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### ► **To cite this version:**

Hortense Moreau, Sabine D Zimmermann, Isabelle Gaillard, Nadine Paris. pH biosensing in the plant apoplast-a focus on root cell elongation. *Plant Physiology*, 2021, 187 (2), pp.504-514. <10.1093/plphys/kiab313>. <hal-03417397>

**HAL Id: hal-03417397**

**<https://hal.science/hal-03417397v1>**

Submitted on 5 Nov 2021

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1                    **pH biosensing in the plant apoplast - a focus on root cell elongation**

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11

12    **Advances**

- 13    - Genetically encoded pH sensors in the range of pH 5.5-8 have been designed and used to  
14    monitor pH in living plant cells.  
15    - Precise spatial localization of sensors can be achieved thanks to our increasing knowledge in  
16    the targeting processes of proteins.  
17    - High-definition imaging capabilities to detect fluorescence signals in living tissue are  
18    available.  
19    - Quantification of local absolute pH has been achieved.

20

21    **Outstanding questions**

- 22    - How far can we stretch the monitoring of proton exchange dynamics using fluorescent  
23    sensors?  
24    - How specific to pH are these biosensors?  
25    - Are there subdomains in the apoplast with specific pH values?  
26    - How can pH be monitored in the cell wall, especially in relation to the pectin methylation  
27    status?  
28    - How do plants perceive pH changes in the soil?

29

30 The study of pH in cellular physiology is of primary importance to understanding its  
31 involvement in a multitude of biological reactions. As plants are sessile organisms, they must  
32 adapt to local environment factors such as light, temperature, atmospheric CO<sub>2</sub>, and humidity,  
33 as well as soil characteristics, for which a knowledge of pH is essential. With respect to plant  
34 nutrition, the soil pH affects the solubility, mobility and availability of minerals. While  
35 several microelements like Al, Fe and Mn are more bioavailable and potentially toxic in  
36 acidic soil ( $\leq$ pH 5.5), major nutrients such as P, K, and N become less bioavailable in these  
37 conditions, causing nutrient deficiency (Ginocchio et al., 2009; Soti et al., 2015).  
38 Furthermore, some membrane ion channels and transporters could be regulated by local  
39 external acidic pH (Zeng et al., 2015). Finally, acidic soil represents 30% of the ice-free land  
40 globally and is therefore a major constraint for most cultivated plants.

41 Understanding how external pH is perceived by roots is essential for plant physiology. Soil  
42 pH is one of the many environmental conditions that affect nutrient availability (Maathuis,  
43 2009). The ability to regulate pH is essential as it participates in the plasma membrane (PM)  
44 proton-motive force generated by the activity of P-type H<sup>+</sup>-ATPases, which are the driving  
45 force for the uptake of minerals by transporters and channels (Nelson, 1994). Moreover,  
46 growth mechanisms such as cell elongation and expansion triggered by high intracellular  
47 turgor pressure are controlled by pH (Rayle and Cleland, 1970). Although the monitoring of  
48 proton concentrations in living tissue has greatly improved in the past two decades thanks to  
49 fluorescent probes, our ability to accurately measure pH variations in an acidic range below  
50 pH 5 remains challenging.

51 In this review, we provide an update on fluorescent pH probes available for live imaging, and  
52 we report on various technical limitations induced by acidic pH. The most adapted sensors to  
53 acidic pH are discussed in the context of pH perception in the apoplast in relation to cell

54 elongation, with a special focus on primary root growth. Finally, we examine the potential for  
55 genetically encoded fluorescent proteins in future studies.

56

### 57 **Apoplastic pH is a master regulator of plant growth processes**

58 In plants, the most acidic pH values reported so far in the endomembrane system correspond  
59 to the lumen of the trans-Golgi network/early endosome (TGN/EE) and the vacuole (Bassil et  
60 al., 2011; Martinière et al., 2013; Luo et al., 2015; Dragwidge et al., 2019). Depending on the  
61 approach, pH levels have been reported in the apoplast of elongating tissue in the range of  
62 4.5-5.5, as shown recently in the apical meristem of *Arabidopsis thaliana* (*Arabidopsis*)  
63 primary root (Barbez et al., 2017). Moreover, pH levels have been predicted to reach values  
64 as acidic as 3.5 in soybean hypocotyls (Yu et al., 2000). Although the mechanisms underlying  
65 the perception of external pH have been elucidated in detail for bacteria, fungi and  
66 mammalian cells, little information is available on whether (and if so, how) external pH is  
67 perceived by plants (Tsai and Schmidt, 2021).

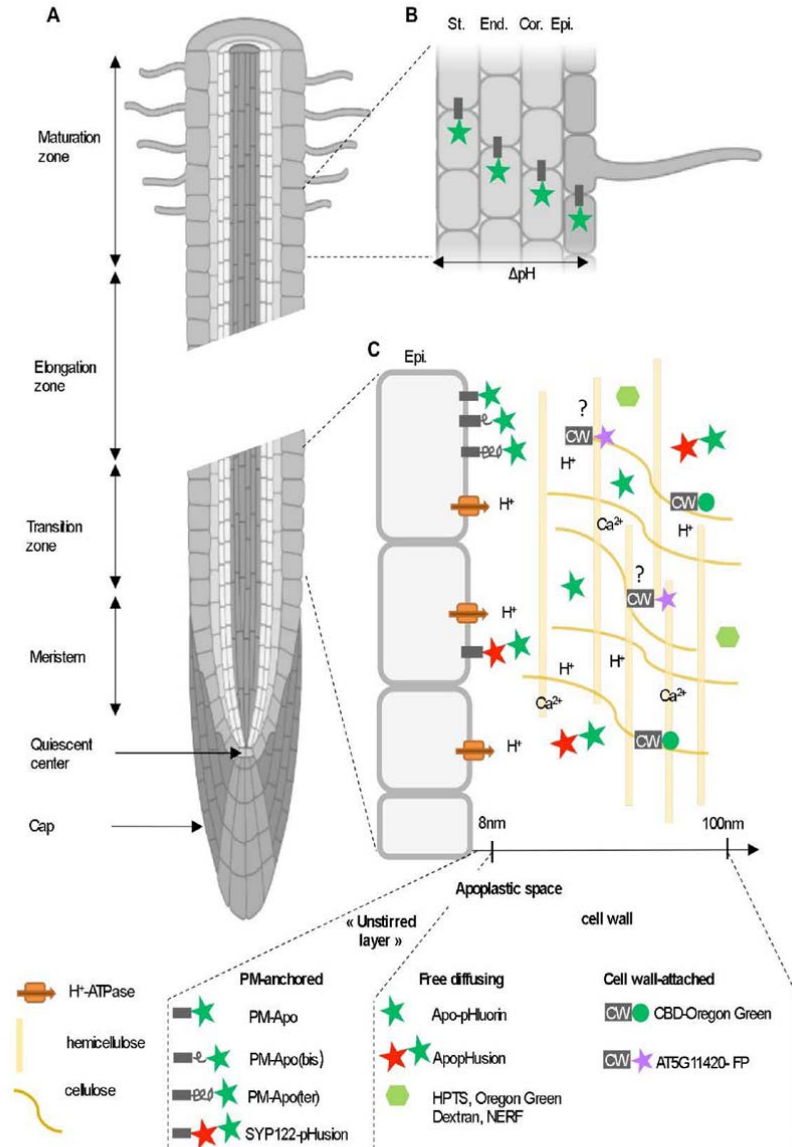
68 Plant cells are surrounded by a cell wall that provides protection and rigidity, but which also  
69 acts as an obstacle to cell elongation. Therefore, cell wall extensibility is required to  
70 accommodate cell expansion driven by turgor pressure. Early plant physiology research  
71 looked at how the cell wall could loosen itself in order to allow cell elongation, resulting in  
72 the “acid growth” theory (Rayle and Cleland, 1970). Since the 1970s, a stunning amount of  
73 data has been obtained, primarily in epicotyls or hypocotyls, resulting in the emergence of cell  
74 elongation models in which the plant hormone auxin plays a central role. Much progress has  
75 been made in our understanding of the molecular mechanisms of auxin signal transduction as  
76 well as the basic machinery involved in its directional distribution (Du et al., 2020). One of  
77 the central responses of auxin is the activation of the PM H<sup>+</sup>-ATPase upon phosphorylation of  
78 its penultimate Thr, resulting in an efflux of protons (Takahashi et al., 2012). This

79 acidification modifies the activity of cell wall enzymes including expansins, xyloglucan  
80 endotransglycosylases/hydrolases, and pectin methylesterases that in turn affect cell wall  
81 extensibility. Increased proton secretion leads to the hyperpolarization of the PM that ensures  
82 solute and consequently water uptake, both of which are required to maintain turgor pressure  
83 for cell elongation (Arsuffi and Braybrook, 2018; Du et al., 2020).

84

### 85 **The acid growth theory in the specific context of the primary roots**

86 In comparison to the hypocotyl, where pH dynamics after gravi-stimulation have been clearly  
87 reported using genetically encoded pH indicators (Fendrych et al., 2016), the primary root tip  
88 model is far more complex (Figure 1A). This structure is composed of the root cap with its  
89 protective cuticle (Berhin et al., 2019); the meristem, separated from the elongation zone by a  
90 transition zone whose position and maintenance determine the final root size; and the  
91 elongation and maturation zones, where most of the ion uptake from the soil takes place,  
92 through the root hairs. In Arabidopsis, the meristem sets its final size 5 days after germination,  
93 when a dynamic equilibrium is established between cell division and cell differentiation  
94 (Pacifci et al., 2015). At the organ scale, proton flux is a complex phenomenon with two  
95 proton-efflux zones at the level of the root cap and the elongation zone, while in the meristem  
96 and the transition zone, protons are taken up by the root (Collings et al., 1992; Staal et al.,  
97 2011). Even more complex, dynamic recordings of pH have demonstrated that growth is a  
98 discontinuous process associated with pulses of acidification, as clearly shown at the organ  
99 level in roots (Monshausen et al., 2011) as well as the cellular level in root hairs (Monshausen  
100 et al., 2007). The acid growth theory is more controversial regarding the root tissue, and it is  
101 important to differentiate root growth from cell elongation. Recent studies on distal  
102 meristematic root cells indicate that high concentrations of auxin induce a transient  
103 alkalinization of the apoplast followed by acidification, but only after several hours (Barbez et



**Figure 1: Schematic representation of a primary root apex with the different published fluorescent sensors for the apoplast.**

A. The different zones of the root, from the apex to the maturation zone.

B. A detailed transverse section of the maturation zone with the plasma membrane-anchored pHluorin, PM-Apo. This sensor is detected down to the stele in living root, and reveals an acidic pH gradient in the apoplast that extends from the surface to the stele. PM-Apo reports pH close to the plasma membrane and is insensitive to short-term changes in membrane polarization (Martinière et al., 2018). Epi: epidermis; Cor: cortex; End: endodermis; St: stele.

C. A detailed longitudinal section of the epidermis in the transition zone, with pH biosensors in various subdomains of the apoplast, including the “unstirred layer” and the cell wall. PM-Apo, PM-Apo(bis) and PM-Apo(ter) are PM-anchored sensors; pHluorin is fused to a transmembrane domain and a linker of variable length. SYP122-pHusion is a chimera of the PM SNARE SYP122 and pHusion (Kesten et al., 2019). CBD: cellulose-binding domain (Fasano et al., 2001). At5g11420-FP is an uncharacterized cell wall protein with unknown fine localization (indicated as ‘?’) that is fused to fluorescent proteins (FP) (Stoddard and Rolland, 2019). Apo-pHluorin is a freely diffusing form of pHluorin.

104 al., 2017). In contrast, cell elongation is altered when the endogenous auxin response is

105 impaired by genetic approaches. These data confirm a complex role of auxin in root growth,  
106 most likely in relation to its local concentration.

107 Plant roots must constantly adjust their growth and development to the local constraints of a  
108 changing soil environment. Peptide hormones play numerous physiological roles during root  
109 development (Hsiao and Yamada, 2021), and one emerging model of apoplastic pH regulation  
110 involves two hormone peptides that have antagonistic actions on H<sup>+</sup>-ATPase activity. The  
111 acidifying plant peptide PSY1 contains sulfated tyrosine 1 and interacts with the receptor  
112 kinase PSY1R to trigger phosphorylation of the auto-inhibitory loop of H<sup>+</sup>-ATPase (Amano et  
113 al., 2007; Fuglsang et al., 2014), while the rapid alkalization factor RALF binds the  
114 *Catharanthus roseus* RLK1-LIKE cell receptor kinase FERONIA, indirectly leading to  
115 inhibition of H<sup>+</sup>-ATPase (Pearce et al., 2001). Interestingly, while directly activating the H<sup>+</sup>-  
116 ATPases and promoting cell elongation, PSY1 also induces the transcription of RALFs. This  
117 in turn triggers a response cascade involving an obligatory increase in cytosolic calcium,  
118 resulting in the inactivation of H<sup>+</sup>-ATPase and consequently apoplast alkalization and  
119 inhibition of cell elongation (Gjetting et al., 2020). This negative feedback mechanism could  
120 act as a switch to control cell elongation, but it remains to be determined how and where  
121 RALF affects the PSY1 response. The availability of genetically encoded biosensors sensitive  
122 to acidic pH would clearly benefit this field of research, as well as help to understand the  
123 physiological significance of the rapid responses to auxins that were observed in plants but  
124 are too fast (i.e. less than two minutes) to be transcriptional in nature (Dubey et al., 2021).

125

### 126 **Apoplastic pH regulation in roots upon biotic interaction**

127 Regarding the interaction with microorganisms, little is known about cell wall-related sensing  
128 and the downstream mechanisms involved in growth and defense responses, although several

129 reports have shown apoplast pH to be involved in these responses. For example, in  
130 *Arabidopsis*, the *Pseudomonas syringae* type III effector protein AvrB induces stomatal  
131 opening through RPM1-interacting protein 4, a negative regulator of plant immunity, by  
132 inducing the PM H<sup>+</sup>-ATPases AHA1 and AHA2 (Liu et al., 2009). The activation of AHA1  
133 and AHA2 results in a hyperpolarization of the PM that initiates activation of the voltage-  
134 dependent channels, including inward rectifying K<sup>+</sup> channels. This entry of positive charges  
135 into the cell leads to water flux into the vacuole, resulting in guard cell turgidity and the  
136 consequent opening of the ostioles.

137 The chitin of the fungus *Serendipita (Piriformospora) indica* has also been described to  
138 induce alkalization in the leaf apoplast (Felle et al., 2009). Recent work has highlighted that  
139 alkalization of the apoplast is not the rule in terms of pathogen response, since rapid  
140 apoplastic acidification has been observed in *Arabidopsis* roots in response to the  
141 fungus *Fusarium oxysporum* (Kesten et al., 2019). In this example, fungal hyphae first attach  
142 to the root surface before penetrating between cells and growing toward the xylem, where the  
143 fungus can proliferate throughout the plant. In the xylem, this fungus secretes proteins named  
144 Secreted In Xylem, which serve to evade plant defense responses. Using a genetically  
145 encoded fluorescent sensor exposed within the root apoplast, the authors could follow the  
146 changes in apoplastic pH in real-time, finding that the *Arabidopsis* root epidermis responds  
147 with a strong acidification of the apoplast after 5 min of contact. The infection facilitation  
148 mechanism, which was attributed to an unknown secreted molecule that counteracts the  
149 alkalization effect of chitin present in the elicitor mix, is mediated by phosphorylation-  
150 based proton pump activation. This acidification in turn triggers a reduction in cellulose  
151 synthesis that surprisingly leads to root growth reduction (Kesten et al., 2019).

152 The more global impact of acidic pH in the soil is also illustrated by transcriptomic studies on  
153 *Arabidopsis* roots (Lager et al., 2010). Indeed, 881 genes were differentially expressed after 8

154 h of exposure to acidic pH media (pH 4.5-6.5). These pH-regulated genes cluster with  
155 transcriptional responses to defense-related hormones (e.g. salicylic acid, auxins and  
156 jasmonate) involved in the cell wall machinery and in calcium signaling. In contrast, no  
157 synergy of treatments was found on pH-induced genes compared to the effect of IAA (indole-  
158 3-acetic acid) or flagellin, supporting the hypothesis that pH acts as a signal for gene  
159 expression regulation. Altogether, pH plays an essential role in the tight interaction between  
160 plants and soil and beneficial microorganisms (Msimbira and Smith, 2020), underlying the  
161 importance of precisely monitoring pH within plant tissues and apoplastic environments.

162

### 163 **Organization of primary root apoplast and the emerging role of pectin methylation in** 164 **cell elongation**

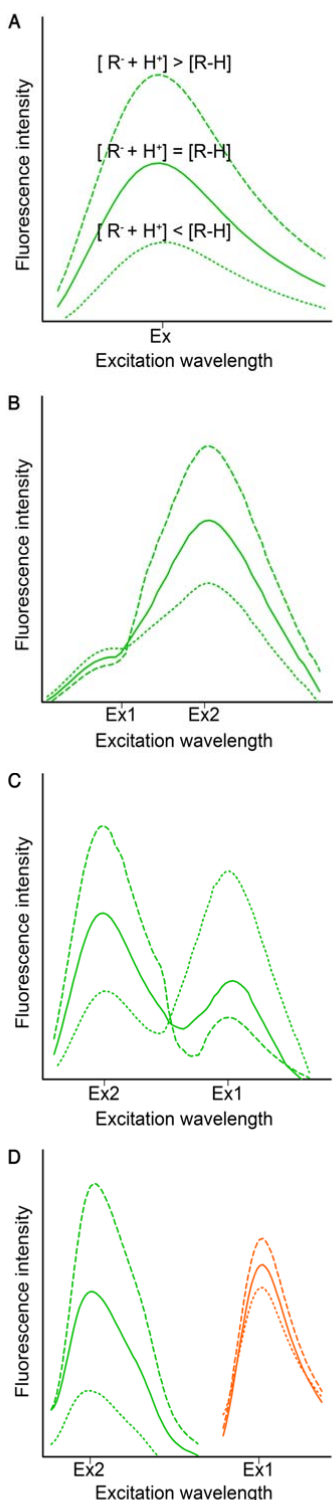
165 The apoplast is the space outside of the PM where water, ions and nutrients can freely diffuse.  
166 Structurally, the apoplast includes the cell wall, the intercellular space and the xylem-lumen  
167 space. Among the major ions, the level of protons in the apoplast is strictly maintained to  
168 energize numerous membrane transport processes and, in turn, to allow long-distance  
169 transport of nutrients within the plant. Depending on the plant species and tissue explored, pH  
170 values have been reported in the range of 3.5-8.3 in the apoplast (Yu et al., 2000). In growing  
171 cells, the primary wall is composed of strong, stable, and inextensible cellulose microfibrils  
172 embedded in a matrix of polysaccharides, the pectins and hemicelluloses (Figure 1C). The  
173 current “biomechanical hotspot” model proposes that cellulose fibrils are connected together  
174 by a xyloglucan-cellulose amalgam with limited enzymatic accessibility (Cosgrove, 2016). In  
175 addition, a major role has recently been proposed for pectins, whose degree of  
176 methylesterification could control the chemistry and rheology of the cell wall (Wolf and  
177 Höfte, 2014; Haas et al., 2020). Since the activity of pectin methylesterases is controlled by  
178 pH, they are proposed to participate in a pH regulation loop (Majda and Robert, 2018; Hocq

179 et al., 2021); additionally, they could be required for acid growth by local, spatial and  
180 temporal fine-tuning of the pectin methylesterification status (Hocq et al., 2017). The  
181 necessity of local and transient acidification of the cell wall is also supported by the fact that  
182 some enzymes with a major role in the acid growth response have an acidic pH optimum.  
183 This is the case for expansin, a key enzyme in cell wall remodeling, which has a maximal  
184 activity at pH ~4 (Cosgrove, 1998).

### 185 **Available fluorescent probes in the acidic pH range**

186 Initially, pH measurements in *Arabidopsis* or maize roots were performed with H<sup>+</sup>  
187 microelectrodes developed in the 1970s (Miller and Gow, 1989; Peters and Felle, 1999; Staal  
188 et al., 2011). Although H<sup>+</sup> microelectrodes are capable of monitoring proton fluxes, they are  
189 difficult to manipulate and suffer from low spatial resolution. In contrast, fluorescent sensor  
190 measurements can be used to investigate the steady state amounts of free protons in a given  
191 position. The use of fluorescent molecules to monitor pH in living tissues has dramatically  
192 expanded in the past 5 years, due in part to technical advances in microscopy that have much  
193 greater sampling capabilities. Fluorescent biosensors can be organic compounds or proteins  
194 targeted specifically to the chosen subcellular location thanks to trafficking signals, i.e.  
195 peptidic motifs/domains that are sufficient to address a protein to a given subcellular  
196 localization. Here, we will present a selection of the most appropriate fluorescent molecules  
197 for monitoring pH in the acidic range, describe their advantages and limitations, and finally  
198 discuss strategies regarding how to expand the panoply of biosensors for pH.

199 Fluorescence is a process by which a molecule that is excited by high-energy light (i.e. a short  
200 wavelength) emits photons at a lower energy (i.e. a longer wavelength). An electron excited  
201 by an external source such as a laser moves from a ground state to an excited electronic  
202 singlet state (S1'). After a lag time of several picoseconds to nanoseconds (i.e. the lifetime),  
203 the excited electrons regain their fundamental equilibrium state (S0) and release their stored



**Figure 2: Schematic excitation spectra associated with the various types of pH biosensors used in plants.**

Hypothetical spectra are presented to illustrate the effect of pH on the protonated and deprotonated forms of a fluorochrome compared to the spectra at pKa (solid line), based on the assumption that fluorescence intensity increases for pH values above the pKa (dashed line) and decreases for pH lower than the pKa (dotted line). The anionic deprotonated state  $[R^{-}+H^{+}]$  is progressively converted to a protonated state  $[R-H]$  as pH decreases. Excitation wavelengths, Ex, Ex1 and Ex2, are indicated for each spectra as ideal wavelengths for either providing pH information (A) or generating a ratiometric pH titration curve (B to D) with Ex2 corresponding to the wavelength with higher pH sensitivity to pH compared to Ex1. Titration curves are obtained by plotting the fluorescence ratio Ex2/Ex1 as a function of applied pH that, in these examples, will increase with pH increase.

(A) Mono-excitation mono-emission type of spectra as for fluorescein. (B and C) Dual-excitation mono-emission spectra as for BCECF and Oregon Green, with Ex1 at the isosbestic point (B) or pFluorin, with two peaks with opposite sensitivity to pH (C). (D) Mix or fusion of two biosensors with mono-excitation mono-emission spectra, but with high (green) or low (orange) sensitivity to pH, as for pHusion.

204 energy as emitted photons. The difference between the maxima excitation and the emission  
 205 energy (wavelength) is known as the Stokes shift. This phenomenon can be affected by the

206 physicochemical parameters of the environment (Abbyad et al., 2007). In the case of  
207 fluorescent probes used for pH measurements, the fluorochrome contains aromatic cycles  
208 with several  $\pi$  bonds that participate in the fluorescence emission. When the pH increases, the  
209 neutral protonated state is progressively converted to an anionic deprotonated form, and in the  
210 case of pH-dependent fluorochromes, the absorption properties of these two forms differ  
211 between each other in the range of visible light. These optical properties are exploited with  
212 imaging techniques to study the local pH that is limited to the range of pH around the pKa of  
213 the fluorophore, i.e. the pH value at which half of the molecules are in a protonated state  
214 (Figure 2A).

215 The preeminent dye in this ratiometric mode is fluorescein and its numerous derivatives.  
216 Fluorescein was discovered and synthesized by Adolf Von Baeyer at the end of the nineteenth  
217 century (Heimann and Wolf, 2007). It presents good absorptivity, fluorescence quantum yield  
218 and solubility, and its intricate fluorescent properties are highly sensitive to pH (pKa=6.4). An  
219 impermeant version of fluorescein coupled to dextran was used to measure external pH in  
220 close vicinity of *Arabidopsis* roots, revealing pH values in the range of pH 5.2-6.2  
221 (Monshausen et al., 2009; Monshausen et al., 2011). A derivate, fluorescein isothiocyanate  
222 (FITC) coupled with dextran, was successfully used to measure apoplastic pH in *Vicia faba*  
223 leaves (pH 5.2) and roots (pH 4.8) (Mühling and Sattelmacher, 1995; Mühling et al., 1998).  
224 Unfortunately, FITC alone does not penetrate tissues well, and requires pressure to be  
225 infiltrated or loaded through a long-term transpiration flux into leaves, which causes cell  
226 stress and modification of the basal pH value (Yu et al., 2000). Furthermore, fluorescein has  
227 known photobleaching issues, and part of its fluorescence is lost below pH 7, making it  
228 unusable for acidic pH measurements.

229 Another fluorescent probe derived from fluorescein is 2',7'-bis-(2-carboxyethyl)-5-(and-6)-  
230 carboxyfluorescein (BCECF). This probe has a pKa around 7 and a pH-dependent emission

231 spectrum with a “shoulder” (Figure 2B) that allows a ratiometric pH measurement  
 232 independent of the molecule’s concentration (Table 1). In its membrane-permeable form,  
 233 BCECF-acetoxymethyl ester (BCECF-AM) enters and accumulates within the vacuole, where  
 234 it is used to measure luminal pH in the range of 5.7 to 5.9 in *Arabidopsis* root tips or in  
 235 mature roots (Krebs et al., 2010; Martinière et al., 2013a). Oregon Green (OG) dye is another  
 236 bimodal excitation-single emission probe. This fluorinated analog of fluorescein has a highly  
 237 useful range of pH measurement in the acidic range (pH 2.5-5.5). In its cell impermeant form,  
 238 OG 488 has been used to study apoplastic pH in living tissue (Table 1).

239 Finally, the bimodal excitation mono-emission probe 8-hydroxypyrene-1,3,6-trisulfonic acid  
 240 (HPTS) is a ratiometric fluorescent probe (Figure 2B) capable of functioning in weakly acidic  
 241 environments (Table 1). HPTS has a pKa of 7.3, and its membrane impermeant properties  
 242 make it useful for monitoring pH in the root apoplast when correlating auxin signaling with  
 243 cell elongation and in response to gravitropism (Barbez et al., 2017).

244

<b>Tool</b>	<b>pH range</b>	<b>Organism/localization</b>	<b>Physiological processes</b>	<b>Ref.</b>
NERF and Texas red dextran mixture	3-6	Arabidopsis root/apoplast	Apoplastic transient acidification at root hair hair initiation	(Bibikova et al., 1998)
		<i>L.angustifolius</i> root elongation zone/apoplast	Response to high pH and to different sources of nitrogen supply.	(Yu et al., 2001)
BCECF-AM	5.5-8.5	Arabidopsis root and tobacco epidermis/vacuole	Native luminal vacuolar pH maintained by V-ATPase activity. Luminal pH gradient and protein trafficking to vacuole.	(Krebs et al., 2010) (Martinière et al., 2013a)
HPTS	5-7.5	Arabidopsis root transition and maturation zone/apoplast	Auxin concentration-dependent role in apoplastic pH regulation controlling cell expansion and gravitropic response. Apoplastic pH estimation in maturation zone.	(Barbez et al., 2017)  (Martinière et al., 2018)
Cellulose binding domain (CBD)-Oregon Green	2.5-5.5	Arabidopsis root/cellulosic cell wall	Rapid apoplastic pH acidification in root cap in response to gravitropism.	(Fasano et al., 2001)
Oregon Green-dextran	2.5-5.5	<i>Vicia faba</i> L. leaf/apoplast	Apoplastic alkalinization is involved in stomatal closure in response to salt stress.	(Geilfus et al., 2015)
		Arabidopsis root maturation	Apoplastic pH measurement.	(Martinière et al., 2018)

		zone/apoplast		
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245 Table 1: Examples of fluorescent probes that have been used to monitor pH in plant acidic  
 246 environments.  
 247

248 Alternatively, a mix of two cell membrane-impermeant dyes with different pKa values (and  
 249 therefore different sensitivities to pH) can be used to monitor pH in a dual excitation/double  
 250 emission mode (Figure 2D). This is the case for NERF-Cl fluorescent rhodol dye (pKa 3.8)  
 251 and Texas Red Dextran (pKa 7.8), which were used to monitor local pH oscillations in root  
 252 hair apoplasts associated with development (Bibikova et al., 1998), as well as in elongation of  
 253 *L. angustifolius* root epidermal cells in response to alkaline pH (Yu et al., 2001).

254

### 255 **Genetically encoded pH biosensors compatible with acidic pH**

256 The array of available genetically encoded fluorescence sensors has dramatically increased in  
 257 the past 5 years, opening up many possibilities for *in vivo* pH studies. Since its first use as a  
 258 gene reporter (Chalfie et al., 1994) and the removal of a cryptic intron by Jim Haselhoff, the  
 259 *Aequorea victoria* green fluorescent protein (GFP) and its derivatives have been widely used  
 260 in plants. After translation and proper folding of the GFP, the fluorophore is self-generated by  
 261 cyclization and oxidation of residues 65 through 67 (Ser-Tyr-Gly), forming a hydroxyphenyl  
 262 ring (Wachter, 2007). Wild type GFP has a bimodal excitation profile (Figure 2C), with  
 263 optima at 395 nm and 475 nm corresponding to the protonated and deprotonated forms,  
 264 respectively. This feature was exploited to develop efficient ratiometric pH-sensitive versions,  
 265 including the sensor pHluorin (Miesenböck et al., 1998). The pHluorin sensor and its  
 266 ratiometric equivalent At-pHluorin (ratioGFP) are sensitive to pH in the range of 5.5-8 (Gao  
 267 et al., 2004; Schulte et al., 2006), and have been used to monitor steady-state pH in various  
 268 cellular subcompartments of the cell, resulting in several breakthroughs in plant physiology  
 269 (Table 2, green).

Tool	pH range	Organism/localization	Physiological processes	Ref.
------	----------	-----------------------	-------------------------	------

pHluorin	5.5-8	Arabidopsis and tobacco epidermis/lumen of secretory organelles	Regulation of luminal pH in relation to vacuolar protein transport.	(Martinière et al., 2013a)
		Arabidopsis root maturation zone/apoplast	Transmembrane pH gradient and ion uptake. Radial apoplastic pH gradient.	(Martinière et al., 2018)
ratioGFP	5.6-7.8	Arabidopsis root/apoplast	Apoplastic pH alkalization in root differentiating cells in response to salt stress.	(Gao et al., 2004)
A-17 pHuji	5.5-8	Arabidopsis root/cytosol	Proton dynamics upon ABA treatment.	(Waadt et al., 2020)
ApopHusion	4.5-7	Arabidopsis root elongation zone/apoplast	pH alkalization in response to auxin in root.	(Gjetting et al., 2012)
		Hypocotyl/apoplast	pH acidification is mediated by the tIR1/AFB-Aux/IAA auxin perception.	(Fendrych et al., 2016)
SYP61-pHusion	4.5-7	Arabidopsis hypocotyl/TGN/EE	Role of V-ATPase in pH homeostasis in TGN/EE in relation to secretion and recycling.	(Luo et al., 2015)
SYFP122-pHusion	4.5-7.5	Arabidopsis root elongation zone/apoplast	pH acidification involved in the regulation of growth/defense balance in response to biotic stress.	(Kesten et al., 2019)

270 Table 2: Examples of available genetically encoded fluorescent proteins for actual or potential  
271 use in taking pH measurements in the acidic range. Green: single molecule with ratiometric  
272 pH properties. Purple: tandem-based pH sensor.

273  
274 Although red versions of pH-sensitive fluorescent proteins have been reported (Tantama et  
275 al., 2011; Li and Tsien, 2012; Shen et al., 2014), their sensitivity is limited to pH 6.  
276 Furthermore, to our knowledge, only A-17, a derivative of pHuji, has been tested in the plant  
277 cytoplasm (Waadt et al., 2020) (Table 2, green).

278 Tandems of fluorescent proteins with different pKa, and therefore different pH sensitivities,  
279 are another strategy that can be applied to generate genetically encoded pH sensors. For  
280 instance, the fusion of mRFP1 (pKa=4.5) with eGFP (pKa=6.15), named pHusion, has a pKa  
281 of 5.8 and allows a slightly improved pH sensibility in the acidic range when compared to  
282 pHluorin (Gjetting et al., 2012) (Table 2, purple). The tandem pHusion has already been  
283 successfully targeted to the apoplast in a freely diffusing form, and more recently it was  
284 anchored in the PM to monitor the real-time response to *Fusarium oxysporum* fungal  
285 infection in roots (Kesten et al., 2019). Despite the ability of pHluorin and pHusion to monitor

286 pH in plants, their pH restrictions within the acidic range limit them to physiological events  
287 occurring at a pH no lower than 4.5-5.

288

### 289 **Potential and limitation of genetically encoded pH sensors**

290 The apoplast is a complex environment with a cell wall structure, as detailed in the transition  
291 zone of the root in Figure 1C. Depending on the probe, the value of the apoplastic pH will  
292 need to be interpreted in the context of the corresponding subregion from which it comes. One  
293 of the main advantages of genetically encoded pH sensors is the nearly infinite possibility of  
294 their targeting to a given subcellular site. In the case of the apoplast, several engineered  
295 fusions have already been shown to efficiently report pH in roots. To monitor pH in close  
296 proximity to the PM, several previous studies anchored pH sensors using either a  
297 transmembrane domain (PM-Apo) together with a long or extra-long linker (PM-Apo(bis) and  
298 PM-Apo(ter), respectively) for pHluorin (Martinière et al., 2018), or they were fused to a  
299 SNARE SYP122 (SYP122-pHusion) (Kesten et al., 2019). Membrane-anchored sensors, as  
300 opposed to their equivalent released in the apoplast (Gjetting et al., 2012; Martinière et al.,  
301 2018), do not diffuse away from the root. In addition, pH from cell layers below the surface is  
302 accessible. This has been shown with PM-Apo, in which pH can be monitored in each cell  
303 layer up to the central cylinder of the root, providing access to the apoplastic pH radial  
304 gradient in the mature portion of living roots (Figure 1B).

305 Freely diffusing sensors, such as HPTS, Apo-pHluorin and ApopHusion, have the potential to  
306 label the entire cell wall (Figure 1C). In practice, HPTS poorly penetrates the space between  
307 the root epidermal cells (Martinière et al., 2018), whereas ApopHusion diffuses away from  
308 the apoplast (Gjetting et al., 2012). While the features that allow a probe to penetrate the cell  
309 wall are difficult to predict, this ability depends in part on the cell wall pores, whose size (30-  
310 50 Å; Carpita et al., 1979) may vary with the degree of pectin methylation (Willats et al.,

311 2001). To circumvent the lack of retention in sensors over time, cell wall-targeting can be  
312 used as previously shown with a cellulose-binding domain tagged with Oregon Green (Fasano  
313 et al., 2001); potentially, this could also be accomplished with an uncharacterized cell wall  
314 protein fused to fluorescent proteins (Stoddard and Rolland, 2019).

315 To convert fluorescence ratios into pH values, a calibration curve must be produced using the  
316 emission ratio obtained after excitation at two wavelengths, in which the dynamic of change  
317 for the excitation profiles sufficiently differs due to pH (see Ex1 and Ex2 in Figure 2B-D).  
318 The pH calibration of genetically encoded fluorescent sensors can be achieved either *in situ* or  
319 *in vivo* (Martinière et al., 2013b). *In situ* calibration uses a sensor produced from the bacteria  
320 *Escherichia coli* that is purified and mixed with different pH buffers. This recombinant  
321 protein can also be used for extensive spectrofluorometry characterization of the sensor under  
322 highly controlled conditions. Indeed, this technique has been used to investigate the  
323 sensitivity of pHluorin to various ionic or redox environments (Martinière et al., 2013a). This  
324 *in situ* calibration is a fast method, taking 10 to 15 min from frozen aliquot of purified sensor  
325 to results and can be repeated as many times as needed during the confocal session with the  
326 exact same settings used to observe the plant samples. Unfortunately, *in situ* calibration does  
327 not address any of the physicochemical parameters inherent to the sample that could  
328 potentially affect the fluorescence ratio and therefore the pH measurements. This issue can be  
329 addressed by comparing the *in situ* value with that obtained from *in vivo* calibration methods.  
330 Essentially, this consists in a direct treatment of the plant sample expressing the sensor with a  
331 sufficient buffering capacity in the presence of a protonophore such as nigericin (Grillo-Hill  
332 et al., 2014) or a weak acid (Kesten et al., 2019), so as to ensure an equilibrium between the  
333 intracellular compartments and the bulk. The time required for equilibration can vary from a  
334 few minutes (Keinath et al., 2015) to an hour (Martinière et al., 2018) or even longer, and  
335 must be determined empirically. It must be noted that the physiological impact of pH

336 clamping on the original *in vivo* environment of the sensor remains unknown. Whichever  
 337 calibration method is chosen, it is important to keep in mind that it may affect the  
 338 measurements. For these reasons, most studies refer to relative changes in pH rather than  
 339 absolute pH.

340

### 341 **Future perspectives and challenges**

342 The tandem strategy that was successfully used for pHusion could very well be extended to  
 343 proteins with low pKa (Table 3). Among the large variety of available fluorescent proteins,  
 344 only a few have physical properties (Benčina, 2013) that make them suitable for monitoring  
 345 pH in the acidic range (Shinoda et al., 2018).

Tool	pKa	Organism/localization	Tolerance to acidic pH	Ref.
Turquoise2	pKa 3.1	Tobacco epidermal cell/cell wall	pH4/pH8 fluorescence intensity 64%	(Stoddard and Rolland, 2019)
mNeonGreen	pKa 5.7		pH4/pH8 fluorescence intensity 52%	
Gamillus	pKa 3.4		pH4/pH8 fluorescence intensity 69%	
TagRFP	pKa 3.8		pH4/pH8 fluorescence intensity 58%	
mCherry	pKa<4.5		pH4/pH8 fluorescence intensity 48%	

346 Table 3: Example of fluorescent proteins with low pKa that are functional in the plant  
 347 endomembrane system that could be used to monitor pH in the acidic range.  
 348

349 To test this possibility, 10 fluorescent proteins with low pKa were chosen and targeted either  
 350 to the cytosol or the apoplast, and their *in vivo* fluorescence properties were examined in  
 351 tobacco epidermal cells (Stoddard and Rolland, 2019). In addition to their proper targeting to  
 352 the apoplast, half of them responded well to bulk pH *in vivo*, ranked here from lowest to  
 353 highest pKa: Turquoise2, Gamillus, TagRFP, mCherry, and mNeonGreen. In particular,  
 354 mTurquoise2 and mCherry show efficient recovery after pH 4 treatments *in vivo* (Table 3).

355 As a proof of concept, the tandem pH-lemon, a fusion of mTurquoise2 with the FRET  
 356 acceptor EYFP, was utilized in human cells, where it was reported to cover *in vitro* a pH

357 range of 3-7 (Burgstaller et al., 2019). For a deeper understanding of apoplastic variations  
358 with regard to cell elongation, future work will need to target acidic tolerant pH sensors to  
359 cell wall polymers, as was done previously with CBD (Fasano et al., 2001). The chitosan  
360 oligosaccharide (COS) has been used to label mainly demethylated polysaccharide  
361 homogalacturonan (Mravec et al., 2014). A fusion of COS with Oregon Green could  
362 potentially provide a way to investigate local pH variation in pectin, in the cell wall. The  
363 recent design of biosensors allowing the direct visualization of auxin distribution in living  
364 tissues (Herud-Sikimić et al., 2021) illustrates our potentially infinite ability to design sensors  
365 for highly specific purposes. Fluorescence-lifetime imaging microscopy (FLIM) can also be  
366 used to measure pH when a higher temporal resolution is needed, since it only uses a single  
367 excitation wavelength. This method, which is insensitive to laser changes or fluorochrome  
368 concentration, exploits the fact that the fluorescence lifetime of a fluorochrome is affected by  
369 pH (including within the acidic range) (Lin et al., 1999). These features have led to the  
370 successful use of FLIM to monitor pH in living tissue (Lin et al., 2003; Linders et al., 2021).  
371 The PM is now viewed as a heterogenous environment, where the formation of protein  
372 platforms is an essential step in plant responses to the environment (Bayle et al., 2021). In  
373 addition, technical advances in fluorescence microscopy allowing super-resolution live  
374 imaging at the subcellular scale (Shimizu et al., 2021) can be used to demonstrate the extreme  
375 dynamics of membranes. Future challenges will certainly involve integrating these fine  
376 plasticity dimensions in our pH sensor approaches. The ability to target tandem-based sensors  
377 with the appropriate range of pH detection at a given subcellular location will most likely  
378 result in very exciting research aimed at further connecting pH information with key  
379 mechanisms of plant responses to the environment.

380

381 **Acknowledgments:**

382 This work was funded by the Homeowall project from the Agence Nationale de la Recherche  
383 (ANR).

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## Parsed Citations

- Abbyad P, Childs W, Shi X, Boxer SG (2007) Dynamic Stokes shift in green fluorescent protein variants. PNAS 104: 20189–20194**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Amano Y, Tsubouchi H, Shinohara H, Ogawa M, Matsubayashi Y (2007) Tyrosine-sulfated glycopeptide involved in cellular proliferation and expansion in Arabidopsis. PNAS 104: 18333–18338**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Arsuffi G, Braybrook SA (2018) Acid growth: an ongoing trip. J Exp Bot 69: 137–146**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Barbez E, Dünser K, Gaidora A, Lendl T, Busch W (2017) Auxin steers root cell expansion via apoplastic pH regulation in Arabidopsis thaliana. PNAS 114: E4884–E4893**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Bassil E, Tajima H, Liang Y-C, Ohto M, Ushijima K, Nakano R, Esumi T, Coku A, Belmonte M, Blumwald E (2011) The Arabidopsis Na<sup>+</sup>/H<sup>+</sup> antiporters NHX1 and NHX2 control vacuolar pH and K<sup>+</sup> homeostasis to regulate growth, flower development, and reproduction. Plant Cell 23: 3482–3497**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Bayle V, Fiche J-B, Burny C, Platre MP, Nollmann M, Martinière A, Jaillais Y (2021) Single-particle tracking photoactivated localization microscopy of membrane proteins in living plant tissues. Nature Protocols 16: 1600–1628**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Benčina M (2013) Illumination of the spatial order of intracellular pH by genetically encoded pH-sensitive sensors. Sensors 13: 16736–16758**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Berhin A, de Bellis D, Franke RB, Buono RA, Nowack MK, Nawrath C (2019) The root cap cuticle: a cell wall structure for seedling establishment and lateral root formation. Cell 176: 1367–1378.e8**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Bibikova TN, Jacob T, Dahse I, Gilroy S (1998) Localized changes in apoplastic and cytoplasmic pH are associated with root hair development in Arabidopsis thaliana. Development 125: 2925–2934**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Burgstaller S, Bischof H, Gensch T, Stryeck S, Gottschalk B, Ramadani-Muja J, Eroglu E, Rost R, Balfanz S, Baumann A, et al (2019) pH-Lemon, a fluorescent protein-based pH reporter for acidic compartments. ACS Sens 4: 883–891**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Carpita N, Sabularse D, Montezinos D, Delmer DP (1979) Determination of the pore size of cell walls of living plant cells. Science 205: 1144–1147**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263: 802–805**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Collings DA, White RG, Overall RL (1992) Ionic current changes associated with the gravity-induced bending response in roots of Zea mays L. 1. Plant Physiol 100: 1417–1426**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Cosgrove DJ (2016) Catalysts of plant cell wall loosening. F1000Res. doi: 10.12688/f1000research.7180.1**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Cosgrove DJ (1998) Cell wall loosening by expansins. Plant Physiology 118: 333–339**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Bassil E, Tajima H, Liang Y-C, Ohto M, Ushijima K, Nakano R, Esumi T, Coku A, Belmonte M, Blumwald E (2011) The Arabidopsis Na<sup>+</sup>/H<sup>+</sup> antiporters NHX1 and NHX2 control vacuolar pH and K<sup>+</sup> homeostasis to regulate growth, flower development, and reproduction. Plant Cell 23: 3482–3497**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Dragwidge JM, Scholl S, Schumacher K, Gendall AR (2019) NHX-type Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporters are required for TGN/EE trafficking and endosomal ion homeostasis in Arabidopsis thaliana. J Cell Sci. doi: 10.1242/jcs.226472**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Luo Y, Scholl S, Doering A, Zhang Y, Irani NG, Di Rubbo S, Neumetzler L, Krishnamoorthy P, Van Houtte I, Myle E, et al (2015) V-ATPase activity in the TGN/EE is required for exocytosis and recycling in Arabidopsis. Nature Plants 1: 1–10**  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)
- Martinière A, Desbrosses G, Sentenac H, Paris N (2013) Development and properties of genetically encoded pH sensors in plants.**

Front Plant Sci. doi: 10.3389/fpls.2013.00523

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Du M, Spalding EP, Gray WM (2020) Rapid auxin-mediated cell expansion. *Annu Rev Plant Biol* 71: 379–402

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Dubey SM, Serre NBC, Oulehlová D, Vittal P, Fendrych M (2021) No time for transcription-rapid auxin responses in plants. *Cold Spring Harb Perspect Biol*. doi: 10.1101/cshperspect.a039891

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Fasano JM, Swanson SJ, Blancaflor EB, Dowd PE, Kao T, Gilroy S (2001) Changes in root cap pH are required for the gravity response of the *Arabidopsis* root. *The Plant Cell* 13: 907–921

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Felle HH, Waller F, Molitor A, Kogel K-H (2009) The mycorrhiza fungus *Piriformospora indica* induces fast root-surface pH signaling and primes systemic alkalization of the leaf apoplast upon powdery mildew infection. *Mol Plant Microbe Interact* 22: 1179–1185

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Fendrych M, Leung J, Friml J (2016) TIR1/AFB-Aux/IAA auxin perception mediates rapid cell wall acidification and growth of *Arabidopsis* hypocotyls. *eLife* 5: e19048

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Fuglsang AT, Kristensen A, Cuin TA, Schulze WX, Persson J, Thuesen KH, Ytting CK, Oehlenschläger CB, Mahmood K, Sondergaard TE, et al (2014) Receptor kinase-mediated control of primary active proton pumping at the plasma membrane. *Plant J* 80: 951–964

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Gao D, Knight MR, Trewavas AJ, Sattelmacher B, Plieth C (2004) Self-reporting *Arabidopsis* expressing pH and [Ca<sup>2+</sup>] indicators unveil ion dynamics in the cytoplasm and in the apoplast under abiotic stress. *Plant Physiol* 134: 898–908

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Geilfus C-M, Mithöfer A, Ludwig-Müller J, Zörb C, Muehling KH (2015) Chloride-inducible transient apoplastic alkalizations induce stomata closure by controlling abscisic acid distribution between leaf apoplast and guard cells in salt-stressed *Vicia faba*. *New Phytol* 208: 803–816

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ginocchio R, De la Fuente LM, Sánchez P, Bustamante E, Silva Y, Urrestarazu P, Rodríguez PH (2009) Soil acidification as a confounding factor on metal phytotoxicity in soils spiked with copper-rich mine wastes. *Environ Toxicol Chem* 28: 2069–2081

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Gjetting SK, Mahmood K, Shabala L, Kristensen A, Shabala S, Palmgren M, Fuglsang AT (2020) Evidence for multiple receptors mediating RALF-triggered Ca<sup>2+</sup> signaling and proton pump inhibition. *The Plant Journal* 104: 433–446

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Gjetting SK, Ytting CK, Schulz A, Fuglsang AT (2012) Live imaging of intra- and extracellular pH in plants using pHusion, a novel genetically encoded biosensor. *Journal of Experimental Botany* 63: 3207–3218

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Grillo-Hill BK, Webb BA, Barber DL (2014) Chapter 23 - Ratiometric imaging of pH probes. In JC Waters, T Wittman, eds, *Methods in Cell Biology*. Academic Press, pp 429–448

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Haas KT, Wightman R, Meyerowitz EM, Peaucelle A (2020) Pectin homogalacturonan nanofilament expansion drives morphogenesis in plant epidermal cells. *Science* 367: 1003–1007

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Heimann H, Wolf S (2007) A practical guide to fluorescein angiography. In AM Jousen, TW Gardner, B Kirchhof, SJ Ryan, eds, *Retinal Vascular Disease*. Springer, Berlin, Heidelberg, pp 193–204

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Herud-Sikimić O, Stiel AC, Kolb M, Shanmugaratnam S, Berendzen KW, Feldhaus C, Höcker B, Jürgens G (2021) A biosensor for the direct visualization of auxin. *Nature* 1–5

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hocq L, Habrylo O, Voxeur A, Pau-Roblot C, Safran J, Sénéchal F, Fournet F, Bassard S, Battu V, Demailly H, et al (2021) The pH-dependent processivity of *Arabidopsis* AtPME2 can control cell wall mechanical properties. *bioRxiv* 2021.03.03.433777

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hocq L, Pelloux J, Lefebvre V (2017) Connecting homogalacturonan-type pectin remodeling to acid growth. *Trends in Plant Science* 22: 20–29

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hsiao Y-C, Yamada M (2021) The roles of peptide hormones and their receptors during plant root development. *Genes* 12: 22

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Keinath NF, Waadt R, Brugman R, Schroeder JI, Grossmann G, Schumacher K, Krebs M (2015) Live Cell Imaging with R-GECO1 Sheds Light on flg22- and Chitin-Induced Transient  $[Ca^{2+}]_{cyt}$  Patterns in Arabidopsis. *Molecular Plant* 8: 1188–1200

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Kesten C, Gámez-Arjona FM, Menna A, Scholl S, Dora S, Huerta AI, Huang H-Y, Tintor N, Kinoshita T, Rep M, et al (2019) Pathogen-induced pH changes regulate the growth-defense balance in plants. *The EMBO Journal* 38: e101822

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Krebs M, Beyhl D, Görlich E, Al-Rasheid KAS, Marten I, Stierhof Y-D, Hedrich R, Schumacher K (2010) Arabidopsis V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. *Proc Natl Acad Sci U S A* 107: 3251–3256

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Lager I, Andréasson O, Dunbar T, Andreasson E, Escobar MA, Rasmusson AG (2010) Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor responses. *Plant Cell Environ* 33: 1513–1528

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Li Y, Tsien RW (2012) pHTomato, a red, genetically encoded indicator that enables multiplex interrogation of synaptic activity. *Nature Neuroscience* 15: 1047–1053

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Lin H-J, Herman P, Lakowicz JR (2003) Fluorescence Lifetime-Resolved pH Imaging of Living Cells. *Cytometry A* 52: 77–89

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Lin H-J, Szmáczinski H, Lakowicz JR (1999) Lifetime-Based pH Sensors: Indicators for Acidic Environments. *Anal Biochem* 269: 162–167

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Linders PTA, Beest M ter, Bogaart G van den (2021) Fluorescence lifetime imaging of pH along the secretory pathway. *bioRxiv* 2021.03.29.437519

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Liu J, Elmore JM, Fuglsang AT, Palmgren MG, Staskawicz BJ, Coaker G (2009) RIN4 functions with plasma membrane H<sup>+</sup>-ATPases to regulate stomatal apertures during pathogen attack. *PLoS Biol* 7: e1000139

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Luo Y, Scholl S, Doering A, Zhang Y, Irani NG, Di Rubbo S, Neumetzler L, Krishnamoorthy P, Van Houtte I, Mylle E, et al (2015) V-ATPase activity in the TGN/EE is required for exocytosis and recycling in Arabidopsis. *Nature Plants* 1: 1–10

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Maathuis FJ (2009) Physiological functions of mineral macronutrients. *Current Opinion in Plant Biology* 12: 250–258

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Majda M, Robert S (2018) The role of auxin in cell wall expansion. *International Journal of Molecular Sciences* 19: 951

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Martinière A, Bassil E, Jublanc E, Alcon C, Reguera M, Sentenac H, Blumwald E, Paris N (2013a) In vivo intracellular pH measurements in tobacco and Arabidopsis reveal an unexpected pH gradient in the endomembrane system. *The Plant Cell* 25: 4028–4043

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Martinière A, Desbrosses G, Sentenac H, Paris N (2013b) Development and properties of genetically encoded pH sensors in plants. *Front Plant Sci*. doi: 10.3389/fpls.2013.00523

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Martinière A, Gibrat R, Sentenac H, Dumont X, Gaillard I, Paris N (2018) Uncovering pH at both sides of the root plasma membrane interface using noninvasive imaging. *PNAS* 115: 6488–6493

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Miesenböck G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394: 192–195

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Miller AL, Gow NAR (1989) Correlation between root-generated ionic currents, pH, fusaric acid, indoleacetic acid, and growth of the primary root of *Zea mays*. *Plant Physiol* 89: 1198–1206

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Monshausen GB, Bibikova TN, Messerli MA, Shi C, Gilroy S (2007) Oscillations in extracellular pH and reactive oxygen species modulate tip growth of Arabidopsis root hairs. *PNAS* 104: 20996–21001

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Monshausen GB, Bibikova TN, Weisenseel MH, Gilroy S (2009) Ca<sup>2+</sup> regulates reactive oxygen species production and pH during mechanosensing in Arabidopsis roots. *Plant Cell* 21: 2341–2356

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Monshausen GB, Miller ND, Murphy AS, Gilroy S (2011) Dynamics of auxin-dependent Ca<sup>2+</sup> and pH signaling in root growth revealed by integrating high-resolution imaging with automated computer vision-based analysis. *Plant J* 65: 309–318

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Mravec J, Kračun SK, Rydahl MG, Westereng B, Miart F, Clausen MH, Fangel JU, Daugaard M, Cutsem PV, Licht HHDF, et al (2014) Tracking developmentally regulated post-synthetic processing of homogalacturonan and chitin using reciprocal oligosaccharide probes. *Development* 141: 4841–4850**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Msimbira LA, Smith DL (2020) The roles of plant growth promoting microbes in enhancing plant tolerance to acidity and alkalinity stresses. *Front Sustain Food Syst.* doi: 10.3389/fsufs.2020.00106**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Mühling KH, Sattelmacher B (1995) Apoplastic ion concentration of intact leaves of field bean (*Vicia faba*) as influenced by ammonium and nitrate nutrition. *Journal of Plant Physiology* 147: 81–86**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Mühling KH, Wimmer M, Goldbach HE (1998) Apoplastic and membrane-associated Ca<sup>2+</sup> in leaves and roots as affected by boron deficiency. *Physiologia Plantarum* 102: 179–184**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Nelson N (1994) Energizing porters by proton-motive force. *Journal of Experimental Biology* 196: 7–13**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Pacifici E, Polverari L, Sabatini S (2015) Plant hormone cross-talk: the pivot of root growth. *J Exp Bot* 66: 1113–1121**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Pearce G, Moura DS, Stratmann J, Ryan CA (2001) RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development. *Proc Natl Acad Sci U S A* 98: 12843–12847**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Peters WS, Felle HH (1999) The correlation of profiles of surface pH and elongation growth in Maize roots. *Plant Physiol* 121: 905–912**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Rayle DL, Cleland R (1970) Enhancement of wall loosening and elongation by acid solutions. *Plant Physiology* 46: 250–253**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Schulte A, Lorenzen I, Böttcher M, Plieth C (2006) A novel fluorescent pH probe for expression in plants. *Plant Methods* 2: 7**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Shen J, Zeng Y, Zhuang X, Sun L, Yao X, Pimpl P, Jiang L (2013) Organelle pH in the Arabidopsis endomembrane system. *Molecular Plant* 6: 1419–1437**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Shen Y, Rosendale M, Campbell RE, Perrais D (2014) pHuji, a pH-sensitive red fluorescent protein for imaging of exo- and endocytosis. *Journal of Cell Biology* 207: 419–432**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Shimizu Y, Takagi J, Ito E, Ito Y, Ebine K, Komatsu Y, Goto Y, Sato M, Toyooka K, Ueda T, et al (2021) Cargo sorting zones in the trans-Golgi network visualized by super-resolution confocal live imaging microscopy in plants. *Nature Communications* 12: 1901**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Shinoda H, Shannon M, Nagai T (2018) Fluorescent proteins for investigating biological events in acidic environments. *Int J Mol Sci.* doi: 10.3390/ijms19061548**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Soti PG, Jayachandran K, Koptur S, Volin JC (2015) Effect of soil pH on growth, nutrient uptake, and mycorrhizal colonization in exotic invasive *Lygodium microphyllum*. *Plant Ecol* 216: 989–998**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Staal M, Cnodder TD, Simon D, Vandenbussche F, Straeten DVD, Verbelen J-P, Elzenga T, Vissenberg K (2011) Apoplastic alkalinization is instrumental for the inhibition of cell elongation in the Arabidopsis root by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid. *Plant Physiol* 155: 2049–2055**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Stoddard A, Rolland V (2019) I see the light! Fluorescent proteins suitable for cell wall/apoplast targeting in *Nicotiana benthamiana* leaves. *Plant Direct* 3: e00112**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Takahashi K, Hayashi K, Kinoshita T (2012) Auxin activates the plasma membrane H<sup>+</sup>-ATPase by phosphorylation during hypocotyl elongation in Arabidopsis. *Plant Physiology* 159: 632–641**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Tantama M, Hung YP, Yellen G (2011) Imaging intracellular pH in live cells with a genetically encoded red fluorescent protein sensor. *J Am Chem Soc* 133: 10034–10037**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Tsai H-H, Schmidt W (2021) The enigma of environmental pH sensing in plants. Nature Plants 7: 106–115**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Waadt R, Köster P, Andrés Z, Waadt C, Bradamante G, Lampou K, Kudla J, Schumacher K (2020) Dual-Reporting Transcriptionally Linked Genetically Encoded Fluorescent Indicators Resolve the Spatiotemporal Coordination of Cytosolic Abscisic Acid and Second Messenger Dynamics in Arabidopsis. The Plant Cell 32: 2582–2601**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Wachter RM (2007) Chromogenic cross-link formation in green fluorescent protein. Acc Chem Res 40: 120–127**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Willats WGT, Orfila C, Limberg G, Buchholt HC, Alebeek G-JWM van, Voragen AGJ, Marcus SE, Christensen TMIE, Mikkelsen JD, Murray BS, et al (2001) Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. J Biol Chem 276: 19404–19413**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Wolf S, Höfte H (2014) Growth Control: A Saga of Cell Walls, ROS, and Peptide Receptors. The Plant Cell 26: 1848–1856**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Yu Q, Kuo J, Tang C (2001) Using confocal laser scanning microscopy to measure apoplastic pH change in roots of *Lupinus angustifolius* L. in response to high pH. Ann Bot 87: 47–52**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Yu Q, Tang C, Kuo J (2000) A critical review on methods to measure apoplastic pH in plants. Plant and Soil 219: 29–40**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Zeng W-Z, Liu D-S, Liu L, She L, Wu L-J, Xu T-L (2015) Activation of acid-sensing ion channels by localized proton transient reveals their role in proton signaling. Scientific Reports 5: 14125**

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)