

Running Title: Plant aquaporins and H₂O₂ signalling

Title: Plant aquaporins: crossroads of hydrogen peroxide signalling

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Abstract

Reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), are important inter- and intracellular signalling molecules involved in various plant physiological processes under biotic and abiotic stress conditions. They are produced in different subcellular compartments such as apoplast, plasma membrane, chloroplasts, peroxisomes, and mitochondria. Under stress, H₂O₂ is mainly produced in the apoplast by plasma membrane localized respiratory oxygen burst oxidase homologs (RBOHs), dismutases, and cell wall peroxidases. The function of H₂O₂ as a signal requires its diffusion through the plasma membrane or internal membranes, a process that is facilitated by the presence of channels named aquaporins (AQPs). In this chapter, we will discuss the mechanisms by which AQPs

24 facilitate and regulate H₂O₂ membrane diffusion, and the relevance of their contribution in
25 organellar, cellular, and cell-to-cell H₂O₂ signalling processes in the context of abiotic and
26 biotic stresses.

27

28 **Keywords:** Aquaporins, Reactive oxygen species, H₂O₂ transmembrane diffusion, H₂O₂
29 signalling, Cellular compartments.

30

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33 **Table:** 1

34

35 **1. Introduction**

36

37 Reactive oxygen species (ROS) play a central role in plant abiotic and biotic stress
38 responses. Among the different ROS species, H₂O₂ is most stable and is a key signal to
39 trigger downstream responses acting in plant defence and tolerance mechanisms. H₂O₂ is
40 produced in most cell compartments. Apoplastic H₂O₂ production is induced in response to
41 abiotic and biotic stimuli i.e., temperature, UV irradiation, ozone, phytohormones (ABA,
42 ethylene, etc.), salt stress, flg22, viruses, and chitin (Hossain et al., 2015; Guo et al., 2019;
43 reviewed in Castro et al., 2021). H₂O₂ is produced mainly by cell wall peroxidases and
44 plasma membrane localized respiratory burst oxidase homologs (RBOHs) (Daudi et al.,
45 2012). But H₂O₂ is also produced in other organelles such as chloroplasts, mitochondria,
46 and peroxisomes (Fig. 1). In chloroplasts, under high light intensity, O₂ is photoreduced to
47 the superoxide anion (O₂^{•-}) (Melher reaction) at the acceptor side of photosystem I during
48 the Water-Water cycle. Reduction of O₂^{•-} then leads to the formation of H₂O₂ through the

49 action of superoxide dismutases (Mullineaux et al. 2018; Asada 1999). This process is
50 important in triggering chloroplast-nucleus retrograde signalling and modification of gene
51 expression and the associated stress response (reviewed in Slesak et al., 2007; Castro et
52 al., 2021). During CO₂ fixation in chloroplasts, 2-phosphoglycolate is produced by the
53 oxygenase activity of RuBisCo, and is converted into glycolate that enters peroxisomes,
54 where it is transformed into glyoxylate, generating H₂O₂. The electron transport chain
55 complexes located in the mitochondrial inner membrane are the main sources of singlet O₂,
56 which is later dismutated to H₂O₂ by the mitochondrial superoxide dismutase. Besides, a
57 flavin adenine dinucleotide oxidase present in the endoplasmic reticulum (ER) could also
58 generate H₂O₂ by reducing oxygen (Tu and Weissman, 2002; Allen and Meyer, 2013).
59 While there are antioxidant enzymes such as catalases and peroxidases that scavenge
60 excess H₂O₂ and regulate its cellular homoeostasis, there are environmental circumstances
61 that perturb its balance, such as drought, high temperature, and/or pathogen attack
62 (reviewed in Waszczak et al., 2018; Smirnoff & Arnaud, 2019). Excessive H₂O₂ triggers
63 oxidative damage to DNA, lipids, and proteins (reviewed in Castro et al., 2021). Therefore,
64 ROS production, distribution, and scavenging in plant cells need to be tightly regulated.

65 H₂O₂ transport between the cell compartments is an inherent component of H₂O₂
66 homoeostasis and ROS signalling. H₂O₂ can permeate cellular membranes by simple
67 diffusion, but this process is slow. The involvement of water channels, named aquaporins
68 (AQPs), in H₂O₂ membrane transport was first demonstrated by heterologous expression in
69 yeast (Bienert et al., 2007; reviewed in Bienert & Chaumont, 2014), and then *in planta* (Tian
70 et al., 2016). The water channel activity of the first mammalian AQP was reported in 1992
71 (Preston & Agre, 1992), and this groundbreaking discovery was recognized by the Nobel
72 Prize in Chemistry to Peter Agre in 2003. AQPs are found in all life kingdoms including
73 plants. While 13 AQP isoforms have been identified in mammals, vascular plant genomes

74 encode many more isoforms (> 30 to more than 100) that differ in terms of tissue and cell
75 distribution, subcellular localization, transport selectivity, and regulation properties
76 (reviewed in Laloux et al., 2018). The water channel activity of the first plant AQP
77 (AtTIP1;1) was demonstrated in 1993 (Maurel et al., 1993). AQPs have attracted
78 considerable interest in plants due to the diverse substrate specificity that is essential for
79 plant growth, development, and physiology. In this chapter, we will provide a
80 comprehensive overview of plant AQPs and their roles in cellular H₂O₂ distribution and
81 signalling upon stress.

82

83 **2. Plant AQP Subfamilies**

84 Plant AQPs play various important physiological roles in long and short distance movement
85 of water, small solutes such as gases (CO₂, NH₃...), and metalloids (reviewed in Bienert &
86 Chaumont, 2011; Chaumont & Tyerman, 2014). Recent research has revealed that AQPs
87 also govern the transmembrane diffusion of H₂O₂ and even cations (Bienert et al., 2007;
88 Dynowski et al., 2008; Bienert et al., 2014; Byrt et al., 2016; Tian et al., 2016). Thus AQP
89 research has spread to diverse plant science fields, from membrane biophysics to cell
90 biology including stress responses to signalling.

91 Vascular plant AQPs are highly diverse and divided into five different subfamilies: (1)
92 plasma membrane intrinsic proteins (PIPs); (2) tonoplast intrinsic proteins (TIPs); (3)
93 Nodulin-26-like intrinsic proteins (NIPs); (4) small basic intrinsic proteins (SIPs), and (5)
94 mischaracterized X intrinsic proteins (XIPs) (Chaumont et al., 2001; Johanson et al., 2001;
95 Danielson & Johanson, 2008). To date, the latter subfamily has not been found in
96 Brassicaceae and in monocots (Danielson & Johanson, 2008; Bienert et al., 2011).
97 Interestingly, two additional subfamilies were found in *Physcomitrella patens* (moss) called
98 hybrid intrinsic proteins (HIPs) and GlpF-like intrinsic proteins (GIPs), which may have

evolved from bacterial origin by a horizontal gene transfer (Gustavsson et al., 2005; Danielson & Johanson, 2008). However, *Selaginella moellendorffii* (spike mosses) have only HIPs along with PIPs, TIPs, NIPs, SIPs, and XIPs (Anderberg et al., 2012). A new subfamily called large intrinsic protein (LIP) was identified in algae that are lacking in seed plants (Khabudaev et al., 2014).

3. AQP structure and solute movement

Three dimensional structures of human and some bacterial AQPs have been obtained, which provide a valuable resource to investigate the molecular mechanisms that govern solute movement through the pore and their regulation. In plants, the spinach SoPIP2;1 structure was obtained at high-resolution and, interestingly, revealed open and closed states (Tornroth-Horsefield et al., 2006; Nyblom et al., 2009), meaning that the channel activity can be regulated by a gating mechanism. Similarly, AtTIP2;1 crystal structure also suggested a possible gating mechanism for ammonia transport (Kirscht et al., 2016).

The general structure of AQPs is highly conserved from bacteria to human. AQPs comprise six transmembrane (TM) α -helices (TM1-TM6), which are connected by five loops (loops A–E), and both the N and C-termini are located in the cytoplasm (Fig. 2a). Loops B and E form short α -helices each containing the very conserved Asn Pro Ala (NPA) motif and dip halfway into the membrane. AQPs form tetramers, but each monomer functions as a channel (Fig. 2b and d). However, the AQP tetrameric assembly forms a central pore that is predicted to be a possible channel for CO₂ and cations in some AQPs (Yool & Weinstein, 2002; reviewed in Henderson et al., 2022; Tyerman et al., 2021) (Fig. 2b and c).

The monomer substrate selectivity is associated with two pore regions: a central region and the aromatic/arginine (ar/R) constriction filter, even if other channel parts, including TM domains can also participate in AQP channel activity (Chaumont et al., 2005;

124 Maurel et al., 2008; Berny et al., 2016). The first constriction occurs at the pore centre by
125 close apposition of the two asparagine residues of the NPA motifs of the loops B and E that
126 preclude proton (H^+) permeation (Murata et al., 2000). The ar/R constriction filter is formed
127 at the extracellular pore aperture by four residues present in TM2, TM5, and loop E (LE1
128 and LE2), respectively (Fu et al., 2000; Sui et al., 2001) (Fig. 2d).

129 The wide range of substrate conductance observed in plant AQPs is assumed to be
130 the result of changes at the ar/R constriction filter (Wallace & Roberts, 2005; Bansal &
131 Sankararamakrishnan, 2007; Gupta & Sankararamakrishnan, 2009; Azad et al., 2012).
132 However, a group of five moderately conserved amino acid residues at the so-called
133 Froger's positions are also important in substrate selectivity (Froger et al., 1998; Heymann
134 & Engel, 2000). More recently, some substrate specificity determining positions have been
135 recommended by combining the plant AQP sequences with the experimentally identified
136 substrates (Hove & Bhawe, 2011; Azad et al., 2016; Ahmed et al., 2020).

137 The specificity and movement of solutes through AQPs is determined by the size
138 and volume of the substrate and by the physicochemical properties of the amino acid
139 residues placed along the pore. Water molecules move as a file through the narrow pore
140 and this process is assisted by a series of backbone carbonyl groups and hydrophilic side
141 chains lining the pore. At the pore centre water is reoriented by the dipoles created by the
142 two half helices of loop B and E. This reorientation breaks the water file connectivity and
143 prevents proton transport via a Grotthus mechanism (Kosinska Eriksson et al., 2013). As
144 H_2O_2 has a slightly larger dipole moment/polarity and molecular diameter compared with
145 water and is able to form hydrogen bonds as water, it was hypothesized to diffuse through
146 AQPs and to mimic the movement of water molecules. Molecular dynamics simulation
147 using SoPIP2;1 indeed indicated that the channel may facilitate transport of H_2O_2 and even
148 the protonated superoxide radicals (HO_2) (Cordeiro, 2015).

150 **4. Determination of H₂O₂ channel activity of AQPs**

151 The first prediction suggesting that H₂O₂ and water diffuse in parallel through plant cell
 152 membranes came from experiments and mathematical modelling performed in internodal
 153 cells of the freshwater algae *Chara corallina* (Henzler & Steudle, 2000). The authors
 154 determined the H₂O₂ permeability coefficient, which is a measure of the speed with which a
 155 compound crosses a membrane, and found that it was very close to that of water. Also,
 156 treatment with the often-used AQP blocker HgCl₂, significantly reduced the influx of both
 157 molecules into the cells (Henzler & Steudle, 2000).

158 Experimental evidence demonstrating that specific AQPs facilitate H₂O₂ diffusion
 159 came from heterologous expression of AQPs in yeast and testing the strain growth
 160 sensitivity to increasing H₂O₂ amounts in the external medium (Bienert et al., 2007). The
 161 presence of H₂O₂ in the medium leads to a decrease in growth and cell survival, depending
 162 on the ability of the strain to detoxify ROS. Expression of human hAQP8 and Arabidopsis
 163 AtTIP1;1 and AtTIP1;2 reduced yeast growth and survival on medium containing H₂O₂
 164 (Bienert et al., 2007). This sensitivity was not due to a decreased ROS detoxification ability
 165 of the strain, demonstrating that the growth and survival impairments were due to the
 166 facilitated diffusion of H₂O₂ into the cells by the expressed AQPs (Bienert et al., 2006).
 167 Using a similar yeast expression system, an increasing number of plant AQPs from four
 168 subfamilies (PIPs, TIPs, NIPs, and XIPs) were reported as H₂O₂ channels and are listed in
 169 Table 1. Actually, it was proposed that all water-permeable AQPs are H₂O₂ channels, but
 170 with variable permeabilities according to the isoform and pore diameter (Almasalmeh et al.,
 171 2014). However, this may not be true for all AQPs. For instance, we demonstrated that the
 172 maize water channel ZmPIP2;5 was able to facilitate H₂O₂ diffusion in yeast, in contrast to
 173 ZmPIP1;2, a channel that, when coexpressed with PIP2;5, assembles in heterotetramers

174 and acts as an active water channel (Fetter et al., 2004; Bienert et al., 2014; Berny et al.,
175 2016). Surprisingly, an inactive water channel can also facilitate H₂O₂ membrane diffusion,
176 as demonstrated for tobacco NtXIP1;1. This isoform had no water channel activity when
177 expressed in *Xenopus* oocytes and present in the plasma membrane, but increased the
178 yeast cell sensitivity to H₂O₂ (Bienert et al., 2011). Other AQPs with water channel activity
179 seem not to facilitate H₂O₂ membrane diffusion when expressed in yeast, such as
180 Arabidopsis AtPIP2;3, AtPIP2;6 and AtPIP2;8 or rice and barley PIPs except HvPIP2;5
181 (Hooijmaijers et al., 2012; Rhee et al., 2016). However, negative results have to be
182 interpreted with caution, and verification of the aquaporin expression, abundance and
183 localisation is required. For instance, most PIPs belonging to the PIP1 subfamily need to
184 interact with PIP2 to reach the plasma membrane (see 5.2 AQP membrane abundance and
185 trafficking). Furthermore, to express maize PIPs in yeast cells, we had to codon optimise
186 the *PIP* cDNAs to detect the protein (Bienert et al., 2014). Nevertheless, these data might
187 indicate that still unknown structural determinants can influence the channel pore properties
188 and make it specific for water and/or H₂O₂. The terms “peroxoporin/peroxiporin” were
189 proposed for AQPs mediating H₂O₂ movement (Henzler & Steudle, 2000; Sies, 2014).
190 However, as these AQPs are also channels for water and/or other solutes, this name can
191 be misleading and should be used carefully depending on the context.

192 While knowledge about the H₂O₂ permeability of plant AQPs was mostly obtained
193 after their expression in yeast, a direct H₂O₂ transport assay in plant cells would be an
194 added value tool. Recently, we took advantage of the H₂O₂ fluorescent probe HyPer to
195 develop an inducible genetic system to evaluate AQP H₂O₂ transport in tobacco BY-2
196 suspension plant cells. This plant cell system allowed us to confirm the H₂O₂ transport
197 ability of ZmPIP2;5 and AtPIP2;1 (Ahmed and Chaumont, in preparation). Furthermore, it
198 has recently been shown that reconstitution of purified AQP proteoliposomes provides an

199 accurate quantitative measurement of AQP driven H₂O₂ transport (Wang et al., 2020a;
200 Wang et al., 2020b). However, this assay required the expression and purification of AQPs,
201 a process that is not straightforward for membrane proteins.

202 While we focus in this chapter on plant AQPs and their roles in cellular H₂O₂
203 membrane diffusion for which recent interesting data have been obtained, this specific role
204 has also been documented in different organisms, including mammals and bacteria (Bienert
205 and Chaumont, 2014). This is not surprising as AQPs share important structural
206 determinants, even if differences in specific amino acid residues within the pore are also
207 observed (Wang et al., 2020a; Montiel et al., 2020). The involvement of mammalian AQPs
208 (AQP0, AQP1, AQP3, AQP5, AQP8, AQP9 and AQP11) as peroxiporins and thus
209 activators of intracellular downstream signalling events has been extended to many diverse
210 physiological processes such as cell survival, cell migration, cancer growth, sterol
211 production, ER redox homeostasis, myocardial remodelling, mitochondrial function, sperm
212 motility, lens transparency, etc. (Bienert and Chaumont, 2014; Hara-Chikuma et al., 2015;
213 Rodrigues et al., 2019; Danielli et al., 2019; Montiel et al., 2020; Bestetti et al., 2020;
214 Varadaraj and Kumari, 2020; Chauvigné et al., 2021; Krüger et al., 2021). Several bacterial
215 or fungal AQPs act also as peroxiporins, such as LpGlpF isoforms in *Lactobacillus*
216 *plantarum*, the H₂O₂-inducible So-AqpA from *Streptococcus oligofermentans*, which
217 facilitates cellular H₂O₂ efflux and reduces oxidative stress, or LbAQP1 from the
218 ectomycorrhizal fungi *Laccaria bicolor* (Bienert et al., 2014; Navarro-RóDenas et al.,
219 2015; Tong et al., 2019). These results obtained in different organisms can certainly provide
220 interesting clues to better understand the function and regulation of plant AQPs as
221 peroxiporins.

222

223 **5. Plant AQP regulation**

224 An important concept to keep in mind is because solute movement through AQPs is
225 passive and only dependent on the solute concentration gradient on both membrane sides,
226 the flow can be bidirectional. This can have implications when considering H₂O₂ movement
227 according to the location of its production and its gradient. It is probably why these channels
228 are highly regulated at different levels to control the membrane permeability to solutes
229 (reviewed in Chaumont & Tyerman, 2014). In addition to *AQP* gene expression regulation,
230 there are different post-translational mechanisms that contribute to membrane permeability
231 to water and H₂O₂.

232

233 **5.1 Gating**

234 The channel activity of plant AQPs is regulated by gating, and this mechanism is dependent
235 on pH, divalent cations, and phosphorylation events (Hedfalk et al., 2006; Tornroth-
236 Horsefield et al., 2006; Verdoucq et al., 2008). A unified model of pore opening and closing
237 was proposed based on SoPIP2;1 molecular structure (Tornroth-Horsefield et al., 2006;
238 Nyblom et al., 2009). PIPs are highly water permeable at neutral or basic pH but largely
239 impermeable at acidic pH, which is sensed by the protonation state of a highly conserved
240 His residue located in loop D (His193 in SoPIP2;1) (Tournaire-Roux et al., 2003; Nyblom et
241 al., 2009). This protonation induced a conformational change of loop D and the occlusion of
242 the pore by a Leu residue (Leu 197 in SoPIP2;1). The channel opening and closing are also
243 controlled by phosphorylation and dephosphorylation of two serine residues located in loop
244 B and the C-terminus (Ser115 and Ser274), respectively, and by interactions between
245 hydrophobic residues from loop D, the N-terminus Asp28 and Glu31 residues, and loop B
246 (Ser115 and Arg118) (Tornroth-Horsefield et al., 2006). Cd²⁺ and/or Ca²⁺ can also bind to
247 the N-terminus and influence the gating mechanism through a network involved in ionic
248 interactions and hydrogen bonding (Tornroth-Horsefield et al., 2006).

249 Oxidative gating of plant AQPs was also proposed. A reduction of the plasma
250 membrane osmotic water permeability by HO radicals produced by a Fenton reaction was
251 reported in *C. corallina*, maize leaf parenchyma and root cortex cells, and *Trandescantia*
252 *fluminensis* epidermal cells, an inhibition which was reversible after removing the radicals
253 (Henzler et al., 2004; Ye & Steudle, 2006; Ye et al., 2008; Kim & Steudle, 2009). The exact
254 mechanism was unknown, but a possible direct action of the radicals within the pore was
255 proposed, even if an indirect effect on the lipid bilayer oxidation affecting AQPs on the
256 outside was not excluded (Henzler et al., 2004). The oxidation of disulfide bonds shown to
257 link the extracellular loops A of two PIP monomers within a tetramer seems not to be
258 responsible for the observed osmotic permeability inhibition, as tested in swelling assays
259 using *Xenopus* oocytes or by molecular dynamics simulations (Bienert et al., 2012;
260 Cordeiro, 2015). Actually, the absence of the effect of H₂O₂ preincubation on the osmotic
261 water permeability of oocytes expressing AtPIP2;1, AtPIP1;2 and AtTIP1;1, suggested that
262 the water permeability inhibition observed in Arabidopsis root cortical cells upon incubation
263 with H₂O₂ is due to another cellular process, the internalization of AQPs present in the
264 plasma membrane (see below) (Boursiac et al., 2008).

265

266 **5.2 AQP membrane abundance and trafficking**

267 AQPs are co-translationally inserted into the ER membrane. From there, they follow the
268 secretory pathway towards the Golgi apparatus and the trans-Golgi network (TGN), to
269 reach their final destinations, mostly the vacuole or the plasma membrane (Chevalier &
270 Chaumont, 2015). However, AQPs appeared to have a large variety of subcellular
271 localization patterns, including chloroplasts and mitochondria (Fig. 1) (Uehlein et al., 2008;
272 Soto et al., 2010; Møller et al., 2020). While the trafficking of AQPs into the latter organelles
273 has not been studied, the transport of PIPs into the secretory pathway to the plasma

274 membrane was thoroughly investigated and shown to depend on several protein-protein
275 interactions and post-translational modifications. Also, while in the plasma membrane,
276 AQPs can be internalized through an endocytic route (reviewed in Hachez et al., 2013).

277 After synthesis in rough ER, PIPs assemble as tetramers that might be stabilized by
278 a disulfide bond between the loop A of two adjacent PIP monomers (Zelazny et al., 2007;
279 Bienert et al., 2012). While most PIP1s are retained in the ER when expressed alone,
280 PIP2s are moving forward to the secretory pathway to reach the plasma membrane
281 (Zelazny et al., 2007). PIP2 ER exit is mostly dependent on a diacidic motif present in their
282 N-terminus, a motif known to interact with the Sec24 coat protein of COPII vesicles
283 (Zelazny et al., 2007; Sorieul et al., 2011), and also on a motif (LxxxA) found in TM3
284 (Chevalier & Chaumont, 2015). When PIP1s are co-expressed with PIP2s, they physically
285 interact in the ER within heterotetramers, and the complexes continue their journey to the
286 plasma membrane, where both PIP1s and PIP2s exhibit channel activities (Fetter et al.,
287 2004; Zelazny et al., 2007; Berny et al., 2016). PIP movement to the plasma membrane
288 also requires physical interaction with SNAREs (soluble N-ethylmaleimide-sensitive factor
289 protein attachment protein receptors), which are proteins involved in vesicle fusion with the
290 target membrane. ZmPIP2;5 physically interacts with SYP121, a plasma membrane Qa-
291 SNARE, regulating their abundance in the plasma membrane and possibly their activity
292 (Besserer et al., 2012; Hachez et al., 2014a). Also, the post-Golgi trafficking of AtPIP2;7
293 involves specific interaction with both SYP121 and the trans-Golgi localized SNARE,
294 SYP61, (Hachez et al., 2014a). ZmPIP2;5 also interacts with the vesicle-associated
295 membrane protein (VAMP)-associated proteins (VAP27s) at ER-plasma membrane contact
296 sites (EPCSs) and endocytic structures upon ionic stress (Fox et al., 2020). Whether this
297 precise location allows a direct transfer of H₂O₂ from the apoplast to the ER or conversely is
298 unknown and would be an interesting aspect for future investigation.

299 Other protein-protein interactions are important in abiotic and biotic stresses.
300 Expression of the E3 ubiquitin ligase (Rma1) and the ubiquitin-conjugating enzyme UBC32,
301 which are produced by drought stress, reduce the AtPIP2;1 amount in the plasma
302 membrane through a degradation process involving the proteasome, avoiding their
303 deleterious impact on drought tolerance (Lee et al., 2009). Another way to regulate PIP
304 abundance under stress is the physical interaction with the plant translocator protein
305 (TSPO), a multi-stress regulator. Upon ABA treatment, TSPO physically interacts with
306 AtPIP2;7 to reduce its plasma membrane abundance and membrane water permeability via
307 the autophagy mediated degradation pathway (Hachez et al., 2014b; Jurkiewicz et al.,
308 2020).

309 Phosphorylation is also involved in the alteration of PIP trafficking. Phosphorylation
310 of the C-terminal Ser283 is required for AtPIP2;1 trafficking to the plasma membrane (Prak
311 et al., 2008). Salicylic acid (SA), salt stress, and/or H₂O₂ treatment induced AtPIP2;1
312 internalization in root cells, a process dependent on the phosphorylation status of Ser208
313 and Ser283 (Boursiac et al., 2008; Prak et al., 2008). An increase in intracellular GFP-
314 PIP2;1 labelling mutated these serine residues (S280A or S280A) in root epidermal cells,
315 and this effect was enhanced upon salt treatment (Prak et al., 2008).

316

317 **6. Plasma membrane AQPs and H₂O₂ signalling in plants**

318 AQPs are found in all plant tissues and are significant players in maintaining water and
319 nutrient balance, and are therefore key actors in many plant physiological processes
320 including transpiration, seed germination, cell elongation, osmoregulation, plant
321 development, etc. (for review, see Heinen et al., 2009; Bienert & Chaumont, 2011;
322 Chaumont & Tyerman, 2014; Maurel et al., 2015). In this part, we will focus on recent

323 physiological evidence for AQP-mediated H₂O₂ diffusion in plants and in signalling
324 processes.

325 Upon biotic and abiotic stresses, H₂O₂ is produced by the activated NADPH
326 oxidases, superoxide dismutases, and peroxidases in the apoplast and enters cells through
327 AQPs to contribute to plant signalling mechanisms. This facilitated H₂O₂ diffusion through
328 the plasma membrane was the first demonstration of a physiological role for AQPs as
329 peroxiporins in plants, and was well documented for stomatal closure upon abscisic acid
330 (ABA) treatment and plant defence upon pathogen interaction (Hirt, 2016; Tian et al., 2016;
331 Qi et al., 2017; Rodrigues et al., 2017; Zhang et al., 2019; Ding et al., 2022).

332

333 **6.1 Stomatal movement**

334 Harsh environmental conditions such as cold, drought, and salt stress induce ABA
335 accumulation, which further activates plant adaptive stress responses. ABA is important in
336 stomatal signalling and closure (Hsu et al., 2021). In Arabidopsis, ABA binds to
337 PYR/PYL/RCAR receptors leading to the inhibition of protein phosphatase (PP2C) activity
338 and the activation of Snf1-related protein kinase 2 (SnRK2.6)/open stomata1 (OST1) kinase
339 (Fig. 3). This activated SnRK2.6/OST1 kinase then phosphorylates several substrate
340 proteins, including membrane transporters, which are involved in stomatal closure such as
341 S-type anion channel SLAC1, NADPH oxidases, and PIP AQPs (Kwak et al., 2003; Hao et
342 al., 2008; Geiger et al., 2009; Sirichandra et al., 2009; Brandt et al., 2012; Grondin et al.,
343 2015; Rodrigues et al., 2017) (Fig. 3). Phosphorylation of the Ser121 of PIP2;1 enhanced
344 its water channel activity, and it was proposed that this process facilitates water exit from
345 the guard cells, resulting in stomatal closure (Grondin et al., 2015). Actually, while the
346 aperture of stomata of *pip2;1* mutated plants is not affected after their transfer to light, their
347 closing response after ABA addition is slower and the stomata remain open compared to

348 the wild-type stomata (Grondin et al., 2015). Also, the use of the genetic probe HyPer
349 revealed that ABA triggers an accumulation of H₂O₂ in wild-type guard cells but not in an
350 *Atpip2;1* KO mutant (Rodrigues et al., 2017), suggesting that AtPIP2;1 contributes to
351 stomatal closure by also facilitating H₂O₂ entry, which in turn acts as a secondary
352 messenger and is sensed by intracellular proteins, leading to activation of inward calcium
353 channels and SLAC1 anion channel (reviewed in Rodrigues & Shan, 2021).

354 The contribution of plasma membrane AQPs in facilitating the membrane diffusion of
355 water and H₂O₂ in guard cells to control stomatal closure dynamics was also documented in
356 maize plants over- and down expressing *ZmPIP2;5* (Ding et al., 2022). Upon mild-water
357 deficit or low ABA concentration treatments, transpiration and stomatal conductance (g_s)
358 decreased more in *PIP2;5* overexpressing (OE), and less in *pip2;5* KO lines, in comparison
359 with WT. In detached epidermis, ABA treatment induced faster stomatal closure in PIP2;5
360 OE lines compared to WT, while *pip2;5* KO stomata were ABA insensitive. These
361 phenotypes were associated with guard cell ROS accumulation, detected by H₂DCFDA
362 staining (Ding et al., 2022).

363 The exact mechanism allowing this dual inverted movement of water and H₂O₂
364 through AQPs during stomatal closure is unknown, but probably requires distinct channels
365 and/or tetramers, and this process might be dependent on local concentration gradients of
366 the transported molecules and/or clustered localization of AQPs and enzymes involved in
367 H₂O₂ production, such as that observed for AtPIP2;1 in membrane microdomains (Wang et
368 al., 2015; Cui et al., 2021). As various AQPs are expressed in the stomatal complexes,
369 determining the contribution of each isoform to the stomatal dynamic behaviour in relation
370 to signalling molecules including H₂O₂ will be important for improving plant water use
371 (Heinen et al., 2014; reviewed in Ding & Chaumont, 2020).

372

6.2 Pathogen infection

Upon pathogen interaction, plants produce apoplastic H₂O₂, which induces an efficient intracellular immunity to overcome the infection. A role of AQPs in this signalling process was clearly demonstrated in several studies (Fig. 3). Upon bacterial infection of *Arabidopsis* leaves, an induction of *AtPIP1;4* gene expression is observed with concomitant H₂O₂ accumulation into the cytoplasm (Tian et al., 2016). This intracellular H₂O₂ accumulation is reduced or increased in *Atpip1;4* KO and *AtPIP1;4* OE plants, respectively, and triggers systemic acquired resistance (SAR) and pathogen-associated molecular patterns-triggered immunity (PTI). In wheat, fungal attack also induces apoplastic H₂O₂ production and its translocation to the cytoplasm through *TaPIP2;10*, resulting in a restriction of fungal attachment by activating PTI (Wang et al., 2021). *TaPIP2;10* overexpression enhances the resistance to powdery mildew and *Fusarium* head blight. In rice, *OsPIP2;2* mediates the membrane diffusion of pathogen-induced apoplastic H₂O₂ into the cell to activate the MAPK cascade and downstream defence responses (Zhang et al., 2022). In response to the bacterial flagellin-derived synthetic peptide flg22 known to induce plant immune responses, the Ser125 of *OsPIP2;2* is phosphorylated to activate H₂O₂ transport, and also triggers the translocation of the membrane-anchored transcription factor *OsmMYB* into the nucleus to regulate gene expression and positively modulate plant innate immunity.

Actually, flg22 peptide is initially perceived by the receptor kinase flagellin sensing 2 (FLS2) in combination with BAK1 and BIK1 kinases (Mersmann et al., 2010), and activates RBOHD and apoplastic H₂O₂ production (Khokon et al., 2011). This process is important to induce cellular signalling and molecular responses and trigger stomatal closure, therefore preventing pathogen entry into the plant (Fig. 3). Rodrigues et al. (2017) showed that, similarly to ABA treatment, flg22-induced cell accumulation of H₂O₂ is dependent on *AtPIP2;1* and its phosphorylation by BAK1.

398 AQP was also shown to be a plant-immunity-related gene. The conserved kinase
399 CRN78 effector from *Phytophthora* promotes infection and physically interacts with
400 NbPIP2;2 from *Nicotiana benthamiana* or GmPIP2;13 from soybean. This protein
401 interaction mediates phosphorylation of the PIP C-terminus and PIP degradation via a
402 proteasome-dependent mechanism (Ai et al., 2021). Interestingly, both NbPIP2;2 and
403 GmPIP2;13 facilitate H₂O₂ membrane diffusion when expressed in *Nicotiana benthamiana*
404 leaves. This effector-mediated mechanism, by targeting AQPs, might prevent H₂O₂ entry
405 and the downstream signalling cascade leading to plant immunity (Ai et al., 2021).

406

407 **6.3 Systemic signalling**

408 An elegant study investigated the role of PIP AQPs in systemic ROS signalling in
409 Arabidopsis (Fichman et al., 2021). In addition to inducing intracellular signalling, the
410 apoplast-to-cytosol translocation of H₂O₂ is also responsible for cell to cell communication
411 of the systemic signal, resulting in systemic acquired acclimation (SAA) throughout the
412 whole plant (Fig. 3) (Miller et al., 2009). This involved the symplastic route through
413 plasmodesmata, which connect the cytoplasm of adjacent cells. Furthermore, the
414 apoplastic H₂O₂ produced by one cell can also move in the apoplastic space and enter
415 neighbouring cells to trigger systemic signalling, acclimation, and defence mechanisms.
416 Using live imaging and ROS sensitive dye, Fichman et al. (2021) showed that, upon local
417 high light stress application, RBOHD and plasmodesmata-localized protein 1 and 5 (PDLP1
418 and PDLP5) increase cell to cell transport and plasmodesmata pore size, and are required
419 for the propagation of the systemic ROS signals and SAA (Fichman et al., 2021). Actually,
420 *Atrobhd* and *Atpdlp5* KO plants display a decrease of carboxyfluorescein spread and
421 plasmodesmata pore area size in response to local high light stress treatment, suggesting
422 that RBOHD-generated ROS promote systemic cell to cell transport through a control of the

plasmodesmata pore size and, therefore, callose deposition in the plasmodesmata edge (Cui & Lee, 2016; Fichman et al., 2021).

The authors also tested the role of AtPIPs in this process. While *Atpip1;4* KO plants exhibited a systemic ROS response similar to wild-type plants, *Atpip2;1* KO plants were completely deficient in the initiation and propagation of this systemic response, and *Atpip1;2* KO enhanced the rate of ROS signal propagation. As mentioned above, AtPIP2;1 facilitates H₂O₂ diffusion (Dynowski et al., 2008; Rodrigues et al., 2017; Ahmed and Chaumont, unpublished). Furthermore, grafting experiments suggested that AtPIP2;1 has a cell-autonomous function and is required for the amplification of the systemic signal in each individual cell along the path of the high light-induced systemic ROS wave (Fichman et al., 2021). The ROS entering cells through PIP2;1 is likely to be mobilized between cells through plasmodesmata. The different effect of *Atpip1;2* and *Atpip2;1* KO on the spread of systemic ROS signals in plants could be due to differences in their channel activity and/or regulation mechanisms such as heterotetrameric assembly, phosphorylation or other post-translational modifications, oxidative status and/or specific protein interaction known to modulate their subcellular localization, fate and activity (see above).

7. AQP-driven chloroplastic H₂O₂ signalling

The chloroplast membranes include the inner and outer envelopes and the thylakoid membrane containing the light-harvesting antenna and the photosynthetic electron transport chain. Oxygenic photosynthesis produces large amounts of ROS, including H₂O₂ produced by thylakoid-bound and stromal dismutases (Asada, 1999). H₂O₂ can move out of the chloroplast and trigger signalling to regulate energy metabolism and genetic and epigenetic responses, which contribute to plant adaptation (Fig. 1) (reviewed in Foyer & Hanke, 2022). This process is associated with retrograde chloroplast-to-nucleus signalling.

448 Different mechanisms are considered to explain this retrograde signalling: physical
449 connections (stromules), organelle proximity, and formation of protein complexes between
450 the organelles forming contact sites (reviewed in Mullineaux et al., 2020; Mittler et al.,
451 2022). This can also involve the ER membrane, which is contiguous with the nuclear
452 envelope and forms physical interaction with chloroplasts (reviewed in Anderson & Hetzer,
453 2007; Breeze & Mullineaux, 2022). While chloroplastic H₂O₂ movement toward the nucleus
454 in stromules does not require crossing any membranes, this is not the case in organelle
455 proximity or contact sites. Whether the latter requires AQP activity is still a matter of debate.
456 The use of the Amplex red probe, which forms fluorescent resorufin when reacting with
457 H₂O₂, showed that the fluorescence outside isolated chloroplasts is suppressed by 60% in
458 the presence of the AQP inhibitor acetazolamide, suggesting that H₂O₂ can diffuse across
459 the chloroplast envelope through AQPs (Borisova et al., 2012).

460 AQP s from the PIP and TIP subfamilies have been identified in chloroplast
461 membranes. *Nicotiana tabacum* NtAQP1 (a PIP1 isoform) is present in both the plasma
462 membrane and chloroplast inner envelope, and was shown to facilitate CO₂ diffusion into
463 chloroplasts (Uehlein et al., 2003; Uehlein et al., 2008). However, to our knowledge, its
464 contribution in H₂O₂ transport is still unknown. AQPs were also identified in proteomic
465 analysis from Arabidopsis chloroplast envelopes and thylakoid membrane (Ferro et al.,
466 2010; Simm et al., 2013). AtPIP1;2, AtPIP1;3, AtPIP2;1, AtPIP2;4, and AtPIP2;7 were
467 detected in the envelope fraction, even if their presence could also be due to putative
468 contamination as proposed for tonoplast AQPs (AtTIP1;1; AtTIP1;2 and AtTIP2;1) detected
469 in the envelope (Ferro et al., 2010). AtPIP2;1, AtPIP2;4, and AtPIP2;7 were shown to be
470 H₂O₂ channels when expressed in yeast and/or plant cells (Dynowski et al., 2008;
471 Hooijmaijers et al., 2012; Rodrigues et al., 2017). Therefore, they could facilitate H₂O₂

transfer from the chloroplasts to the cytosol or to the associated organelles, and contribute to the retrograde ROS signalling.

AtTIP1;2 and AtTIP2;1 were specifically detected in the thylakoid fraction. The presence of AQPs in the thylakoid membrane has been considered for their water channel activity, and are proposed to regulate volume changes of the thylakoid lumen in light (Beebo et al., 2013). However, as H₂O₂ is also generated inside thylakoid membranes (Mubarakshina et al., 2006), a role in H₂O₂ membrane diffusion for the stroma is not excluded, and would need to be investigated in detail.

480

8. AQPs and their putative contribution in ROS signalling in mitochondria, peroxisomes, and ER membranes

ROS production in other organelles such as mitochondria, ER, and peroxisomes, and the subsequent signalling also imply H₂O₂ movement across membranes (Fig. 1). While no plant AQPs have been identified in peroxisomal membranes, proteomics analysis showed that six Arabidopsis AQPs (AtTIP1;1, AtTIP2;1, AtTIP5;1, AtPIP1;4, AtPIP2;2, and AtPIP2;7) localize to the inner mitochondria (Møller et al., 2020). AtPIP5;1 was also in the mitochondria of pollen tubes, but was proposed to be involved in nitrogen recycling (Soto et al., 2010). Human AQP8 was demonstrated to be a H₂O₂ pore in the inner mitochondrial membrane of different cell lines (Marchissio et al., 2012; Chauvigné et al., 2015; Krüger et al., 2021), making plant AQPs also plausible channels to regulate mitochondrial H₂O₂ fate and signalling.

AQPs are also found in the ER membrane. As previously explained, PIP1s expressed alone are retained in the ER (Zelazny et al., 2007) but are mainly found in the plasma membrane in intact tissues, due to their interaction with PIP2s. However, there is a

subfamily of plant AQPs that are mostly present in ER, the SIPs (Ishikawa et al., 2005). These proteins are ubiquitously expressed in all plant tissues. Curiously, yeast strains expressing *Glycine max* GmSIP1;3, maize ZmSIP1;1, or ZmSIP2;1 are more resistant to increased H₂O₂ concentrations in the medium (Zhang et al., 2017; Bienert, Lebrun and Chaumont, unpublished data). *GmSIP1;3* overexpression confers growth retardation with shorter root length. Also, *GmSIP1;3* OE seeds germinate earlier than wild type in the presence of H₂O₂, indicating that the protein confers tolerance to H₂O₂ and therefore plays a role in protecting plants from oxidative stress (Zhang et al., 2017). As mentioned above, the plasma membrane ZmPIP2;5 was also found in EPCS in direct interaction with the ER resident VAP27;1 (Fox et al., 2020). This localization was proposed to stabilize AQPs and guide their endocytosis in response to salt stress. However, we cannot exclude that this specific EPCS location also plays a role in the direct transfer of H₂O₂ from the apoplast to the ER.

509

510 **9. AQPs at the crossroads of Ca²⁺ and H₂O₂ signalling**

During stress conditions, a challenge for plants is to maintain water and solute homoeostasis (Mahdieh et al., 2008). Therefore, plants require elaborate adaptive responses to tackle water limitation in harsh environments. AQPs are certainly important actors in these responses and contribute in maintaining water and solute balance upon stress conditions (reviewed in Chaumont & Tyerman, 2014). An important aspect is the multiple substrate channel activity of AQPs that led us to hypothesize that each function is interlinked. For instance, salt stress induces ionic, osmotic, and oxidative stress leading to H₂O₂ production in root tissues that functions as an intermediate between salt stress perception and regulation of root water transport by AQPs (Demidchik et al., 2003; Leshem et al., 2006; Boursiac et al., 2008). Also, AQP-mediated H₂O₂ membrane diffusion activates

521 H₂O₂ dependent signalling pathways, which also affect AQP phosphorylation status (Prak et
522 al., 2008; Rodrigues et al., 2017). Indeed, H₂O₂ treatment alters the AtPIP2;1 C-terminus
523 phosphorylation state (Prak et al., 2008). Also, receptor-like kinase (RLK) and FERONIA
524 potentially physically interact with AtPIP2;1 in response to NaCl and H₂O₂ treatments to
525 modify PIP phosphorylation status by unknown partners (Bellati et al., 2016). Furthermore,
526 salt stress induces PIP internalization, which is mediated by H₂O₂ (Boursiac et al., 2008;
527 Pou et al., 2016). Altogether, these observations suggest that H₂O₂ is a key signalling
528 intermediate during salt stress that regulates PIP phosphorylation, activity, and subcellular
529 dynamics to contribute to plant salt tolerance.

530 AQP-driven H₂O₂ transport possibly activates Ca²⁺ channels by indirectly modifying
531 their phosphorylation status (Fig. 3). Activation of Ca²⁺ channels increases the Ca²⁺ influx
532 into cells, which directly activates Ca²⁺ dependent kinases (CPKs), leading to the activation
533 of RBOHD to continue the positive feedback loop (Dubiella et al., 2013). Besides, pathogen
534 infection induces oxidative burst leading to transient Ca²⁺ increases, possibly by activating
535 Ca²⁺ permeable channels. The plasma membrane localized AtOSCA1.3, a newly identified
536 calcium channel, is rapidly phosphorylated upon flg22 perception (Thor et al., 2020).
537 Possibly, this phosphorylation event activates OSCA1.3, triggers Ca²⁺ influx, and ultimately
538 activates further RBOHD via another phosphorylation event (Fig. 3). Indeed Ca²⁺ cytosolic
539 import triggers the activation of several membrane components including RBOHD and
540 AQPs through CPK-dependent phosphorylation events to initiate the cytoplasmic import of
541 H₂O₂ and activate PTI (Lee et al., 2020; reviewed in Marcec et al., 2019).

542 Several studies proposed that intracellular Ca²⁺ and ROS signals are interlinked
543 (Choi et al., 2014; reviewed in Gilroy et al., 2016; Fichman & Mittler, 2020). During stress
544 conditions, RBOHD generated H₂O₂ increased the Ca²⁺ wave by activating glutamate
545 receptor-like Ca²⁺ channels (Gilroy et al., 2014). ROS waves are also involved in generation

of electric signals (Suzuki et al., 2013). Mechano-activated Ca^{2+} channels were proposed to be involved in hydraulic waves initiation (reviewed in Gilroy et al., 2016; Fichman & Mittler, 2020). Altogether, Ca^{2+} , ROS, and electric and hydraulic waves are proposed to interplay together. Also, electron transfer from the cell to the apoplast and RBOHD mediated H_2O_2 production could modify the pH and the membrane potential and regulate plasma membrane proteins such as H^+ -ATPase (AHA1), AQPs, or other channels (reviewed in Fichman & Mittler, 2020). However, several components of this complex signalling network still need to be experimentally validated. Furthermore, as explained above, AQP-driven cytoplasmic H_2O_2 entry might influence the cell-to cell connectivity by regulating plasmodesmata aperture (Fichman et al., 2021). Plasmodesmata are directly involved in cell to cell transport of plant nutrients and pathogens (Cui & Lee, 2016). For instance, plant viruses move from cell to cell by plasmodesmata, which could restrict the virus movement upon H_2O_2 -mediated plasmodesmata closure (Tilsner et al., 2013; Wang, 2021). Systemic ROS signalling is also regulated by Ca^{2+} permeable channels (GLR, CNGC, and MSL) in addition to RBOHD, PLD1, PLD5, and PIP2;1 (Fichman et al., 2021). All these observations suggest that AQP-mediated H_2O_2 transport might play an important signalling intermediate in ROS and Ca^{2+} signalling.

563

564 **10. Conclusion and perspectives**

565 Current knowledge strongly suggests that AQPs are mediating facilitated membrane
566 diffusion of H_2O_2 into and within plant cells, and that this activity triggers intra- and
567 intercellular ROS signalling involved in many physiological processes, notably in relation to
568 biotic and abiotic stress. This AQP-driven H_2O_2 transport activity seems to be an important
569 process for initiating common stress signalling to the plant cell for plant acclimation, in
570 interaction with other redox enzymes, Ca^{2+} channels, and other transporters. However, how
571 all these actors are interconnected and the molecular mechanisms behind are still poorly

understood. Recent technological advances such as genetically encoded ROS probes, super-resolution microscopy, and genome editing techniques will provide ways to explain the role of specific AQP isoforms and their potential partners in plant stress responses. A major difficulty will be to differentiate the H₂O₂ channel activity from the other substrate specificities of AQPs that might contribute to the observed phenotypes. Indeed, a plant AQP with a unique peroxiporin activity was not identified yet, and the H₂O₂ substrate specificity is expected to be very difficult to engineer. An interesting aspect to investigate is the contribution of AQPs in organelle to organelle ROS retrograde signalling (mitochondria, chloroplast, or ER to nucleus), which is an important component of plant response to environmental stimuli. While AQPs are found in the organellar membranes, their role as peroxiporins in these membranes was not demonstrated yet. Currently, the functional relationship between AQPs and H₂O₂ in plant immunity is well established. However, how pathogens escape plant immunity still needs to be investigated. It was recently proposed that pathogens target AQPs to inhibit their H₂O₂ channel activity and escape the host plant defence system (Ai et al., 2021), but this attractive mechanism would need additional experiments to be validated.

Overall, a better understanding of AQPs in mediating H₂O₂ cell autonomous and plant systemic signalling, together with characterization of molecular mechanisms/partners regulating their peroxiporin activity would be very useful to improve/select plants with better abiotic and/or biotic stress tolerance.

592

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AQP name	Subcellular localization	Model used to test the channel activity	Physiological roles	References
AtPIP1;4	PM	Yeast	Plant immunity	(Tian <i>et al.</i> , 2016)
AtPIP2;1	PM	Yeast, Stomata	Stomatal closure (H ₂ O ₂ Signalling)	(Dynowski <i>et al.</i> , 2008; Hooijmaijers <i>et al.</i> , 2012; Rodrigues <i>et al.</i> , 2017)
AtPIP2;2	PM	Yeast	?	(Hooijmaijers <i>et al.</i> , 2012)
AtPIP2;3	PM	Yeast	?	(Dynowski <i>et al.</i> , 2008; Israel <i>et al.</i> , 2022)
AtPIP2;4	PM	Yeast Reconstitution in liposomes	?	(Hooijmaijers <i>et al.</i> , 2012; Wang <i>et al.</i> , 2020a)
AtPIP2;5	PM	Yeast	?	(Hooijmaijers <i>et al.</i> , 2012)
AtPIP2;7	PM	Yeast	?	(Hooijmaijers <i>et al.</i> , 2012)
GmPIP2;13	PM	<i>N. benthamiana</i>	Plant immunity	(Ai <i>et al.</i> , 2021)
HvPIP2;5	PM	Yeast	?	(Rhee <i>et al.</i> , 2017)
NbPIP2;2	PM	<i>N. benthamiana</i>	Plant immunity	(Ai <i>et al.</i> , 2021)
OsPIP2;2	PM	Yeast	Plant immunity	(Zhang <i>et al.</i> , 2022)
OsPIP2;3	PM	Yeast	?	(Zhang <i>et al.</i> , 2022)
OsPIP2;6	PM	Yeast	?	(Zhang <i>et al.</i> , 2022)
SoPIP2;1	PM	Reconstitution in liposomes	?	(Wang <i>et al.</i> , 2020a)
TaPIP2;10	PM	Yeast	Plant immunity	(Wang <i>et al.</i> , 2021)
ZmPIP2;5	PM	Yeast Stomata	Stomatal movement	(Bienert <i>et al.</i> , 2014;

AtTIP2;3	Tonoplast	Yeast	?	Ding <i>et al.</i> , 2022)
TgTIP2;1	Tonoplast	Yeast	?	(Dynowski <i>et al.</i> , 2008)
TgTIP4;1	Tonoplast	Yeast	?	(Azad <i>et al.</i> , 2012)
AtNIP1;2	ER	Yeast	?	(Azad <i>et al.</i> , 2012)
NtXIP1;1	PM	Yeast	?	(Dynowski <i>et al.</i> , 2008)
SIXIP1;1	PM	Yeast	?	(Bienert <i>et al.</i> , 2011)
				(Bienert <i>et al.</i> , 2011)

Figure legends

Fig. 1 Control of H₂O₂ (red and white sphere) cell homoeostasis, including production and transport. Extracellular H₂O₂ production is induced by the cell wall bound peroxidases and respiratory burst oxidase homologs (RBOHs). Its diffusion to the cell uses AQPs (shown in purple ball). Major intracellular H₂O₂ production sites such as mitochondria, peroxisomes, and chloroplasts were shown. Intraorganelle H₂O₂ movement is proposed to be mediated by AQPs (shown in purple oval) and putative channels in peroxisomal and nuclear membranes (shown in blue cylinder). Black solid arrow indicates transport; magenta dashed arrow indicates putative transport.

Fig. 2 Plant AQP general structure. (a) The AQP topology consists of six TM α -helices (TM1-TM6), which are linked by five loops (loops A–E), with both the N and C-termini located on the membrane cytoplasmic side. Several posttranslational modification sites are indicated including potential phosphorylation sites (purple), pH sensing residues (green), Ca²⁺ responsive residues (blue), mechano-sensing residues (magenta), and cGMP sensing residue (yellow). The aromatic arginine (ar/R) residues are shown in black stars and

1085 Froger's positions are shown in red triangle. (b) Tetrameric assembly of maize plasma
1086 membrane AQP (PIP2;5); individual and proposed central pore are indicated with a black
1087 and purple circle, respectively. (c) Cross section of a representative maize AQP (PIP2;5)
1088 tetramer and the path of the central pore is shown in a red ball. The figure was generated
1089 with PoreWalker software. (d) Monomeric maize PIP2;5 3D structure and the pore are
1090 indicated with red spheres. Ar/R filter residues are shown in magenta spheres and NPA
1091 motifs are shown in cyan spheres.

1092
1093 **Fig. 3** AQP-mediated H₂O₂ and its signalling roles. ABA activates the SnRK2.6/OST1
1094 kinases, which phosphorylate several membrane proteins including RBOHD, calcium
1095 channels such as OSCA1s, and AQPs. Conversely, Flg22 is perceived by the FLS2 that
1096 later phosphorylates RBOHD with the help of BAK1 and BIK1 kinases. Wu et al., 2020 first
1097 identified HPCA1 as an extracellular H₂O₂ sensor in plants, which senses the apoplastic
1098 H₂O₂ that activates PM localized calcium channels (for instance OSCA1.3) and leads to
1099 cytoplasmic Ca²⁺ elevation that activates RBOHD, which contributes to produce apoplastic
1100 H₂O₂, which is further taken up to the cell via PIPs. Stresses induce elevated intracellular
1101 H₂O₂ that could activate several signalling cascades, such as activation of PM localized
1102 Ca²⁺ channels such as OSCA1s, and cell-to-cell communication through open
1103 plasmodesmata (PD) by activation of callose synthase genes (CalS1/8) (indicated as a light
1104 blue ball) and PD localized proteins (PDLPs) (indicated as yellow ball) at PD.