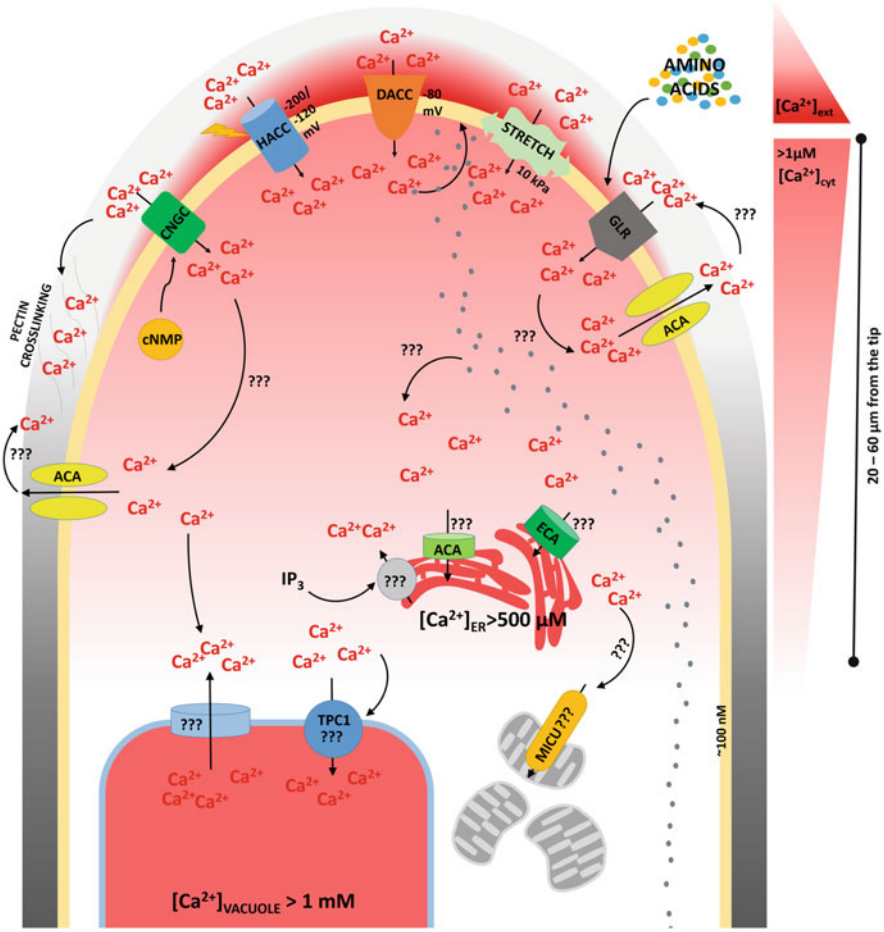

Fig. 9.4 Graphical representation of calcium transport and storage systems governing root hair tip growth. *Question marks* refer to hypothetically involved components and mechanisms. *Red colours* represent calcium concentrations. *Grey outline* = cell wall, *yellow outline* = plasma membrane, *grey spheres* = exocytic vesicles. All abbreviations are referred to in Sect. 9.4.1

(Schiefelbein et al. 1992; Pierson et al. 1996; Holdaway-clarke et al. 1997; Felle and Hepler 1997; Michard et al. 2008; Fan et al. 2011). Relative to the RH tip, this gradient extents inward for 20–60 μm (Felle and Hepler 1997; Monshausen et al. 2008). In rapidly growing RHs, the $[Ca^{2+}]_{cyt}$ ranges from 100 nM at the basal part (RH shank) to more than 1 μM at the apex (Wymer et al. 1997; Felle and Hepler 1997). In PTs, $[Ca^{2+}]_{cyt}$ higher than 3 μM have been reported at the tip (Pierson et al. 1996).

Several pharmacological experiments showed that the existence of this tip-focused Ca^{2+} gradient is crucial for proper elongation of tip-growing cells. For instance, treatment of RHs with Ca^{2+} channel blockers such as verapamil, nifedipine or La^{3+} or the Ca^{2+} chelator EGTA resulted in dissipation of the Ca^{2+} gradient and subsequent RH growth inhibition or a sudden increase in growth rate followed by bursting of the RH tip (Herrmann and Felle 1995; Bates and Lynch 1996; Wymer et al. 1997; Monshausen et al. 2008).

The localization of the Ca^{2+} gradient also determines the directionality of growth in RHs and PTs. Using a caged Ca^{2+} ionophore (A23187) approach, a local and transient Ca^{2+} increase can be induced extracellularly, which stops RH growth immediately (Monshausen et al. 2008). Conversely, it was also shown that both RHs and PTs direct their growth towards a transiently induced $[Ca^{2+}]_{ext}$ maximum (Malhó and Trewavas 1996; Bibikova et al. 1997). Curiously, whereas RHs redirect towards the original growth direction after reestablishment of the normal $[Ca^{2+}]_{ext}$ distribution, PTs do not. At the time of writing, the mechanisms that lie at the basis of this difference between RH and PT development remain unidentified.

Together, these results show that the Ca^{2+} gradient is a key component of the tip growth machinery. Importantly however, tip growth does not occur as a linear process. Instead, RH and PT growth rates oscillate, with alternate periods of slower growth followed by periods of fast expansion. In RHs, growth rates range between 1 and 3.2 μm min^{-1} (Monshausen et al. 2008; Cárdenas 2009). PTs grow considerably faster, with growth rates ranging between 6 and 30 μm min^{-1} (Messerli et al. 1999, 2000). The tip-focused $[Ca^{2+}]_{cyt}$ oscillates at the same frequency as the growth rate, but with a phase delay of several seconds. For instance, *Arabidopsis* RH growth rates and apical $[Ca^{2+}]_{cyt}$ oscillate at a frequency of 2–4 min^{-1}. By using the ratiometric calcium marker Yellow Cameleon 3.6 (YC3.6), Monshausen et al. (2008) demonstrated that the peak Ca^{2+} level lags the growth rate peak by approximately 5 s. It seems that the timing of these events is conserved between PT and RH development and among species. For instance, whereas the growth rate oscillations of PTs (period of 15–50 s) occur much faster compared to RHs, the phase lag in lily PTs was also found to be approximately 4–5 s (Pierson et al. 1996; Messerli et al. 2000).

9.4.1.3 Ca^{2+} Fluxes

An Oscillating, Tip-Focused Influx of Extracellular Ca^{2+}

The presence of an oscillating tip-focused Ca^{2+} gradient in RHs and PTs relies on active Ca^{2+} transport of extracellular Ca^{2+} across the apical PM. Using non-invasive ion-selective vibrating probe analysis, several independent experiments reported on the existence of an apical Ca^{2+} influx both in RHs and PTs of several species. In RHs, the Ca^{2+} influx ranged from approx. 0.01 to 0.04 amol μm^{-2} s^{-1} at the tip, until reaching basal levels at a distance of ± 20 μm from the apex (Schiefelbein et al. 1992; Jones et al. 1995; Felle and Hepler 1997). In growing PTs, inward Ca^{2+} fluxes ranged from 0.003 to 0.5 amol μm^{-2} s^{-1} (Kuhtreiber and Jaffe 1990; Pierson et al. 1996; Holdaway-clarke et al. 1997; Messerli et al. 1999; Michard et al. 2008, 2011). The study of Messerli et al. (1999) revealed that PTs grew at a steady rate (0.2 μm s^{-1}) until they reached a certain length (approx. 1 mm); after that PT growth rate and Ca^{2+} influx switched to an oscillating pattern. Similarly, Michard et al. (2011) observed strong Ca^{2+} influx oscillations at the tip of tobacco PTs (fluctuating between 0.06 and 0.5 amol μm^{-2} s^{-1}). The rate of Ca^{2+} influx oscillations seemed to be correlated to the PT growth rate (Pierson et al. 1996) and is sensitive to extracellular stimuli such as D-serine (Michard et al. 2011).

Similar to the $[Ca^{2+}]_{cyt}$, the tip-focused Ca^{2+} influx also exhibits the same period, but cross-correlation analysis showed that the peak Ca^{2+} influx followed the peak growth rate with a lag time of approx. 15 s (Holdaway-Clarke et al. 1997; Messerli et al. 1999; Holdaway-Clarke and Hepler 2003).

The structural properties of the cell wall largely depend on the presence of Ca^{2+} (see Sect. 9.4.1.4). As such, the observed Ca^{2+} influx at the tip of growing RHs and PTs is expected to simultaneously provide Ca^{2+} for the cell wall and Ca^{2+} for active transport into the cytosol. Recently, Hepler et al. (2012) calculated the species-specific Ca^{2+} influx in growing PTs that would be needed to provide the required amount of structural Ca^{2+} to the cell wall (Hepler et al. 2012). Their findings implicate that the majority of vibrating microelectrode measurements likely underestimate the actual fluxes involved in tip growth (Table 9.1). Holdaway-Clarke et al. (1997) performed a similar analysis for *Lilium* PTs and found that, in perfect agreement with their experimental data, a flux of approx. 0.35 amol μm^{-2} s^{-1} would be needed to supply the cell wall with the required amount of Ca^{2+} (Holdaway-Clarke et al. 1997).

Ca^{2+} Transporters

Plants have evolved specific Ca^{2+} transport proteins, different from those found in other eukaryotic organisms (Steinhorst and Kudla 2014). Both in PTs and RHs, a Ca^{2+} influx is maintained by a specialized set of Ca^{2+} transport proteins (Fig. 9.4). Given the heterogeneity of the extracellular environment, it is expected that

Table 9.1 Observed and predicted Ca^{2+} flux densities to sustain pollen tube growth (Adopted from Hepler et al. 2012)

Species	PT diameter (μm)	Growth rate (μm min^{-1})	Observed flux (amol μm^{-2} s^{-1})	Predicted flux needed for cell wall (amol μm^{-2} s^{-1})	Predicted flux needed for 10 μM $[Ca^{2+}]_{cyt}$ (amol μm^{-2} s^{-1})
Arabidopsis	4	0.3–1.5	0.01–003	0.03–0.16	0.002
Nicotiana	10	2–10	0.05–0.5	0.09–0.43	0.006
Lilium	18	6–30	0.02–0.15	0.14–0.72	0.005

tip-growing cells contain several types of Ca^{2+} transporters that would allow for Ca^{2+} influx in different conditions. Great scientific effort has gone into identifying the precise characteristics of the Ca^{2+} influx, in order to narrow the search for the involved Ca^{2+} channels in both RH and PT development. The ability to perform patch-clamp analysis on either isolated protoplasts or intact tip-growing cells leads to the identification of a series of candidates, some of which are yet to be confirmed and others which are now believed to be crucial tip growth regulators.

Cyclic nucleotide-gated channels (CNGCs) are non-specific, Ca^{2+}-permeable cation channels regulated by cyclic nucleotides such as cAMP (Maser et al. 2001; Talke et al. 2003; Ward et al. 2009). CNGCs also have a binding site for calmodulin (Kohler and Neuhaus 2000). As such, they can form a direct relay system between cNMP and Ca^{2+} signal transduction pathways. The *Arabidopsis* genome contains 20 CNGCs, some of which have been shown to be involved in Ca^{2+}-mediated PT growth. The latter is consistent with the fact that cNMP signals in pollen can affect growth in a Ca^{2+}-dependent manner (Moutinho et al. 2001; Rato et al. 2004; Wu et al. 2011). Publicly available transcriptomics data shows that at least six *Arabidopsis* CNGCs are strongly expressed in in vivo grown PTs and nine CNGCs show strong expression in trichoblast cell files (Table 9.2). Alterations in expression levels of the PT-expressed CNGCs resulted in dramatic growth defects and reduced male fertility (Tunc-Ozdemir et al. 2013; Frietsch et al. 2007; Gao et al. 2016). The transcription of several CNGC genes is altered in a number of well-characterized RH developmental mutants (Bruex et al. 2012; Simon et al. 2013). Most notably, CNGC6 and CNGC9 show very high and specific transcription in RH cell files, and their transcription is strongly affected in several RH developmental mutants. At the time of writing, however, no studies have focused on the role of CNGCs in RH development.

Hyperpolarization- and depolarization-activated Ca^{2+} channels (HACCs and DACCs) have been identified in the RH and PT apical PM. Over a decade ago, Véry and Davies identified a hyperpolarization-activated Ca^{2+} conductance in growing RH protoplasts, operative specifically at membrane potentials equal to and lower than the resting potential of growing *Arabidopsis* RHs (between −160 and −200 mV; Lew 1996), and within the range of physiological $[Ca^{2+}]_{cyt}$ (Wymer et al. 1997; Felle and Hepler 1997; Véry and Davies 2000). Consistent with the model of tip-localized Ca^{2+} influx, this hyperpolarization-activated Ca^{2+} conductance was found to be much lower in the subapical RH region. Interestingly, the conductance increased in the presence of higher physiologically relevant $[Ca^{2+}]_{cyt}$ (100–900 nM). The latter suggest that Ca^{2+} influx across the PM and subsequent Ca^{2+} accumulation in the cytosol could induce the Ca^{2+} influx even further. This mechanism might be part of a feedback loop, regulating Ca^{2+} oscillations. In 2003, Foreman et al. found a ROS-dependent HACC conductance in RHs, related to ROS originating from NADPH oxidase activity (RHD2; Foreman et al. 2003). The mechanism of ROS-controlled Ca^{2+} influx was also found in PTs (e.g. Wu et al. 2010), and is now considered to be a pivotal part of tip growth in general

Table 9.2 Transcription level of *Arabidopsis* cyclic nucleotide-gated calcium channels in tip-growing cells (no < yes < strong < very strong)

CNGC ID	Transcription in PTs (Qin et al. 2009)	Transcription in trichoblasts (Brady et al. 2007)	Transcription in atrichoblasts (Brady et al. 2007)	Altered transcription in RH developmental mutants
1				
2				
3		Yes		No
4				
5				
6		Very strong		Yes
7	Yes	Yes		No
8	Yes		Yes	No
9	Yes	Very strong		Yes
10	Yes	Yes		Yes
11		Yes		No
12			Yes	No
13				
14		Strong		Yes
15			Yes	Yes
16	Yes			
17		Yes		Yes
18	Yes	Yes		No
19				
20				

(see Sect. 9.3.2). HACCs have also been identified in PT protoplasts of several species, indicating a voltage-gated Ca^{2+} influx as a central player in establishing tip growth-related Ca^{2+} dynamics (Shang et al. 2005; Qu et al. 2007; Wu et al. 2007, 2011). Comparable to RHs, these channels typically open at a threshold voltage of approximately −100 mV, similar to the resting potential of PTs (Messerli et al. 1999). Next, a DACC conductance was identified in the apical region of *Arabidopsis* RHs (Miedema et al. 2008). DACCs are typically most active at −80 mV (in the presence of 30 mM external Ca^{2+}; Thion et al. 1998, 1996).

Given the known membrane potential observed in growing RHs, HACCs seem to be the main component for tip growth-related Ca^{2+} influx. However, the co-occurrence of CNGCs, HACCs and DACCs could provide RHs with a mechanism for Ca^{2+} influx at a broad range of membrane potentials and intra- as well as extracellular Ca^{2+} concentrations. For instance, increase of the tip-focused $[Ca^{2+}]_{cyt}$ by CNGC-mediated Ca^{2+} influx at moderately negative voltage values would lower the activation voltage needed for HACC activity (Demidchik et al. 2002). Despite the identification of HACC conductances in RHs, actual identification and thus functional characterization of the genes coding for these channels is still lacking.

Notably, the presence of a cAMP-activated HACC was shown in *Pyrus pyrifolia* PTs using patch-clamp analysis (Wu et al. 2011). This finding might hint towards the family of CNGCs in the quest for HACCs.

Mechanosensitive calcium channels are also involved in regulating Ca^{2+} fluxes in PT growth. *Tradescantia virginiana* PTs were found to reorient upon touch or relocalization of the Ca^{2+} maximum, strongly suggesting that PTs contain mechanosensitive regulation of Ca^{2+} fluxes (Bibikova et al. 1997). Indeed, a mechanosensitive Ca^{2+} conductance was identified in protoplasts of *Lilium longiflorum* PTs (Dutta and Robinson 2004). Upon application of 10 kPa suction force, these channels would open and preferentially allow Ca^{2+} to enter the cytosol. Interestingly, Bibikova et al. (1997) found that in RHs and PTs, the Ca^{2+} gradient maximum is reoriented away from a mechanical obstacle or a touch induction site. More so, local mechanical perturbation of RH cells elicits a transient Ca^{2+} peak 1–18 s after application of the stimulus (Monshausen et al. 2009). Finally, Wang and colleagues found that impalement of RH cells with a glass microelectrode results in a similar transient increase of the $[Ca^{2+}]_{cyt}$ (Wang et al. 2015b). These data strongly suggest that also in RHs, despite the lack of further evidence, a mechanosensitive Ca^{2+}-linked mechanism is in place. Identification of the possible gene(s) related to this mechanosensitive Ca^{2+} conductance in PTs and RHs is still lacking.

The consensus regarding Ca^{2+} dynamics and growth rate oscillations states that the phase of Ca^{2+} oscillations follows that of growth oscillations with a lag of approximately 4–5 s. It is therefore easy to speculate that a growth spurt would result in subsequent opening of mechanosensitive Ca^{2+} channels due to mechanical deformation of the PM at the tip.

The search for identification of mechanosensitive channels involved in tip growth is progressing. Recently, Hamilton and colleagues identified an anion-selective mechanosensitive channel named MSCS-LIKE 8 (MSL8), with a crucial role in male fertility through regulation of osmotic forces during pollen development (Hamilton et al. 2015). MID1-COMPLEMENTING ACTIVITY 1 and 2 (MCA1, MCA2) were shown to be Ca^{2+} conducting mechanosensitive channels involved in root growth (Nakagawa et al. 2007; Yamanaka et al. 2010). Transcriptomics data shows that at least MCA1 is expressed in RH cell files (Brady et al. 2007). More so, transcription of MSL2 and MSL3 was detected in both RHs and PTs, making these genes worthwhile candidates for further investigation.

Glutamate receptor-like channels (GLRs) are other candidates for generating the Ca^{2+} influx in PTs and RHs. In *Arabidopsis*, 20 genes code for proteins with strong similarity to the mammalian Ca^{2+} conducting glutamate receptor (Ward et al. 2009). Pollen transcriptome data revealed that at least six *GLRs* are preferentially expressed in pollen and one in PTs. Interestingly, at least four *GLRs* are preferentially expressed in trichoblast cell files (Brady et al. 2007). Of these four genes, three (*GLR3.5*, *GLR3.7* and *GLR2.1*) are differentially expressed in several RH developmental mutants (Bruex et al. 2012; Simon et al. 2013). Despite the implications for these genes in their involvement in tip growth, current knowledge on their function is still mostly lacking. Only *GLR1.2* and *GLR3.7* were shown to be

important in PT growth (Michard et al. 2011). Loss-of-function mutations in both genes resulted in shorter PTs and reduced fertility. Moreover, *glr1.2* mutant pollen resembled the morphology associated with GLR inhibition by CNQX treatment, a well-known inhibitor of mammalian glutamate receptors. Crucially, the results of Michard and colleagues show that GLRs are responsible for perception of D-serine and subsequent Ca^{2+} influx in PTs followed by growth stimulation. Upon exogenous application of D-serine (a rare amino acid present in the pistil and ovules) to growing PTs, YC3.6 monitoring of the $[Ca^{2+}]_{cyt}$ revealed an increase in the amplitude of Ca^{2+} oscillations in the tip. This effect of D-serine was not seen in the presence of GLR inhibitors, and the Ca^{2+} signature was not detected in response to other amino acids such as glutamate. These data suggest that GLRs form another component of the apical Ca^{2+} influx machinery. Importantly, it also shows that the specificity of GLRs is diverse and not limited to glutamate. The latter is supported by the finding that GLR3.4 expressed in HEK cells shows highly selective Ca^{2+} transport in response to the amono acids Asn, Gly and Ser (Vincill et al. 2012).

Calcium-efflux transporters have been largely neglected when it comes to studying tip growth. This is largely due to the fact that no outward Ca^{2+} currents have been observed at the PT or RH tip. However, Schiott and colleagues identified the autoinhibited Ca^{2+}-ATPase efflux pump ACA9 as a crucial component of PT tip growth (Schiott et al. 2004). *Aca9* knockout PTs grew slower in vivo and in vitro, eventually resulting in shorter PTs (75% reduction in length) and a strong reduction in fertilization efficiency. Given the fact that ACA9 is localized at the PT PM, Schiott et al. (2004) suggest that it might have a role in recycling cytosolic Ca^{2+} towards the cell wall during tip growth. Interestingly, *ACA9* is strongly expressed in RH cell files in *Arabidopsis*. It would be interesting to see what in-depth analysis of Ca^{2+} dynamics in *aca9* mutant RHs and PTs could reveal. In total, ten autoinhibited efflux Ca^{2+}-ATPases are found in the genome of *Arabidopsis thaliana*. Besides ACA9, ACA7 is also localized to the PM and expressed (albeit relatively low) in PTs and RHs. Current knowledge states that ACA7 is involved in microsporogenesis (Lucca and León 2012), but no experiments have been performed on PT and RH development. Finally, ACA12 was recently found to be localized to the plant PM (Limonta et al. 2014). Ectopic expression of ACA12 in *aca9* plants led to the rescue of the *aca9* male sterility phenotype, showing that ACA12 is a functional PM-localized Ca^{2+}-efflux pump. No *ACA12* transcription has been documented in in vivo grown PTs. However, in roots, *ACA12* is preferentially expressed in trichoblast cells (Brady et al. 2007).

9.4.1.4 Ca^{2+} Sources and Sinks

A large influx of Ca^{2+} is in place during RH and PT growth; however the subsequent fate of incoming Ca^{2+} is unknown. The nature of fluctuating Ca^{2+} dynamics in tip-growing cells inherently relies on active transport and sequestration of Ca^{2+} ions from and to Ca^{2+} sources and sinks (Fig. 9.4). Solely based on the major

concentration differences observed between the cytosol, cell wall and subcellular organelles, it is expected that inter-compartment Ca^{2+} allocation takes place inside the cell. Illustratively, the typical resting $[Ca^{2+}]_{cyt}$ is kept around 100–200 nM, whereas the free Ca^{2+} concentration in some of the major subcellular Ca^{2+} stores and the cell wall can easily reach 10 mM (Bush 1995). So which compartments are known to or could contribute to Ca^{2+} dynamics in tip growth?

The cell wall has been considered the main source for tip-focused cytosolic Ca^{2+} in tip-growing cells. In support of this consensus is the presence of multiple Ca^{2+} channels and Ca^{2+} conductances, together with the discovery of a growth-correlated oscillating inward Ca^{2+} influx. More so, already very early in the research of PT tip growth, it was shown that radioactive $^{45}Ca^{2+}$ incorporates mostly in the PT cell wall (Kwak 1967). The extracellular environment is considered to form the main source for cell wall accumulating Ca^{2+} in both RHs and PTs. This is illustrated by the fact that in vitro growth of RHs and PTs relies on a $[Ca^{2+}]_{ext}$ optimum. Importantly, instead of simply diffusing through the cell wall, a large quantity of Ca^{2+} is used as a means of providing dynamic structural cell wall support during tip growth. As such, normal cell wall dynamics at the tip of growing PTs and RHs inherently rely on the maintained presence of Ca^{2+}. Thus, in order to provide sufficient Ca^{2+} to the cytosol while maintaining proper Ca^{2+} levels in the cell wall, an intricate balance must be kept between Ca^{2+} export and import from and to the cell wall. An array of Ca^{2+} transport proteins provides tight control over the Ca^{2+} efflux (cytosolic influx) from the cell wall (see Sect. 9.4.1.3). But how is Ca^{2+} retained and released from the cell wall at the tip? The RH and PT cell wall consists partly of pectins which alternate between their methylesterified and de-esterified form during tip growth (Bosch and Hepler 2005). The Ca^{2+}-binding capacity of pectins changes accordingly, as does the availability of Ca^{2+} for maintaining the influx into the cytoplasm. An autoregulatory feedback loop thus links oscillations in Ca^{2+} influx, Ca^{2+} cell wall cross-linking and $[Ca^{2+}]_{cyt}$ (Franklin-Tong 1999). As such, experimental evidence suggests that a self-regulating interplay between cytosolic and extracellular Ca^{2+} controls Ca^{2+} fluxes from and to the cell wall and into the cytosol. Hepler et al. (2012), however, calculated that in theory most of the Ca^{2+} flux at the PT tip is used for structural cell wall support and only a small quantity can be used for cytosolic influx.

Hereafter, the potential role of subcellular compartments in Ca^{2+} release and sequestration is discussed. However, contrary to the belief that the cell wall acts solely as a source of cytosolic Ca^{2+}, it is rarely considered that the cell wall might also act as a sink, receiving Ca^{2+} from the intracellular environment through active transport. As discussed earlier, a Ca^{2+}-efflux pump was shown to be involved in regulating PT tip growth (Schiott et al. 2004). The authors hypothesized that ACA9 could be responsible for recycling Ca^{2+} back to the cell wall, thereby regulating peak/trough Ca^{2+} levels at the tip.

The vacuole extents throughout tip-growing cells and is thought to be crucial for the generation of an outward-directed osmotic pressure that drives cell expansion (Schiefelbein et al. 1993). In general the vacuole maintains a much higher Ca^{2+}

concentration than that found in the cytoplasm (Bush 1995). The role of the vacuole in regulating Ca^{2+} release/sequestration in plants has been questioned several times (Lommel and Felle 1997; Britto and Kronzucker 2002; Rocha and Vothknecht 2012; Schönknecht 2013; Nomura and Shiina 2014). Also in RH and PT tip growth, a prominent role might exist for vacuolar Ca^{2+} release and sequestration (Carol and Dolan 2002). However, given the distance between the vacuole and the tip-focused clear zone, it is unlikely that the vacuole would directly contribute to the fine regulation of Ca^{2+} oscillations in the tip. However, massive vacuolar Ca^{2+} release could have a pivotal role in Ca^{2+}-induced Ca^{2+} release and subsequent intracellular signal transduction. Unfortunately, direct Ca^{2+} dynamics within the vacuolar lumen have not been observed at the time of writing, due to the fact that the vacuolar pH interferes with Ca^{2+} reporters. A hopeful study came from Wang and colleagues, where they describe vacuolar ion conductance and channel activity in response to cytosolic Ca^{2+} alterations (Wang et al. 2015b). Their findings suggest a vacuolar Ca^{2+} release in RHs following a cytosolic Ca^{2+} peak. Wang and colleagues found that a BAPTA- or FURA-2 induced $[Ca^{2+}]_{cyt}$ peak evoked a transient 2.5-fold increase in tonoplast ion conductance, whereas slow and small Ca^{2+} peaks did not elicit this response. The Ca^{2+} induced increase in tonoplast ion conductance is thought to be due to activation of voltage-independent channels.

TWO-PORE CHANNEL 1 (TPC1), a tonoplast localized depolarization-activated Ca^{2+} influx channel, was shown to be important for salt stress-induced Ca^{2+} signal propagation through the root (Choi et al. 2014). TPC1 is activated by high $[Ca^{2+}]_{cyt}$ and is sensitive to both cytosolic and vacuolar Ca^{2+} levels, which suggests that it might be involved in regulating the Ca^{2+} balance across the vacuolar membrane (Dadacz-Narloch et al. 2011). It might, however, act indirectly through the generation of a vacuolar membrane potential which in turn would activate other voltage-gated Ca^{2+} channels (Choi et al. 2014). The role of TPC1 in tip growth remains unexplored, but transcript levels are selectively high in trichoblast cell files in *Arabidopsis* (Brady et al. 2007). As such, it will be interesting to see if TPC1 might be involved in regulating Ca^{2+} dynamics in RH cells (Konrad et al. 2011).

Finally, patch-clamp measurement of ion conductance on intact vacuole has revealed the existence of several types of Ca^{2+}-regulated cation channels in the tonoplast (Allen and Sanders 1996; Isayenkov et al. 2010; Hedrich and Marten 2011). At the time of writing, no data exists on their involvement in RH or PT development.

Elements of the endoplasmic reticulum (ER) are continuously replenished through the clear zone, at the tip of growing PTs and RHs (Lovy-Wheeler et al. 2007). They move along the 'reverse fountain' cytoplasmic streaming typical of tip-growing cells. This finding, together with several implications that the ER is able to sequester vast amounts of Ca^{2+}, makes it an ideal candidate organelle for a Ca^{2+} source/sink in tip-growing cells. The ER has been hypothesized to function as a capacitive Ca^{2+} store (Trewavas and Malho 1997) and is one of the possible organelles that could contribute to the oscillating Ca^{2+} dynamics observed in the apical cytoplasm. Following a $[Ca^{2+}]_{cyt}$ peak, Ca^{2+} sequestration in the ER could bring the Ca^{2+}

level back to the basal level. The ER in *Arabidopsis* PTs sequesters up to 500 μM of Ca^{2+} (Iwano et al. 2009) and contains a protein called calreticulin which is able to bind 25 mol of Ca^{2+} per mol of protein (Michalak et al. 2009). Even more, the dissipation of tip-localized Ca^{2+} is thought to be regulated by ER-localized Ca^{2+}-ATPases which would allow Ca^{2+} ions to move against the concentration gradient into the ER lumen (Obermeyer and Weisenseel 1991; Sze et al. 1999; Franklin-Tong 1999). Using an ER-targeted YC3.6, it was shown that cyclopiazonic acid (an inhibitor of ER-type Ca^{2+}-ATPases) decreased the $[Ca^{2+}]_{ER}$ and inhibited the growth of PTs (Iwano et al. 2009). Several Ca^{2+}-transporting proteins such as ACA2 (autoinhibited Ca^{2+}-ATPase) and ECA1 (ER-type Ca^{2+}-ATPase) are expressed in PTs (Sze et al. 2006).

Finally, despite the strong influx of Ca^{2+} from the cell wall, the ER might also release Ca^{2+} at the tip. A possibility is that elements in the ER would facilitate IP_3-induced Ca^{2+} release (Malhó 1998; Franklin-Tong 1999). A role for IP_3-regulated Ca^{2+} release is well established in PTs (Malhó 1998; Monteiro et al. 2005). More so, the ER membrane contains inositol triphosphate (IP_3) receptors which, in response to low $[Ca^{2+}]$, relay information to the PM ultimately resulting in an influx of extracellular Ca^{2+} (Putney et al. 2001).

Vesicles are densely packed in the tip of growing RHs and PTs and occupy the entire clear zone. Vesicle fusion relies strongly on a high $[Ca^{2+}]_{cyt}$ micro-environment (Zorec and Tester 1992). However, despite their obvious role in the exo- and endocytic pathway, they might also function as a Ca^{2+} store. In neuroendocrine cells, some vesicles were shown to harbor Ca^{2+} that can be exchanged in a cyclic ADP ribose-dependent manner (Mitchell et al. 2001). In plants, another hint towards a role as Ca^{2+} stores for vesicles is the fact that a calcium pump (ECA3) was shown to be localized to the post-Golgi endomembranes, which give rise to secretory vesicles (Sze et al. 2006).

Mitochondria are not found in the clear zone of growing RHs and PTs. The mitochondrial proteome contains several Ca^{2+}-binding, EF-hand-containing proteins and Ca^{2+}-dependent protein kinases (Day et al. 2002; Heazlewood et al. 2004). Mitochondria have been shown to release Ca^{2+} to the cytosol, however, in a way that is too slow to relate to the rapid Ca^{2+} spikes observed in tip-growing cells (Loro et al. 2012).

Contrastingly, accumulated evidence implicates that mitochondria might have a pivotal role in Ca^{2+} sequestration instead. Plant mitochondria were shown to contain a Ca^{2+} uptake system (Hanson et al. 1965; Dieter and Marmé 1980; Akerman and Moore 1983; Zottini and Zannoni 1993). For Ca^{2+} to be pumped into the mitochondrial matrix, the Ca^{2+} acquisition system must be able to function at an inner-membrane potential of approx. 180 mV. Using targeted YC3.6 Ca^{2+} sensors to simultaneously monitor Ca^{2+} dynamics in the cytoplasm and mitochondria of *Arabidopsis* root cells, it was shown that a cytoplasmic Ca^{2+} increase related directly to mitochondrial Ca^{2+} accumulation (Loro et al. 2012). Importantly, the observed Ca^{2+} dynamics were particularly different from those in the cytosol (Logan and Knight 2003; Loro et al. 2012). Ca^{2+} dynamics also responded to

exogenous application of ATP, which has also been implicated in the regulation of RH growth. A hint towards the protein(s) that might regulate mitochondrial Ca^{2+} dynamics came from the functional characterization of the *Arabidopsis* MICU protein, an EF-hand mitochondrial Ca^{2+} uniporter (Wagner et al. 2015). MICU is involved in fine-tuning the Ca^{2+} influx into the mitochondrial lumen. The *micu* loss-of-function mutations resulted in a higher than normal mitochondrial Ca^{2+} resting concentration and a reduced ability to dampen auxin and ATP-triggered Ca^{2+} influx into the mitochondrial matrix. Most notably, *MICU* is expressed in in vivo grown PTs and throughout the root (Brady et al. 2007; Qin et al. 2009).

The hereby mentioned Ca^{2+} sources and sinks do not function separately. It is important to acknowledge that Ca^{2+} dynamics in tip-growing cells are the result of a complex pathway that connects all involved Ca^{2+} stores and transport mechanisms. Localized Ca^{2+} concentrations depend on diffusion through the cell wall; retention in the cell wall; active inward transport; uptake into the vacuole, ER, mitochondria and vesicles; and transport outward towards the apoplast.

9.4.1.5 Direct Targets of Calcium Signalling

The intrinsic properties of Ca^{2+} signatures are interpreted by the action of several Ca^{2+}-sensing proteins. The Ca^{2+} signal is then 'decoded', resulting in a specific cellular response (e.g. long-distance Ca^{2+}-mediated signal transduction from root to shoot leads to changes in shoot gene transcription; Choi et al. 2014). In tip growth, Ca^{2+} signal transduction has been shown to ultimately regulate most of the key components related to the tip growth machinery (Fig. 9.5). It has been found that Ca^{2+} may regulate membrane trafficking since loss of the Ca^{2+} gradient disrupts the tip-focused accumulation of RabA4b and affects RH growth (Preuss et al. 2006). In addition, cytosolic Ca^{2+} can modulate F-actin dynamics by controlling the activity of diverse actin-binding proteins (ABPs) such as profilins, villins and actin-depolymerizing factors (Hussey et al. 2006), thereby indirectly influencing vesicle movement. Moreover, Ca^{2+} has been implicated as a crucial component of several feedback loops regulating ROP-GTPase activity (Yan et al. 2009) and ROS production at the tip (Takeda et al. 2008).

Multiple Ca^{2+}-sensing/-binding proteins are expressed in PTs and RHs (Fig. 9.5). Upon Ca^{2+} binding, a conformational change is induced leading to signal transduction. Signal transduction is often the result of consecutive phosphorylation cascades initiated by Ca^{2+}-dependent protein kinases (CDPKs). Such CDPKs containing an enzymatic domain are commonly referred to as Ca^{2+} responders, whereas proteins that lack direct Ca^{2+}-dependent catalytic activity (calmodulin proteins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases, (CIPKs)) are designated sensor relays (Sanders et al. 2002). The latter 'relay' the Ca^{2+} signal by conformation-induced

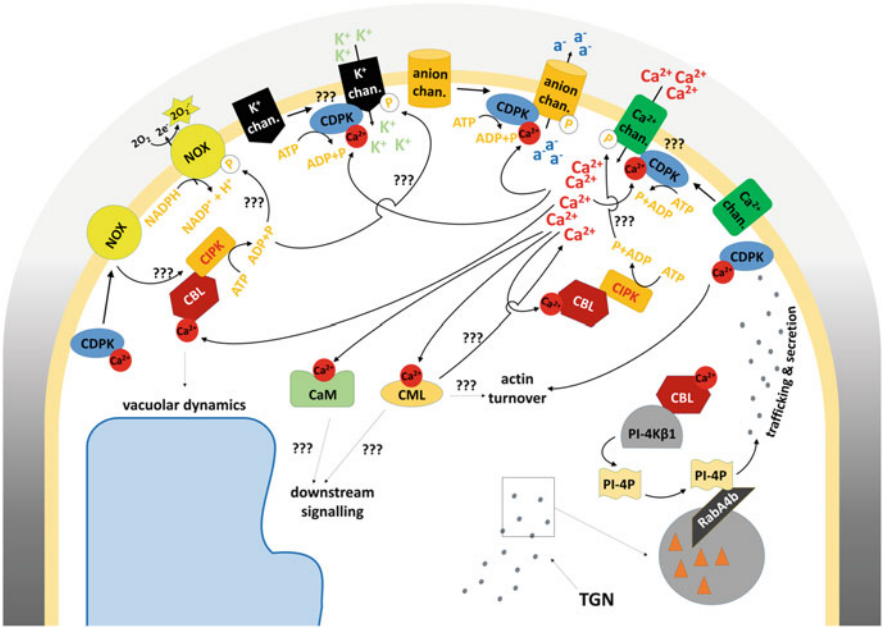

Fig. 9.5 Graphical representation of direct targets of calcium signalling during root hair tip growth. *Question marks* refer to hypothetically involved components and mechanisms. *Grey outline* = cell wall, *yellow outline* = plasma membrane, *grey spheres* = exocytic vesicles, a^- = anion, e^- = electron. All abbreviations are referred to in Sect. 9.4.1

protein-protein interactions. We will shortly discuss these proteins in more detail, in relation to RH and PT development.

Calcium-dependent protein kinases have both a CaM and kinase domain, allowing them to 'sense' Ca^{2+} and immediately relay the Ca^{2+} signal through phosphorylation of one or more target proteins. The CDPK family consists of 34 members, most of which contain N-myristoylation and/or N-palmitoylation sites. The latter reflects a probable membrane association, consistent with their role as Ca^{2+}-sensing proteins. Importantly, one fourth of the known *Arabidopsis CDPK*s is preferentially expressed in pollen (Pina et al. 2005), suggesting a major role in tip growth. Moreover, CDPKs are considered active at Ca^{2+} concentrations in the physiological range detected at the tip of growing RHs and PTs (100–1000 nM; Myers et al. 2009). Multiple studies have indeed shown that CDPKs are important regulators of tip growth. For instance, Myers et al. (2009) have shown that the Ca^{2+}-dependent protein kinases 17 and 34 (CPK17 and CPK34) are localized at the PM at the tip of growing PTs and that they act redundantly to regulate PT elongation and fertilization. Most interestingly, a recent study found that a CDPK-regulated negative anion gradient exists at the tip of growing RHs (Gutermuth et al. 2013). More specifically, CDPK activated anion efflux at the tip resulted in the occurrence

of an anion decrease parallel with each Ca^{2+} peak. These anion oscillations are due to the interaction of CPK2 and CPK20 and their ability to phosphorylate SLAC1 HOMOLOGUE 3 (SLAH3) in growing PTs. Importantly, *CPK2* is transcribed in trichoblast cell files in *Arabidopsis* roots (Brady et al. 2007). More evidence showing that CDPKs can couple different ion dynamics comes from Zhao et al. (2013a). The PM-localized proteins CPK11 and CPK24 negatively regulate PT growth through Ca^{2+}-mediated control of the SHAKER POLLEN INWARD K^+ CHANNEL (SPIK; Zhao et al. 2013a). Interestingly, CPK32 was shown to interact with CNGC18, a crucial Ca^{2+} channel in PTs (Zhou et al. 2014). Furthermore, several CDPKs were shown to regulate actin cytoskeleton organization, polarity and even self-incompatibility in *Zea mays, Nicotiana alata* and *Petunia hybrid* (Estruch et al. 1994; Kunz et al. 1996; Allwood et al. 2001; Yoon et al. 2006).

Clearly, most research about the role of CDPKs in tip growth has focused on PTs. However, in *Medicago truncatula* CDPK1 was shown to control RH growth and morphology (Ivashuta et al. 2005). MtCDPK1 loss of function leads to dissipation of the tip-focused ROS gradient and disorganization of the actin cytoskeleton. Interestingly, Dubiella et al. (2013) showed that in response to a Ca^{2+} peak, CPK5 phosphorylates the NADPH oxidase RBOHD, thereby leading to apoplastic ROS production and cell-to-cell Ca^{2+}-ROS signal transduction (Dubiella et al. 2013). Publicly available transcriptomics data shows that *CPK5* is also expressed in RH cell files. Could it be involved in trichoblast-to-trichoblast Ca^{2+} signal transduction? Similarly, *CPK32* and its interaction partner *CNGC18* in PTs were also found to be expressed in RHs. Could both proteins be working together in RHs to regulate Ca^{2+} dynamics at the tip? Finally, *CPK11* but not *CPK24* (which together regulate K^+ channel activity in PTs) was found to be highly expressed in RH cells in *Arabidopsis*. Investigating RH growth and morphology in *cpk11* knockout plants could be an interesting avenue in identifying novel players in Ca^{2+}-regulated RH development.

Calmodulin (CaMs), calmodulin-like (CMLs), calcineurin B-like (CBLs) and CBL-interacting protein kinases (CIPKs) are also likely to have a prominent role in tip growth regulation. However, little is known about these proteins and their involvement in RH and PT development. The *Arabidopsis* genome contains seven *CaM*-coding sequences, three of which code for the same protein (CaM2, CaM3 and CaM5). Besides a potential role for CaM2 in pollen development and fertilization efficiency (Landoni et al. 2010), none of the CaM proteins have been functionally characterized in relation to tip growth. Based on their preferential expression in RHs and PTs, *CaM3* and potentially *CaM7* could be involved in regulating the tip growth process.

The family of CMLs contains 50 members in *Arabidopsis*. However, most of them remain uncharacterized at the time of writing. Two CML proteins have been shown to be involved in pollen development. *CML24/TOUCH2* loss of function leads to delayed pollen germination, slower PT growth and a shorter final PT length (Yang et al. 2014). *Cml24/touch2* PTs exhibit a higher $[Ca^{2+}]_{cyt}$ and a disorganized actin cytoskeleton. Interestingly, *CML24/TOUCH2* transcription is

induced by touch, suggesting it might function in the pathway of mechanosensitive Ca^{2+} channels. In the root, *TCH2* is specifically expressed in atrichoblast cell files. *CML25* knockout plants have a similar phenotype to that of *cml24* plants (Wang et al. 2015a). In addition, *cml25* plants were shown to regulate K^+ influx into PTs. Importantly, *CML25* is also specifically expressed in RH cell files, implying a general involvement in tip growth. Lin and colleagues showed that *cml25* plants grow longer RHs under phosphate starvation (Lin et al. 2011b). However, no additional information is known on the role of this protein in RH development. Public transcriptomics data shows that at least eight CML proteins could be involved in RH development. The transcription of *CML4*, *CML7*, *CML12*, *CML18*, *CML25*, *CML32*, *CML37* and *CML48* is high and specifically directed towards RH cells (Brady et al. 2007). As such, the current knowledge of CMLs might heavily underestimate their role in RH tip growth.

Ten *CBL*s are found in the *Arabidopsis* genome. CBL1 is the only CBL protein with an established role in RH development. Preuss et al. (2006) found that CBL1 interacts in vivo with PI-4Kβ1 (phosphatidyl-inositol 4-OH kinase which generates the membrane-trafficking regulator phosphoinositide PI-4P), which in turn interacts with RabA4b. RabA4b is found on apically localized trans-Golgi-derived vesicles which are thought to transport new cell wall material to the tip. As such, the authors provided a link between CBL1-mediated Ca^{2+} signalling and cell wall delivery at the tip. Despite the fact that *CBL3*, *CBL4*, *CBL6* and *CBL9* show preferential transcription in RH cell files, none of these have been investigated for a role in RH tip growth. However, interesting results have highlighted an important role for CBL1, *CBL2*, *CBL3* and *CBL9* in PT development. Knowing that CBL3 and CBL9 might also be present in RH cells, it could be very interesting to see if these findings also apply for RH development. CBL2 and CBL3 regulate vacuolar dynamics in PTs through interaction with CIPK12 (Steinhorst et al. 2015). Overexpression of *CBL2* and *CBL3* results in lower pollen germination frequency and slower PT growth, whereas single- or double-knockout mutant exhibits impaired PT growth in vivo and in vitro. Upon interaction of the CBLs with CIPK12, the latter protein relocalized from the cytosol to the vacuole. Overexpression of *CIPK12* results in a marked vacuolar phenotype, whereas loss of function results in complete loss of polar PT growth.

Similarly, *CBL1* and *CBL9* overexpression leads to impaired PT growth (Mähs et al. 2013). This phenotype was shown to be related to overexpression-induced hypersensitivity to high K^+ levels. As such, in knockout PTs, low K^+ availability resulted in a reduction of PT growth. Most interestingly, Drerup et al. (2013) subsequently showed that CBL1 and CBL9 interact with CIPK26 which in turn phosphorylates and activates the NADPH oxidase RBOHF, leading to induction of ROS production. Notably, *CBL9* and *RBOHF* are also expressed in RHs, implying that a similar mechanism might act during RH growth.

Twenty-six *CIPK* genes have been identified in the genome of *Arabidopsis*. Again, little is known about the function of these genes in plant development. However, recent effort has identified CIPKs as important regulators of PT growth. As highlighted before, CIPK12 was shown to be involved in PT growth through

interaction with CBL2 and CBL3 (Steinhorst et al. 2015). CIPK19 is likely involved in regulating the Ca^{2+} influx at the tip of growing PTs (Zhou et al. 2015a). Overexpression of *CIPK19* led to higher apical $[Ca^{2+}]_{cyt}$, the formation of bulged PT tips, loss of PT polarity and decreased fertilization competitiveness. Treatment with La^{3+} (a calcium channel blocker) rescued the phenotype, showing that CIPK19 affect tip-focused Ca^{2+} concentration through regulation of the apical Ca^{2+} influx. In the *Arabidopsis* root, *CIPK19* is specifically, albeit mildly transcribed in RH cell files, implying a common role in RH development. *CIPK23*, which is expressed in RH cells, was shown to be involved in HIGH-AFFINITY K^+ TRANSPORTER 5/K^+ TRANSPORTER 1 (HAK5/AKT1)-mediated K^+-uptake through interaction with CBL1/CBL9 in roots (Ragel et al. 2015; Wang et al. 2016). A possible role for CIPK26 was also discussed previously. Despite these efforts to characterize the role of CIPKs in tip growth, no studies have focused on RH growth. However, at least 12 out of 25 *CIPK*s are strongly expressed in RH cell files (*CIPK2*, *CIPK5*, *CIPK6*, *CIPK8*, *CIPK9*, *CIPK10*, *CIPK13*, *CIPK19*, *CIPK21*, *CIPK22*, *CIPK23*, *CIPK24*), suggesting a major role in RH development.

9.4.2 Proton Oscillations Regulate Tip Growth

9.4.2.1 Proton Concentration

Intra- and extracellular pH regulation is of crucial importance for several developmental processes. Cytosolic and apoplastic pH homeostasis allows nutrient and sugar transport across the PM, organ development and cell elongation (Gjetting et al. 2012). More so, the existence of local pH gradients controls secondary transport in plant cells (Sze et al. 1999; Felle 2001), enzymatic reactions mostly rely on a pH optimum, differences in pH allow for compartmentalization (e.g. cytosol vs. vacuolar lumen) and pH differences across membranes lay the basis of hyperpolarization-driven signal transduction.

Local pH alterations depend on (1) the buffering capacity of the considered compartment, (2) active transport of protons (H^+) across plant membranes and (3) local H^+ production or consumption by metabolic processes. Scientific research has led to the understanding that well-coordinated interaction between these processes can lead to the formation of local H^+ accumulation or depletion. As a result, intracellular pH gradients are formed, which have been shown to function as secondary messengers in plant development. Much like the characteristics of Ca^{2+} signatures, pH differences often occur in a highly spatially and temporally organized manner. Quantitative analyses of these pH signatures has been challenging for long. Consecutively, our understanding of pH dynamics is still limited compared to what has been observed for Ca^{2+}. However, the recent development of several genetically encoded ratiometric pH sensors (Choi et al. 2012; Zhang et al. 2012; Gjetting et al. 2012) is promising and will most definitely further contribute to our understanding.

H^+ translocation across membranes occurs in two directions, depending on specific transporters. As such, transient acidification or alkalinization has been observed both in the cytosol and the apoplast. With regard to cell growth, H^+ translocation between the intra- and extracellular environment is key to the 'acid growth' theory which states that cells are able to expand faster following acidification of the cell wall (Rayle and Cleland 1992). This finding is now largely attributed to the acidic pH optimum of cell wall loosening expansins (McQueen-Mason et al. 1992; McQueen-Mason and Cosgrove 1995).

Crucially, the extracellular pH (pH_{ext}) has a pivotal role in tip growth, too. For instance, when placed in different pH buffers, the growth of PT can be inhibited (Feijó et al. 1999). Similarly, a sudden increase in the pH_{ext} led to cessation of RH growth, whereas a sudden decrease caused RH bursting (Monshausen et al. 2007). Consistent with the acid growth theory, upon RH initiation the cytosolic pH (pH_{cyt}) locally increases, while the cell wall locally acidifies (Bibikova et al. 1998). Until the switch to tip growth is made, this configuration is maintained. Next, during fast tip growth, a tightly controlled mechanism regulates cytosolic and apical apoplastic H^+ dynamics (Fig. 9.6). The resulting spatial and temporal pH changes are pivotal for controlling oscillating tip growth.

9.4.2.2 A Tip-Focused Oscillating H^+ Gradient Controls PT and RH Growth

The diffusion coefficient is much higher for H^+ compared to that of Ca^{2+}. Moreover, experimental evidence has shown that a change in H^+ concentration of 25–90 mM is required for a one unit pH_{cyt} increase (Plieth et al. 1997; Plieth and Hansen 1998; Oja et al. 1999). Despite H^+ diffusion and the high passive buffering capacity of the cytosol (Felle 2001), a pH_{cyt} gradient exists at the tip of growing PTs and RHs (Fig. 9.6). In RHs, the tip-localized pH is slightly alkaline (pH 7.4–7.6) compared to the rest of the cytoplasm (Monshausen et al. 2007; Bai et al. 2014a). In PTs on the other hand, the pH at the tip is slightly acidic, followed by the presence of a subapical alkaline band (Feijó et al. 1999; Michard et al. 2008, 2009). As such, the maintained presence of alkaline micro domains suggests that in both RHs and PTs a mechanism must be in place that actively removes H^+ from the apical region. Indeed, both in RHs and PTs, scientific evidence points towards polarized H^+ efflux in the subapical regions (Fig. 9.6). More specifically, Feijó et al. (1999) reported on a H^+ efflux (0.004–0.01 amol μm^{-2} s^{-1}) at the base of the clear zone in PTs. Similarly, a H^+ efflux (0–0.02 amol μm^{-2} s^{-1}) was detected starting 20 μm from the tip in tobacco PTs (Certal et al. 2008). Further away from the tip, this pattern would revert to a continuous small influx with a peak close to the pollen grain. At the surface of the pollen grain itself, a strong H^+ efflux was again present. Certal et al. (2008) showed that, considering the entire process ranging from early pollen germination (10 μm) to prolonged PT growth (>500 μm), flux dynamics are much more complex than previously thought. Originally, a strong H^+ efflux is present

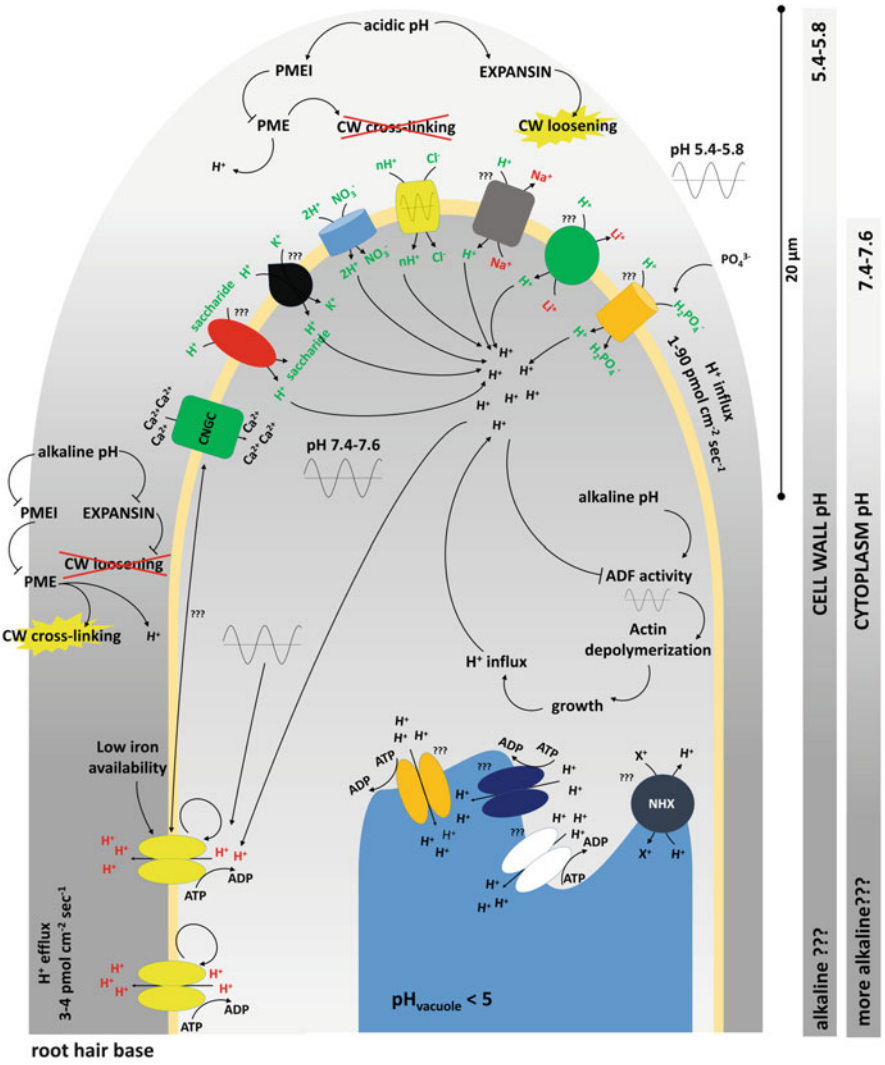

Fig. 9.6 Graphical representation of proton transport and sink systems governing root hair tip growth. *Question marks* refer to hypothetically involved components and mechanisms. The upper 20 µm represents the clear zone. *Sigmoidal curves* represent oscillatory behaviour. *Outer grey area* = cell wall, *yellow outline* = plasma membrane, *inner grey area* = cytoplasm. *Grey gradient* represents pH differences (magnitude depicted on the *right*). All abbreviations are referred to in Sect. 9.4.2

at the pollen grain's surface. During PT growth, efflux of H^+ is observed along the subapical region of the PT. Subsequently, callose plugs are formed in spatially defined regions where no net flux is detected. Upon formation of the primary callose

plug, the efflux ranging from the plug to the grain gradually converts to an influx which finally causes this part of the PT to die.

Compared to PTs, current knowledge in RHs suggests that the spatial distribution of H^+ fluxes in RHs is far less complex. In contrast with PTs, no subapical alkaline band was observed in growing RHs. However, H^+ efflux was found to be the highest at the base of the growing RH (0.03–0.04 amol μm^{-2} s^{-1}; Jones et al. 1995).

Like for the tip-localized Ca^{2+} gradient, the apical pH gradient found in RHs and PTs also occurs in an oscillatory manner (Lovy-Wheeler et al. 2006; Monshausen et al. 2007; Michard et al. 2008). During RH tip growth, the pH_{cyt} oscillates between 7.4 and 7.6. These oscillations have the same frequency as the growth rate oscillations (2–4 min^{-1}), but each growth pulse is preceded by an acidification with a lead of 7 s. Similarly, the apical pH_{cyt} in tobacco PTs was found to oscillate with a magnitude of at least 0.3 pH units and a period of 1–4 min (Michard et al. 2008). In *Lilium* PTs a cytosolic alkalinization preceded a growth pulse by approx. 11 s, and a subsequent acidification followed growth by approx. 8 s (Lovy-Wheeler et al. 2006). These transient acidifications are the result of H^+ influx at the extreme apex of growing PTs and RHs. As such H^+ are (at least partly) cycled through the growing tip. Hence, they are imported at the tip and subsequently exported back to the apoplast either at the base of the clear zone (PTs) or at the base of the shank (RHs).

9.4.2.3 Oscillating H^+ Influx from the Cell Wall

Each cytosolic acidification event is the result of active H^+ transport across the PM at the tip. As such, transmembrane H^+ fluxes also oscillate. Vibrating microelectrode analysis indeed revealed a H^+ influx at the very tip of growing RHs and PTs. However, the measured fluxes strongly differ between studies. Monshausen et al. (2007) reported inward H^+ fluxes up to 0.9 amol μm^{-2} s^{-1} at the tip of growing RHs. Jones et al. (1995) reported on a H^+ influx at the tips of growing *Limnobium stoloniferum* RHs in the range of 0.01 amol μm^{-2} s^{-1}. Feijó et al. (1999) reported on an oscillating H^+ influx in the range of 0–0.04 amol μm^{-2} s^{-1}, whereas Michard et al. (2008) and Messerli et al. (1999) report on a pulsatile influx of 0.1–0.4 and 4.89 $\pm$ 0.81 amol μm^{-2} s^{-1}, respectively, in growing PT tips.

The buffering capacity of the apoplast is generally considered to be much lower than that of the cytosol (in the range of 4 mM H^+ per pH unit; Oja et al. 1999). Logically, periodic efflux of H^+ from the cell wall to the cytosol thus causes pH_{ext} oscillations. Monshausen et al. (2007) found that each cytosolic acidification/H^+ influx peak was indeed accompanied by an extracellular alkalinization. The pH_{ext} was found to oscillate between 5.8 and 5.4 (double the amplitude of pH_{cyt} oscillations). An extracellular alkalinization coincided with cytosolic acidification and followed each growth pulse by 7 s. The nature of transient extracellular alkalinization/acidification events is considered crucial for regulating cell wall dynamics (see section “Cell Wall Pectin Dynamics”).

9.4.2.4 H^+ Transporter Proteins Regulate H^+ Fluxes During Tip Growth

Apical and subapical H^+ fluxes are generated by specific H^+ pumps and non-specific H^+ symporter or antiporter proteins (Fig. 9.6). Several types of H^+ pumps have been reported, either specifically dedicated to H^+ transport (PM-, tonoplast- or endomembrane-localized H^+-ATPases) or relying on H^+ symport for active translocation of nutrients across the PM. The former ATPases actively generate H^+ electrochemical gradients (Palmgren 2001), which are then exploited for active nutrient transport by H^+/nutrient symport or antiporter proteins. Holdaway-Clarke and Hepler (2003) hypothesized that H^+ influx at the tip of growing PTs is regulated by non-specific cation channels, whereas H^+ efflux relates to the specific activity of H^+-ATPases. At the time of writing, little is known about the exact nature of H^+ transporting proteins that act in RH tip growth. However, a quick survey of public transcriptomics data shows that many putative RH-specific proteins might be involved in generating H^+ fluxes across membranes.

H^+ Influx Proteins at the Tip

Whereas H^+-specific microelectrode experiments revealed inward H^+ fluxes across the apical PM, the exact nature of the H^+ transporting proteins is still largely unknown. H^+ influx is generally facilitated by non-specific H^+ symporter or antiporter proteins, which use the transmembrane electrochemical gradient to co-transport nutrients alongside with H^+. Several putative H^+ symport and antiport proteins were shown to be transcribed in pollen, but functional characterization is generally lacking (Bock et al. 2006). A PM-localized pollen-specific H^+/broad-spectrum monosaccharide symporter SUGAR TRANSPORTER 6 (STP6) was identified in *Arabidopsis* (Scholz-Starke et al. 2003). However, *STP6* loss-of-function pollen exhibited no obvious phenotype. Notably, pollen-specific monosaccharide and sucrose transport proteins were also identified in *Petunia* and tobacco, suggesting that a conserved saccharide-based H^+-symport system exists in pollen (Ylstra et al. 1998; Lemoine et al. 1999). More so, in vitro RH and PT tip growth rely on the presence of sucrose. The *Arabidopsis* genome contains 9 sucrose and 14 monosaccharide transport proteins (Scholz-Starke et al. 2003).

At the tip of growing PTs, a tip-focused K^+ influx (6.88 ± 1.44 amol μm^{-2} s^{-1}) coexists alongside the H^+ influx (Messerli et al. 1999). H^+ and K^+ fluxes oscillate in phase relative to each other, but H^+/K^+ peaks lag growth pulses by ± 11 s. Might this suggest the existence of a H^+/K^+ symport system? Interestingly, both in RHs and PTs, several K^+ influx transporter proteins have been identified and characterized, but none of them exhibit H^+ symport activity (Rigas et al. 2001; Ivashikina et al. 2001; Reintanz et al. 2002; Mouline et al. 2002; Desbrosses et al. 2003; Ahn et al. 2004; Lu et al. 2011). Most likely, the coupling of H^+ and K^+ fluxes is due to the strong pH sensitivity of K^+ uptake channels, rather than the existence of a H^+/K^+ symport system (Mouline et al. 2002; Griessner and Obermeyer 2003; Lu et al. 2011). However, the latter option cannot be ruled out.

The current knowledge on H^+ symport proteins in RHs is also quite limited. Patch-clamp analysis revealed the existence of an H^+/NO_3^- symport system in the PM of *Maize* roots (Ruiz-Cristin and Briskin 1991). Similar NO_3^- transport was observed at the PM of *Arabidopsis* RHs (Meharg and Blatt 1995). The latter NO_3^- transport occurred in a pH-dependent manner and involved the co-transport of two protons per single NO_3^- molecule, providing strong evidence for a dedicated H^+/NO_3^- RH-located symport system. Ion-sensitive microelectrode measurements also revealed a H^+/Cl^- symporter current in RHs of *Sinapis alba* (Felle 1994). This current has not been assigned to a specific locus; however, the evidence strongly suggests symport-based coupling of H^+ and Cl^- transport. Upon lowering the pH_{ext}, the $[Cl^-]_{cyt}$ increases and the cytoplasm acidifies. Complementary, the same response was observed when increasing the $[Cl^-]_{ext}$. More so, the Cl^- fluxes were also shown in PTs to oscillate in phase with growth pulses (Zonia et al. 2001).

Hamam and colleagues described a Na^+/H^+ exchange protein at the PM (Hamam et al. 2016), and it is expressed specifically in RH cell files. Interestingly, a Li^+/H^+ exchange protein was characterized at the PM, which is also expressed in RHs (An et al. 2007; Brady et al. 2007).

Finally, a $H^+/H_2PO_4^-$ symporter protein named LePT1 was identified in tomato (Daram et al. 1998). The gene is strongly expressed in RHs and upregulated under phosphate deficiency.

To our knowledge, no other H^+ influx proteins have been characterized at the PM of growing PTs and RHs until now. Thus, a major knowledge gap exists regarding the mechanisms controlling H^+ influx in tip-growing cells.

Subapical H^+ Efflux Proteins

H^+-ATPases constitute the main family of H^+ export proteins. They perform active H^+ transport across the PM through the hydrolysis of ATP. H^+-ATPases are thought to lie at the basis of plant nutrient uptake. Hence, the H^+-ATPase-mediated generation of a PM-localized electrochemical gradient is known to activate nutrient-specific secondary active transporters and membrane channels (Gilroy and Jones 2000; Ashcroft et al. 2009). The *Arabidopsis* genome codes for 12 H^+-ATPase genes (*AHA1-12*). Seven P-type H^+ pumps were found to be expressed during pollen germination and PT growth (Song et al. 2009), and several H^+-ATPases were found to be expressed in RHs (Moriau et al. 1999; Palmgren 2001; Santi and Schmidt 2009; Młodzińska et al. 2015). Indeed, public transcriptomics data confirms that the transcription of several *AHA* genes is restricted to RHs, PTs or both (Table 9.3).

A role for H^+-ATPases in controlling tip growth was first confirmed through a series of pharmacological experiments. PTs grew faster in the presence of fusicoccin (a H^+-ATPase agonist) but seized to grow when supplemented with the H^+-ATPase antagonists vanadate or N-ethylmaleimide (Rodriguez-Rosales et al. 1989; Feijó et al. 1992; Fricker et al. 1997; Pertl et al. 2001; Sun et al. 2009; Lang et al. 2014).

Table 9.3 Transcription of H^+-ATPases in RHs and PTs

AHA gene ID	Transcription in RHs (Brady et al. 2007)	Transcription in PTs (Qin et al. 2009)
1	Yes, highly specific	Yes, low
2	Yes, highly specific	No
3	No	No
4	Yes, highly specific	No
5	Yes, low	No
6	No	Yes
7	Yes, highly specific	Yes
8	No	Yes, highly specific
9	Yes, low	Yes
10	Yes, low	Yes
11	No	No
12	No	No

More so, treatment of tobacco PTs with orthovanadate inhibited the subapical H^+ efflux by 80%, strongly suggesting that H^+-ATPases maintain the latter (Certal et al. 2008). Few studies have focused on the functional annotation of H^+-ATPases in tip growth. Loss of function of *AHA7* was shown to cause a decrease in the number of RHs (Santi and Schmidt 2009). A tobacco pollen-specific AHA with homology to *Arabidopsis* AHA9, AHA8 and AHA6 was found at the subapical PM of PTs (Certal et al. 2008). The fact that it was excluded from the PT tip is consistent with the subapical H^+ effluxes observed in tip-growing cells. Overexpression of *NtAHA* led to the formation of abnormal callose plugs, aberrant H^+ fluxes and overall shorter PTs. Protein-reporter constructs showed that AHA2 is expressed in RHs (Fuglsang et al. 2007; Młodzińska et al. 2015). Santi and Schmidt (2009) found that AHA2 could be involved in controlling iron mobilization through rhizosphere acidification in response to iron deficiency. Most interestingly, both AHA2 and AHA1 were found to interact with CNGC17 (Ladwig et al. 2015). CNGC channels are putative Ca^{2+} channels with the potential of regulating Ca^{2+} dynamics in tip-growing cells (see Sect. 9.4.1.3). *CNGC17* is also specifically expressed in RH cells. As such this raises the question: 'might there be a direct link between the H^+ efflux machinery and the regulation of Ca^{2+} import?'.

A recent study by Veshaguri and colleagues added another layer of complexity to the regulation and dynamics of H^+-ATPase-mediated H^+ fluxes (Veshaguri et al. 2016). Based on single-molecule characterization, they showed that AHA2 switches between three discrete functional states: pumping, inactive and leaky. The intrinsic properties of pH gradients directly determine in which state the pump resides. As such, it is easy to image that also in RHs and PTs, the tip-focused pH signatures regulate the functioning of subapical H^+-ATPases. In addition, all P-type H^+-ATPases contain an autoregulatory domain, which is subject to phosphorylation, thus adding another layer of control.

The Vacuole as H^+ Source/Sink

The role of the vacuole is often neglected when it comes to allocation of H^+. However, (1) several H^+ pumps are thought to localize preferentially to the tonoplast, (2) the vacuolar pH is strongly acidic in comparison to the pH_{cyt} and (3) the vacuole extents until right beneath the clear zone. Several families of putative vacuolar H^+-ATPases exist in the *Arabidopsis* genome, and despite the fact that none of these pumps have been investigated towards their possible involvement in tip growth, transcriptome data leaves no doubt that they might have a pivotal role in controlling RH development. At least 20 vacuolar H^+-ATPase subunits are specifically transcribed in RH cell files (Table 9.4; Brady et al. 2007).

More so, the Na^+/H^+ EXCHANGER 1-4 (NHX1-4) H^+ efflux proteins also localize to the tonoplast (Reguera et al. 2015). Transcriptome analysis shows that *NHX1* is strongly and preferentially expressed in RH cells. Albeit low, *NHX4* is preferentially transcribed in trichoblasts.

9.4.2.5 Signalling Downstream of pH Oscillations

Actin Remodelling

The actin cytoskeleton is a highly spatially organized and dynamic component in tip-growing cells. Experimental evidence derived from studying PTs strongly supports pH-dependent regulation of actin turnover during tip growth. This is based on the facts that (1) an actin fringe of cortical microfilaments co-localizes with the alkaline band in growing PTs, (2) cytosolic acidification disturbs or abolishes this actin fringe and (3) pH-regulated actin depolymerization factor (ADF) proteins co-localize with the actin fringe (Lovy-Wheeler et al. 2006). Lovy-Wheeler and colleagues carefully studied the pH-dependent dynamics of the actin cytoskeleton in growing *Lilium* PTs. They found that artificially lowering the tip-focused pH caused the actin fringe to move closer to the growing tip and resulted in 80% growth reduction (10 mM sodium acetate) or caused the fringe to dissipate completely and growth to stop entirely (100 mM sodium acetate). In addition, treatment of PTs with latrunculin B (LatB; destabilizes actin filaments) causes the dissipation of the acidic domain at the tip and the allocation of the alkaline band towards the apex (Cárdenas et al. 2008). These findings support a role for a feedback loop between tip pH_{cyt} and apical actin dynamics (Fig. 9.6). Furthermore, they showed that ADF co-localizes strongly with the actin fringe and allocates similarly in response to acidification. ADF proteins are thought to promote actin-polymerization in an alkaline environment (Gungabissoon et al. 1998; Allwood et al. 2002; Chen et al. 2002). Contrary to PTs, and consistent with the RH base-positioned H^+ efflux, a subapical alkaline band has not been observed in growing RHs. However, whereas in PTs the apical pH gradient is slightly acidic (located above the alkaline band), in RHs the apical pH gradient is slightly alkaline (Monshausen et al. 2007; Bai et al.

Table 9.4 RH-specific transcription of vacuolar ATPase subunits

V-ATPase domain	V-ATPase subunit	Gene name	Locus ID	Family
V_1	A	AtVHA-A	At1g78920	Vacuolar H^+-pyrophosphatase family
	B2	AtVHA-B2	AT4g38510	V-type ATPase family
	C	AtVHA-C	At1g12840	V-type ATPase family
	D	AtVHA-D	AT3g58730	V-type ATPase family
	F	AtVHA-F	AT4g02620	V-type ATPase family
	H	AtVHA-H	AT3g42050	V-type ATPase family
V_0	a1	AtVHA-a1	At2g28520	V-type ATPase family
	a2	AtVHA-a2	At2g21410	Putative vacuolar H^+-ATPase subunit 1 family
	a3	AtVHA-a3	AT4g39080	Putative vacuolar H^+-ATPase subunit 1 family
	c1	AtVHA-c1	AT4g34720	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c2	AtVHA-c2	At1g19910	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c3	AtVHA-c3	AT4g38920	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c4	AtVHA-c4	At1g75630	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c5	AtVHA-c5	At2g16510	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c″1	AtVHA-c″1	AT4g32530	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c″2	AtVHA-c″2	At2g25610	Vacuolar H^+-ATPase 16 kDa proteolipid family
	d1	AtVHA-d1	At3g28710	V-type ATPase family
	d2	AtVHA-d2	AT3g28715	V-type ATPase family
	e1	AtVHA-e1	AT5g55290	V-type ATPase family
	e2	AtVHA-e2	AT4g26710	V-type ATPase family

2014a). As such, we suggest that ADF activity could also regulate actin dynamics in RHs. More so, both *ADF8* and *ADF11* were shown to be transcribed specifically in RH cell files (Brady et al. 2007; Kandasamy et al. 2007; Ruzicka et al. 2007) and were previously found to be part of the transcriptional core RH machinery (Bruex et al. 2012).

Lovy-Wheeler et al. (2006) suggested a self-sustained cycle controlling pH-mediated actin-remodelling at the tip. An alkaline pH would promote ADF-activity, which in turn favours increased actin depolymerization. The latter would result in faster growth and an increase of the H^+ influx at the tip, leading to cytoplasmic acidification. Subsequent inactivation of ADF would then lead to a decrease of actin polymerization, growth and H^+ influx, allowing for the pH to gradually rise again. Logically, such an autoregulatory loop would result in actin polymerization/depolymerization oscillations at the tip (see Sect. 9.5.1.2).

Cell Wall Pectin Dynamics

The cell wall of tip-growing cells is a highly dynamic compartment, strongly relying on pH oscillations to fine-tune the spatial and temporal balance of cell wall rigidification/loosening (Fig. 9.6). This process is now thought to largely rely on the presence of several cell wall-modifying proteins whose action is pH dependent. In general, RH and PT elongation seem to be consistent with the theory of acid growth, where a more acidic environment promotes cell wall loosening (Rayle and Cleland 1992). More H^+ would generally increase the activity of expansins while inhibiting the action of pectin methylesterases (Cosgrove 2000; Michelli 2001; see Sects. 9.6.2 and 9.6.3).

9.5 The Cytoskeleton

9.5.1 Actin Dynamics Control Apical Cell Growth in RHs

9.5.1.1 Introduction

RH and PT tip growth rely on highly polarized delivery of secretory vesicles to the growing apex and strict subcellular localization of specific organelles. In RHs, directional vesicle and organelle transport is facilitated by the presence of a dynamic actin cytoskeleton (Fig. 9.7) similar to that observed in growing PTs (see Chap. 3).

When RH growth seizes, thick actin bundles protrude the entire cytoplasm, including the apex (Ketelaar et al. 2003; He et al. 2006). It has been shown that treatment with the actin polymerization inhibitor CytD terminates RH growth (Miller et al. 1999). Moreover, like in PTs, depolymerization of F-actin with LatB inhibits RH growth (Gibbon et al. 1999; Baluška et al. 2000). In *Arabidopsis*, there are at least eight isovariants of actin. Ectopic expression of individual isoforms

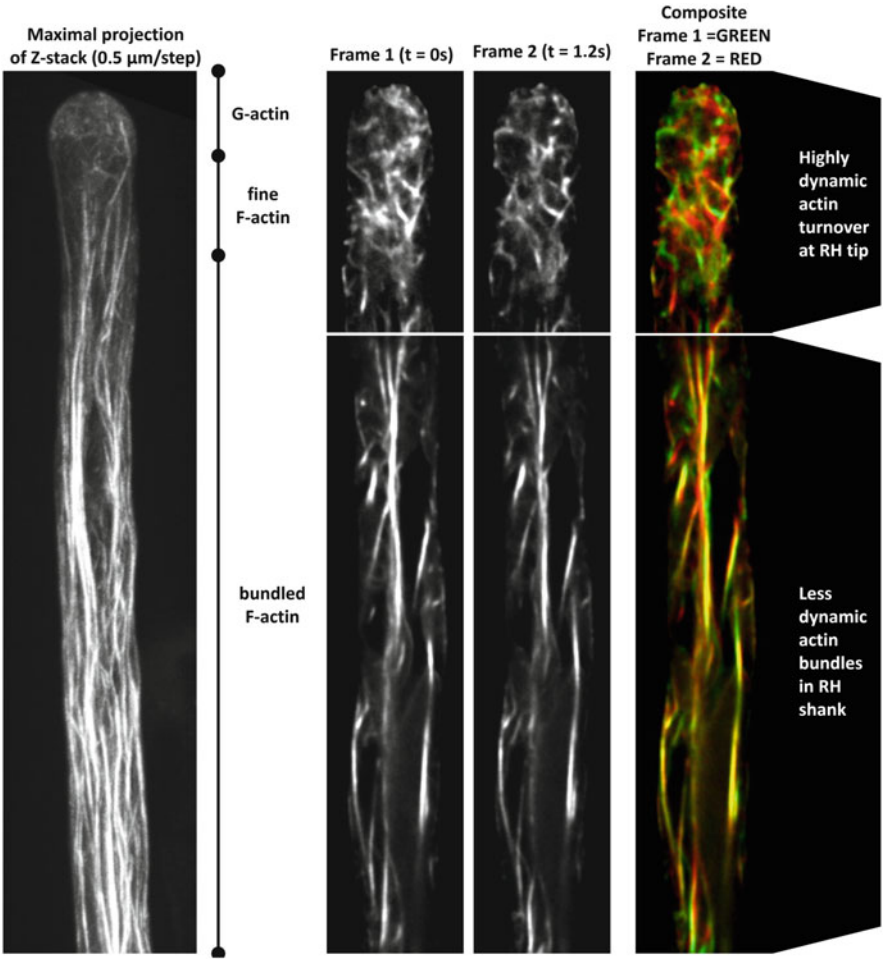

Fig. 9.7 Spinning disc confocal images of the actin cytoskeleton in growing *Arabidopsis* root hairs stably expressing Lifeact-Venus (first employed by Riedl et al. 2008). (*Left*) Maximal projection of a Z-stack (0.5 µm step size) of the actin cytoskeleton. (*Right*) Consecutive frames (taken at 1.2 s interval) showing actin dynamics in the RH tip and shank

resulted in disruption of the actin cytoskeleton and severe developmental defects (An et al. 1996a, b; Huang et al. 1997; Kandasamy et al. 2002, 2007). As such, different actin isoforms seem to relate to different functions. The latter might also imply that they are each associated with different means of regulation. However, at the time of writing, evidence for such isoform-specific control in both RHs and PTs is rather limited. Kandasamy and colleagues did reveal that ectopic expression of *ACT1* resulted in strong developmental defects, likely associated with incorrect interaction with endogenous vegetative actin-binding proteins, since coexpression with the native ACT1-specific ABPs partially rescued the phenotypes.

ACT2, *ACT7* and *ACT8* are expressed in vegetative tissues including RHs. ACT2 and ACT8 are essential for proper tip growth since *act2-1* (aka *enl2* or *der1*) and *act8-2* mutants exhibit stunted RHs, while the *act2-1/act8-2* double mutant is even hairless (Gilliland et al. 2002; Ringli et al. 2002; Nishimura et al. 2003; Kandasamy et al. 2009). Even partial loss of ACT2 functionality results in drastic RH swelling and premature growth arrest (Diet et al. 2004). *ACT7* transcription is auxin inducible and found throughout the root epidermis rather than being restricted to trichoblast cell files (McDowell et al. 1996a; An et al. 1996b; Kandasamy et al. 2001). Its role, however, seems to be restricted to early RH establishment, given that the RH density is decreased in *act7* knockout plants. Together with ACT2, ACT7 regulates planar polarity establishment in early RH development (reviewed in Balcerowicz et al. 2015; Kiefer et al. 2015).

In PTs, several more actin isoforms are expressed besides *ACT2* and *ACT8* (Wang et al. 2008). The remaining isoforms are referred to as the class of reproductive actins (*ACT1*, *ACT3*, *ACT4*, *ACT11* and *ACT12*; McDowell et al. 1996b; McKinney and Meagher 1998), and surprisingly, not much is known about their individual functions. ACT11 was shown to regulate actin turnover and subsequent vesicle turnover rates in *Arabidopsis* PTs (Chang and Huang 2015).

A continuous effort towards the characterization of the actin cytoskeleton and its key regulators has led us to acknowledge its role in PT (see Chap. 3) and RH development. Future research will greatly benefit from novel in vivo labelling and imaging techniques and the availability of a vast number of T-DNA insertional mutants. Here we describe the current state of knowledge regarding the molecular players controlling actin dynamics in growing RHs.

9.5.1.2 Do Actin Polymerization/Depolymerization Oscillations Exist in RHs?

The regulation of polymerization/depolymerization of AFs has been shown to depend on Ca^{2+}, pH, phosphoinositide and ROP-GTPase dynamics, most of which oscillate during tip growth (see Sects. 9.2 and 9.4). As such, it is easily conceivable that actin turnover at the tip could also exhibit an oscillatory pattern. In RHs, initial data revealed clear fluctuations in labelling intensity of tip-localized F-actin + ends (Vazquez et al. 2014). Quantitative analysis of these fluctuations on a broader timescale might reveal the temporal and spatial dynamics of actin oscillations in growing RH. For now, however, the hypothesis that oscillatory actin turnover might exist during RH development is solely based on what has been observed in PTs. Treatment of growing PTs with low [LatB] (2 nM) resulted in reversible inhibition of growth rate and $[Ca^{2+}]_{cyt}$ oscillations (Cárdenas et al. 2008). Importantly, however, PTs did not seize to grow but instead continued to elongate at a basal growth rate. This finding illustrates that actin polymerization is needed to sustain oscillatory but not linear growth, strongly suggesting that actin turnover itself exhibits oscillatory changes. Indeed, through the development of a G-actin-specific ratiometric dye, the authors were able to show that tip-focused G-actin oscillates with the same period

as growth oscillations, but with a phase lag of ±10 s. As such, a low level of G-actin/an increase in F-actin polymerization anticipated an increase in growth rate. Ca^{2+} oscillations lag growth oscillations by approx. 5 s (Messerli et al. 2000) and thus anticipate G-actin oscillations. It is therefore tempting to propose that Ca^{2+} dynamics could regulate oscillatory actin turnover through direct control of ABPs (Pei et al. 2012). Moreover, the ABPs VILLINS and ADFs have been shown to regulate RH growth in a Ca^{2+}-dependent manner. For instance, ZmADF3's actin-severing activity is regulated by a Ca^{2+}-dependent protein kinase (Smertenko et al. 1998; Allwood et al. 2001). VILLIN4 promotes capping and severing of AFs at high (5 μM) but not at low (0.5 μM) [Ca^{2+}] (Zhang et al. 2011a). Considering that these concentrations lie within the physiological range associated with the tip-focused Ca^{2+} gradient, it is easy to imagine that VILLIN activity oscillates together with Ca^{2+} oscillations.

In addition to Ca^{2+}-dependent regulation of actin oscillations, pH oscillations might also play an important role. Based on pharmacological and localization studies, there seems to be a feedback loop controlling actin and pH dynamics at the apex of tip-growing cells (Lovy-Wheeler et al. 2006). Moreover, research has shown that certain ADF proteins are preferentially functional in an alkaline environment, consistent with the base of the clear zone.

9.5.1.3 Actin-Binding Proteins Regulate RH Tip Growth

Emerging evidence shows that the control of actin dynamics by several ABPs is tightly embedded with the core tip growth machinery. This is illustrated by the existence of actin-regulating factors with a specific sensitivity towards Ca^{2+}, pH and phospholipid signalling (Fig. 9.8). Here, we provide an overview of the ABPs shown to be involved in regulating RH tip growth.

Control of Actin Nucleation

Formins are proteins that directly bind to the elongating barbed end of AFs, where they enhance filament elongation while protecting the growing end from capping proteins (Kovar and Pollard 2004; Romero et al. 2004). Their function largely depends on their ability to interact with a variety of proteins, thereby forming large multiprotein complexes generally including profilins (discussed later). Notably, several formin isoforms contain ROP-GTPase-binding domains (Watanabe et al. 1997; Petersen et al. 1998) and were shown to associate with MTs (Deeks et al. 2010; Li et al. 2010; Wang et al. 2013). Formins have a pivotal role in mammals, fungi and plants and were shown to be important for RH and PT development (Deeks et al. 2005; Yi et al. 2005; Ye et al. 2009; Cheung et al. 2010; Huang et al. 2013b). Twenty-one formin-related sequences were identified in the *Arabidopsis* genome alone (Deeks et al. 2002; Cvrckova et al. 2004). Several formins seem to be preferentially transcribed in RH cells (relatively high transcription, *AtFH5*,

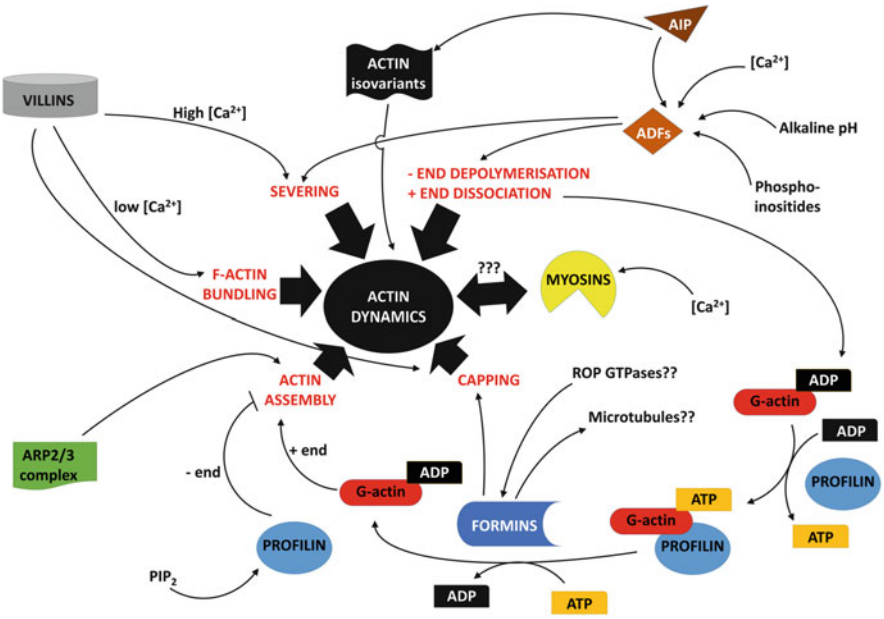

Fig. 9.8 Simplified scheme illustrating the mechanisms controlling actin dynamics in growing root hairs. *Question marks* refer to hypothetically involved components and mechanisms. All abbreviations are referred to in Sect. 9.5.1

AtFH8 and *AtFH1*; moderate transcription, *AtFH10*, *AtFH13*, *AtFH16*, *AtFH19*, *AtFH20*), and overexpression of *AtFH8* lacking its FH2 (actin-binding) domain led to aberrant RH development, with RHs being shorter, seizing growth at the bulge stage or being completely absent from the epidermis (Deeks et al. 2005). The latter suggest that AtFH8 could be involved in both bulge formation (reviewed in Balcerowicz et al. 2015) and tip growth. The authors suggest that in vivo the truncated protein would compete with the interacting proteins of the functional AtFH8, thereby preventing the latter from nucleating the actin cytoskeleton. An additional study revealed that constitutive *AtFH8* overexpression resulted in short, waved, bulged and/or branched RHs with an over-accumulation of AFs (Yi et al. 2005). FH8 was shown to nucleate actin barbed ends in vitro and, curiously, to induce actin severing instead of polymerization. Yet it enhances polymerization in the presence of profilin. The latter has not been shown in any other formin isoform.

AtFH5 is also strongly and specifically expressed in RH cell files (Brady et al. 2007). Corresponding functional characterization in RHs is however still lacking. In PTs, AtFH3 and AtFH5 both regulate apical expansion, but they seem to do so in different ways (Ye et al. 2009; Cheung et al. 2010). Interestingly, present data clearly supports a role for FH5 in subapical vesicle targeting.

OsFH1 (*FH1 Oryza sativa* homologue) knockout plants display very short RHs when no structural support is present (liquid media instead of solid; Huang et al.

2013a, b). When grown on a solid surface, RHs appeared normal. Besides OsFH1 and AtFH8, no other formins have been attributed to regulating RH growth.

Just like formins, the ACTIN-RELATED PROTEIN 2/3 (ARP2/3) complex mediates barbed end actin assembly and thus promotes actin nucleation (Hussey et al. 2006). The seven-protein complex binds to existing AFs and stimulates the formation of new side branches. The complex consists of seven subunits (Arp2, Arp3, ArpC1/p41, ArpC2/p34, ArpC3/p20, ArpC4/p20, ArpC5/p16), which are all expressed in plants (Mathur et al. 2003a). The function of the ARP2/3 complex is considered to be highly conserved between different biological systems, given that *Arabidopsis* subunits can complement their yeast homologues and mammalian homologues can complement mutations in plant subunits (Le et al. 2003; Mathur et al. 2003b). The currently described plant-specific mutations associated with loss of function of *Arp2/3* subunits all resulted in morphogenesis-related phenotypes (Mathur et al. 1999, 2003a; Li et al. 2003) or related to regulation of stomatal aperture (Li et al. 2013, 2014) or salt stress-induced Ca^{2+} signalling (Zhao et al. 2013b). The evidence regarding involvement in RH and PT tip growth is very scarce. It was shown that *AtARP2* is strongly transcribed in pollen grains (Klahre and Chua 1999), and Mathur et al. (2003b) revealed that RHs of the *crooked* mutants, harbouring a loss-of-function mutation in the *ARPC5* subunit, were either short and stunted or curvy and up to double as wide as wild-type RHs. In addition, the occasional presence of two bulges on a single trichoblast cell suggests that the ARP2/3 complex might regulate both RH initiation and elongation. The actin cytoskeleton in *crooked* RHs consisted of alternating regions with thick actin patches (associated with growth restriction) and fine F-actin bundles (associated with growth). The *wurm* (*ARP2* knockout) and *distorted1* (*ARP3* knockout) mutations exhibit a similar short and wavy RH phenotype, as a result of F-actin aggregation (Mathur et al. 2003a). Given the fact that loss of function of each of three *ARP2/ARP3* single subunits causes the same phenotype, it is conceivable that the observed defect is due to loss of function of the entire complex. As such, it can be hypothesized that the ARP2/3 complex regulates the formation of fine filamentous actin cables in RHs, thereby controlling RH polarity and final RH length.

Profilins are G-actin-binding nucleotide exchange factors that catalyse the exchange of ADP to ATP, thereby returning actin monomers to the profiling-actin-ATP pool (Valenta et al. 1993; Gibbon et al. 1998; Pollard and Borisy 2003). They can constitute a large part of the total protein pool (up to 127 μM), largely equimolar with the concentration of G-actin, as observed in *Maize* pollen (Gibbon et al. 1999). In the presence of profilins, plant formins can enhance filament elongation rates by a factor of 10–42 (Zhang et al. 2016). However, profilins can either inhibit spontaneous nucleation of AFs in the absence of formins (at the minus-ends) or promote AF elongation through interaction formation of profilin-G-actin-formin complexes at plus-ends (Kovar et al. 2003; Michelot et al. 2005; Vidali et al. 2009; Zhang et al. 2011b; van Gisbergen and Bezanilla 2013; Ketelaar 2013). Minus-end inhibition of polymerization might be actin isoform specific (Kijima et al. 2016). Five profilin isoforms are transcribed in *Arabidopsis* (Huang et al. 1996) yet only

one is trichoblast specific (*PRF1*; Brady et al. 2007), whereas at least three profilins are expressed in in vivo grown PTs (*PRF1*, *PRF4* and *PRF5*). In RHs, profilin specifically localized to the apex of emerging RH bulges and growing RHs (Braun et al. 1999). This pattern is altered when RH growth stops or upon treatment with the microfilament disruptor CytD and the phosphatidylinositol 4,5-biphosphate [$PI(4,5)P_2$] signalling disruptor mastoparan, strongly suggesting that profilin is involved in controlling tip growth in a $PI(4,5)P_2$-dependent manner. *Prf1* knockout plants exhibit a variety of RH phenotypes related to increased cell elongation (McKinney et al. 2001). The authors suggest that the absence of PRF1 protein could result in more free G-actin, which could enhance filament polymerization. However, the actual mechanism by which PRF1 controls RH elongation has not been characterized. Surprisingly, Vidali and Hepler (1997) found that in PTs profilin localization differs from that observed in RHs. In PTs, profilins were found to localize to the peripheral cytoplasm of the cell and accumulate mostly in the central region of the shank.

Control of Actin Depolymerization

Actin-depolymerizing factors show affinity for both filamentous actin and actin monomers. They enable severing of ADP-actin filaments and promote ADP-actin filament dissociation at the minus-end (Maciver and Hussey 2002; Pollard and Borisy 2003). The latter means that ADF proteins enhance actin turnover by replenishing the monomer pool, and hence, released minus-end monomers can be recycled for plus-end growth. In addition, ADFs might also promote AF bundling and nucleation (Dong et al. 2001a; Andrianantoandro and Pollard 2006; Tholl et al. 2011). Their action is regulated by cytosolic Ca^{2+} (through phosphorylation by CDPKs), pH dynamics and phosphoinositide signalling (Gungabissoon et al. 1998; Smertenko et al. 1998; Allwood et al. 2001, 2002; Tholl et al. 2011; Dong and Hong 2013). As such, it is easy to imagine that ADFs perceive related cytosolic signatures and relay them towards fine-tuning of actin dynamics. The *Arabidopsis* genome encodes at least 11 ADF proteins (Ruzicka et al. 2007). *ADF8* and *ADF11* are specifically transcribed in trichoblast cell files (Brady et al. 2007; Ruzicka et al. 2007; Bruex et al. 2012). However, both genes have not been functionally described in relation to RH tip growth. Overexpression of *AtADF1*, which is expressed in the root epidermis but not specifically in trichoblasts, caused shorter and wider RHs with irregular actin distribution, whereas *adf1* knockout RHs were longer and contained more actin cables (Dong et al. 2001b). These observations are consistent with the function of ADF proteins and indicate that ADF-mediated actin depolymerization is important for RH development. Not *ADF8* and *ADF11* but *ADF7* and *ADF10* are predominantly expressed in growing PTs, and both proteins are associated with the actin fringe and thick actin cables in the PT shank (Ruzicka et al. 2007; Wang et al. 2008; Qin et al. 2009; Bou Daher et al. 2011). ADF7 was shown to regulate AF severing and depolymerization rates in vivo (Zheng et al. 2013).

The actin-interacting protein1 (AIP1) enhances ADF depolymerization activity and has a crucial role in directing RH tip growth (Allwood et al. 2002; Ketelaar et al. 2007, 2004). It caps barbed ends and disassembles ADF-bound filaments (Ono et al. 2004; Okada et al. 2006). Ethanol-induced RNA interference of *AIP1* transcript resulted in drastically shorter RHs and a dramatic decrease of the RH growth rate (Ketelaar et al. 2004). Moreover, consistent with the presumable lack of ADF activity and subsequent absence of actin depolymerization, thick actin bundles reached all the way into the RH apex. Surprising though, the *AIP1* loss-of-function phenotype (short RHs) differs considerably from that observed in *ADF* knockout plants (long RHs; Dong et al. 2001b). This difference might imply an additional role of AIP1 besides stimulation of ADF activity. Ethanol-induced *AIP1* overexpression on the other hand also inhibited RH growth, resulting in short and swollen RHs and thick actin cables (Ketelaar et al. 2007). Interestingly, AIP1 and ADF seem to function in a feedback loop, where AIP1 functioning also depends on ADF presence (Augustine et al. 2011). A recent study showed that AIP1 interacts directly with ACT7 (Kiefer et al. 2015), and together they regulate RH initiation through ROP-mediated planar polarity establishment (reviewed in Balcerowicz et al. 2015), downstream of *WEREWOLF* (*WER*) in an ethylene-dependent manner.

Control of Actin Bundling

The *Arabidopsis* genome encodes 5 *villin-type proteins* (Huang et al. 2005; Khurana et al. 2010), shown to be involved in regulating actin bundling, but also severing, nucleation and plus-end capping (e.g. Yokota et al. 2005; Zhang et al. 2010). As such, villins are considered to be important in regulating actin turnover, and some do so in a Ca^{2+}-dependent manner (Yokota et al. 2005; Zhang et al. 2011a), whereas others are Ca^{2+} insensitive (Khurana et al. 2010). The villin-specific Ca^{2+}-dependency coincides with the presence of at least three Ca^{2+}-binding sites (Huang et al. 2005), and their function regulates tip growth, both in RHs and PTs.

In RHs, double loss of function of *VLN2* and *VLN3* did not result in a RH phenotype (van der Honing et al. 2012). However, GFP-VLN3 was shown to decorate shank-localized F-actin bundles. Loss of function of the RH-specific Ca^{2+}-sensitive *VLN4* that regulates severing and capping, resulted in slowing of RH growth, shorter RHs and alterations of cytoplasmic streaming, due to reduction of actin bundles (Zhang et al. 2011a). Given their apparent redundancy, it would be very interesting to see how RH growth is affected in a triple *vln2 vln3 vln5* knockout mutant.

In PTs VLN2 and VLN5 are thought to act redundantly in regulating actin turnover and construction of actin collars at the PT tip (Zhang et al. 2010; Qu et al. 2013). The two *Lilium* villin homologues P-115-ABP and P-135-ABP regulate Ca^{2+}-dependent AF bundling (Yokota et al. 2005), and P-135-ABP can promote actin turnover. Together, these results identify villins as important regulators of PT and RH growth.

Control of Actin-Mediated Trafficking

Myosins are motor proteins that, by means of ATP hydrolysis, transport vesicles and organelles along AFs (e.g. Reisen and Hanson 2007; Sweeney and Houdusse 2010). Seventeen myosin-class genes are transcribed in *Arabidopsis*, of which 13 belong to the XI-myosin class with an established role in plant development (Hashimoto et al. 2005). Functional characterization of myosin proteins has been challenging due to a high degree of redundancy (Peremyslov et al. 2008, 2010). However, recent advances have provided increasing evidence for their involvement in RH tip growth. The first evidence came from Hashimoto and colleagues, showing that MYOSIN 2 (MYA2) was localized to RHs, appeared as punctate dots (Hashimoto et al. 2005), co-sedimented with F-actin and potentially transported peroxisomes. In addition, MYA2 was suggested to also transport vesicles (Reisen and Hanson 2007; Walter and Holweg 2008). The RH length of *mya2* knockout plants was over 50% decreased compared to wild-type, in parallel with a two times reduction in Golgi, peroxisome and mitochondrial trafficking speeds in RHs (Peremyslov et al. 2008; Avisar et al. 2009). *XI-K* loss of function resulted in the same RH and trafficking defects (Ojangu et al. 2007; Peremyslov et al. 2008; Avisar et al. 2012). *Xi-k* RHs grew slower and seized to grow earlier (Park and Nebenführ 2013). Together with myosin-binding proteins 1 and 2 (MyoB1/MyoB2), XI-K co-localized with yet uncharacterized non-ER-derived endomembrane vesicles along F-actin cables (Peremyslov et al. 2012). Fluorescently tagged XI-K and MyoB1/2 protein exhibited a highly polarized localization in growing RH, which disappeared when growth seized (Peremyslov et al. 2012, 2013). Together, these findings support a role for myosins in regulating fast RH tip growth.

However, at least six myosins are transcribed specifically in RHs, implying that besides MYA2 and XI-K, XI-B/XI-C/XI-D and XI-H might also regulate RH development (Brady et al. 2007). Peremyslov et al. (2010) generated a number of triple and quadruple knockout mutants of XI-family myosins and illustrated that simultaneous loss of function could result in branched, thick or mislocalized RH morphology and a decrease in RH length of up to a factor 10. The fact that RH morphology strongly differed in different knockout combinations suggests that individual myosins serve distinct roles in RH development. Moreover, their results indicate that myosins might also be involved in planar polarity establishment during RH initiation (Peremyslov et al. 2010; reviewed in Balcerowicz et al. 2015).

Also in PTs, at least six XI myosins are expressed (Madison et al. 2015), and PTs of *xi-c xi-e* double-knockout plants grew much slower, resulting in a severely decreased fertilization efficiency. Peroxisome movement and AF organization were disturbed in mutant PTs. Interestingly, a similar myosin-dependent effect on F-actin organization was observed in RHs (Peremyslov et al. 2010; Park and Nebenführ 2013). As such, a feedback loop seems to exist in both RHs and PTs, with F-actin organization regulating myosin functioning, and *vice versa*. The mechanism that controls myosin to actin signalling has not yet been identified.

Finally, cytoplasmic Ca^{2+} might be involved in regulating myosin activity. Myosin XI motility and actin association strongly depend on the Ca^{2+} concentration (Tominaga et al. 2012). At concentrations higher than 1 μM Ca^{2+}, the motility and ATPase activity of a myosin isolated from *Lilium* PTs were strongly decreased (Yokota et al. 1999). Crucially, calmodulin-dependent reversible regulation of myosin motility was observed in the range of 1–10 μM Ca^{2+}, consistent with the $[Ca^{2+}]_{cyt}$ fluctuations observed at the apex of tip-growing cells (Pierson et al. 1996; Wymer et al. 1997; Felle and Hepler 1997). As such, it is tempting to imagine that in tip-growing cells, myosin trafficking could be regulated by the Ca^{2+} signatures present in the apex.

9.5.2 The Microtubule Cytoskeleton

9.5.2.1 Organization of Microtubules in Root Hairs

In RHs, two distinct populations of MTs have been identified. Cortical MTs (CMTs) are present throughout all stages of RH development and, similar to what has been observed in PTs (see Chap. 3), are oriented longitudinally along the shank (Van Bruaene et al. 2004). Studies from several plant species suggest that CMTs, like F-actin, are absent from RH apices (Sieberer et al. 2002; Weerasinghe et al. 2003; Van Bruaene et al. 2004; Vassileva et al. 2005). Endoplasmic MTs (EMTs), which exhibit a more irregular arrangement in comparison to CMTs, form when tip growth begins and accumulate in the subapical region and around the nucleus. EMTs are extremely dynamic and display higher sensitivity to depolymerizing drugs than CMTs (Sieberer et al. 2002; Van Bruaene et al. 2004). As for PTs, the role of MTs in regulating RH development remains poorly understood. It is suggested that EMTs might be involved in positioning of AFs and transport of nucleation complexes from the nucleus; however, their exact role remains unclear (Van Bruaene et al. 2004). Pharmacological analysis revealed that EMTs are required for nuclear positioning within RHs of *Medicago* (Sieberer et al. 2002) but not of *Arabidopsis* (Ketelaar et al. 2002).

9.5.2.2 Disruption of Microtubules Affects Root Hair Morphology

Both pharmacological and genetic studies have shown that MTs play a role in RH development by maintaining the direction of tip growth. Treatment of *Arabidopsis* roots with the MT stabilizing drug taxol and the MT depolymerizing drug oryzalin results in wavy RH growth and occasionally causes formation of multiple tips (Bibikova et al. 1999; Ketelaar et al. 2002). In addition, manipulations of the Ca^{2+} gradient and application of a touch stimulus trigger formation of new growth points in taxol-treated RHs, whereas in the control RHs, this only leads to the transient reorientation of growth (Bibikova et al. 1999). Consistent with the effects

of MT disturbing drugs, crooked and/or branched RH have also been reported for mutants in α-tubulin (Bao et al. 2001), *MICROTUBULE ORGANIZATION 1* (*MOR1*; Whittington et al. 2001) and *ARMADILLO REPEAT-CONTAINING KINESIN 1/MORPHOGENESIS OF ROOT HAIR 2* (*ARK1/MRH2*; Jones et al. 2006; Sakai et al. 2008). *MOR1* encodes a MT-associated protein (MAP) with several HEAT repeats that might serve as MT-binding sites. The point mutation in the N-terminal HEAT motif of *MOR1* leads to temperature-dependent disruption of CMTs indicating that MOR1 plays a role in stabilizing MTs (Whittington et al. 2001). In contrast, ARK1/MRH2 has recently been shown to promote MT disassembly through a yet unknown mechanism. Low concentrations of oryzalin were able to partially restore normal RH growth in *ark1-1* mutants, whereas taxol caused even more severe RH defects. As such, the role of ARK1/MRH2 in RH tip growth seems to be maintaining the pool of free tubulin which is required for rapid MT polymerization (Eng and Wasteneys 2014). However, it is important to note that a previous study suggested that ARK1/MRH2 acts as a MT-stabilizing factor (Yang et al. 2007). Interestingly, in contrast to RHs, taxol and oryzalin do not have a significant effect on PT growth indicating possible differences in the role of the MT cytoskeleton in these two cell types (Gossot and Geitmann 2007; Poulter et al. 2008; Bou Daher and Geitmann 2011; Zhou et al. 2015b).

9.5.2.3 The Microtubule/Actin Interface

There is growing evidence that MTs contribute to tip growth through interaction with the actin cytoskeleton. It has been demonstrated that administration of the actin-disrupting agent cytochalasin B (CytB) causes an inhibition of reverse fountain streaming in *Hydrocharis* RHs and that the effect of the drug is fully reversible (Shimmen et al. 1995). However, simultaneous treatment with CytB and the MT-destabilizing herbicide propyzamide followed by removal of CytB does not lead to recovery of long actin cables and cytoplasmic streaming (Tominaga et al. 1997). It therefore appears that the presence of MTs is essential for re-establishment of AFs. On the other hand, in *Medicago*, longer treatment with the actin-depolymerizing drug LatB leads to formation of MT bundles in the (sub)apical region of RH cells (Timmers et al. 2007). Consistently, in tobacco PTs, long MTs organized as bundles replace the short and randomly oriented ones in the presence of a low dose of LatB (Idilli et al. 2012). Thus, the actin cytoskeleton has an influence on MT dynamics too. In *Arabidopsis* RHs, the cross-talk between MTs and AFs might involve the action of ARK1/MRH2 since an ARM domain-containing fragment of this kinesis has the ability to bind polymerized actin in vitro and *mrh2–mrh3* mutant displays increased sensitivity to LatB (Yang et al. 2007). Moreover, a mutation in *ARK1* enhances the phenotype of CA-ROP2 (Yang et al. 2007), and ROP2 regulates actin organization in RHs (Jones et al. 2002).

9.6 The Cell Wall as a Dynamic System

Much like in PTs, the protoplasm of RHs is surrounded by a dynamic cell wall that consists of cellulose, hemicellulose, pectin and several types of proteins (Somerville et al. 2004). In addition, the basic mechanisms controlling cell wall deposition in RHs are similar to those found in PTs, as well as the resulting biomechanical properties along the RH axis. More so, several cell wall-related genes are transcribed in both RH and PT cells (Becker et al. 2014). Nevertheless, despite these commonalities, the detailed molecular composition of the RH cell wall seems to differ considerably from that of PTs. Here we review the current knowledge on the RH cell wall.

9.6.1 Cellulose Deposition in Root Hair Cell Walls

The main load-bearing network of plant cell walls, cellulose microfibrils tethered by hemicelluloses such as xyloglucan, is partly responsible for the restraining feature in the RH's shank (Cosgrove 2005). Electron microscope experiments revealed that most terrestrial plants have a random and short cellulose microfibril organization in the primary wall deposited at the RH tip (Newcomb 1965; Emons and van Maaren 1987; Akkerman et al. 2012; Rounds and Bezanilla 2013). In the shank of the RH, a second layer of more ordered cellulose microfibrils is deposited at the inside of the random wall, helicoidal or parallel to the RH axis (Akkerman et al. 2012). Addition of the cellulose synthase inhibitor, 2,6-dichlorobenzene (DCB) or cellulose-specific endo-(1→4)-β-glucanase (cellulase) to RHs resulted in rapid cessation of growth and even induced cell rupture at the tip, suggesting that deposition of cellulose or cellulose-like polysaccharides is a necessity for controlled tip growth of RHs (Park et al. 2011).

While most of the cell wall components like pectin, xyloglucan and other hemicelluloses are delivered by secretory vesicles, cellulose is synthesized at the PM by cellulose synthase (CESA) complexes (CSCs; Cosgrove 2005). From the 10 *CESA* genes present in *Arabidopsis* (Doblin et al. 2002), only *CESA1, CESA3* and *CESA6* are involved in primary cellulose synthesis (Arioli et al. 1998; Fagard et al. 2000; Scheible and Pauly 2004). Nevertheless, several lines of evidence suggest that other proteins than these three CESAs are involved in cellulose synthesis at the hair's tip. When the temperature-sensitive mutant *radial swollen 1* (*rsw1*), mutated in the *CESA1* gene, was grown at restrictive temperature, RH morphology was altered, but the tip growth process was not completely abolished (Baskin et al. 1992; Arioli et al. 1998; Williamson et al. 2001), which suggests that at least CESA1 is not essential for tip growth in RHs. Fluorescent fusion proteins of the two other CESAs involved in cellulose synthesis in primary cell walls, CESA3 and CESA6, do not localize to the apical PM of growing RHs, nor to the vesicle-rich zone in the hair apex, which are the sites one expects when these proteins would play

an essential role in cellulose deposition at the tip (Park et al. 2011). In addition, isoxaben, interfering with CESA3 and CESA6, was not able to alter RH elongation. All data so far suggest that CESA1, CESA3 and CESA6 are not required for RH tip growth, but it cannot be ruled out that other CESAs take part in cellulose deposition at the tip. Interestingly, mutants of two members of the cellulose-like super family, *CSLD2* and *CSLD3* (*KOJAK*), produce RHs with a range of abnormalities, with many RHs rupturing late in development (Bernal et al. 2008), or contain RHs that burst prematurely (Favery et al. 2001; Wang et al. 2001), respectively. Additionally, both YFP-tagged proteins are localized to the PM and in the vesicle-rich zone in the expanding tip. The *CSLD* family is the closest one of the *CSLs* to the *CESA* genes, with 35% identity at the amino acid level (Richmond and Somerville 2001), and even though the (1→4)-β-glucan synthase activity of CESA6 can functionally replace the catalytic activity of the CSLD3 domain, it is not clear at the moment whether the products of CSLD3, (1→4)-β-glucan polysaccharides, assemble into cellulose-like microfibrils (Galway et al. 2011; Park et al. 2011).

Most importantly, unlike in RHs where cellulose is the predominant cell wall component, PTs have a lower amount of cellulose but a very high amount of callose in their cell walls (see Chap. 3). In contrast to RHs, PT cellulose synthesis does seem to rely on CESA6 (Chebli et al. 2012). Yet here also the CSLD family plays a role since mutants of *CSLD1* and *CSLD4* show reduced pollen germination ability in vitro (Bernal et al. 2008), although it is not sure whether this is a pre- or post-germination effect.

9.6.2 Xyloglucan

Xyloglucan (XyG), the most abundant form of hemicellulose in dicots, interacts with cellulose microfibrils forming the load-bearing cellulose-xyloglucan network (Carpita and Gibeaut 1993). As it tethers adjacent cellulose microfibrils (Cosgrove 2005), it is expected that alterations in XyG might result in changes in mechanical properties of the cell walls and hence potential growth defects. Pena et al. (2012) reported that a unique acidic XyG is exclusively present in the *Arabidopsis* RH cell wall and that a loss-of-function mutation in *ROOT HAIR SPECIFIC8/XYLOGLUCAN-SPECIFIC GALACTURONOSYLTRANSFERASE1* (*RHS8/XUT1*), catalysing the synthesis of this galacturonic acid-containing XyG, causes a short RH phenotype, indicating the importance of this form of XyG in polarized expansion. Furthermore, Cavalier et al. (2008) have shown that double mutants in xylosyltransferases (*XXTs*), *xxt1/xxt2*, that catalyse the addition of xylosyl to the glucan backbone of XyG lack detectable amounts of XyG and produce short RHs with swollen bases. The single mutant *xxt5* and the triple mutant *xxt1 xxt2 xxt5* have short and swollen RHs as well (Zabotina et al. 2008, 2012), but do not show rupturing tips. Selective degradation of XyG in growing RHs using xyloglucanase leads to the same conclusion that XyG absence results in reduced

RH elongation, but not to rupture as was the case when cellulose was targeted (Park et al. 2011).

Besides altered synthesis, modifications of XyG can also affect cell wall mechanical properties. Xyloglucan endotransglucosylases/hydrolases (XTHs) are cell wall enzymes that cut XyG chains and reform bonds with water (hydrolase activity, XEH) and other xyloglucan acceptor substrates (endotransglycosylase activity, XET), potentially altering the distance between adjacent cellulose microfibrils and hence loosening cell walls (Nishitani and Vissenberg 2007; Van Sandt et al. 2007). High XET activity was indeed found in elongating epidermal cells and RHs at all stages of growth (Vissenberg et al. 2000, 2001, 2003), yet *xth* mutants with a detectable RH phenotype are still absent. Surprisingly, addition of recombinant XTH proteins leads to RH swelling, reduction and even cessation of RH growth (Maris et al. 2009), although one should take care since the XTH proteins are experimentally added from the outside of the wall, while under normal circumstances, they are secreted at the inside and younger part of the cell wall. Expansins are another class of proteins that interact with XyG and break their hydrogen bonds with cellulose at more acidic pH, thereby weakening the wall (Cosgrove 2005). A reduction of *expansin A7* (*EXPA7*) expression resulted in shorter RHs (Lin et al. 2011a), confirming that expansin A7 likely performs active cell wall modification activities during the elongation of the hair tip.

In PTs, fucosylated XyG epitopes were uniform all along the cell wall of straight growing PT, with a slightly lower abundance at the tip compared with the distal region (Chebli et al. 2012). At the moment, no effects of XyG absence are described on PT and pollen development.

Taken together, XyG plays a role in the growth of RHs, but its absence does not lead to cell rupture, suggesting that cellulose is not only connected to XyG. Indeed, Dick-Pérez et al. (2011) demonstrated interactions between pectin and cellulose microfibrils, and both pectic polysaccharides and xylans seem to have an enhanced role in the walls from the *xxt1 xxt2* double mutant that lacks detectable XyG (Park and Cosgrove 2012).

9.6.3 The Pectin Matrix

The above findings demonstrate that next to the cellulose/XyG load-bearing network, other cell wall components such as pectin, forming a highly hydrated matrix for the cellulose-xyloglucan framework, greatly contribute to RH growth. Enzymatic degradation of pectin using pectate lyase indeed inhibited RH elongation, but did not induce RH rupture (Park et al. 2011). In *Arabidopsis*, mutations in *UDP-4-KETO-6-DEOXY-D-GLUCOSE-3,5-EPIMERASE-4-REDUCTASE 1* (*UER1*) and *UDP-D-GLUCURONATE 4-EPIMERASE 6* (*GAE6*), both enzymes involved in the synthesis of pectic substrates, cause a short RH phenotype that can be rescued by applying exogenous precursors to the growth media (Pang et al. 2010). Furthermore, plants lacking LRR-extensin1 (LRX1) are defective in RH cell wall formation and

produce short, collapsed and branched RHs. A suppressor screen has revealed that the *lrx1* phenotype can be reversed by mutation in *RHAMNOSE BIOSYNTHESIS1* (*RHM1*). Since rhamnose is a major component of pectin and the *rhm1* mutation changes the expression of other cell wall-related genes, the suppression of the *lrx1* phenotype is likely due to changes in cell wall composition (Diet et al. 2006). In addition, the pectin cell wall dynamics in tip-growing cells has been analysed using propidium iodide. In RHs and PT the fluorescence intensity of apical propidium iodide shows an oscillatory pattern in which each peak precedes growth rate oscillations. However, the distribution of fluorescence in both cell types is distinct, with RHs exhibiting predominant labelling along the shanks, whereas in PT this is clearly seen at the apex (Rounds et al. 2011). These results are consistent with previous findings that the tip region of growing PTs is mainly composed of pectins, especially homogalacturonan (HG). HGs are deposited in a highly methylesterified form at the apex through exocytosis and they become demethylated by pectin methylesterase (PME) activity (see Sect. 9.3.5). Spatial and temporal regulation of PME activity depends strongly on PMEIs, the Ca^{2+} concentration in the cell wall and local pH differences. Inactivation of a pollen-specific PME, PPME1, resulted in curved, irregularly shaped PTs that were dramatically stunted because of reduced elongation rates (Tian et al. 2006). Similar unstable and retarded PT growth with occasional tube bursting was seen in mutants of *VANGUARD1* (*VGD1*), another pollen-expressed PME (Jiang et al. 2005). Surprisingly, at the moment, evidence for the involvement of PMEs in regulating RH growth is largely lacking.

9.6.4 Control by Second Messengers and Cell Wall Sensing/Modifying Proteins

The presence and correct assembly of extensins (EXTs), cell wall structural proteins, is crucial in the development of RHs. As mentioned before, LRX1 needs to interact with the RH cell wall, and this depends on the oxidative cross-linking of tyrosine (Tyr) residues in its EXT domain (Baumberger et al. 2001; Ringli 2010). Absence of this interaction results in impaired RH development. It is known that during plant defence reactions, EXT cross-linking can be mediated by peroxidases, and they have also been predicted to form the required cross-links with EXTs during RH formation (Almagro et al. 2009). Furthermore, cell wall self-assembly of EXTs requires correct O-glycosylation, and this is needed for normal RH elongation (Velasquez et al. 2011; Boron et al. 2014). Localization and interference with degrading enzymes indicate that EXTs are present in the PT wall and that they are required for normal PT elongation as well (García-Hernández and Cassab López 2005; Zhang et al. 2014).

The abundance and arrangements of apoplastic components determine the cell wall mechanical properties at the RH and PT tip, yet this can be changed by the activity of several other actors. Ca^{2+} can interact with demethylesterified pectin to

stiffen the wall, and local changes in apoplastic pH can change the activity of cell wall remodelling enzymes and proteins (Cosgrove 2005; Maris et al. 2009, 2011). Furthermore, ROS seem to play dual roles, as hydroxyl radicals can cleave cell wall polymers, thereby loosening the wall, whereas hydrogen peroxide can cause the opposite by helping peroxidases to cross-link certain cell wall components. On top of that, the complexity rises as certain cell wall defects in RHs and PT might not be the direct cause of reduced or ceased elongation, since certain RLK family members can serve as cell wall integrity sensors (Guo et al. 2009; Wolf et al. 2012), conferring information from the cell wall to the inside of the cell, regulating, for example, ROS production through ROPs (Duan et al. 2010), which affects the elongation of tip-growing cells.

9.7 Conclusion and Perspective

The number of identified players in the molecular regulation of RH tip growth is increasing rapidly, but their specific interactions and the mechanisms that integrate internal and environmental signals to fine-tune RH growth remain far from clear. Comparing the more comprehensive knowledge on PTs with that of RH tip growth, we identified many possible targets for future RH research. However, many challenges remain to be addressed in order to better understand the vast complexity and integrated nature that lie at the basis of RH tip growth and tip growth in general. A problem of particular concern regarding the study of RH development lies within the fact that RH cells are an integral part of the total root system. Unlike for PTs, it is currently very challenging to acquire a pure or undiluted RH cell extract without employing harsh techniques such as protoplast isolation. Consecutively, high throughput -omics techniques which cannot be performed on single cells have been left unexploited, despite the fact that they could provide enormous insight and identify several new targets in studying RH tip growth. Similarly, the cell wall has proven to be a very complex, dynamic and difficult structure to probe. Most importantly, however, the outcome of intracellular-extracellular signalling pathways is the targeted secretion, synthesis and modification of new cell wall material. Despite its importance, very little is known about tip-related cell wall dynamics. Plants have been shown to contain a diverse set of putative cell wall sensing proteins (CrRLK1Ls), which likely perceive specific information on cell wall composition and subsequently relay the latter towards the cell wall-controlling tip growth machinery. How do these proteins sense cell wall composition, and is RH growth disturbance/cessation in cell wall mutants a direct consequence of aberrant cell wall structure or is it the result of CrRLK1L-mediated signal transduction?

In addition, we now know that cell wall dynamics largely depend on extracellular changes in ion and ROS concentrations. However, it is most likely that highly localized ion transport and ROS generation lead to the existence of micro domains in which cell wall modification strongly differs from the surrounding environment, much like the regulatory micro domains observed at the surface of membranes. The

fluxes leading to these extra- as well as intracellular ion accumulation/depletion dynamics have been experimentally observed in several independent experiments. Based on their characteristics, they are thought to relate to a vast array of ion-transporting transmembrane proteins localized either to the tip or the shank of growing RHs. The identification of the loci corresponding to plasma membrane and endomembrane-localized transporters is however still largely lacking. More so, whereas different ion oscillations have been shown to influence one another, the way in which they are interconnected is poorly understood. For instance, it is unclear how Ca^{2+}, ROS and pH dynamics interact to control RH elongation.

Furthermore, the signals encoded by oscillatory ion signatures are mostly unknown. Why do ion concentrations oscillate, and how do these oscillations translate into oscillatory growth rates? How is the oscillatory nature of ion transport regulated? Recent research efforts are gradually providing insight into these processes. However, the possible involvement of Ca^{2+} and pH-sensitive proteins, which interpret and relay the ion-specific signal towards downstream targets, has been largely neglected. Feedback loops involving several of these proteins are likely embedded throughout the signalling cascade leading up to successful RH and PT growth. Importantly, ROP GTPases are now thought to function as signalling hubs, 'sensing' and integrating the key molecular RH growth machinery mentioned above. But what are the direct and downstream targets of these proteins, and how is their signal relayed to govern tip growth?

ROP GTPases have been shown to regulate cytoskeleton dynamics, but despite the well-established role of the actin cytoskeleton, the role of microtubules in both RH and PT elongation is still dubious. Finally, the cytoskeleton has a pivotal role in the establishment of a highly organized subcellular organization in tip-growing cells. However, the role of individual subcellular compartments and the way in which they might interact to regulate RH elongation are poorly understood.

It is clear that many challenges lie ahead to better understand RH development. Here we have identified many interesting targets for future research. Studying the molecular pathways governing tip growth and integrating them into a network that leads up to successful RH elongation will have to go hand in hand with the development of new techniques which will allow us to probe the complexity of RH cells and their building blocks. Many questions remain open, yet combined efforts on PTs and RHs will lead to exciting discoveries and a better understanding of the tip growth process.

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