

1 Short title: Chemical genetics of auxin-induced Ca²⁺ entry

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6 Title:

7 Identification of novel inhibitors of auxin-induced Ca²⁺ signaling via a
8 plant-based chemical screen

9

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24 One-sentence Summary:

25 Screening of an annotated library of biologically active molecules for inhibitory effects on
26 auxin-induced Ca²⁺ entry in BY-2 cells yielded several new inhibitors for investigating Ca²⁺
27 signaling.

28

29 **Footnotes:**

30 Author contributions:

31 K.D.V., E.H. and K.D. designed and performed the experiments and analyzed the data; A.D.
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33 supervised the experiments; S.V. and T.B. conceived the project and wrote the article with
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52 **Abstract**

53 Many signal perception mechanisms are connected to Ca^{2+} -based second messenger
54 signaling to modulate specific cellular responses. The well-characterized plant hormone
55 auxin elicits a very rapid Ca^{2+} signal. However, the cellular targets of auxin-induced Ca^{2+} are
56 largely unknown. Here, we screened a biologically annotated chemical library for inhibitors of
57 auxin-induced Ca^{2+} entry in plant cell suspensions to better understand the molecular
58 mechanism of auxin-induced Ca^{2+} and to explore the physiological relevance of Ca^{2+} in auxin
59 signal transduction. Using this approach, we defined a set of diverse, small molecules that
60 interfere with auxin-induced Ca^{2+} entry. Based on annotated biological activities of the hit
61 molecules, we found that auxin-induced Ca^{2+} signaling is, among others, highly sensitive to
62 disruption of membrane proton gradients and the mammalian Ca^{2+} channel inhibitor bepridil.
63 Whereas protonophores nonselectively inhibited auxin-induced and osmotic-stress-induced
64 Ca^{2+} signals, bepridil specifically inhibited auxin-induced Ca^{2+} . We found evidence that
65 bepridil severely alters vacuolar morphology and antagonized auxin-induced vacuolar
66 remodeling. Further exploration of this plant-tailored collection of inhibitors will lead to a
67 better understanding of auxin-induced Ca^{2+} entry and its relevance for auxin responses.

68

69 **Introduction**

70 The plant hormone auxin is a potent regulator of a diverse set of developmental processes,
71 ranging from embryogenesis, postembryonic organogenesis, and regeneration to tropic
72 growth responses (Vanneste and Friml, 2009). These pluripotent effects in plant
73 development make auxin a key player in the plant's developmental plasticity. Moreover,
74 auxin is subject to extensive cross-talk with many other signaling pathways for flexible
75 integration in auxin-regulated development (Chaiwanon et al., 2016; Liu et al., 2017).
76 Decades of extensive research have led to the formulation of a canonical auxin signaling
77 pathway. In short, the perception of auxin occurs via the auxin-induced stabilization of a
78 coreceptor complex constituted by a TIR1/AFB F-box protein and Aux/IAA proteins, resulting
79 in the ubiquitination and proteolysis of the latter. Consequently, Aux/IAA-interacting AUXIN
80 RESPONSE FACTORS (ARFs) can become active (Lavy and Estelle, 2016; Weijers and
81 Wagner, 2016). This auxin signaling mechanism can explain many of the plant's responses
82 to auxin. In addition, a nontranscriptional branch of TIR1/AFB-based auxin perception was
83 recently connected to the nontranscriptional inhibition of elongation (Fendrych et al., 2018),
84 vacuolar remodeling (Lofke et al., 2015), and activation of Ca²⁺ signaling (Dindas et al.,
85 2018).

86 A large body of literature describes the role of Ca²⁺ in a variety of cellular processes in plants
87 in the context of responses to light, and biotic and abiotic stress (reviewed in Tuteja and
88 Mahajan, 2007; Kudla et al., 2010; Kudla et al., 2018). However, little is known about the role
89 of Ca²⁺ signaling downstream of auxin. Interestingly, a few reports connect Ca²⁺ to auxin
90 transport regulation (Dela Fuente and Leopold, 1973; Benjamins et al., 2003; Zhang et al.,
91 2011; Rigo et al., 2013). More recently, auxin-induced cytosolic Ca²⁺ increase was proposed
92 to contribute to auxin's inhibitory effect on root growth and auxin-regulated root hair growth
93 via the nonselective cation channel CNGC14 (Shih et al., 2015; Dindas et al., 2018). Jointly,
94 these reports illustrate the importance of Ca²⁺ in auxin physiology. Despite this recent
95 progress, it is clear that much remains to be uncovered about the underlying signaling
96 mechanism and its cellular targets.

97

98 Several types of plant Ca^{2+} channel types exist in relatively large gene families, as illustrated
99 in a few examples in *Arabidopsis* (*Arabidopsis thaliana*): 20 CYCLIC NUCLEOTIDE-GATED
100 CHANNELS (CNGCs; Ma and Berkowitz, 2011), 20 GLUTAMATE RECEPTOR-LIKE
101 CHANNELS (GLRs; Forde and Roberts, 2014), and 16 OSCA/TMEM63 channels (Murthy et
102 al., 2018; Zhang et al., 2018). This genetic complexity greatly hinders pinpointing the Ca^{2+}
103 channels that are involved in a given Ca^{2+} regulated process. Therefore, the involvement of
104 Ca^{2+} in any process in plants is often deduced from using Ca^{2+} chelators such as EGTA and
105 BAPTA or very nonselective Ca^{2+} channel blockers such as La^{3+} and Gd^{3+} . However, these
106 treatments do not reveal anything about the molecular nature of the Ca^{2+} channel involved.
107 Moreover, some important side effects need to be considered when using these treatments
108 to manipulate Ca^{2+} , for example, acidification caused by EGTA and BAPTA releasing four H^+
109 when binding two Ca^{2+} and the efficient precipitation of phosphates by La^{3+} (reviewed in De
110 Vriese et al., 2018).

111 The importance of Ca^{2+} in human physiology and neurology led to the development of an
112 extensive pharmacological toolbox to manipulate specific groups of Ca^{2+} channels. Simple
113 drug treatments thus allow manipulation of specific Ca^{2+} channels and evaluation of its effect
114 on any process of interest. Unfortunately, the Ca^{2+} signaling machinery in plants has
115 diverged significantly from the one in the animal kingdom, with many of the Ca^{2+} signaling
116 components of animals being absent in plants and *vice versa* (Nagata et al., 2004; Edel et
117 al., 2017). For instance, channels associated with muscle and nerve Ca^{2+} signal transduction
118 in animals, such as L-type voltage-dependent Ca^{2+} channels (VDCCs; Zuccotti et al., 2011),
119 inositol 1,4,5-triphosphate receptors (IP_3Rs ; Nixon et al., 1994), and ryanodine receptors
120 (RyRs ; Lanner et al., 2010) are either missing or significantly different in plants. Hence, many
121 Ca^{2+} channel inhibitors established in animal systems are arguably of limited use in plants.
122 Only a few ionotropic GLR inhibitors (DNQX, CNQX, MNQX, and AP5) were also shown to
123 have inhibitory effects on specific plant GLR-based Ca^{2+} channeling activities (reviewed in
124 De Vriese et al., 2018), but the molecular nature and specificity of these inhibitory effects

125 remain to be characterized. These examples illustrate that there is an important need to
126 develop Ca²⁺ channel inhibitors directly in plant systems to stimulate plant Ca²⁺ research.
127 Here, we set out to identify small molecules that modify the shape and amplitude of auxin-
128 induced cytosolic Ca²⁺ dynamics and use them to further explore the role of Ca²⁺ signaling in
129 cellular auxin responses. We screened a collection of biologically active and structurally
130 diverse compounds for inhibitors of auxin-induced Ca²⁺ signaling in transgenic tobacco
131 (*Nicotiana tabacum*) BY-2 cell lines that express YFP-apoaequorin as a reporter of Ca²⁺
132 signaling. Our primary screen identified 80 potential inhibitors, of which 67 were reconfirmed
133 in a confirmation screen. Based on annotated biological functions, we found that
134 protonophores nonselectively interfered with auxin-induced Ca²⁺. Moreover, we found that
135 auxin-induced Ca²⁺ signals were much more sensitive to bepridil than hyperosmotic-stress-
136 induced Ca²⁺ signals. This differential drug sensitivity is consistent with distinct Ca²⁺ channel
137 types being involved in the Ca²⁺ response (Yuan et al., 2014; Shih et al., 2015; Dindas et al.,
138 2018). Interestingly, we found that bepridil had a severe impact on vacuolar morphology, to
139 the extent that it antagonized auxin-induced vacuolar remodeling. The resulting set of
140 inhibitors thus represents a valuable expansion of the toolbox with nonspecific and
141 semiselective inhibitors for exploring the role of Ca²⁺ signaling in plants.

142

143 **Results**

144 **Assay development and chemical screen**

145 Unlike whole plants, which are composed of complex mixtures of cell types with different
146 stimulus sensitivities, tobacco BY-2 cell suspension cultures are highly homogenous in terms
147 of cell type and developmental stage, thus making them highly suited for high-throughput
148 screening in a multiwell format. A BY-2 cell suspension line expressing YFP-fused
149 apoaequorin (YA) (Mehlmer et al., 2012) was established for luminescence-based
150 quantification of Ca²⁺ signals (Shimomura et al., 1962; Knight et al., 1991). Aequorin is a

151 bioluminescent photoprotein that emits blue light upon Ca^{2+} binding and has been used
152 extensively as a Ca^{2+} sensor (Shimomura et al., 1962; Shimomura, 2005) (Fig. 1A). The YFP
153 fused to the aequorin was used to visualize expression of the probe in the BY-2 cells but did
154 not contribute to the sensor activity. The assay was miniaturized to 96-well plate format and
155 validated by assessing the effect of 14 known elicitors of Ca^{2+} signaling. The known elicitors
156 induced distinct signals in this BY-2 cell culture (Fig. 1B-O), corroborating the suitability of
157 the cells to assess the effect on Ca^{2+} signaling.

158 The BY-2 cell suspensions were poorly responsive to exogenous application of auxins (Fig.
159 1H-J). This probably reflects an auxin insensitivity that is associated with prolonged culturing
160 of the cells in relatively high 2,4-D concentrations ($0.2 \text{ mg/L} \approx 1 \text{ } \mu\text{M}$ in the growth medium).
161 Therefore, the 2,4-D concentration was increased to $500 \text{ } \mu\text{M}$, which resulted in a robust
162 auxin-induced Ca^{2+} response. Immediately after 2,4-D addition, the luminescent signal rapidly
163 increased and reached a maximum after approximately 90 seconds, which attenuated to
164 close to baseline levels around 300 seconds after addition (Fig. 1J). By using the viability
165 stains propidium iodide (PI) and fluorescein diacetate (FDA), no obvious difference in cellular
166 integrity was observed after 1-hour treatment with 2,4-D, suggesting that the immediate Ca^{2+}
167 response to 2,4-D (within minutes) is unlikely the result of a defect in cellular viability
168 (Supplemental Fig. S1).

169 Next, we aimed to evaluate the quality of our assay by calculating its Z-prime (Z') factor,
170 which is a commonly used statistical indicator of quality for high-throughput assays (Zhang et
171 al., 1999). It allows estimation of the quality of the bioassay in discriminating the effect of hit
172 molecules from read-out variation based on two parameters: 1) the 'separation band' of the
173 assay: the difference between the mean of the negative control plus three times the standard
174 deviations of the negative control and the mean of positive control minus three times the
175 standard deviations of the positive controls, and 2) the dynamic range of the assay: the
176 difference between the mean of the positive and negative controls. In a good assay, the
177 means of the positive and negative controls differ strongly from each other, while the

178 standard deviations are very low (Fig. 2A). An ideal assay has a Z' factor close to 1, and
179 assays with a Z' factor between 0.5 and 1 are generally considered to be excellent (Zhang et
180 al., 1999). Here, we calculated the Z' value of our assay based on the means and standard
181 deviations of the peak intensities of mock-treated cells (DMSO) and cells treated with a
182 nonspecific Ca²⁺ channel inhibitor (GdCl₃) (Fig. 2A). The Z' factor was 0.54, suggesting that
183 our assay is robust enough to reliably distinguish potential inhibitors from well-to-well
184 variation. Subsequently, we used this experimental setup to screen the Spectrum collection
185 (MicroSource Discovery Systems) of 2,320 annotated compounds (Fig. 2B). This resulted in
186 the identification of 80 molecules that reduced the maximal response induced by 500 μM 2,4-
187 D to less than 55% of that of the respective DMSO controls. Next, we used a more sensitive
188 well-per-well reconfirmation assay via a plate reader, which also allowed assay of the viability
189 of the cells at the end of the assay by adding a discharge solution. This treatment causes
190 disruption of the membrane integrity and flooding of the cytoplasm with Ca²⁺, which binds the
191 remnant reconstituted aequorin, and thus generates a sharp, strong luminescence peak
192 (discharge peak) (Fig. 2C). Out of the 80 primary hits, 67 were reconfirmed for reducing the
193 auxin-responsive Ca²⁺ signal by more than 45% (Supplemental Table S1). Of these 67
194 confirmed hits, 39 showed no obvious short-term cytotoxicity, as evidenced by the presence
195 of a large Ca²⁺ discharge peak (Fig. 2C (green); Supplemental Table S1 (confirmed)).
196 However, 28 confirmed hits caused a reduction (Fig. 2C (yellow); Supplemental Table S1
197 (semi-confirmed)) or even complete absence of the Ca²⁺ discharge peak, indicating a defect
198 in Ca²⁺ compartmentalization mechanisms and/or cell viability (Fig. 2C (blue); Supplemental
199 Table S1 (cytotoxic)). Based on structural features, the 67 hits retained after the confirmation
200 screen could be organized into 43 clusters of structurally similar compounds (Supplemental
201 Table S1).

202 Given that both the primary screen and confirmation screen represented single-well
203 analyses, we aimed to further validate a part of our dataset using multiple biological repeats.
204 Therefore, we selected 13 commercially available hit molecules representing a large

205 chemical diversity for further validation (Table 1). The auxin-induced Ca^{2+} responses were
206 analyzed in 4-8 replicates on YFP-apoaequorin-expressing BY-2 cells (Mehlmer et al., 2012).
207 Out of the 13 tested chemicals, 10 could be confirmed to strongly modify the 2,4-D induced
208 Ca^{2+} signature, while maintaining a robust discharge peak (Fig. 2D). Together, these data
209 highlight that our set of 67 hits after the confirmation screen is rich in potent modifiers of
210 auxin-induced Ca^{2+} signaling.

211
212
213

Fenamates alter the shape of auxin-induced Ca^{2+}

214 Among the 67 confirmed hit compounds, we found four highly related fenamate-type
215 chemicals: flufenamic acid (FFA **(1)**), niflumic acid (NFA **(2)**), tolfenamic acid (TFA **(3)**), and
216 flunixin meglumine **(4)** (structures of all compounds described in the manuscript are shown in
217 Supplemental Fig. S2). Unlike any other tested hit compounds, which simply reduced the
218 amplitude of the Ca^{2+} response, these fenamates had a dramatic effect on the shape of the
219 Ca^{2+} response. Treatment with FFA, NFA, or TFA reduced the maximum of the Ca^{2+} signal,
220 but also revealed a novel Ca^{2+} signal that preceded the maximum (Fig. 3A-C). Such an effect
221 on the Ca^{2+} signal shape was not observed for any of the other hit compounds that were
222 selected for validation.

223 Seedlings grown for 7 days in the presence of 20 μM FFA, NFA, or TFA had significantly
224 shorter roots than seedlings grown on control plates and displayed a reduced gravitropic root
225 growth, as indicated by a reduced vertical growth index (Fig. 3D-E; Supplemental Fig. S3).
226 Consistently, we observed spreading of the expression of the synthetic auxin response
227 reporter DR5rev::VENUS-N7 in the columella and stem cell niche (Fig. 3F-H), reminiscent of
228 an inhibitory effect on auxin transport. However, neither of the two known auxin transport
229 inhibitors, 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid (BUM **(8)**) and 1-N-
230 naphthylphthalamic acid (NPA **(9)**), had an obvious effect on auxin-induced Ca^{2+} (Fig. 4A-C),

231 suggesting that a block in auxin transport does not explain the effect of fenamates on Ca²⁺
232 signaling.

233 The inhibition of cyclooxygenase activity by fenamates renders them suitable for use as
234 nonsteroidal anti-inflammatory drugs (NSAIDs) (Graham, 2016), and NFA has reported anion
235 channel inhibitory activities (Diatloff et al., 2004; Gilliam and Tester, 2005). Based on these
236 reported bioactivities, we investigated whether other NSAIDs and anion channel inhibitors
237 have a similar effect on Ca²⁺ signaling (Fig. 4A). Of two structurally unrelated NSAIDs
238 (ibuprofen **(10)** and oxaprozin **(11)**) and two unrelated anion channel inhibitors, 5-nitro-2-(3-
239 phenylpropylamino) benzoic acid (NPPB **(12)**) and 9-anthracenecarboxylic acid (9-ACA **(13)**),
240 only NPPB inhibited auxin-induced Ca²⁺ (Fig. 4D-G). However, the effect of NPPB was
241 clearly distinct from the effect of fenamates on Ca²⁺ response dynamics (Fig. 4G), suggesting
242 that the observed effects of fenamates on Ca²⁺ signaling are independent of any additional
243 effects on cyclooxygenases or anion channels.

244 These analyses suggest that the effect of fenamates on Ca²⁺ signaling is not related to any of
245 their known (cyclooxygenase inhibition and anion channel inhibition) and observed (auxin
246 transport inhibition) biological activities but is rather a distinct feature associated with the
247 chemical structure. Therefore, we also tested the effect of a range of commercially available
248 structurally similar compounds to see whether the effect of the fenamates could be explained
249 by the presence of a specific substructure (Fig. 4A). However, every tested molecular variant
250 (diphenylamine **(14)**, diphenylmethane **(15)**, α -phenyl-o-toluic acid **(16)**, aniline **(17)**, and
251 anthranilic acid **(18)**) failed to inhibit/modify auxin-induced Ca²⁺ signals at 50 μ M (Fig. 4H-L),
252 suggesting that the fenamate-core structure represents the biological activity on Ca²⁺.
253 Moreover, when revisiting the primary screen data of all fenamates that were included in the
254 Spectrum library, we found that fenamic acid **(5)** and mefenamic acid **(6)** showed a tendency
255 to reduce the Ca²⁺ response changes, albeit with a lower potency than the validated
256 fenamates, while the effect of meclofenamate sodium **(7)** was negligible (Fig. 4A).

257 When we evaluated the 2,4-D-sensitive root growth, no obvious resistance or hypersensitivity
258 could be observed, suggesting that fenamates do not affect auxin perception or 2,4-D uptake
259 (Supplemental Fig. S4A). Similarly, no obvious resistance or hypersensitivity to the ethylene
260 precursor 1-aminocyclopropane-1-carboxylic acid (ACC) could be observed in seedlings
261 grown in the presence of these fenamates (Supplemental Fig. S4A). In addition, we also
262 evaluated the effects of NPPB, 9-ACA, BUM, NPA, and the fenamate analogues on root
263 growth and their sensitivity to 2,4-D and ACC (Supplemental Fig. S4B). Interestingly, unlike
264 FFA, NFA, and TFA, diphenylamine, aniline, and anthranilic acid caused a significant 2,4-D
265 resistance, which further illustrates a completely different mode of action between the
266 effective fenamates and diphenylamine, aniline, and anthranilic acid.

267

268 **Protonophores impair the Ca²⁺ response to distinct stimuli and render roots** 269 **insensitive to 2,4-D**

270 Inspection of the 67 confirmed hits revealed a total of at least 13 molecules with reported
271 protonophore activities in different organisms (Supplemental Table S1), suggesting an
272 important contribution of H⁺ gradients to auxin-induced Ca²⁺. We selected niclosamide (**19**),
273 (+)-usnic acid (**20**), and cloxyquin (**21**) as structurally diverse representatives of this group of
274 hits. Both niclosamide and (+)-usnic acid could be confirmed as potent inhibitors of auxin-
275 induced Ca²⁺ signals (Fig. 5A-B), while cloxyquin only caused a modest reduction in the peak
276 of the Ca²⁺ signal (Fig. 5C). To test whether the suspected protonophore activities of
277 niclosamide and (+)-usnic acid are the underlying cause of their observed inhibitory effect on
278 Ca²⁺ signals, we also analyzed three well-characterized, but structurally unrelated
279 protonophores: carbonyl cyanide m-chlorophenyl hydrazone (CCCP (**22**)), tyrphostin A23
280 (TyrA23 (**23**)) and endosidin9 (ES9 (**24**)) (Dejonghe et al., 2016). In addition, we included the
281 K⁺ selective ionophore valinomycin (**25**) to account for general disruption of gradients of
282 monovalent cations. Similar to niclosamide and (+)-usnic acid, the three tested

283 protonophores were potent inhibitors of 2,4-D-induced Ca^{2+} signaling (Fig. 5D). Importantly,
284 protonophore treatment did not have obvious defects in Ca^{2+} discharge profiles in the BY-2
285 cells, suggesting that an impaired Ca^{2+} compartmentalization cannot explain the inhibition of
286 2,4-D-induced Ca^{2+} . Unlike the protonophores, valinomycin did not reduce the maximal Ca^{2+}
287 responses (Fig. 5D), suggesting that 2,4-D-induced Ca^{2+} signaling specifically requires
288 proton-based gradients, rather than K^+ gradients. These findings are entirely consistent with
289 the recently reported very tight interdependence between H^+ and Ca^{2+} dynamics (Behera et
290 al., 2018).

291 Because protonophores dissipate H^+ -gradients across membranes, including the
292 mitochondrial membranes, they are expected to interfere with ATP production due to
293 mitochondrial uncoupling. Therefore, we evaluated the evolution of ATP content in BY-2 cells
294 during 60-minute treatments with 20 or 50 μM niclosamide, (+)-usnic acid, or cloxyquin (Fig.
295 5E). As a positive control we included the well-described protonophore CCCP (20 μM).
296 Within 2 minutes, niclosamide, (+)-usnic acid, and CCCP caused a significant reduction of
297 cellular ATP levels compared to DMSO-treated cells. Niclosamide had a milder effect on the
298 ATP levels than (+)-usnic acid and CCCP. In contrast, cloxyquin did not interfere with ATP
299 production, even at prolonged incubation times. This suggests that niclosamide and (+)-usnic
300 acid have protonophore activities in plants, which could explain how they inhibit 2,4-D-
301 induced Ca^{2+} signals.

302 Next, we evaluated the effects of these molecules on root growth (Fig. 5F). While cloxyquin
303 did not cause any noticeable defects, plants treated with niclosamide had on average slightly
304 longer primary roots, while (+)-usnic acid had a strong inhibitory effect on the primary root
305 length, suggesting that they have distinct cellular targets. Moreover, (+)-usnic acid induced
306 DR5rev::VENUS-N7 expression in the lateral root cap, which was not observed with
307 niclosamide and cloxyquin (Fig. 5G-J). When cotreated with 100 nM 2,4-D and niclosamide
308 or cloxyquin (but not (+)-usnic acid), the seedlings had significantly longer roots than the
309 controls (Fig. 5F). However, as cloxyquin only caused a slight inhibition of 2,4-D-induced

310 Ca^{2+} signaling and did not disturb ATP production in BY-2 cells, the observed resistance to
311 2,4-D is likely unrelated to impairment of Ca^{2+} signaling or protonophore activity. Importantly,
312 the protonophores did not render root growth resistant to ACC (Fig. 5F), as seen for 2,4-D
313 uptake-defective *aux1* mutants (Swarup et al., 2001), suggesting that the observed 2,4-D
314 resistance is not due to impaired AUX1-mediated auxin uptake.

315

316 **Bepridil is a potent inhibitor of auxin-induced Ca^{2+} signaling and modifies vacuolar**
317 **morphology**

318 Bepridil (**26**) is the only molecule inhibitor with reported Ca^{2+} channel inhibitory effects that
319 we identified in our chemical screen (Yatani et al., 1986; Sarajarvi et al., 2012; Lipsanen et
320 al., 2013). Also, in the validation, bepridil robustly interfered with the auxin-induced Ca^{2+}
321 response compared to mock-treated cells, while maintaining a strong response to the
322 discharge solution (Fig. 6A). This suggests that bepridil is a potent inhibitor of 2,4-D-induced
323 Ca^{2+} signaling in BY-2 cells. Moreover, bepridil potently inhibited the rapid IAA-induced Ca^{2+}
324 response in roots of Arabidopsis seedlings expressing the intensiometric Ca^{2+} sensor R-
325 GECO1 (Fig. 6B-C; Supplemental Video S1).

326 Bepridil treatment on Arabidopsis seedlings caused a dose-dependent reduction in primary
327 root length (Fig. 6D), which was associated with altered DR5rev::VENUS-N7 expression in
328 the lateral root cap (Fig. 6E-F). Cotreatment of seedlings with 20 μM bepridil and either 100
329 nM 2,4-D, 20 μM ACC, 150 nM NAA, or 250 nM NAA did not result in noticeable growth
330 resistance to any of the hormone treatments (Fig. 6G). Because vacuolar remodeling was
331 proposed to be part of the mechanism by which auxin inhibits root growth (Lölfke et al., 2015)
332 and because bepridil interferes with auxin-induced Ca^{2+} signaling, we next investigated the
333 effect of bepridil on vacuoles by analyzing the localization of the tonoplast marker VAMP711-
334 YFP (Fig. 6H-I). Bepridil dramatically altered vacuolar morphology, inducing swollen and
335 roundish central vacuoles in Arabidopsis roots (Fig. 6H). Notably, auxin treatments induce

336 smaller luminal vacuoles, which consequently impact on cellular elongation rates (Löpfke et
337 al., 2015; Scheuring et al., 2016). However, in the presence of bepridil, the auxin effect on
338 vacuolar morphology was abolished (Fig. 6H-I), tentatively suggesting that auxin-regulated
339 vacuolar remodeling could depend on a bepridil-sensitive step.

340 During the vacuolar morphology experiments, the tonoplast marker VAMP711-YFP seemed
341 to display some ectopic subcellular pattern. Therefore, we also analyzed several fluorescent,
342 late endosomal markers. Each of these markers had an aberrant localization pattern after
343 bepridil treatment (Supplemental Fig. S5). These observations suggest that bepridil has
344 pleiotropic effects on late endosomal trafficking, which could complicate the interpretation of
345 the auxin resistance of vacuolar remodeling after bepridil treatment. When lowering the
346 bepridil concentration, we found that vacuolar morphology was still aberrant at 10 μM , but
347 was no longer obviously impaired at 5 μM (Supplemental Fig. S6).

348

349 **Sucrose-induced Ca^{2+} signals are highly sensitive to fenamates and sterol** 350 **biosynthesis inhibitors, but not to bepridil**

351 Next, we sought to evaluate the specificities of the identified inhibitors. The auxin response
352 requires CNGC14 for eliciting Ca^{2+} (Shih et al., 2015; Dindas et al., 2018), while
353 hyperosmotic stress is predicted to activate OSCA/TMEM63-type mechanosensitive Ca^{2+} -
354 permeable channels (Yuan et al., 2014; Murthy et al., 2018; Zhang et al., 2018). Thus, auxin
355 and hyperosmotic stress activate two distinct Ca^{2+} entry mechanisms.

356 We used 0.5 M sucrose as a hyperosmotic stimulus. When eliciting YFP-apoaequorin-
357 expressing Arabidopsis seedlings, we observed a very fast and transient rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig.
358 7; Supplemental Fig. S7), as was previously described for such hyperosmotic treatments
359 (Furuichi et al., 2001; Stephan et al., 2016). The sucrose-induced Ca^{2+} signal was
360 characterized by an initial large peak in free $[\text{Ca}^{2+}]_{\text{cyt}}$ that reached a maximum value (1.5-2
361 μM range) within seconds. After reaching the maximum peak value, the Ca^{2+} signal quickly

362 decreased until it reached an elevated steady-state concentration 30-40 seconds after elicitor
363 addition (Fig. 7; Supplemental Fig. S7).

364 We evaluated the fenamates (FFA, NFA, and TFA), the protonophores (niclosamide, (+)-
365 usnic acid, ES9, and CCCP), and bepridil for their ability to inhibit hyperosmotic-stress-
366 induced Ca^{2+} . Additionally, we also tested two imidazole-type fungicides (clotrimazole (**27**)
367 and oxiconazole nitrate (**28**)), which were initially identified as inhibitors of 2,4-D-induced
368 Ca^{2+} signals but were not further pursued in the context of auxin responses due to a poor
369 reproducibility in follow-up experiments (Supplemental Fig. S8). All tested fenamates, three
370 protonophores ((+)-usnic acid, ES9, and CCCP) and, surprisingly, both imidazoles potently
371 interfered with sucrose-induced Ca^{2+} (Fig. 7; Supplemental Fig. S7). This suggests that
372 hyperosmotic stress Ca^{2+} entry is much more sensitive to inhibition of sterol biosynthesis
373 than 2,4-D-induced Ca^{2+} responses. On the other hand, while bepridil and niclosamide were
374 potent inhibitors of auxin-induced Ca^{2+} , they did not inhibit the sucrose-induced Ca^{2+}
375 response (Fig. 7D, H). Niclosamide was the only one of the tested protonophores that could
376 not inhibit sucrose-induced Ca^{2+} , suggesting that its inhibitory effect on auxin-induced Ca^{2+}
377 may not be related solely to its protonophore activity. Together, these data illustrate that
378 auxin and hyperosmotic stress Ca^{2+} responses show different pharmacological sensitivities.

379

380 Discussion

381 The molecular mechanism by which auxin regulates transcriptional changes is largely
382 captured by the very well characterized TIR1/AFB-based degradation of Aux/IAA
383 transcriptional corepressors. This pathway accounts for much of the auxin-regulated
384 transcriptional changes and, thus, the cellular response. Recently, TIR1/AFB-based auxin
385 perception was also found to be required for nontranscriptional cellular responses, such as
386 rapid and reversible inhibition of root growth (Fendrych et al., 2018). The inhibitory effect of
387 auxin root elongation is correlated with alkalinization of the apoplast (Barbez et al., 2017),

388 activation of Ca^{2+} signals through CNGC14 (Shih et al., 2015), and remodeling of the vacuole
389 (Löfke et al., 2015). Alkalinization of the apoplast is possibly the result of the coordinated
390 inhibition of plasma membrane H^+ -ATPase activity and AUX1-mediated H^+ /IAA⁻ uptake from
391 the apoplast (Dindas et al., 2018). Moreover, auxin-induced alkalinization of the apoplast is
392 coupled to CNGC14-dependent auxin-induced Ca^{2+} signaling in the epidermis (Shih et al.,
393 2015). Importantly, root elongation of *cngc14* mutants is mildly insensitive to inhibitory auxin
394 levels (Shih et al., 2015), suggesting that auxin-induced cytosolic Ca^{2+} increase is part of the
395 root growth inhibitory auxin signaling pathway. We identified bepridil as a potent inhibitor of
396 auxin-induced cytosolic Ca^{2+} increase and found that bepridil interferes strongly with auxin-
397 induced vacuolar remodeling. This makes it tempting to speculate that auxin-induced
398 cytosolic Ca^{2+} increase controls vacuolar morphology. However, the strong pleiotropic effects
399 on late endosomal compartments preclude drawing such strong conclusions. Therefore, it
400 will be interesting to identify the molecular target(s) of bepridil and characterize its function in
401 auxin-regulated vacuolar remodeling.

402 The identification of bepridil as a potent inhibitor of auxin-induced Ca^{2+} is consistent with its
403 reported Ca^{2+} channel blocker function in animals (Yatani et al., 1986; Sarajarvi et al., 2012;
404 Lipsanen et al., 2013) and suggests, in line with *cngc14*'s defects in auxin-induced cytosolic
405 Ca^{2+} increase (Shih et al., 2015), that CNGC14 might be a bepridil target. However,
406 electrophysiological experiments demonstrated that bepridil inhibits outward rectified K^+
407 currents in plant protoplasts (Thomine et al., 1994). This hints at a functional coupling of
408 auxin-induced Ca^{2+} entry with outward rectified K^+ currents as described for Ca^{2+} spiking
409 during nodulation (Ane et al., 2004; Charpentier et al., 2008; Charpentier et al., 2016).
410 However, auxin activates inward rectified K^+ currents in Arabidopsis and maize (Thiel and
411 Weise, 1999; Philippar et al., 2004), and we found that the K^+ selective ionophore
412 valinomycin had no effect on auxin-induced Ca^{2+} signals. Instead, it seems more likely that
413 bepridil targets CNGC14 and/or other CNGCs via structural features that they share with
414 outward rectifying K^+ channels.

415 Recently, the protonophore carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP)
416 was shown to completely inhibit the ATP-induced Ca^{2+} response, while pretreatment with the
417 ionophore nigericin only had marginal effects on the Ca^{2+} and pH transients (Behera et al.,
418 2018). These observations are perfectly in line with our findings showing that protonophores
419 are potent inhibitors of auxin- and osmotic-stress-induced Ca^{2+} signaling and strengthen
420 previous notions that Ca^{2+} and pH are functionally coupled in several cellular processes in
421 plants, including root hair and pollen tube growth (Herrmann and Felle, 1995; Monshausen et
422 al., 2008; Michard et al., 2017), cold stress response (Gao et al., 2004), and touch response
423 (Monshausen et al., 2009). Also, disruption of the transmembrane pH gradient via
424 alkalinization of the apoplast interfered with auxin-induced Ca^{2+} entry in root hairs (Dindas et
425 al., 2018). This was explained by a need for a proton gradient to drive AUX1-mediated
426 H^+/IAA^- symport into the cell. In this model, the inability of IAA to enter the cell in *aux1*
427 prevents TIR1-mediated CNGC14 activation (Dindas et al., 2018). In turn, *cngc14* has a
428 defect in AUX1 activity, providing a mechanistic model for the coupling of Ca^{2+} and H^+
429 dynamics during auxin response. However, NAA-induced cytosolic Ca^{2+} increase was
430 recently also coupled to a cytoplasmic acidification (Behera et al., 2018), which cannot be
431 explained by AUX1-mediated H^+ uptake, as NAA is not a good substrate for AUX1 (Yang et
432 al., 2006).

433 In summary, by exploring a small subset of hits, we readily identified several new inhibitors of
434 auxin-induced Ca^{2+} . Further exploration of these inhibitors will thus lead to novel insights in
435 the mechanism (e.g. protonophores) and the physiological relevance of auxin-induced Ca^{2+}
436 (e.g. vacuolar remodeling). Importantly, the use of a library of annotated molecules has the
437 added advantage that many of the compounds are commercially available and thus are
438 easily accessible to researchers for further characterization and analysis of structural
439 derivatives with a higher inhibitory potency and specificity. Identification of the molecular
440 targets will be key for further refining the inhibitors in terms of specificity and affinity.

441

442 **Materials and methods**

443 **BY-2 cell lines and Arabidopsis plant lines**

444 We stably transformed wild-type tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cell
445 suspensions with a kanamycin-resistant construct for constitutive expression of YFP-fused
446 apoaequorin (AEQ) driven by an ubiquitin (UBQ10) promoter as previously described
447 (Mehlmer et al., 2012). The transgenic BY-2 cell lines were maintained by weekly dilution
448 (1:40) in modified Linsmaier and Skoog (LS) medium. The cell cultures were agitated on a
449 rotary shaker at 130 rpm at 25°C in the dark and used in experiments 5 days after
450 subculture.

451 Transgenic Col-0 Arabidopsis seedlings that carry a proUBQ10::YFP-apoAEQ cassette were
452 used in our experiments (Mehlmer et al., 2012). These lines were generated using
453 *Agrobacterium*-mediated transformation via the floral-dip method (Clough and Bent, 1998).
454 Transformants were selected based on BASTA resistance and YFP expression levels and
455 were made homozygous in subsequent generations.

456 The other used plant lines expressing R-GECO1 (Keinath et al., 2015), DR5rev::VENUS-N7
457 (Heisler et al., 2005), Ara7-mRFP (Jia et al., 2013), VAMP727-YFP (Ebine et al., 2008),
458 2xFYVE-YFP (Vermeer et al., 2006), VAMP711-YFP (Geldner et al., 2009), VAMP711-
459 mCherry (Geldner et al., 2009) have been described previously.

460

461 **Compounds**

462 The compounds used for the primary screen and confirmation screen belong to the Spectrum
463 compound library (MicroSource Discovery Systems) and were dissolved in DMSO. The
464 compounds used in follow-up experiments (FFA, NFA, TFA, diphenylamine,
465 diphenylmethane, α -phenyl-o-toluic acid, aniline, anthranilic acid, NPPB, 9-ACA, NPA, BUM,
466 clotrimazole, oxiconazole nitrate, artemether, niclosamide, (+)-usnic acid, cloxyquin, bepridil,

467 TyrA23, CCCP, and valinomycin) were obtained from Sigma-Aldrich (Overijse, Belgium) and
468 dissolved in DMSO. Coelenterazine-h was obtained from Promega (Leiden, The
469 Netherlands) and dissolved in methanol.

470

471

472 **Primary screen setup**

473 The Spectrum library of 2320 compounds with a wide range of reported biological activities
474 and structural diversity (MicroSource Discovery Systems) was screened for inhibitors of 2,4-
475 D-induced Ca^{2+} signaling. The individual compounds and controls were added to 100 μL
476 YFP-apoaequorin-expressing BY-2 cells in white 96-well plates, with a final concentration of
477 50 μM per well. Negative (0.5% DMSO) and positive (10 mM GdCl_3) controls were added to
478 both outer columns of each multiwell plate. After a 30-minute incubation period in the dark to
479 reduce background signals, 100 μL 2,4-D (final concentration 500 μM) was added to each
480 well with a liquid handling robot (Tecan Freedom EVO200 with 96-channel head) and
481 immediately transferred to the luminescence imaging system (NightSHADE LB 985 *in vivo*
482 Plant Imaging System, Berthold Technologies) to ensure capturing the peak signal in each
483 well. The induced luminescent signal was measured for 30 cycles with a 10-second exposure
484 time per cycle. The maximum signal in each well was calculated and normalized to the
485 average of the negative control of the corresponding multiwell plate.

486

487 **Luminescence measurements of Ca^{2+} responses in BY-2 cells during confirmation** 488 **screen and follow-up experiments**

489 Five days after subculturing, YFP-apoAEQ-expressing BY-2 cells were collected by
490 centrifugation, washed, and resuspended in fresh BY-2 medium. Aequorin was reconstituted
491 in the BY-2 cells by adding 2.5 μM of coelenterazine-h (Promega, Leiden, The Netherlands)

492 for 3 hours under agitation in the dark. Afterwards, 100 μL of reconstituted BY-2 cells was
493 added to each well of a white 96-well plate (PerkinElmer), and the cells were preincubated
494 with the hits identified in the primary screen for 30 minutes to 1 hour in the dark. For
495 measuring elicitor-induced Ca^{2+} responses per well, 100 μL elicitor solution was added to the
496 cells, after which the aequorin-induced light emission was measured every 1.5 seconds for
497 200 seconds (every 2 seconds for 240 seconds during the confirmation screen) with a
498 luminescence plate reader (all measurements were done in a LUMIstar Galaxy, BMG
499 LABTECH, unless mentioned otherwise). Immediately after this measurement, the remaining
500 reconstituted aequorin was discharged by the addition of 50 μL of discharge solution (0.1 M
501 CaCl_2 and 20% ethanol (v/v)), and luminescence was measured every 1.5 seconds for an
502 additional 100 seconds (every 2 seconds for 160 seconds during the confirmation screen).
503 The bioluminescence signal of our transgenic BY-2 cell lines could not be converted to
504 absolute $[\text{Ca}^{2+}]_{\text{cyt}}$ values because the total luminescent signal after aequorin discharge could
505 not be completely detected *in situ* due to saturation of the plate reader detector. Therefore,
506 all treatments were always evaluated relative to controls within each corresponding multiwell
507 plate. This normalization also accounted for day-to-day variation in amplitude and shape of
508 the Ca^{2+} signals.

509

510 **Luminescence measurements of Ca^{2+} responses in Arabidopsis seedlings**

511 Gas-sterilized seeds were grown on plates containing half-strength Murashige and Skoog
512 (MS) medium for 3 days until germination. Freshly germinated seedlings were transferred
513 individually to wells of sterile, white, 96-well microplates (PerkinElmer) containing 130 μL of
514 medium composed of $\frac{1}{2}$ MS salts (2.2 g/l), 0.5 g/l MES, 1% sucrose, and 0.08% phyto agar.
515 This low phyto agar concentration provided modest support for the growing seedlings while
516 still allowing rapid mixing of injected pretreatment compounds and elicitor solutions. The
517 transparent lids of the 96-well plates were sealed with Parafilm to prevent medium

518 evaporation. The seedlings were grown in the plates for 2 to 3 days in a growth chamber
519 under continuous light conditions at 21°C. The evening before measurement, 130 µL of water
520 containing 2.5 µM coelenterazine-h (Ctz-h; Promega, Leiden, The Netherlands) was added
521 to each well to allow overnight reconstitution of apoaequorin into functional aequorin. During
522 this incubation, the plate was covered in aluminum foil to prevent light-induced degradation
523 of Ctz-h. The following day, 130 µL of the medium was removed and replaced with 130 µL of
524 a solution containing 2x the final concentrations of the compounds in water. After a 1-hour
525 preincubation period, 130 µL medium was removed just before the luminescence
526 measurements, to allow the addition of elicitor solutions.

527 The seedling-containing 96-well plates were analyzed in a luminescence plate reader
528 (LUMIstar Galaxy, BMG LABTECH). Luminescence was first measured every 0.3 seconds
529 for 6 seconds to establish a baseline reading, after which 100 µL of a 2x elicitor solution was
530 automatically added and luminescence was further measured every 0.3 seconds for 54
531 seconds. Subsequently, the plate was removed from the plate reader and 100 µL of solution
532 was removed from each well. The plate was then placed into the plate reader again and
533 luminescence was measured every 0.3 seconds for 6 seconds to establish a baseline signal.
534 Subsequently, 100 µL of discharge solution (2 M CaCl₂ and 50% ethanol) was automatically
535 added and luminescence was further measured every 0.3 seconds for 54 seconds in order to
536 determine the remaining aequorin in the seedlings.

537

538 **Quantification of Ca²⁺ response data in transgenic YFP-apoaequorin-expressing**
539 ***Arabidopsis thaliana* seedlings**

540 Raw light data measured by the LUMIstar Galaxy plate reader were converted to calcium
541 concentration by applying the empirically determined formula $pCa = 0.332588(-\log k) +$
542 5.5593 (Knight et al., 1996). The rate constant k in this formula equals the elicitor-induced

543 luminescence counts per second divided by the total remaining counts. The total remaining
544 counts were determined after adding discharge solution (2 M CaCl₂ + 50% ethanol).

545

546 **Phenotyping**

547 Wild-type gas-sterilized *Arabidopsis thaliana* seeds were plated on ½ MS medium
548 supplemented with the appropriate compounds and/or hormones (3 rows/plate, 0.5 cm
549 between seeds). For the primary root length experiments, the plated seeds were first
550 stratified for 3 days in the dark at 4°C and subsequently transferred to a growth chamber
551 under continuous light conditions at 21°C. After 7 days of growth, the plates were scanned
552 and the primary root lengths of the seedlings were measured with ImageJ. For each
553 treatment 10-61 individual roots were counted.

554

555 **Late endosomal marker localization**

556 Three- to four-day-old seedlings from the endomembrane marker lines (2xFYVE-YFP, Ara7-
557 mRFP, and VAMP727-YFP) were pretreated with control medium or 50 µM bepridil for 5
558 hours. The control medium (CaPLUS) consists of the following components dissolved in
559 MilliQ (for 0.5 liters): 25 mL MS basal salt micronutrient solution, 5 g sucrose, 0.05 g
560 myoinositol, 0.25 g MES, 0.413 g NH₄NO₃, 0.045 g MgSO₄, 0.475 g KNO₃, 0.043 g H₂KO₄P⁻,
561 and 0.083 g CaCl₂, with pH set to 5.7.

562 For imaging of the endomembrane markers, the confocal laser scanning microscopes Leica
563 SP2 (Leica Microsystems) and Zeiss 710 (Zeiss) were used. Fluorescence emission of
564 mRFP (ex 561 nm/em 570-630 nm) and YFP (ex 514 nm/em 520-565 nm) was detected
565 using a 63x water objective (NA 1.2, digital zoom 1,2x). Images were analyzed using Fiji
566 (Schindelin et al., 2012).

567

568 **R-GECO1 visualization**

569 Seedlings from the R-GECO1 line were pretreated for 30 minutes with 50 μ M bepridil or
570 0.1% DMSO and mounted in a specialized imaging chamber as previously described
571 (Himschoot et al., 2018). The imaging chamber was mounted on the stage of an Ultra View
572 Vox spinning disc microscope (PerkinElmer) and 250 nM IAA was added to elicit a Ca^{2+}
573 response in the samples. R-GECO1 fluorescence intensity was monitored for 8 minutes after
574 elicitor addition and processed as previously described (Himschoot et al., 2018). Per
575 treatment, 3-4 individual measurements were performed.

576

577 **DR5rev::VENUS-N7 visualization**

578 DR5rev::VENUS-N7 seedlings were grown for 5 days on $\frac{1}{2}$ MS plates containing the
579 appropriate compounds or 0.1% DMSO. For each treatment, 5 seedlings were stained with
580 freshly prepared PI solution (15 μ M in distilled water) for 2 minutes, rinsed twice in water, and
581 subsequently spread on a glass microscope slide. Fluorescence emission of
582 DR5rev::VENUS-N7 (ex 514 nm/em 535-590 nm) and PI (ex 514 nm/em 570-630 nm) was
583 visualized and imaged using a Leica SP2 confocal microscope (Leica Microsystems).

584

585 **Analysis of vacuolar morphology**

586 Analysis of vacuolar morphology was carried out on 6-day-old seedlings of a tonoplast
587 marker line (pUBQ10::VAMP711-YFP) that were grown on solid $\frac{1}{2}$ MS medium. The samples
588 were pretreated for 5 hours with 50 μ M bepridil or solvent control (DMSO). Subsequently, the
589 seedlings were transferred to plates containing either DMSO, 50 μ M bepridil, 250 nM IAA, or
590 bepridil and IAA (50 μ M and 250 nM, respectively). Afterwards, seedlings were grown for
591 another 3 hours prior to image acquisition. Roots were mounted in PI solution (0.02 mg/mL)
592 to counterstain cell walls and report viability. YFP was excited at 514 nm (fluorescence

593 emission: 525 nm - 555 nm) and PI at 561 nm (fluorescence emission: 644 - 753 nm) using a
594 Leica TCS SP5 confocal laser scanning microscope equipped with a Leica HCX PL APO CS
595 63 × 1.20 water-immersion objective. Confocal images were analyzed using ImageJ. To
596 calculate the vacuolar morphology index, the longest and widest distance of the biggest
597 luminal structure was measured and multiplied (Lofke et al., 2015). The atrichoblast cells
598 were quantified before the onset of elongation (late meristematic). To depict this region, the
599 first cell being twice as long as wide was considered as the onset of elongation. Starting from
600 this cell, the next cell towards the meristem was excluded (as it usually shows either partial
601 elongation and/or already substantial vacuolar expansion), and vacuoles of the subsequent 4
602 cells were quantified as described previously (Dünser et al., 2017).

603

604 **BY-2 viability assay**

605 Five-day-old BY-2 cells were treated with H₂O, 1% DMSO, 500 μM 2,4-D, or a combination
606 of 1% DMSO and 500 μM 2,4-D. After 1 hour of treatment, the cells were stained with freshly
607 prepared FDA-PI staining solution (15 μM FDA and 15 μM PI in BY-2 medium) for 5 minutes
608 in the dark. Afterwards, the cells were washed with fresh medium and 100 μL of cells was
609 spread on a glass microscope slide per treatment. For each treatment, approximately 100
610 cells were counted using an Ultra View Vox spinning disc microscope (PerkinElmer) and
611 classified as alive or dead based on their individual uptake of FDA and PI. For each
612 treatment, 2-4 such individual measurements were performed.

613

614 **ATP content determination**

615 Wild-type tobacco BY-2 cell cultures were used and maintained as described earlier. Five
616 days after subculturing, the BY-2 cell suspension was diluted 5 times in modified LS medium
617 and preconditioned in the dark for 1 hour on a shaker before use. The cells were

618 subsequently distributed in white 96-well plates (95 μL /well) and 5 μL of the appropriate
619 compounds (DMSO, cloxyquin (50 and 20 μM final concentration), niclosamide (50 and 20
620 μM final concentration), (+)-usnic acid (50 and 20 μM final concentration), and CCCP (20 μM
621 final concentration)) was added with a Freedom EVO robot (Tecan). All compounds were
622 dissolved in DMSO with the final DMSO concentration for each treatment being 0.66%.
623 There were 8 repeats per treatment. The ATP levels were detected by adding 80 μL of the
624 ATPlite 1step Luminescence Assay System (PerkinElmer) after incubation of the cells in the
625 presence of the compounds for the indicated time. Luminescence was measured with an
626 EnVision 2104 Multilabel Reader (PerkinElmer).

627

628 **Accession Numbers**

629 Sequence data from this article can be found in the GenBank/EMBL data libraries under
630 accession numbers E14214.1 (apoaquorin), JN258411.1 (R-GECO1), NM_115290.5
631 (VAMP727-At3g54300) and NM_119367.3 (VAMP711- AT4G32150).
632

633 **Supplemental Material**

634 **Supplemental Fig. S1.** DMSO and 2,4-D have no significant impact on BY-2 cell viability.

635 **Supplemental Fig. S2.** Overview of chemical structures.

636 **Supplemental Fig. S3.** Higher magnification of root phenotypes of fenamate-treated plants.

637 **Supplemental Fig. S4.** Diphenylamine, aniline, and anthranilic acid make roots resistant to
638 2,4-D.

639 **Supplemental Fig. S5.** Bepridil has a profound impact on endomembrane trafficking.

640 **Supplemental Fig. S6.** Bepridil has a profound impact on vacuolar morphology.

641 **Supplemental Fig. S7.** The protonophores ES9 and CCCP alter sucrose-induced Ca^{2+}
642 signals.

643 **Supplemental Fig. S8.** Imidazoles are not robust inhibitors of 2,4-D induced Ca^{2+} .

644 **Supplemental References. References for Supplemental Table S1.**

645 **Supplemental Table S1.** List of confirmed hit compounds (excel file).

646 **Supplemental Video S1. Bepridil inhibits the IAA-induced Ca²⁺ response in Arabidopsis**
647 **roots expressing R-GECO1 (.avi file)**

648

649

650 **Tables**

651 **Table 1. Thirteen compounds selected for further validation experiments**

Name	Reported Bioactivities	Reference
Artemether	antimalarial agent, HMGCoA inhibitor	Korade et al., 2016
Bepridil hydrochloride	calcium-blocking agent, antiarrhythmic, antihypertensive, calmodulin antagonist	Narahara et al., 1992
Clotrimazole	antifungal, antibacterial, sterol biosynthesis inhibitor	Qiu et al., 2017
Cloxyquin	antibacterial, antifungal	Hongmanee et al., 2007
Dicyclomine hydrochloride	anticholinergic	Ali et al., 2018
Flufenamic acid	anti-inflammatory, analgesic, antipyretic	Habjan and Vandenberg, 2009
Nicosamide	anthelmintic, teniacide	Monin et al., 2016
Niflumic acid	analgesic, anti-inflammatory	Hogg et al., 1994
Oxiconazole nitrate	antifungal, sterol biosynthesis inhibitor	Jegasothy and Pakes, 1991
Tannic acid	nonspecific enzyme/receptor blocker	Isenburg et al., 2005
Tolfenamic acid	anti-inflammatory, analgesia	Pentikainen et al., 1981
Triclosan	anti-infective, antibacterial, antifungal	Heath et al., 1999
(+)-Usnic acid	antibacterial	Latkowska et al., 2006

652

653

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656

657 **Figure Legends**

658 **Fig. 1. Fourteen elicitors induce a distinct Ca^{2+} signal in aequorin-expressing BY-2**
659 **cells. (A)** Schematic representation of aequorin complex formation and bioluminescent
660 reaction. A functional aequorin complex is formed upon binding of apoaequorin with its
661 substrate coelenterazine (CTZ) in the presence of O_2 . The binding of three Ca^{2+} ions leads to
662 the conversion of CTZ into coelenteramide (CTA) and CO_2 , upon which blue light ($\lambda = 469$
663 nm) is emitted. **(B-O)** Ca^{2+} response of YFP-apoaequorin-expressing BY-2 cells treated with
664 various potential elicitors: **(B)** 0.5 M D-glucose, **(C)** 0.5 M sucrose, **(D)** 0.5 M D-mannitol, **(E)**
665 0.5 M D-sorbitol, **(F)** 0.3 M NaCl, **(G)** 167 μM and 500 μM ATP, **(H)** 167 μM and 500 μM IAA,
666 **(I)** 20 μM and 500 μM NAA, **(J)** 167 μM and 500 μM 2,4-D, **(K)** 5 mM salicylic acid (SA), **(L)**
667 167 μM and 500 μM gibberellin (GA_3), **(M)** 167 μM and 500 μM 6-benzylaminopurine (6-
668 BAP), **(N)** 125 nM flg22, and **(O)** 5 mM H_2O_2 . The data represent average luminescence
669 values of 3 individual measurements in the same multiwell plate. Error bars represent \pm SEM.

670

671 **Fig. 2. Schematic representation of primary screen for Ca^{2+} signaling inhibitors. (A-B)**
672 Multiwell setup for screening inhibitors of auxin-induced Ca^{2+} responses via YFP-
673 apoaequorin-expressing BY-2 cells. Based on the means and standard deviations of both the
674 positive (10 mM GdCl_3 , purple) and negative (0.5% DMSO, cyan) controls of a test run, a Z'
675 score of 0.54 could be calculated **(A)**, supporting the robustness of the assay. Using this
676 setup, the Spectrum library of 2320 compounds was screened for inhibitors of 2,4-D-induced
677 Ca^{2+} signaling **(B)**. The outer columns contained positive (10 mM GdCl_3 , purple) and
678 negative (0.5% DMSO, cyan) controls, with the assay compounds in the 10 inner columns
679 (50 μM ; white). Addition of auxin induced a rapid luminescence-based signal that was
680 detected with the NightSHADE luminescence imaging system. From this library, 80 hit
681 compounds were retained that caused a maximum signal less than 55% of that of the
682 DMSO-treated control cells. **(C)** Confirmation screen of the 80 hit compounds in a multiwell

683 plate reader. After 240 seconds, 50 μ L discharge solution (0.1 M CaCl_2 and 20% ethanol)
684 was added and luminescence was measured for the remaining 160 seconds. Based on their
685 Ca^{2+} response and burst patterns, the tested compounds could be further categorized into 4
686 groups: confirmed (green), semiconfirmed (yellow), cytotoxic (blue), and false-positive (red)
687 compounds. **(D)** Maximum 2,4-D-induced Ca^{2+} response signal of YFP-apoaequorin-
688 expressing BY-2 cells pretreated with 50 μ M of 13 selected hit compounds. The data
689 represent average maximum luminescence values of 4-8 individual measurements in
690 comparison to the average of 4-8 DMSO controls in the same multiwell plate. Error bars
691 represent \pm SEM. Bars are color-coded based on the underlying Ca^{2+} response and burst
692 patterns. Student's t-test p-values: *p < 0.05, **p < 0.01, and ***p < 0.001. Bepr., bepridil;
693 FFA, flufenamic acid; NFA, niflumic acid; TFA, tolfenamic acid; Clot., clotrimazole; Ox. Nit.,
694 oxiconazole nitrate; Art., artemether; Nicl., niclosamide; U.A., (+)-usnic acid; Clox., cloxyquin;
695 Dic. Hyd., dicyclomine hydrochloride; T.A., tannic acid; Tricl., triclosan.

696

697 **Fig. 3. Fenamates alter the shape of auxin-induced Ca^{2+} .** **(A-C)** 2,4-D-induced Ca^{2+}
698 response of YFP-apoaequorin-expressing BY-2 cells treated with 50 μ M FFA **(A)**, NFA **(B)**,
699 or TFA **(C)**. Discharge solution was added after 200 seconds. The data represent average
700 luminescence values of 4-8 individual measurements (solid lines) in comparison to the
701 average of 4-8 DMSO controls (dotted lines) in the same multiwell plate. Error bars represent
702 \pm SEM. **(D)** Phenotype of WT Col-0 seedlings grown for 7 days in presence of 0.1% DMSO
703 or 20 μ M FFA, NFA, or TFA. **(E)** Vertical Growth Index (VGI) values for the roots from (D).
704 For each treatment, 42 to 47 roots were measured. Student's t-test p-values: ***p < 0.001.
705 **(F-H)** Confocal microscopy images of 5-day-old DR5rev::VENUS-N7 seedlings grown on
706 0.1% DMSO **(F)**, 20 μ M FFA **(G)**, and 20 μ M TFA **(H)**. Green: DR5rev::VENUS-N7 signal;
707 red: propidium iodide staining.

708

709 **Fig. 4. Functional and structural fenamate analogs do not alter the shape of auxin-**
710 **induced Ca²⁺.** (A) Overview of a small-scale SAR analysis of the fenamates. Based on
711 known and observed functions and structures of the hit fenamates, a set of functional and
712 structural analogs was investigated. (B-C) 2,4-D-induced Ca²⁺ response of YFP-
713 apoaequorin-expressing BY-2 cells treated with 50 μM of the auxin transport inhibitors BUM
714 (B) and NPA (C). Discharge solution was added after 200 seconds. The data represent
715 average luminescence values of 4 individual measurements (solid lines) in comparison to the
716 average of 4 DMSO controls (dotted lines) in the same multiwell plate. Error bars represent ±
717 SEM. (D-E) 2,4-D-induced Ca²⁺ response of YFP-apoaequorin-expressing BY-2 cells treated
718 with 50 μM of the NSAIDs ibuprofen (D) and oxaprozin (E) (note: measurements were done
719 with a new plate reader (GloMax Navigator - Promega) because the old one was defective).
720 The data represent average luminescence values of 8 individual measurements (solid lines)
721 in comparison to the average of 8 DMSO controls (dotted lines) in the same multiwell plate.
722 Error bars represent ± SEM. (F-L) 2,4-D-induced Ca²⁺ response of YFP-apoaequorin-
723 expressing BY-2 cells treated with 50 μM of 2 anion channel inhibitors (9-ACA and NPPB; F-
724 G) and various compounds structurally similar to fenamates (H-L). Discharge solution was
725 added after 200 seconds. The data represent average luminescence values of 4 individual
726 measurements (solid lines) in comparison to the average of 4 DMSO controls (dotted lines)
727 in the same multiwell plate. Error bars represent ± SEM. BUM, 2-[4-(diethylamino)-2-
728 hydroxybenzoyl]benzoic acid; NPA, 1-N-naphtylphtalamic acid; 9-ACA, 9-
729 anthracenecarboxylic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; α-ph.-o-tol.
730 acid, α-phenyl-o-toluic acid; A.U., arbitrary units.

731

732 **Fig. 5. Protonophores impair the 2,4-D-induced Ca²⁺ response and render roots**
733 **insensitive to 2,4-D.** (A-C) 2,4-D-induced Ca²⁺ response of YFP-apoaequorin-expressing
734 BY-2 cells treated with 50 μM niclosamide (A), (+)-usnic acid (B), or cloxyquin (C). Discharge
735 solution was added after 200 seconds. The data represent average luminescence values of 4

736 individual measurements (solid lines) in comparison to the average of 4 DMSO controls
737 (dotted lines) in the same multiwell plate. Error bars represent \pm SEM. **(D)** Maximum 2,4-D-
738 induced luminescence in YFP-apoaequorin-expressing BY-2 cells treated with 50 μ M of the
739 hit protonophores niclosamide and (+)-usnic acid, the ionophore valinomycin, or the non-hit
740 protonophores tyrphostin A23, CCCP, and ES9. Four individual measurements were
741 performed for each treatment and compared to the average of 8 DMSO controls in the same
742 multiwell plate. Error bars represent \pm SEM. Student's t-test p-values: **p < 0.01 and ***p <
743 0.001. **(E)** ATP measurements of BY-2 cells pretreated with 0.5% DMSO (white), 20 μ M
744 CCCP (black), or 20 μ M or 50 μ M cloxyquin (dots), niclosamide (diagonal stripes), or (+)-
745 usnic acid (horizontal stripes). ATP was measured 2, 10, 30, and 60 minutes after compound
746 treatment. Error bars represent \pm SEM. Student's t-test p-values: *p < 0.05, **p < 0.01, and
747 ***p < 0.001. **(F)** Average primary root length of WT Col-0 seedlings grown for 7 days on $\frac{1}{2}$
748 MS medium in presence of 0.1% DMSO or 20 μ M niclosamide, cloxyquin, or (+)-usnic acid
749 (grey bars) and supplemented with 100 nM 2,4-D (red bars) or 20 μ M ACC (blue bars). Root
750 lengths represent average of 12-21 roots. Error bars represent \pm SEM. Student's t-test p-
751 values: *p < 0.05, **p < 0.01, and ***p < 0.001. **(G-J)** Confocal microscopy images of 5-day-
752 old DR5rev::VENUS-N7 seedlings grown on 0.1% DMSO **(G)**, 20 μ M niclosamide **(H)**, 20 μ M
753 cloxyquin **(I)**, or 20 μ M (+)-usnic acid **(J)**. Green: DR5rev::VENUS-N7 signal; red: propidium
754 iodide staining. The image in **(G)** is identical to Fig. 3F.

755

756 **Fig. 6. Bepridil is a potent inhibitor of auxin-induced Ca²⁺ signaling. (A)** 2,4-D-induced
757 Ca²⁺ response of YFP-apoaequorin-expressing BY-2 cells treated with 50 μ M bepridil.
758 Discharge solution was added after 200 seconds. The data represent average luminescence
759 values of 4 individual measurements (solid lines) in comparison to the average of 4 DMSO
760 controls (dotted lines) in the same multiwell plate. Error bars represent \pm SEM. **(B)**
761 Fluorescent intensity in R-GECO1-expressing Arabidopsis seedlings after IAA treatment.
762 Seedlings were pretreated for 30 minutes with 50 μ M bepridil (solid lines) or 0.1% DMSO

763 (dotted lines). IAA (250 nM) was added at time point 0, and R-GECO1 fluorescence intensity
764 was monitored for 8 minutes. The data represent average fluorescence values of 4 individual
765 measurements in comparison to the average of 3 DMSO controls. Error bars represent \pm
766 SEM. **(C)** Snapshot of the peak Ca^{2+} signal elicited in R-GECO1 seedlings by 250 nM IAA
767 after a 30-minute pretreatment with 0.1% DMSO (left) or 50 μM bepridil (right). The
768 snapshots were taken from Supplemental Video S1 30 seconds after IAA addition. **(D)**
769 Average primary root length of WT Col-0 seedlings grown for 7 days on $\frac{1}{2}$ MS medium in
770 presence of 0.1% DMSO or 10 μM , 20 μM , or 50 μM bepridil. Root lengths represent the
771 average of 54-60 roots. Error bars represent \pm SEM. Student's t-test p-value: *** $p < 0.001$.
772 **(E-F)** Confocal microscopy images of 5-day-old DR5rev::VENUS-N7 seedlings grown on
773 0.1% DMSO **(E)** and 20 μM bepridil **(F)**. Green: DR5rev::VENUS-N7 signal; red: propidium
774 iodide staining. **(G)** Average primary root length of WT Arabidopsis seedlings grown for 7
775 days on $\frac{1}{2}$ MS medium in presence of 20 μM bepridil or 0.1% DMSO (grey) and
776 supplemented with 100 nM 2,4-D (red), 20 μM ACC (blue), 150 nM NAA (yellow), or 250 nM
777 NAA (green). Root lengths represent the average of 51-61 roots. Error bars represent \pm
778 SEM. Student's t-test p-values in comparison to DMSO: ** $p < 0.01$ and *** $p < 0.001$. **(H-I)**
779 The effect of bepridil on vacuolar morphology. Six-day-old pUBQ10::VAMP711-YFP
780 seedlings were pretreated with 50 μM bepridil or solvent control for 5 h, followed by 3-h
781 treatments with (DMSO), 250 nM IAA (IAA), 50 μM bepridil (Bepr), or 50 μM bepridil and 250
782 nM IAA (Bepr + IAA). Tonoplast-localized VAMP711-YFP (orange) as vacuolar marker and
783 propidium iodide stain (green) for decorating the cell wall were used for confocal imaging of
784 atrichoblast cells **(H)**. The quantification of the vacuolar morphology index was performed
785 with 4 vacuoles of late meristematic atrichoblasts per root, with 10-14 roots used for each
786 treatment **(I)**. Statistical analysis was performed using one-way ANOVA (Kruskal-Wallis test)
787 followed by Dunn's multiple comparison test, b: $p < 0.05$, c: $p < 0.001$.
788

789 **Fig. 7. Sucrose-induced Ca²⁺ signals are highly sensitive to fenamates, protonophores,**
790 **and imidazoles. (A-H)** Sucrose-induced Ca²⁺ responses of YFP-apoaequorin-expressing
791 Arabidopsis seedlings treated with fenamates **(A-C)**, protonophores **(D-E)**, imidazoles **(F-G)**,
792 or bepridil **(H)**. The data represent average luminescence values of 4 individual
793 measurements (solid lines) in comparison to the average of 4 DMSO controls (dotted lines)
794 in the same multiwell plate. Error bars represent ± SEM. FFA, flufenamic acid; NFA, niflumic
795 acid; TFA, tolfenamic acid; Nicl., nicosamide; U.A., (+)-usnic acid; Clot., clotrimazole; Ox.
796 Nit., oxiconazole nitrate; Bepr., bepridil.

797

798

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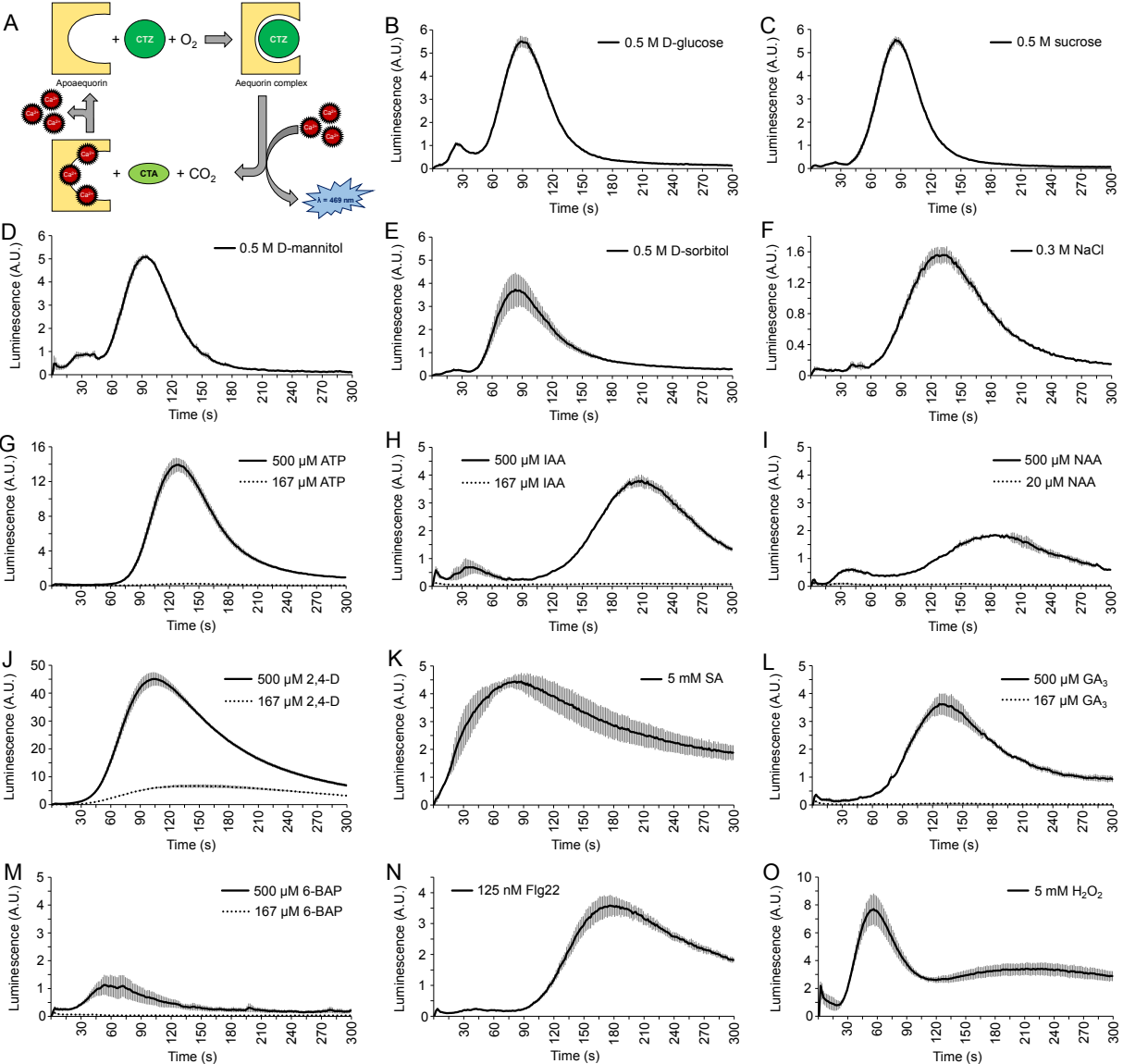
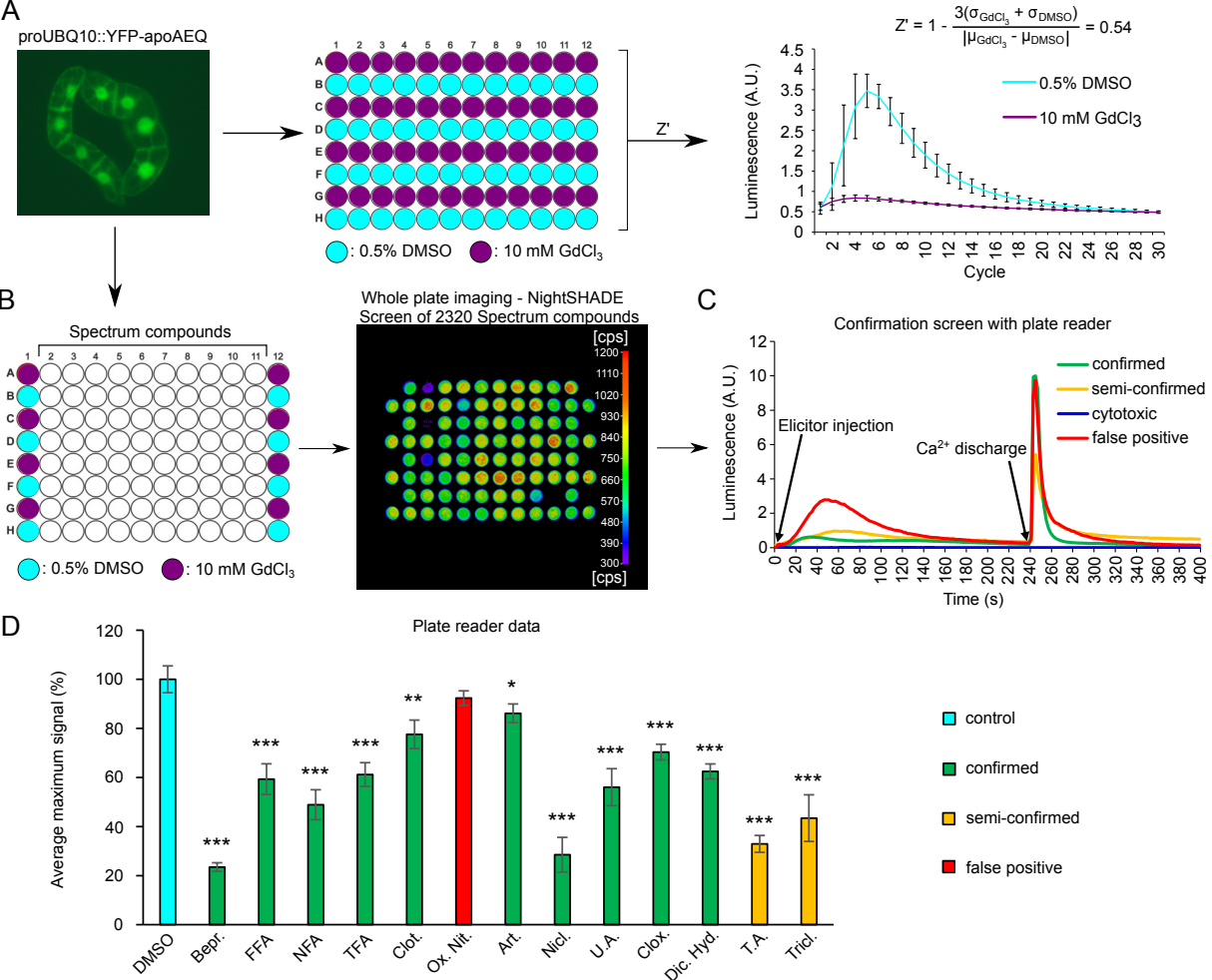


Fig. 1. 14 elicitors induce a distinct Ca²⁺ signal in aequorin-expressing BY-2 cells. (A) Schematic representation of aequorin complex formation and bioluminescent reaction. A functional aequorin complex is formed upon binding of apoequorin with its substrate coelenterazine (CTZ) in presence of O₂. The binding of three Ca²⁺ ions leads to the conversion of CTZ into coelenteramide (CTA) and CO₂, upon which blue light (λ = 469 nm) is emitted. (B-O) Ca²⁺ response of YFP-apoequorin-expressing BY-2 cells treated with various potential elicitors: (B) 0.5 M D-glucose, (C) 0.5 M sucrose, (D) 0.5 M D-mannitol, (E) 0.5 M D-sorbitol, (F) 0.3 M NaCl, (G) 167 μM and 500 μM ATP, (H) 167 μM and 500 μM IAA, (I) 20 μM and 500 μM NAA, (J) 167 μM and 500 μM 2,4-D, (K) 5 mM salicylic acid (SA), (L) 167 μM and 500 μM gibberellin (GA₃), (M) 167 μM and 500 μM 6-benzylaminopurine (6-BAP), (N) 125 nM flg22, (O) 5 mM H₂O₂. The data is represented by average luminescence values of 3 individual measurements in the same multi-well plate. Error bars represent ± SEM.



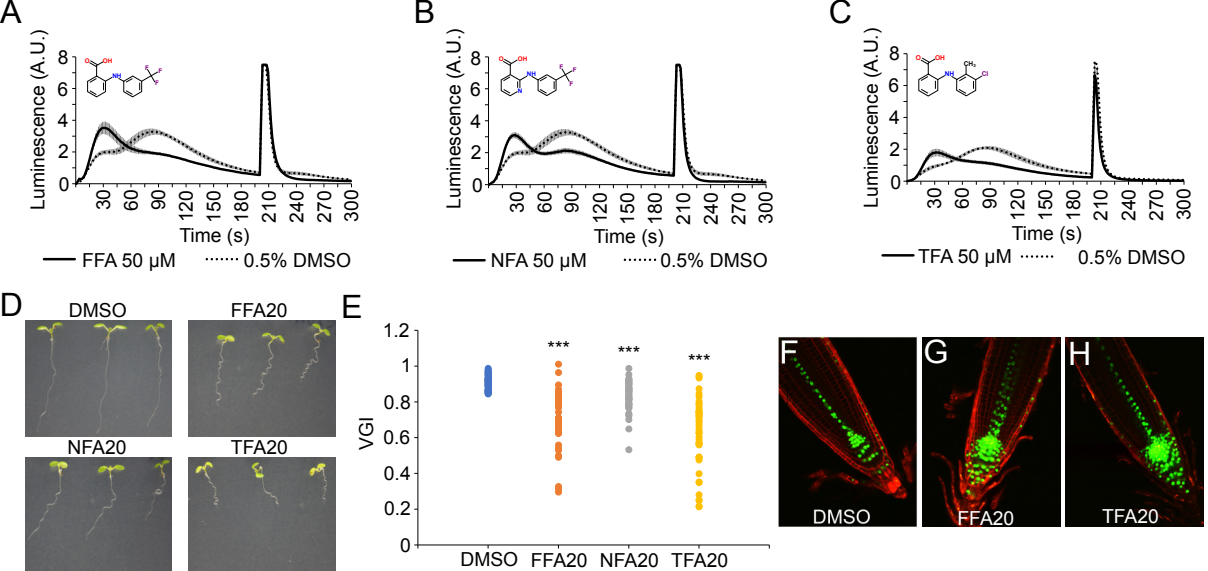


Fig. 3. Fenamates alter the shape of auxin-induced Ca^{2+} . (A-C) 2,4-D-induced Ca^{2+} response of YFP-*apocaequorin*-expressing BY-2 cells treated with 50 μM FFA (A), NFA (B) or TFA (C). Discharge solution was added after 200 seconds. The data is represented by average luminescence values of 4-8 individual measurements (solid lines) in comparison to the average of 4-8 DMSO controls (dotted lines) in the same multi-well plate. Error bars represent \pm SEM. (D) Phenotype of WT Col-0 seedlings grown for 7 days on $\frac{1}{2}$ MS medium in presence of 0.1% DMSO or 20 μM FFA, NFA or TFA, respectively. (E) The Vertical Growth Index (VGI) values for the roots from (D). For each treatment, 42 - 47 roots were measured. Student's *t*-test values: *** $p < 0.001$. (F-H) Confocal microscopic images of 5-day-old DR5rev::VENUS-N7 seedlings grown on 0.1% DMSO (F), 20 μM FFA (G) and 20 μM TFA (H), respectively. Green: DR5rev::VENUS-N7 signal; Red: propidium iodide staining.

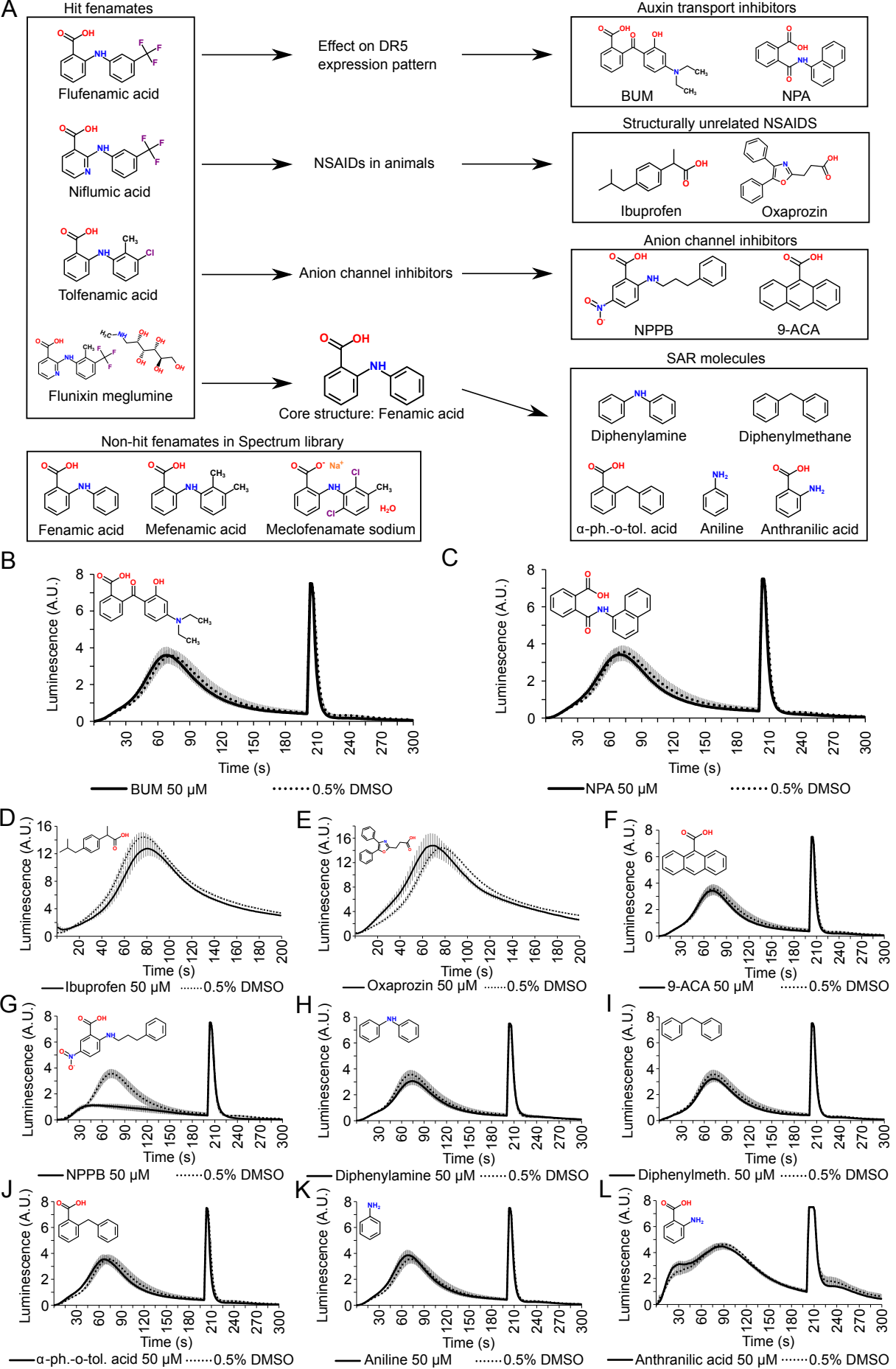


Fig. 4. Functional and structural fenamate analogs do not alter the shape of auxin-induced Ca^{2+} . (A)

Overview of a small-scale SAR analysis of the fenamates. Based on known and observed functions and structures of the hit fenamates, a set of functional and structural analogs was investigated. (B-C) 2,4-D-induced Ca^{2+} response of YFP-apoaequorin-expressing BY-2 cells treated with 50 μM of the auxin transport inhibitors BUM (B) and NPA (C). Discharge solution was added after 200 seconds. The data is represented by average luminescence values of 4 individual measurements (solid lines) in comparison to the average of 4 DMSO controls (dotted lines) in the same multi-well plate. Error bars represent \pm SEM. (D-E) 2,4-D-induced Ca^{2+} response of YFP-apoaequorin-expressing BY-2 cells treated with 50 μM of the NSAIDs ibuprofen (D) and oxaprozin (E) (Measurement in GloMax Navigator platereader (Promega)). The data is represented by average luminescence values of 8 individual measurements (solid lines) in comparison to the average of 8 DMSO controls (dotted lines) in the same multi-well plate. Error bars represent \pm SEM. (F-L) 2,4-D-induced Ca^{2+} response of YFP-apoaequorin-expressing BY-2 cells treated with 50 μM of 2 anion channel inhibitors (9-ACA and NPPB; F-G) and various compounds structurally similar to fenamates (H-L). Discharge solution was added after 200 seconds. The data is represented by average luminescence values of 4 individual measurements (solid lines) in comparison to the average of 4 DMSO controls (dotted lines) in the same multi-well plate. Error bars represent \pm SEM. BUM, 2-(4-(diethylamino)-2-hydroxybenzoyl)benzoic acid; NPA, 1-N-naphthylphthalamic acid; 9-ACA, 9-anthracenecarboxylic acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; α -ph.-o-tol. acid, α -phenyl-o-toluic acid; A.U., arbitrary units.

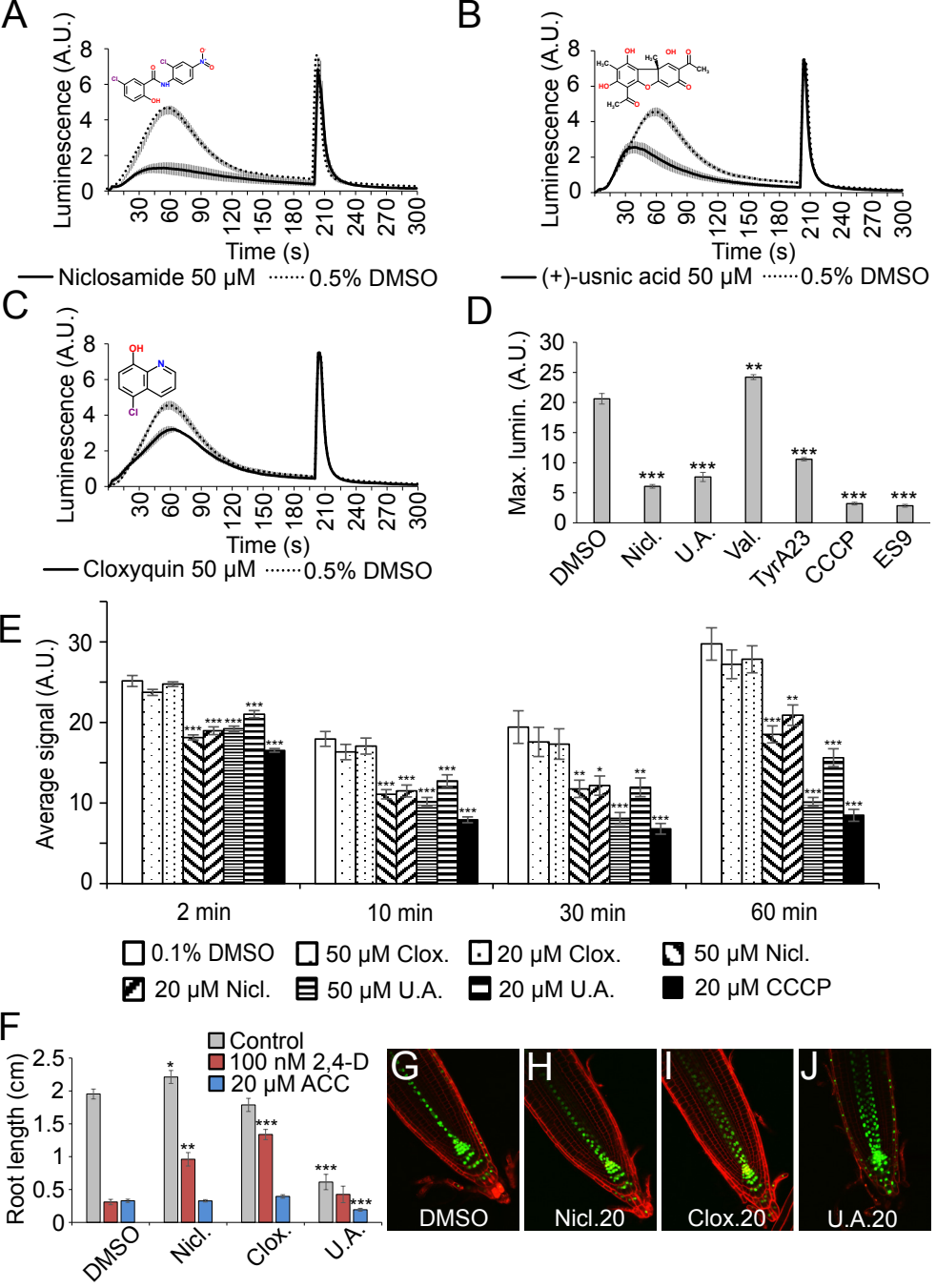


Fig. 5. Protonophores impair the 2,4-D-induced Ca^{2+} response, and render roots insensitive to 2,4-D. (A-C) 2,4-D-induced Ca^{2+} response of YFP-apoaequorin-expressing BY-2 cells treated with 50 μM niclosamide (A), (+)-usnic acid (B) or cloxyquin (C). Discharge solution was added after 200 seconds. The data is represented by average luminescence values of 4 individual measurements (solid lines) in comparison to the average of 4 DMSO controls (dotted lines) in the same multi-well plate. Error bars represent \pm SEM. (D) Maximum 2,4-D-induced luminescence in YFP-apoaequorin-expressing BY-2 cells treated with 50 μM of the hit protonophores niclosamide and (+)-usnic acid, the ionophore valinomycin, or the non-hit protonophores tyrphostin A23, CCCP and ES9. Four individual measurements were performed for each treatment and compared to the average of 8 DMSO controls in the same multi-well plate. Error bars represent \pm SEM. Student's t-test p-values: ** $p < 0.01$, *** $p < 0.001$. (E) ATP measurements of BY-2 cells pre-treated with 0.5% DMSO (white), 20 μM CCCP (black), or 20 μM or 50 μM of cloxyquin (dots), niclosamide (diagonal stripes) or (+)-usnic acid (horizontal stripes), respectively. ATP was measured 2, 10, 30 and 60 minutes after compound treatment. Error bars represent \pm SEM. Student's t-test p-values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (F) Average primary root length of WT Col-0 seedlings grown for 7 days on $\frac{1}{2}$ MS medium in presence of 0.1% DMSO or 20 μM niclosamide, cloxyquin or (+)-usnic acid (grey bars), and supplemented with 100 nM 2,4-D (red bars) or 20 μM ACC (blue bars). Root lengths represented as average of 12-21 roots. Error bars represent \pm SEM. Student's t-test p-values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (G-J) Confocal microscopic images of 5-day-old DR5rev::VENUS-N7 seedlings grown on 0.1% DMSO (G), 20 μM niclosamide (H), 20 μM cloxyquin (I) or 20 μM (+)-usnic acid (J), respectively. Green: DR5rev::VENUS-N7 signal; Red: propidium iodide staining. The image in (G) is identical to Fig. 3F.

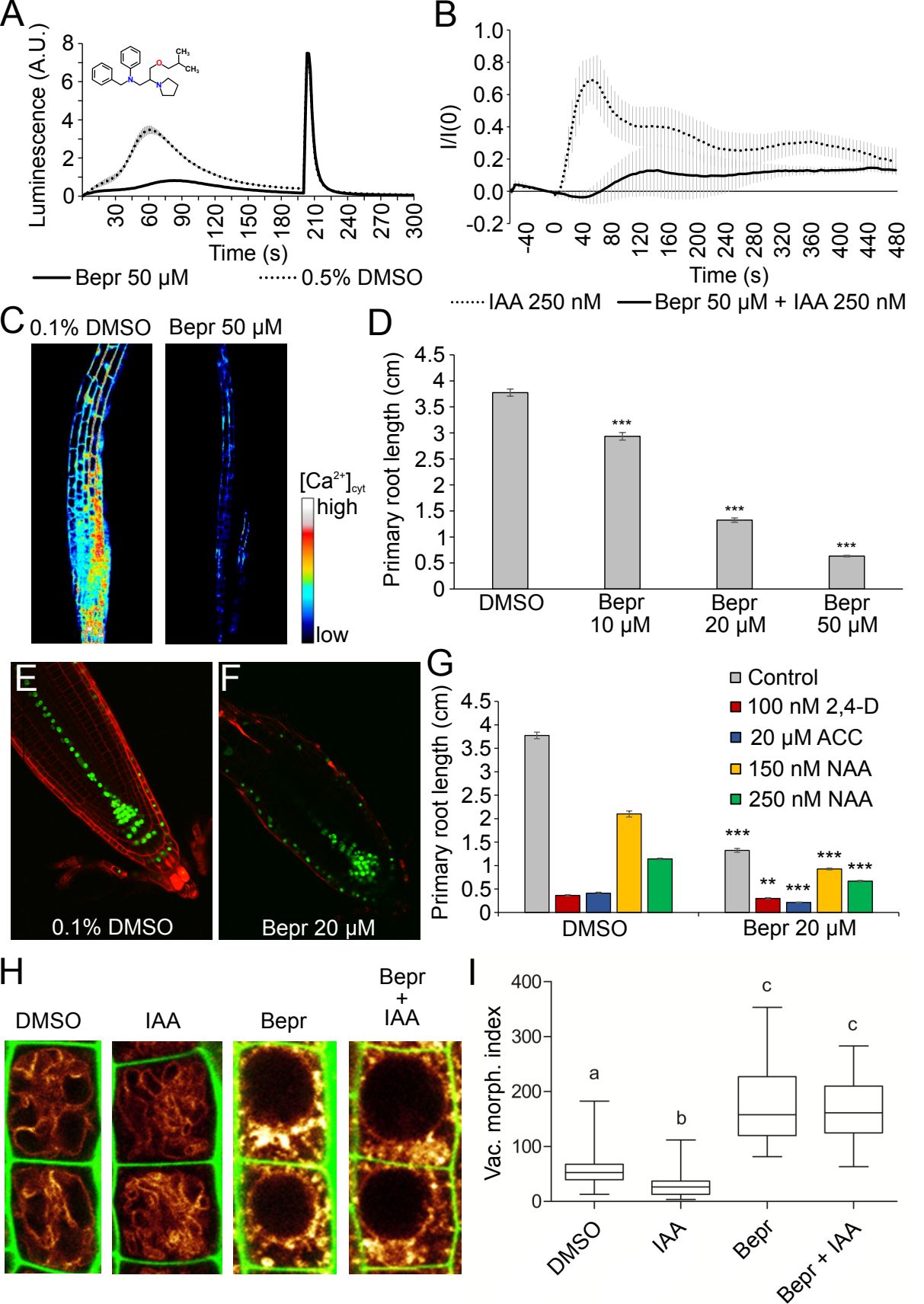


Fig. 6. Bepridil is a potent inhibitor of auxin-induced Ca²⁺ signaling. (A) 2,4-D-induced Ca²⁺ response of YFP-apoaequorin-expressing BY-2 cells treated with 50 μ M bepridil. Discharge solution was added after 200 seconds. The data is represented by average luminescence values of 4 individual measurements (solid lines) in comparison to the average of 4 DMSO controls (dotted lines) in the same multi-well plate. Error bars represent \pm SEM. (B) Fluorescent intensity in R-GECO1-expressing *Arabidopsis* seedlings after IAA treatment. Seedlings were pre-treated for 30 minutes with 50 μ M bepridil (solid lines) or 0.1% DMSO (dotted lines). IAA (250 nM) was added at time point 0, and R-GECO1 fluorescence intensity was monitored for 8 minutes. The data is represented by average fluorescence values of 4 individual measurements in comparison to the average of 3 DMSO controls. Error bars represent \pm SEM. (C) Snapshot of the peak Ca²⁺ signal elicited in R-GECO1 seedlings by 250 nM IAA after a 30 minute pre-treatment with 0.1% DMSO (left) or 50 μ M bepridil (right), respectively. The snapshot was taken 30 seconds after IAA addition. (D) Average primary root length of WT Col-0 seedlings grown for 7 days on $\frac{1}{2}$ MS medium in presence of 0.1% DMSO or 10 μ M, 20 μ M or 50 μ M of bepridil, respectively. Root lengths represented as average of 54-60 roots. Error bars represent \pm SEM. Student's t-test p-values: ***p < 0.001. (E-F) Confocal microscopic images of 5 day old DR5rev::VENUS-N7 seedlings grown on 0.1% DMSO (E) and 20 μ M bepridil (F), respectively. Green: DR5rev::VENUS-N7 signal; Red: propidium iodide staining. (G) Average primary root length of WT *Arabidopsis thaliana* seedlings grown for 7 days on $\frac{1}{2}$ MS medium in presence of 20 μ M bepridil or 0.1% DMSO (grey), and supplemented with 100 nM 2,4-D (red), 20 μ M ACC (blue), 150 nM NAA (yellow) or 250 nM NAA (green). Root lengths represented as average of 51-61 roots. Error bars represent \pm SEM. Student's t-test p-values in comparison to DMSO: **p < 0.01, ***p < 0.001. (H-I) The effect of bepridil on vacuolar morphology. Six-day old pUBQ10::VAMP711-YFP seedlings were pre-treated with 50 μ M bepridil or solvent control for 5 h, followed by 3-h treatments with (DMSO), 250 nM IAA (IAA), 50 μ M bepridil (Bepr) or 50 μ M bepridil and 250 nM IAA (Bepr + IAA). Tonoplast localized VAMP711-YFP (orange) as vacuolar marker and propidium iodide stain (green) for decorating the cell wall were used for confocal imaging of atrichoblast cells (H). The quantification of the vacuolar morphology index was performed with 4 vacuoles of late meristematic atrichoblasts per root, with 10-14 roots used for each treatment (I). Statistical analysis was performed using one-way Anova (Kruskal-Wallis test) followed by Dunn's multiple comparison test, b: p < 0.05, c: p < 0.001.

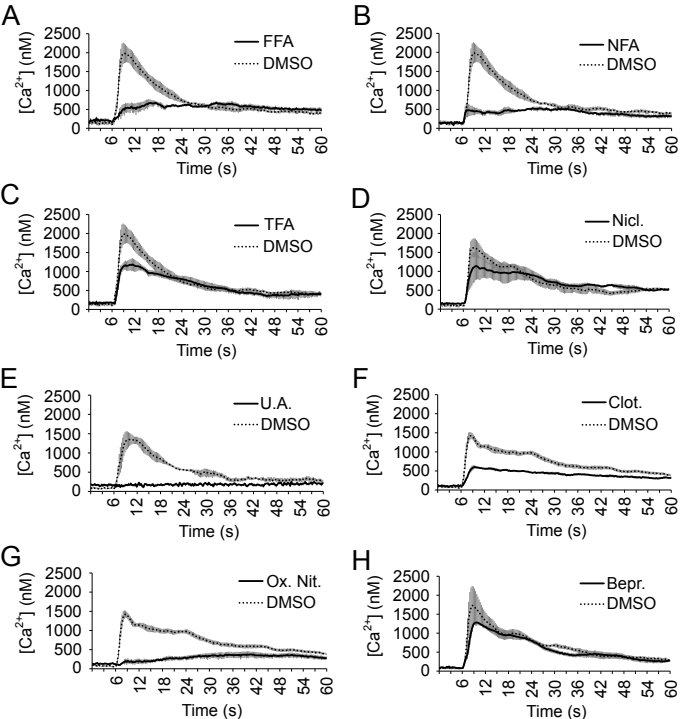


Fig. 7. Sucrose-induced Ca²⁺ signals are highly sensitive to fenamates, protonophores and imidazoles. (A-H) Sucrose-induced Ca²⁺ responses of YFP-apoaequorin-expressing *Arabidopsis thaliana* seedlings treated with fenamates (A-C), protonophores (D-E), imidazoles (F-G), or bepridil (H). The data is represented by average luminescence values of 4 individual measurements (solid lines) in comparison to the average of 4 DMSO controls (dotted lines) in the same multi-well plate. Error bars represent \pm SEM. FFA, flufenamic acid; NFA, niflumic acid; TFA, tolfenamic acid; Nicl., niclosamide; U.A., (+)-usnic acid; Clot., clotrimazole; Ox. Nit., oxiconazole nitrate; Bepr., bepridil.

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