The latest HyPe(r) in plant H2O2 biosensing

José Manuel Ugalde, Michelle Schlösser, Armelle Dongois, Alexandre Martinière, Andreas Meyer

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Dear Editor,

Hydrogen peroxide (H_2O_2) is widely used as a signaling molecule in plants during development, wounding, pathogen or symbiotic interaction and a wide range of abiotic stresses (Waszczak et al., 2018; Smirnoff and Arnaud, 2019). For better understanding its role as a messenger, it is critical to measure H_2O_2 with high spatial and temporal resolution (Gilroy et al., 2016). Stress-induced oxidation has frequently been shown with oxidation-sensitive fluorescent chemical probes, most prominently 2',7'-dihydrodichlorofluorescein diacetate (Fichman et al., 2019). These probes, however, have the disadvantage of limited specificity for H_2O_2 and the fact that they may report only the accumulation of oxidants over time without any dynamic and spatial information (Winterbourn, 2014; Ortega-Villasante et al., 2018). In addition, these probes need to be loaded into the cells, where they need to be deesterified to become active, and they can be analyzed only intensiometrically with excitation at a single wavelength. Altogether, this renders the observed fluorescence sensitive to several non-controlled factors and thus often ambiguous. Genetically encoded probes for H_2O_2 have the promise of overcoming these limitations (Meyer and Dick, 2010; Schwarzländer et al., 2016). Probes of the HyPer family consist of a circularly-permuted yellow fluorescent protein (cpYFP) core and a sensing domain constructed from the bacterial H_2O_2-sensitive transcription factor OxyR (Belousov et al., 2006; Bilan and Belousov, 2016). cpYFP, however, has the disadvantage of a pronounced pH-sensitivity (Schwarzländer et al., 2014). With a more alkaline cytosolic pH in illuminated green tissues as measured with cpYFP, HyPer readouts of H_2O_2 fluxes become highly ambiguous and demand elaborate controls (Exposito-Rodriguez et al., 2017). In contrast, roGFP2-Orp1 is pH-insensitive and has successfully been used to sense H_2O_2 originating from an elicitor-induced oxidative burst in the apoplast and from chloroplasts in which ROS production was boosted by methyl viologen (MV) in combination with light (Nietzel et al., 2019; Ugalde et al., 2021). Small dynamic changes in H_2O_2 in the low nanomolar range may, however, not be easily detectable because of a limited responsiveness of Orp1 to H_2O_2 and it may be overridden by a strong reducing effect of glutathione. Indeed, mutants with a less negative glutathione redox potential (E_{GSH}) render the sensor more responsive due to the diminished reducing power (Marty et al., 2009; Nietzel et al., 2019).

Recently, a novel ultrasensitive HyPer variant, HyPer7, consisting of the OxyR protein of *Neisseria meningitidis* and a mutated cpYFP, which in contrast to its earlier versions is largely pH-insensitive, was reported (Pak et al., 2020). These features sparked much interest across biology and thus we are currently observing multiple laboratories considering the use of HyPer7. To test its ability for reporting dynamic changes of H_2O_2 in the cytosol of *Arabidopsis thaliana*, we generated stable HyPer7 reporter lines. These plants did not show any obvious phenotype (Supplemental Materials and Methods; Supplemental Fig. S1). Ratiometric analysis of HyPer7 indicated that the sensor was largely reduced in all tissues except the root meristem, where HyPer7 appeared slightly oxidized (Supplemental Figs. S1 and S7). Depletion of glutathione by germinating seeds on L-buthionine sulfoximine (BSO) (Meyer et al., 2007) did cause only a slight ratio increase in HyPer7 while the original HyPer (Costa et al., 2010) was unresponsive and roGFP2-Orp1 (Nietzel et al., 2019) was fully oxidized (Supplemental Fig. S2). This suggests that reduction of HyPer7 is largely independent of E_{GSH}, but that with GSH depletion, H_2O_2 increase to levels that can be sensed by HyPer7. To further elucidate the responsiveness of HyPer7 to an externally imposed oxidation, we perfused seedlings with H_2O_2, buffer and DTT and compared its response with reporter lines expressing roGFP2-Orp1 and HyPer (Supplemental Materials and Methods; Fig. 1A). When perfused with H_2O_2, all three sensors showed a concentration-dependent increase of the fluorescence ratio with HyPer7 showing the largest fold-change of about 4 compared

to 3.2 for roGFP2-Orp1 and less than 2 for HyPer with 1 mM H_2O_2 perfusion (Fig. 1B-D). The speed of oxidation of HyPer7 after perfusion with H_2O_2 was slightly faster than for the other sensors. Cytosolic changes in H_2O_2 after perfusion with 0.01 mM H_2O_2 were only visible with HyPer7 (Fig. 1E-G). Subsequent perfusion with buffer consistently led to a decline in the fluorescence ratio. Addition of 10 mM DTT 6 minutes after the washout of H_2O_2 caused a further drop in ratio back to the starting value for roGFP2-Orp1 consistent with the response of other roGFP2-based probes (Marty et al., 2009), but did not cause any distinct change for HyPer7 (Fig. 1B-D; Supplemental Fig. S3A). This suggests that DTT is not capable of efficiently reducing the OxyR domain. Consistent with this observation, 10 mM DTT exerted only a minor reducing effect on recombinant HyPer7 while 5 mM of the stronger reductant Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) reduced the protein completely (Supplemental Fig. S3B).

Direct comparison of HyPer and HyPer7 for their response to externally added H_2O_2 in roots consistently showed the more pronounced response of HyPer7 (Supplemental Fig. S4). While HyPer showed an oxidation response to 10 mM H_2O_2 in leaves, the same H_2O_2 concentration did not cause a ratio increase in roots, or even caused a complete lack of excitability at 488 nm preventing any ratiometric analysis. A pronounced drop in the fluorescence ratio of cpYFP with H_2O_2 identifies the decrease of the HyPer ratio to be caused by H_2O_2-induced acidosis in the cytosol (Supplemental Fig. S5). pH-clamp experiments on roots showed the pronounced pH-dependence of HyPer while HyPer7 is largely pH-independent at neutral and slightly alkaline pH (Supplemental Fig. S6). This pH-insensitivity of HyPer7 avoids ambiguities in the interpretation of measured ratio values.

To explore the capability of HyPer7 for sensing stress-induced H_2O_2 in live plant cells, we tested the response to different stress factors. In roots, addition of 100 mM NaCl caused a slight oxidation in the meristematic zone, but not in the elongation zone (Supplemental Fig. S7). In leaves, 50 µM MV in combination with light causes the formation of H_2O_2, which is detectable with roGFP2-based probes (Ugalde et al., 2021). The response of HyPer7 suggests that HyPer7 is more sensitive than roGFP2-Orp1 and that 50 µM MV already resulted in a pronounced cytosolic oxidation with the normal plate reader excitation light (Supplemental Fig. S8A). Continuous illumination with actinic light for 1 h caused a strong oxidation, which quickly disappeared after the illumination period. Similarly, roGFP2-Orp1 can be used to monitor the cytosolic response to an apoplastic oxidative burst elicited by flg22 (Nietzel et al., 2019). While HyPer under the conditions used was not sensitive enough to monitor this burst, the response of HyPer7 was more pronounced than that of roGFP2-Orp1 (Supplemental Fig. S8B). The response of HyPer7 also indicated an additional, yet to be characterized, earlier oxidation peak about 30 min after addition of flg22, which was not observed with roGFP2-Orp1. HyPer7 thus may allow to generate information that complements previous findings.

In pavement cells of cotyledons, illumination by laser light during scanning already caused a gradual oxidation of HyPer7, which increases depending on the zoom factor (Fig. 2; Supplemental Fig. S9). Surprisingly, this oxidation was almost completely abolished when seedlings were pretreated with 20 µM DCMU as an inhibitor of photosynthetic electron transport (Fig. 2B, D, E). These results, suggests that laser light used for excitation is sufficient to induce H_2O_2-release from chloroplasts, and that these low amounts of H_2O_2 can be detected by HyPer7. Laser-induced oxidation of HyPer declined within minutes indicated by a decreasing fluorescence ratio (Fig. 2F-H). The capability of sequentially repeating this oxidation-reduction cycle emphasizes the dynamic response of HyPer7 albeit with a slower reduction phase.

In summary, the results show that HyPer7 is largely pH-insensitive and more sensitive to H_2O_2 than current probes. Nonetheless, the Arabidopsis cytosol appears to lack an efficient reduction system for HyPer7, which may limit its use for fully dynamic studies. With an increased sensitivity, HyPer7 offers the possibility of detecting physiological H_2O_2 fluxes and thus opens the door for further studies on

intracellular H_2O_2 signaling during normal development and under environmental stress. At the same time the high sensitivity, at least in green tissues, bears the risk of generating artefacts if non-photosynthesis-related H_2O_2 signaling processes are to be investigated. Knowing these potential artefacts should help designing appropriate control experiments and with that generate data that help elucidating H_2O_2 signaling.

Supplemental Data

The following supplemental materials are available:

Supplemental Figure S1. Expression of the HyPer7 sensor in *Arabidopsis thaliana***.**

Supplemental Figure S2. Effect of GSH depletion on the oxidation of genetically encoded H_2O_2 probes**.**

Supplemental Figure S3. Excitation spectra of HyPer7 *in vivo* and *in vitro*.

Supplemental Figure S4. Determination of minimum and maximum oxidation of different H_2O_2 probes.

Supplemental Figure S5. Hydrogen peroxide causes acidification in the cytosol.

Supplemental Figure S6. Response of HyPer and HyPer7 to changes in intracellular pH.

Supplemental Figure S7. HyPer7 ratio changes in root cells treated with salt.

Supplemental Figure S8. Methyl viologen (MV)-induced photo-oxidative stress and the **e**licitor flg22 causes oxidation of H_2O_2 probes in the cytosol.

Supplemental Figure S9. Oxidation of HyPer7 depends on laser light intensity reaching the scanned area**.**

Supplemental Materials and Methods. Methodology used in this study

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Author contributions

A.J.M., A.M., A.D. and J.M.U. designed experiments and analyzed data; M.S., A.D., A.M. and J.M.U. performed experiments; A.J.M. and A.M. supervised experiments; A.J.M. wrote the article.

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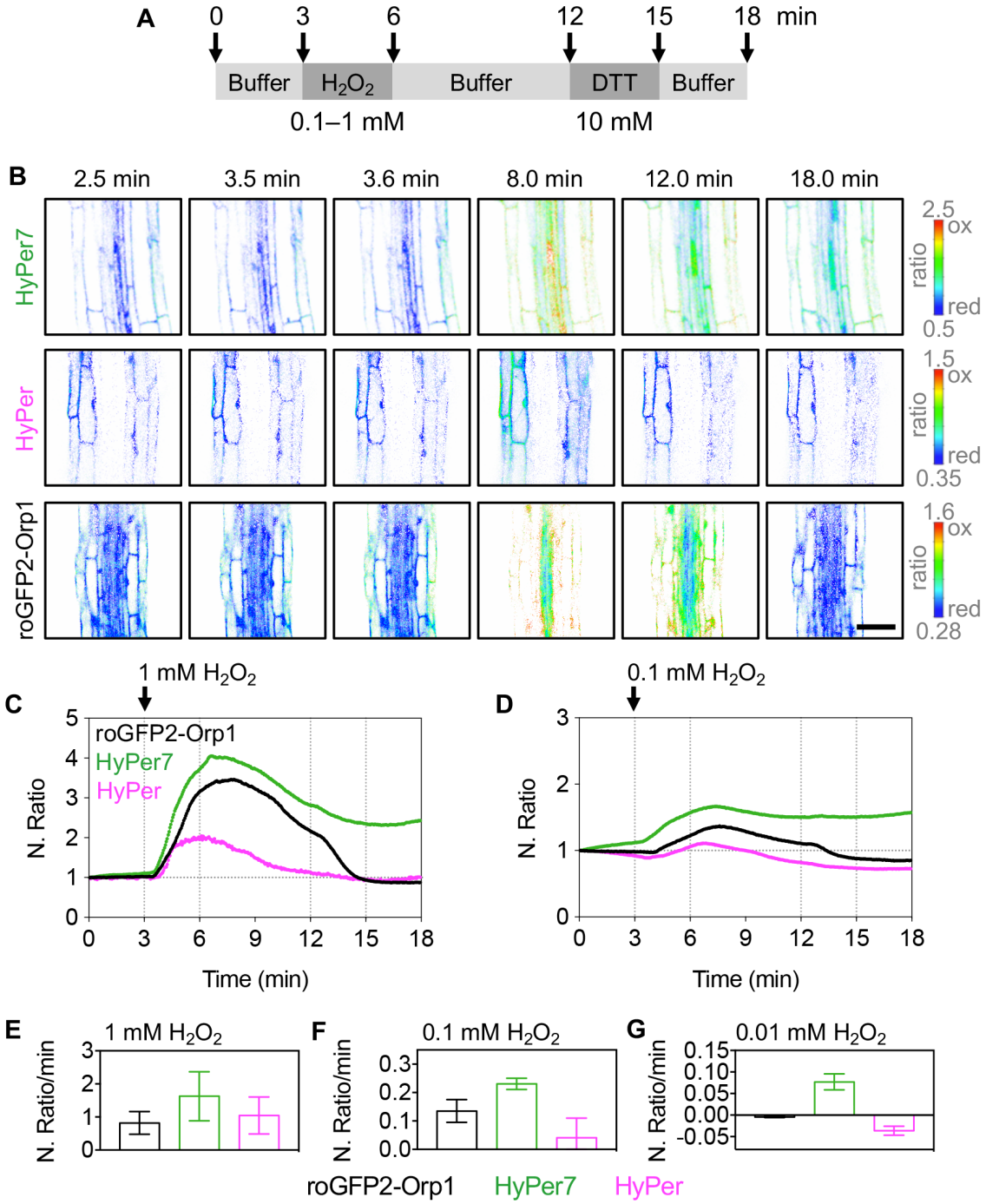
Figure Legends

Figure 1. Oxidation and reduction of different genetically encoded H_2O_2 probes in the cytosol of wild-type Arabidopsis seedlings. A, Experimental design for sequential perfusion with different concentrations of H_2O_2, imaging buffer and 10 mM DTT. B, Confocal microscopy images of root cells from 7-day-old seedlings expressing HyPer7, HyPer or roGFP2-Orp1, all targeted to the cytosol. The false color ratio images show the fluorescence ratios calculated from two separate images collected with excitation at 488 nm and 405 nm. Emission was collected at 508-535 nm. Ratios are 488 nm/ 405 nm for HyPer7 and HyPer, and 405 nm/488 nm for roGFP2-Orp1. Bar, 50 µm. C, D, Typical time courses showing the dynamic response of the probes to transient oxidation by the indicated H_2O_2 treatments (arrows). All ratios are normalized to the starting values at t = 0 min. n = 3–4 replicates. E–G, Slopes of the ratio value changes after H_2O_2 perfusion in the indicated concentrations. The slopes were calculated from ratio values between 3.9–4.7 min. Data indicates the mean values ± SD. n = 2–4 replicates.

Figure 2. Laser illumination of green tissues during scanning is sufficient to produce release of H_2O_2 form chloroplasts. A, B, Laser-induced oxidation is more visible in cells expressing HyPer7 compared to cells with HyPer or roGFP2-Orp1. Plants expressing cpYFP were included to test for putative concomitant pH changes. The false color ratio images show the fluorescence ratios calculated from two separate images collected with excitation at 488 nm and 405 nm. Emission was collected at 508-535 nm. Ratios are 488 nm/ 405 nm for HyPer7, HyPer and cpYFP, and 405 nm/488 nm for roGFP2-Orp1. Bar, 20 µm. C, Ratio values of purified recombinant protein imaged with the same instruments settings as in (A). The protein was reduced with TCEP prior to the measurement. D, E, Inhibition of photosynthetic electron transport blocks laser induced oxidation of HyPer7 in the cytosol. Seedlings were incubated in imaging buffer supplemented with 20 µM DCMU for 45 minutes in the dark prior to the measurement. Bar, 20 µm. F–H, Laser-induced oxidation of HyPer7 in a defined region of interest (magenta square) is reversible. Letters indicate time windows for laser-induced oxidation caused by high frequency imaging at high zoom (a-b and c-d) and a recovery phase with intermittent imaging at low zoom (b-c and d-e). G, Fluorescence measured from the two independent channels (405 nm and 488 nm), normalized to the value at t = 0 min. All ratios in B, C, E and H are normalized to the ratio value at t = 0 min. Mean ratios + SD , n = 4–11 replicates.



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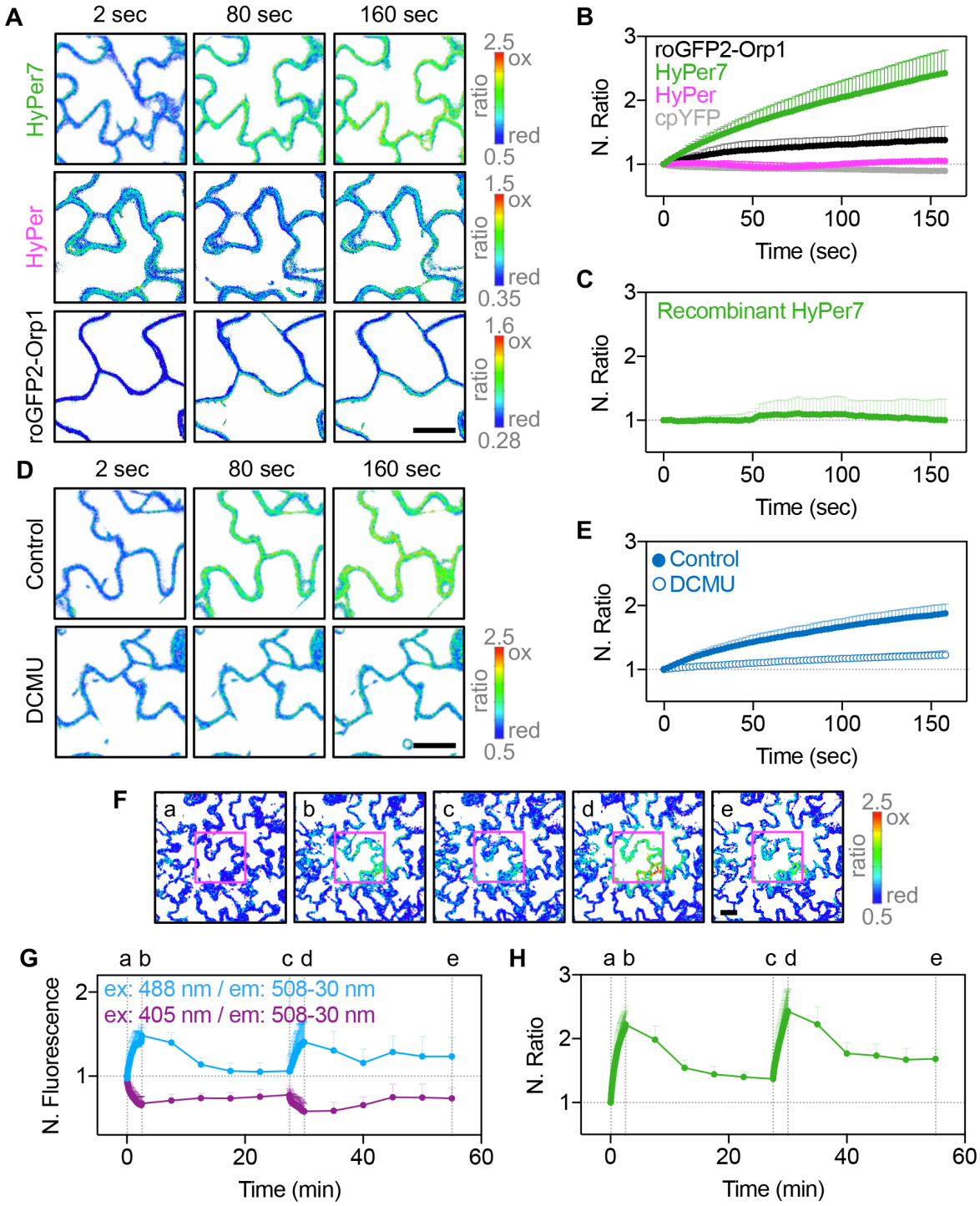

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