BREAKTHROUGH REPORT

Real-time Monitoring of Subcellular H$_2$O$_2$ Distribution in Chlamydomonas reinhardtii

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Short title: H$_2$O$_2$ Dynamics in Chlamydomonas

One-sentence summary: The establishment of a hypersensitive H$_2$O$_2$ sensor in six major compartments of the Chlamydomonas cell reveals steep intracellular H$_2$O$_2$ gradients under normal physiological conditions with limited diffusion into other compartments.

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Abstract

H$_2$O$_2$ is recognized as an important signaling molecule in plants. We sought to establish a genetically encoded, fluorescent H$_2$O$_2$ sensor that allows H$_2$O$_2$ monitoring in all major subcompartments of a Chlamydomonas cell. To this end, we used the Chlamydomonas Modular Cloning toolbox to target the hypersensitive H$_2$O$_2$ sensor roGFP2-Tsa2ΔCR to the cytosol, nucleus, mitochondrial matrix, chloroplast stroma, thylakoid lumen, and endoplasmic reticulum (ER). The sensor was functional in all compartments, except for the ER where it was fully oxidized. Employing our novel sensors, we show that H$_2$O$_2$ produced by photosynthetic linear electron transport (PET) in the stroma leaks into the cytosol but only reaches other subcellular compartments if produced under non-physiological conditions. Furthermore, in heat-stressed cells, we show that cytosolic H$_2$O$_2$ levels closely mirror temperature up- and downshifts and are independent from PET. Heat stress led to similar up- and downshifts of H$_2$O$_2$ levels in the nucleus and, more mildly, in mitochondria but not in the chloroplast. Our results thus suggest the establishment of steep intracellular H$_2$O$_2$ gradients under normal physiological conditions with limited diffusion into other compartments. We anticipate that these sensors will greatly facilitate future investigations of H$_2$O$_2$ biology in plant cells.

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Introduction

In plant cells, hydrogen peroxide \( (\text{H}_2\text{O}_2) \), or its precursor superoxide \( (\text{O}_2^-) \), is produced as a side product of cellular processes including photosynthetic linear electron transport (PET), the mitochondrial respiratory chain, or substrate oxidation, for example by the photorespiration enzyme glycolate oxidase (Cheeseman, 2007; Foyer and Noctor, 2016). \( \text{H}_2\text{O}_2 \) is a relatively stable molecule and is largely unreactive with most proteins. It is therefore well-suited as a second messenger in cellular signaling cascades (Waszczak et al., 2018). Such signaling cascades can be fueled either with \( \text{H}_2\text{O}_2 \) produced as a side product, e.g., from PET (Exposito-Rodriguez et al., 2017), or with \( \text{H}_2\text{O}_2 \) produced specifically for a signaling cascade. One such example is the respiratory burst oxidase homolog D (RBOHD) located in the plasma membrane that produces \( \text{H}_2\text{O}_2 \) for local and systemic signaling upon stimulation by wounding, cold, heat, high light, and salinity (Miller et al., 2009; Lew et al., 2020). RBOHD also plays a role during abscisic acid (ABA)-mediated stomatal movement (Pei et al., 2000; Kwak et al., 2003).

The recognition of \( \text{H}_2\text{O}_2 \) as a second messenger has generated a demand for tools to measure \( \text{H}_2\text{O}_2 \) dynamics within plant cells. While the development of synthetic dyes for \( \text{H}_2\text{O}_2 \) detection has proven useful, several problems are inherently associated with their use, including variable uptake and efflux, lack of subcellular compartment specificity, lack of redox species specificity, and the irreversibility of their reaction with \( \text{H}_2\text{O}_2 \) (Bilan and Belousov, 2018; Roma et al., 2018). A major methodological advance for the subcellular detection of \( \text{H}_2\text{O}_2 \) came with the creation of genetically encoded fluorescent protein sensors that allow for subcellular compartment-specific monitoring of \( \text{H}_2\text{O}_2 \) in real time in living organisms. The first of these sensors was HyPer (Belousov et al., 2006). HyPer (from hydrogen peroxide) consists of a circularly permuted (cp) yellow fluorescent protein (YFP) positioned between the two halves of the \( \text{H}_2\text{O}_2 \)-sensitive \( \text{E.coli} \) transcription factor OxyR. The presence of \( \text{H}_2\text{O}_2 \) leads to the formation of a disulfide bond between the \( \text{H}_2\text{O}_2 \)-reactive cysteine on one OxyR domain and the resolving cysteine residue on the other domain. This disulfide bond results in a structural change that is transmitted to cpYFP, thereby inducing a ratiometric change in its fluorescence excitation spectrum and allowing
measurements that are independent of probe concentration. Several improved versions of HyPer have been generated since that expand its dynamic range, its oxidation and reduction rates, or employ a red fluorescent protein (Bilan and Belousov, 2018).

A second family of genetically encoded fluorescent H$_2$O$_2$ sensors is based on reduction-oxidation sensitive green fluorescent protein (roGFP2), which contains two cysteines in adjacent β-strands on the surface of the protein β-barrel (Hanson et al., 2004). The formation of a disulfide bond between these cysteines results in small structural changes that induce a ratiometric change in the fluorescence excitation spectrum. As with HyPer, roGFP2 allows measurements that are independent of sensor concentration. Since the cysteines of roGFP2 do not readily react with H$_2$O$_2$, roGFP2 needs to be coupled with an H$_2$O$_2$-reactive enzyme in a redox relay system. The first such sensor was roGFP2-Orp1, which involves a fusion between roGFP2 and the glutathione peroxidase-like enzyme Orp1 from budding yeast (Saccharomyces cerevisiae), with a short interspacing polypeptide linker (Gutscher et al., 2009). In this sensor, Orp1 sensitively reacts with H$_2$O$_2$ to form an intramolecular disulfide bond between its peroxidatic and resolving cysteine, followed by a thiol-disulfide exchange reaction to roGFP2.

Many cellular peroxiredoxins contain peroxidatic cysteine residues that react with H$_2$O$_2$ two to three orders of magnitude faster than that of the Orp1 peroxidase (Roma et al., 2018). This observation led to the generation of roGFP2-Tsa2ΔC$_R$, a fusion between the typical yeast 2-Cys peroxiredoxin Thiol-Specific Antioxidant 2 (Tsa2) and roGFP2 (Morgan et al., 2016). The resolving cysteine in Tsa2 was mutated to alanine such that reduction of the sensor by thioredoxin is impeded and sensor sensitivity further enhanced. In yeast, this sensor was shown to be approximately 20-fold more sensitive towards H$_2$O$_2$ than either HyPer or roGFP2-Orp1 (Morgan et al., 2016). Furthermore, roGFP2-Tsa2ΔC$_R$ makes a negligible contribution to the cellular H$_2$O$_2$-scavenging capacity, has a low propensity for hyperoxidation, and is unaffected by changes in pH between 6.0 and 8.5 (Morgan et al., 2016; Roma et al., 2018). Its pH insensitivity distinguishes roGFP2-Tsa2ΔC$_R$ from HyPer, for which additional pH probes need to be implemented to account for changes in pH, as observed for
example in the stroma upon illumination (Schwarzlander et al., 2014; Exposito-Rodríguez et al., 2017). A pH-insensitive version of HyPer (HyPer7) has recently been established, but has not been tested in plant cells (Pak et al., 2020).

The aim of this work was to establish a sensitive H$_2$O$_2$ sensor for the unicellular green alga Chlamydomonas (Chlamydomonas reinhardtii), a traditional model system for plant cell biology and photosynthesis (Sasso et al., 2018). To this end, we engineered roGFP2-Tsa2ΔC$_R$ as a genetic part for the Chlamydomonas Modular Cloning (MoClo) toolbox for synthetic biology. We targeted the sensor to six cell compartments: the cytosol, nucleus, mitochondrial matrix, chloroplast stroma, thylakoid lumen, and endoplasmic reticulum (ER); and show that changes in H$_2$O$_2$ concentrations can be monitored in real time in all compartments, except for the ER. Our data also revealed the establishment of strong intracellular H$_2$O$_2$ gradients in response to physiological stresses.

Results

Construction of an H$_2$O$_2$ sensor for Chlamydomonas

To develop a genetically encoded, fluorescent H$_2$O$_2$ sensor for Chlamydomonas, we synthesized the coding sequence of roGFP2-Tsa2ΔC$_R$ (Morgan et al., 2016) with codons optimized for expression in Chlamydomonas and containing the three introns of the nuclear RuBisCO small subunit gene $RBCS2$, as a standard gene part (level 0) for the Chlamydomonas Modular Cloning (MoClo) kit (Crozet et al., 2018) (Figure 1A). The MoClo strategy is based on Type IIS restriction enzymes and allows the directed assembly of standard gene parts (promoters, coding sequences, untranslated regions) in a single reaction into modules (transcriptional units, level 1) (Weber et al., 2011). Likewise, level 1 modules can be further assembled into multi-gene constructs termed devices (level 2). Following this strategy, the roGFP2-Tsa2ΔC$_R$ part was assembled into level 1 modules. These included either the PHOTOSYSTEM I SUBUNIT D (PSAD) promoter (PSADpro) or the HEAT SHOCK PROTEIN 70A (HSP70A)-RBCS2 (AR) fusion promoter (ARpro), the RIBOSOMAL PROTEIN L23 (RPL23) terminator, various sequences encoding N-terminal targeting peptides or retention signals to facilitate localization to defined subcellular
compartments, and in one case the sequence of a C-terminal 3xHA tag. The resulting level 1 modules were then assembled into level 2 devices together with a spectinomycin resistance cassette (aadA) under the control of the PSAD promoter and terminator (Figure 1B). We then transformed the level 2 devices into the UVM4 expression strain (Neupert et al., 2009). As a control, we generated a level 0 part containing only the roGFP2 coding sequence, harboring the first RBCS2 intron, and placed it under control of the AR promoter (roGFP2, Figure 1B). The best promoters and targeting peptides to use in Chlamydomonas are still a subject of debate. We therefore tested different promoters and targeting peptide combinations. For each device, we picked at least 13 spectinomycin-resistant transformants and screened them for sensor accumulation by immunoblotting with an anti-GFP antibody. Between 23% and 70% of the transformants accumulated the sensor protein to readily detectable levels and with the expected size (Supplemental Figure S1). To compare the accumulation levels of the various sensors, we separated whole-cell protein extracts from the best accumulating transformants on the same SDS-PAGE and probed the resulting immunoblot using an anti-GFP antibody (Figure 1C).

With respect to the constructs for cytosolic localization, we observed that the sensor driven by the AR promoter accumulated to ~2-fold higher levels than the PSAD promoter-driven sensor (Figure 1C; the size shift seen for the PSAD promoter-driven sensor comes from the 3xHA tag). Furthermore, unfused roGFP2 accumulated to about the same level as the full roGFP2-Tsa2ΔC<sub>R</sub> sensor (Figure 1C). For targeting to the mitochondrial matrix, ER, and nucleus, we employed targeting sequences from HSP70C, Binding immunoglobulin Protein (BiP1), and the nuclear localization signal from Simian Virus 40 (SV40), respectively. For each construct, we detected a single protein band by immunoblotting and confirmed the correct localization of the sensor by confocal fluorescence microscopy (Figure 1C and 1D). For targeting to the chloroplast stroma, we tested three different transit peptides derived from universal stress protein A (USPA), HSP70B, and chloroplast DnaJ homolog 1 (CDJ1). We obtained a double band for all USPA transformants and detected some GFP fluorescence in the cytosol (Figure 1C and 1D; Supplemental Figure S1). We therefore reasoned that the USPA transit peptide was not efficiently driving chloroplast import. Likewise, as judged from the protein double band and residual
GFP fluorescence in the cytosol, the HSP70B transit peptide was also unable to efficiently drive sensor import into chloroplasts (Figure 1C and 1D). By contrast, we observed a single protein band in immunoblots and an exclusive chloroplast localization, as judged by fluorescence microscopy, when using the CDJ1 transit peptide (Figure 1C and 1D). We observed a punctate signal for the stromal sensor that might be derived from heterooligomerization with (a) native peroxidase(s), as was reported in yeast (Morgan et al., 2016). We noticed no punctate signal in a transformant with weaker sensor accumulation (Supplemental Figure S2A and S2B). We also wished to target the sensor to the thylakoid lumen; to this end, we created level 0 parts encoding the bipartite signaling sequences from the PSAN and PSBO proteins, which are nucleus-encoded subunits of photosystem I and II, respectively. PSAN uses the twin arginine protein translocation (Tat) pathway for the import of folded proteins, while PSBO uses the Sec pathway for the import of unfolded proteins (Albiniak et al., 2012). Immunological detection of the sensor in the highest accumulating PSAN and PSBO transformants showed that the sensor reaches only about half of the levels detected with the best ARpro transformant. Moreover, we obtained multiple protein bands that migrated with larger apparent mass than expected for the mature protein in PSAN transformants (Figure 1C, Supplemental Figure S1). We also observed GFP fluorescence all over the chloroplast and even in the cytosol (Figure 1D), indicating incomplete import of the sensor into both chloroplast and thylakoid lumen by the PSAN bipartite targeting sequence. By contrast, we observed only one major protein band migrating with the expected mass in PSBO transformants, with GFP fluorescence restricted to the thylakoid lumen, indicating full functionality of the PSBO bipartite targeting signal (Figure 1C and 1D, Supplemental Figure S1).

**RoGFP2-Tsa2ΔC** functions as an **ultra-sensitive H$_2$O$_2$ sensor in Chlamydomonas**

Having generated a suite of transformants targeting the sensor to distinct subcellular compartments, we first tested whether we could detect the cytosolic roGFP2-Tsa2ΔC sensor and monitor its oxidation by exogenously applying H$_2$O$_2$ in real time. To this end, we took cells of the highest accumulating PSADpro transformant. We concentrated cells by centrifugation in a 96 well microtiter plate and monitored GFP
fluorescence in control samples (with no added H$_2$O$_2$) and upon the addition of 0.1, 0.5, or 1 mM H$_2$O$_2$ in a fluorescence plate reader. Note that these H$_2$O$_2$ concentrations are initial exogenous concentrations; in HeLa cells, the resulting intracellular H$_2$O$_2$ concentrations were estimated to be several hundred-fold lower (Huang and Sikes, 2014). As shown in Figure 2A, we indeed detected sensor oxidation in a dose-dependent manner, but the signal was very noisy. This noise was mainly derived from the fluorescence signal gained from excitation at 405 nm, where the difference between oxidized and reduced sensor is considerably smaller than at 488 nm (Meyer and Dick, 2010; Supplemental Figure S3). However, we observed a clear H$_2$O$_2$ concentration-dependent sensor response in the ARpro transformant (Figure 2B), presumably because the sensor accumulated to about two-fold higher levels than in the PSADpro transformant. The cytosolic roGFP2-Tsa2ΔC$_R$ sensor was ~25% oxidized at steady-state at the beginning of our assays. Upon H$_2$O$_2$ addition, we observed a rapid probe oxidation, which slowly recovered over a period of ~80 min, presumably in a glutathione (GSH)/glutaredoxin-dependent manner, as previously shown in yeast (Morgan et al., 2016). The unfused cytosolic roGFP2 was completely unresponsive to the same concentrations of H$_2$O$_2$ used above, confirming the requirement of the Tsa2 moiety to couple roGFP2 oxidation with changes in H$_2$O$_2$ availability (Figure 2C). Notably, the kinetics of roGFP2-Tsa2ΔC$_R$ oxidation and reduction were very similar to those observed in yeast (Morgan et al., 2016), including a reduction of the sensor over time in untreated samples, which in yeast was shown to be caused by cell-dependent oxygen consumption from the assay buffer. That this is also true for Chlamydomonas is suggested by the observation that reduction of the sensor occurs faster at higher cell numbers used in the assay, just as in yeast (Supplemental Figure S4). These data strongly suggest that roGFP2-Tsa2ΔC$_R$ functions as an ultra-sensitive sensor in Chlamydomonas and is capable of sensing and responding to changes in basal cytosolic H$_2$O$_2$ levels, as it does in yeast.

For comparison, we synthesized the roGFP2-Orp1 sensor (Gutscher et al., 2009) with the Chlamydomonas codon usage and with three RBCS2 introns as a level 0 construct, equipped it with the AR promoter and RPL23 terminator in a level 1 construct, and at level 2 with the aadA cassette (Supplemental Figure S5A and S5B).
Transformants accumulated the roGFP2-Orp1 sensor in the cytosol up to ~86% of levels of roGFP2-Tsa2ΔC_R in the highest accumulating ARpro transformant (Supplemental Figure S5C and S5D). The sensor was fully reduced under steady-state conditions and was barely oxidized when H_2O_2 was added exogenously. These results clearly demonstrate the superior sensitivity of the roGFP2-Tsa2ΔC_R sensor compared to the roGFP2-Orp1 sensor in Chlamydomonas (Supplemental Figure S5E and S5F).

Having confirmed the feasibility of H_2O_2 measurements with cytosolic roGFP2-Tsa2ΔC_R, we next tested the response to exogenously added H_2O_2 of the sensor targeted to the five additional cellular compartments. Under steady-state conditions, stromal and mitochondrial sensors were more oxidized than those in the cytosol or nucleus (Figure 2B, D, F, G). However, as for the cytosolic sensor, we observed a rapid dose-dependent oxidation followed by slow reduction in response to exogenous H_2O_2 for sensors targeted to the chloroplast stroma, thylakoid lumen, mitochondrial matrix and the nucleus (Figure 2D–G). The response for the stromal sensor accumulating to lower levels and not producing fluorescent puncta was very similar to that of the highly accumulating sensor, although much more noisy (Supplemental Figure S2C). Measurements were not possible in the ER, as the sensor was fully oxidized at steady-state (Figure 2H). In general, signal noise inversely correlated with the accumulation level of the sensor: the highest noise was detected for the thylakoid lumen sensor, whose protein levels were about two-fold less abundant than those for the sensors in the stroma and nucleus, which showed the least noise (Figure 1C and 2D, E, G). The nuclear, stromal, and thylakoid lumen-localized sensors were reduced at faster rates than their counterparts in the cytosol and mitochondrial matrix (Figure 2B, D-G). Overall, the cytosolic sensor exhibited the highest sensitivity to exogenously added H_2O_2 and the nuclear sensor the lowest, as judged from their responsiveness to the lowest H_2O_2 concentration employed (0.1 mM) (Figure 2B, G). This result may indicate that H_2O_2 scavenging enzymes in the cytosol limit the diffusion of exogenous H_2O_2 to the intracellular organelles.

**RoGFP2-Tsa2ΔC_R reveals the formation of light-dependent subcellular H_2O_2 gradients**
With these Chlamydomonas lines with sensors in different subcellular compartments, we asked whether we could detect changes in H$_2$O$_2$ levels under challenging environmental conditions. We intended to first test whether exposing cells to high light would result in increased H$_2$O$_2$ levels in the chloroplast stroma and whether H$_2$O$_2$ would diffuse into other subcellular compartments. The fluorescence assay used up to this point allowed for monitoring of the sensor oxidation state in real time. However, as we had no way to illuminate cells in our plate reader assay, we turned to a 'redox trapping' approach. We adopted a protocol from yeast that allows for a rapid 'trapping' of the sensor oxidation state with the membrane permeable alkylation agent N-ethylmaleimide (NEM) (Morgan et al., 2016). NEM irreversibly alkylates free thiol groups, thus preventing further probe oxidation or reduction. To test the applicability of NEM trapping for Chlamydomonas, we added H$_2$O$_2$ exogenously to the ARpro transformant, followed by the addition of NEM immediately (0 s), after 37 s and after 1,850 s, and monitored sensor oxidation over time. As shown in Figure 3, the oxidation state of the sensor was instantaneously trapped after NEM addition and remained stable over the time course of the experiment, thus demonstrating the applicability of NEM trapping in Chlamydomonas. With this method at hand, we next grew transformants accumulating the sensor in the cytosol, nucleus, matrix, stroma, and thylakoid lumen under a low light intensity of 30 µmol photons m$^{-2}$ s$^{-1}$ to mid-log phase. Cultures were then kept in low light or exposed to 1,000 µmol photons m$^{-2}$ s$^{-1}$ in the absence or presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to block PET. We harvested cells before and during the time course and measured the oxidation state of the sensor by plate reader after NEM trapping. In these experiments (Figure 4), we observed a higher oxidation state of the sensor under steady-state conditions in all compartments when compared to the experiments shown in Figure 2, for which oxygen consumption of cells during centrifugation and preparation for the plate reader is likely to result in lower cellular H$_2$O$_2$ levels. Hence, NEM trapping of cells directly from the culture circumvented this technical limitation and revealed the true steady-state probe oxidation. Upon shifting cells into high light, H$_2$O$_2$ levels in the stroma increased rapidly within minutes and remained at higher levels when compared to cells kept under low light intensity during the 20-min time course (Figure 4A). The cytosolic probe also responded rapidly, with a non-significant trend for probe recovery after 20 min. With slower kinetics, we also observed a slight,
A non-significant trend towards higher H₂O₂ levels in high light in the mitochondrial matrix, while we failed to detect an increase in the nucleus (Figure 4C and 4D). The increase in H₂O₂ levels in all tested compartments was largely abolished when cells were exposed to high light in the presence of DCMU, indicating that H₂O₂ in high light was produced by PET. Because of its low signal to noise ratio (Figure 2E), we were unable to monitor the response of the thylakoid lumen sensor in this assay.

That we did not detect any increase in H₂O₂ levels in the nucleus of Chlamydomonas cells exposed to high light appeared surprising because such an increase was previously observed in Nicotiana benthamiana epidermal cells exposed to 1,000 µmol photons m⁻² s⁻¹ (Exposito-Rodriguez et al., 2017). We therefore repeated our experiment at higher light intensities of 1,500 µmol photons m⁻² s⁻¹ for 30 min but again were unable to detect any increase of H₂O₂ levels in the nucleus (Figure 4E).

Metronidazole and paraquat (also known as methyl viologen) both facilitate the Mehler reaction in intact cells, i.e., the transfer of electrons from PSI to oxygen to produce superoxide, which is then converted to H₂O₂ by superoxide dismutase (Mehler, 1951; Asada, 2000). While paraquat is directly reduced by PSI, metronidazole is reduced by ferredoxin (Schmidt et al., 1977). Paraquat is active at much lower concentrations than metronidazole. However, in contrast to metronidazole, paraquat can kill Chlamydomonas cells even when grown in the dark and therefore must affect other cellular processes in addition to PET (Schmidt et al., 1977). We employed both drugs to test whether the enhanced rates of H₂O₂ produced in their presence even under low light intensities might be detected in the nucleus. As shown in Figure 4E, this was indeed the case following a 30-min incubation with either drug. We will note that such short treatment leads to a growth retardation but does not kill the cells (Supplemental Figure S6). We also monitored H₂O₂ production upon metronidazole treatment in the stroma, cytosol, and matrix and discovered that H₂O₂ levels increase in all subcompartments. In all cases, DCMU decreased the accumulation of H₂O₂ but did not abolish it, most likely because plastoquinone reduction occurs from starch breakdown via NADH dehydrogenase 2 (Ndh2) mediating the so-called pseudo-linear electron transport under these conditions (Bulté et al., 1990; Mus et al., 2005; Desplats et al., 2009). Similar lower
H$_2$O$_2$ levels were also observed in Arabidopsis seedlings treated with paraquat and DCMU in the light (Ugalde et al., 2021).

In summary, our data show a rapid, PET-dependent increase in H$_2$O$_2$ levels in the chloroplast stroma. While H$_2$O$_2$ levels produced by metronidazole or paraquat feeding were so high that H$_2$O$_2$ readily diffused into all other subcompartments, H$_2$O$_2$ production under high light was of sufficient magnitude to increase cytosolic H$_2$O$_2$ but did not substantially influence H$_2$O$_2$ levels in the mitochondrial matrix or nucleus. Thus, these results are consistent with our exogenous H$_2$O$_2$ application experiments, suggesting that the cytosol acts as an effective barrier that limits the intracellular diffusion of H$_2$O$_2$ concentrations typically produced under physiological conditions.

**Heat stress-derived H$_2$O$_2$ is independent of photosynthetic electron transport**

We chose heat stress as a second environmental challenge. To test whether H$_2$O$_2$ levels within Chlamydomonas cells changed when exposed to heat stress, we employed the cell line accumulating the H$_2$O$_2$ sensor in the cytosol, as this sensor exhibited the highest signal-to-noise ratio (Figure 2B). To allow us to monitor real-time changes in cytosolic H$_2$O$_2$ during heat stress, we developed an experimental setup in which cells were cultivated under agitation and continuously circulated to a quartz cuvette inserted into a spectrofluorometer via connected tubing and a peristaltic pump. The culture flask, equipped with a temperature sensor, was kept on a platform well above the water surface of a 40°C water bath, placed into the water bath 20 min after the fluorescence recording had started, and placed back onto the platform 30 min later for recovery. Notably, sensor oxidation and thus H$_2$O$_2$ production occurred immediately after the temperature increased and immediately declined when the temperature dropped (Figure 5A). When the cultures reached the initial starting temperature 30 min into recovery, probe oxidation had not fully recovered. To verify that the change in fluorescent light emission under heat stress was not caused by a heat-induced conformational change of the sensor, we trapped the sensor with NEM in steady-state as well as in the fully oxidized and fully reduced states and recorded fluorescent light emission after excitation at 405 nm and 488 nm at 25°C, 30°C, 35°C, 40°C, and 45°C. As shown in Supplemental Figure S7, we observed no change in fluorescence ratios, indicating that the sensor is stable at this
range of temperatures and that the changes in fluorescence emission under heat stress are indeed caused by changes in roGFP2 oxidation.

As a further test of our observations, we used the same setup employed for the high light experiments (Figure 4). Specifically, we harvested cells from a culture accumulating the cytosolic sensor before shifting the temperature from 23°C to 40°C, after exposure to 40°C for 30 min, and 30 min after shifting the culture back to 23°C. Again, we measured the redox state of the sensor after trapping with NEM. As shown in Figure 5B, we confirmed that heat stress leads to a transient increase in sensor oxidation that was abolished when the temperature dropped again. To rule out that the heat-induced increase in sensor oxidation is due to an impairment of reduction systems rather than higher H$_2$O$_2$ levels, we performed the experiment with cells accumulating the cytosolic roGFP2-Orp1 sensor. This sensor barely reacts to H$_2$O$_2$ (Supplemental Figure S5E and S5F) but is reduced by the same systems as roGFP-Tsa2ΔC$_R$. As shown in Supplemental Figure S8, the oxidation state of roGFP2-Orp1 did not increase under heat stress, leaving increased H$_2$O$_2$ concentrations as the source for roGFP-Tsa2ΔC$_R$ oxidation.

To test whether increased H$_2$O$_2$ levels under heat are derived from PET, we performed the experiment in the presence of DCMU. As the increase in H$_2$O$_2$ levels was the same in the presence of DCMU (Figure 5B), we concluded that H$_2$O$_2$ produced during heat stress does not derive from PET. Another possible source of H$_2$O$_2$ might be NAD(P)H oxidases at the plasma membrane, two of which are encoded by the Chlamydomonas genome: RESPIRATORY BURST OXIDASE 1 (RBO1, Cre03.g188300) and RBO2 (Cre03.g188400). To test their potential involvement, we performed the heat stress experiment with cells accumulating roGFP-Tsa2Δ$_R$ or roGFP2-Orp1 in the cytosol in the presence of the NAD(P)H oxidase inhibitor diphenyleneiodonium chloride (DPI). DPI led to an increased oxidation of both sensors under ambient and heat stress conditions, whereas it did not affect the increased oxidation of roGFP-Tsa2Δ$_R$ in response to heat (Supplemental Figure S8). Hence, DPI appears to impair cytosolic reducing systems in Chlamydomonas and does not allow conclusions about the source of H$_2$O$_2$ produced during heat stress.
We also applied the heat stress-recovery regime to cells accumulating the sensor in the stroma, the matrix, and the nucleus (Figure 5B). Heat stress led to a significant increase in \( \text{H}_2\text{O}_2 \) levels in the nucleus and a non-significant increase in the matrix; higher \( \text{H}_2\text{O}_2 \) levels in both compartments were abolished after recovery. By contrast, \( \text{H}_2\text{O}_2 \) levels in the stroma dropped slightly after heat stress, and significantly after the recovery period. These data point to a source of heat-induced \( \text{H}_2\text{O}_2 \) production outside of the chloroplast and mitochondria, and once again to limited intracellular diffusion of \( \text{H}_2\text{O}_2 \) concentrations.

Discussion

**RoGFP2-Tsa2ΔC\(_R\), a sensor for the detection of \( \text{H}_2\text{O}_2 \) in five Chlamydomonas compartments**

Here we report on the development of Chlamydomonas reporter strains accumulating the ultra-sensitive, genetically-encoded \( \text{H}_2\text{O}_2 \) sensor RoGFP2-Tsa2ΔC\(_R\) (Morgan et al., 2016) in five different subcellular compartments (cytosol, nucleus, mitochondrial matrix, chloroplast stroma, and thylakoid lumen). The establishment of the sensor in Chlamydomonas was facilitated by using the Modular Cloning (MoClo) technology (Weber et al., 2011) and the recently generated Chlamydomonas MoClo kit (Crozet et al., 2018). The interchangeability of individual parts and the ability to assemble them in a single reaction, combined with the short generation time of Chlamydomonas, enabled iterative cycles of construct production and testing in a very short time frame. Accordingly, problems encountered with insufficient promoter strength or inefficient targeting of the encoded sensor to the chloroplast and thylakoid lumen were rapidly solved.

We demonstrate the suitability of RoGFP2-Tsa2ΔC\(_R\) to monitor intracellular \( \text{H}_2\text{O}_2 \) levels in Chlamydomonas with three different experimental setups, each having inherent advantages and limitations. First, we developed an assay using microtiter plates in a plate reader (Figures 2 and 3; Supplemental Figures S2C, S3, S4, S5E, and S5F). The advantage of this first setup is that changes in \( \text{H}_2\text{O}_2 \) levels can be monitored in real-time and at high throughput. Limitations of this assay are that cells
need to be concentrated by centrifugation and are in the dark, which precludes the use of this approach to the monitoring of responses to changes in light intensity. In a second setup, we continuously circulated cells between culture flask and a cuvette in a spectrofluorometer (Figure 5A). The advantage of this setup is that changes in \( \text{H}_2\text{O}_2 \) levels can be monitored in real-time under physiological conditions (if the dark period in the cuvette is not a problem), but this setup suffers from low throughput. In a third option, we trapped the sensor oxidation state with NEM (Figures 4 and 5B; Supplemental Figures S7 and S8). The advantage here is that measurements are under fully physiological conditions and at medium throughput. The limitation of NEM trapping is that \( \text{H}_2\text{O}_2 \) levels are not monitored in real time and therefore rapid \( \text{H}_2\text{O}_2 \) dynamics may be missed. The decision as to which setup to employ needs to be made depending on the specific biological question being asked.

**Properties of the roGFP2-Tsa2ΔCRC sensor in Chlamydomonas cells**

Overall, the kinetics of oxidation and reduction of roGFP2-Tsa2ΔCRC after the exogenous addition of \( \text{H}_2\text{O}_2 \) were very similar to those observed for this sensor in yeast (Morgan et al., 2016) and for the roGFP2-Orp1 sensor in Arabidopsis seedlings (Ugalde et al., 2021). Consistent with previous observations in yeast, we observed that cytosolic roGFP2-Tsa2ΔCRC is more than 20% oxidized at steady-state and that the oxidation of the untreated control sample decreases over time. In yeast, this decrease in sensor oxidation was shown to be caused by decreasing oxygen levels in the assay buffer (Morgan et al., 2016) and this is also likely the case in Chlamydomonas (Supplemental Figure S4). Thus, our observations indicate that, as in yeast, roGFP2-Tsa2ΔCRC functions as an ultra-sensitive \( \text{H}_2\text{O}_2 \) sensor in Chlamydomonas that enables the monitoring of ‘basal’ cellular \( \text{H}_2\text{O}_2 \) levels. The exquisite sensitivity of the roGFP2-Tsa2ΔCRC in Chlamydomonas is reflected by the finding that upon the exogenous addition of \( \text{H}_2\text{O}_2 \), a roGFP2-Orp1 sensor accumulating in the Chlamydomonas cytosol to levels similar as the roGFP2-Tsa2ΔCRC sensor was barely responsive (Supplemental Figure S5). Accordingly, while 0.1 mM of exogenously added \( \text{H}_2\text{O}_2 \) induced a strong roGFP2-Tsa2ΔCRC response in Chlamydomonas, 2 mM \( \text{H}_2\text{O}_2 \) was required to elicit a roGFP2-Orp1 response in Arabidopsis seedlings, albeit a comparison of single cells with cells within the confine of tissues might not be entirely fair (Ugalde et al., 2021).
RoGFP2-Tsa2ΔC_R targeted to the ER was fully oxidized (Figure 2H). Full oxidation was also observed for an unfused roGFP2 targeted to the ER of tobacco (*Nicotiana tabacum*) leaf cells (Meyer et al., 2007) and for HyPer targeted to the ER in mammalian cells (Mehmeti et al., 2012). This is almost certainly due to the oxidative milieu in the ER lumen rather than to high ER luminal H_2O_2 concentrations (Mehmeti et al., 2012). Specifically, the ER maintains an active disulfide-generating machinery and a relatively oxidized glutathione pool (in the absence of glutaredoxins) that does not allow sensor reduction (Schwarzlander et al., 2016). Hence, in this compartment disulfide bond formation is largely favored over disulfide reduction.

Cytosolic roGFP2 alone was insensitive to exogenously added H_2O_2 and was constantly in a highly reduced state (Figure 2C). RoGFP2 has been shown to be in equilibrium with the 2GSH/GSSG redox couple, which is catalyzed by enzymatically active glutaredoxins (Gutscher et al., 2008; Marty et al., 2009; Morgan et al., 2013). This points to a large excess of reduced glutathione (GSH) over oxidized glutathione (glutathione disulfide, GSSG) in the Chlamydomonas cytosol, as is the case for the cytosol of a wide variety of other cell types (Schwarzlander et al., 2016).

The expression of HyPer has been reported to be susceptible to gene silencing beyond the cotyledon stage in Arabidopsis (Exposito-Rodriguez et al., 2013). Although silencing can be circumvented, for example by transient expression in *Nicotiana benthamiana* abaxial epidermal cells (Exposito-Rodriguez et al., 2017), this is not ideal. We observed no silencing of our roGFP2-Tsa2ΔC_R constructs over a period of 1–2 years in Chlamydomonas. However, although we employed all ‘tricks’ for high-level transgene expression, which included using the strongest promoter currently available (*HSP70A-RBCS2*), a codon-optimized ORF containing the three *RBCS2* introns, the *RPL23* terminator, and the UVM4 expression strain (Schroda, 2019), sensor expression was only just sufficient to achieve a good signal-to-noise ratio in most compartments. When expression levels were reduced by about half, i.e., when the *PSAD* promoter was used instead of the *AR* promoter, or when the sensor was targeted to the thylakoid lumen, the signal became too noisy for reliable measurements, mainly because of the 405 nm channel (Supplemental Figure S3).
Hence, for future applications of this sensor, care must be taken that accumulation levels of the sensor in the chosen Chlamydomonas strain are sufficiently high.

**H$_2$O$_2$ produced by PET in the stroma under physiological conditions readily diffuses into the cytosol but not to other subcellular compartments**

Our observations demonstrated the suitability of roGFP2-Tsa2ΔC$_R$ to monitor H$_2$O$_2$ levels under physiologically relevant conditions. For example, we observed a rapid, PET-dependent increase in stromal H$_2$O$_2$ levels under high light intensities (1,000 μmol photons m$^{-2}$ s$^{-1}$), together with a concomitant increase in cytosolic H$_2$O$_2$. Changes in H$_2$O$_2$ in the mitochondrial matrix were barely detectable and no change was observed in the nucleus. We also failed to detect an increase in H$_2$O$_2$ levels in the nucleus, even when we increased the light intensity to 1,500 μmol photons m$^{-2}$ s$^{-1}$ (Figure 4D and 4E). However, we did detect H$_2$O$_2$ in the nucleus and mitochondria of cells supplemented with paraquat and/or metronidazole under low light intensities (Figure 4E). Thus, our findings partially corroborate earlier studies that reported leakage of PET-dependent H$_2$O$_2$ from the chloroplast into other compartments (Mubarakshina et al., 2010; Caplan et al., 2015; Exposito-Rodriguez et al., 2017; Ugalde et al., 2021). However, our results also indicate that the H$_2$O$_2$ scavenging capacity of the cytosol is sufficiently high to quench the comparably low H$_2$O$_2$ concentrations resulting from high light exposure, which effectively limits H$_2$O$_2$ diffusion into other subcellular compartments. By contrast, the cytosolic H$_2$O$_2$ scavenging capacity appears to become overwhelmed by the high H$_2$O$_2$ concentrations produced in the presence of metronidazole or paraquat (or exogenously added H$_2$O$_2$) and therefore H$_2$O$_2$ is not prevented from reaching other subcellular compartments.

In summary, our results strongly support the conclusion that cellular H$_2$O$_2$ scavenging enzymes limit the intracellular diffusion of H$_2$O$_2$, thereby leading to the establishment of steep intracellular H$_2$O$_2$ concentration gradients. This interpretation would be in line with the observation that efficient transfer of H$_2$O$_2$ from chloroplasts to the nucleus in *Nicotiana benthamiana* epidermal cells requires that the two compartments be in close proximity (Exposito-Rodriguez et al., 2017). This is possible because of the mobility of chloroplasts in land plant cells and appears unlikely for the architecture of Chlamydomonas cells with a single, large chloroplast.
H$_2$O$_2$ is produced rapidly under heat stress and does not derive from PET

Cytosolic and nuclear H$_2$O$_2$ levels increased rapidly and transiently during heat stress (Figure 5). Rapid accumulation of H$_2$O$_2$ has been previously reported in tobacco seedlings, spinach (*Spinacia oleracea*) leaves, or Arabidopsis and tobacco cell cultures when exposed to heat stress and therefore appears to be a conserved response in plant cells (Foyer et al., 1997; Vacca et al., 2004; Volkov et al., 2006; Gómez et al., 2008). In Chlamydomonas, cytosolic H$_2$O$_2$ levels closely paralleled the temperature change in the culture, indicating that H$_2$O$_2$ appears to be derived from a constitutive temperature-dependent cellular process (Figure 5A). As the increase in cytosolic H$_2$O$_2$ levels was also observed in the presence of DCMU, it cannot derive from PET, a hypothesis that is confirmed by the observed decline of H$_2$O$_2$ levels in the stroma during heat stress and recovery (Figure 5B). The mild, transient increase of H$_2$O$_2$ levels in mitochondria also argues against this organelle as a source for heat-induced H$_2$O$_2$ production. However, there are many alternative production sites for H$_2$O$_2$ in plant cells that might be temperature-controlled, such as limited substrate oxidases (with glycolate, xanthin, urate, sulfite, mono- and polyamines as substrates), type III peroxidases, or NAD(P)H oxidases at the plasma membrane (Cheeseman, 2007; Sun and Guo, 2016). Photorespiration is an unlikely source for H$_2$O$_2$ in Chlamydomonas, as it depends on input of electrons from PET and Chlamydomonas has a glycolate dehydrogenase that does not produce H$_2$O$_2$, rather than a glycolate oxidase (Kern et al., 2020).

Despite the rapid increase in H$_2$O$_2$ levels upon heat stress, Chlamydomonas cells accumulate scavenging enzymes such as superoxide dismutases, catalase, peroxiredoxins, or dehydroascorbate reductase only between 3 h and 24 h after the temperature shift (Mühlhaus et al., 2011; Hemme et al., 2014; Schroda et al., 2015). This observation indicates that the increased levels of H$_2$O$_2$ upon heat exposure are not detrimental to the cells during the first few hours at elevated temperatures and might play a role in signaling.

In summary, we report the generation of constructs enabling the accumulation of the ultra-sensitive H$_2$O$_2$ probe, roGFP2-Tsa2ΔC$_R$, in six different subcellular
compartments in Chlamydomonas. We show that these sensors respond readily to both exogenously added and endogenously produced H$_2$O$_2$ in five of these compartments and reveal that, by following the response of sensors targeted to multiple subcellular compartments, the existence of intracellular H$_2$O$_2$ gradients can be inferred. We anticipate that the future application of these sensors will allow exciting new insights into subcellular H$_2$O$_2$ homeostasis and dynamics.
Methods

Strains and Culture Conditions

*Chlamydomonas reinhardtii* UVM4 cells (Neupert et al., 2009) were grown in Tris-Acetate-Phosphate (TAP) medium (Kropat et al., 2011) on a rotatory shaker at a constant light intensity of ~40 μmol photons m$^{-2}$ s$^{-1}$ provided by MASTER LEDtube HF 1200 mm UO 16W830 T8 and 16W840 T8 (Philips). For high-light exposure, cells were grown to a density of ~1x10$^6$ cells/mL, transferred to an open 1-L beaker, placed on an orbital shaker, and exposed to 1,000 or 1,500 μmol photons m$^{-2}$ s$^{-1}$ provided by CF Grow® (CXB3590-X4). Transformation was performed with the glass beads method (Kindle, 1990) as described previously (Hammel et al., 2020) with constructs linearized with NotI digestion. Transformants were selected on TAP medium containing 100 μg mL$^{-1}$ spectinomycin.

Cloning of sensor and signal peptide coding sequences

The *roGFP2-Tsa2ΔC_R* and *roGFP2-Orp1* sequences (Morgan et al., 2016; Gutscher et al., 2009) were reverse-translated using the most highly used Chlamydomonas codons and equipped with the three Chlamydomonas RBCS2 introns as previously suggested for foreign genes (Schroda, 2019), but using as much of the native flanking sites of these introns (CAA-Intron 1-GA, ACG-Intron 2-GC, and GC-Intron 3-CTG) as possible. For *roGFP2-Tsa2ΔC_R*, an AAG Lys codon was converted into the suboptimal AAA Lys codon to eliminate a GAAGAC BpiI recognition site. The sequences were flanked with BsaI restriction sites such that upon BsaI digestion, fragments with AATG and TTCG overhangs are generated for the B3/4 position of level 0 parts according to the Modular Cloning (MoClo) syntax for plant genes (Weber et al., 2011; Patron et al., 2015) (Figure 1A). Synthesis and cloning into the pUC57 vector was accomplished by GeneCust (Luxembourg), giving rise to pMBS418 (*roGFP2-Orp1*) and pMBS419 (*roGFP2-Tsa2ΔC_R*). The latter construct was used as template for PCR to amplify a 898-bp fragment containing only the *roGFP2* coding sequence with the first RBCS2 intron. The PCR product was combined with the destination vector pAGM1287 (Weber et al., 2011), digested with BpiI and assembled by ligation into the level 0 construct pMBS467. The level 0 part encoding the HSP70B chloroplast transit peptide was generated via PCR using plasmid pMBS197 as template. This plasmid harbors the sequence encoding the
HSP70B transit peptide with an intron, in which a BsaI recognition site was eliminated. The resulting 251-bp product and plasmid pAGM1276 (Weber et al., 2011) were digested with BpiI and ligated to yield pMBS639. The level 0 part for the chloroplast transit peptide of CDJ1 was similarly produced by PCR using genomic DNA as template, generating a 166-bp product and giving rise to pMBS640. The same procedure was followed to produce level 0 parts for bipartite transit peptides for stroma and thylakoid lumen of PSAN and PSBO, to generate PCR products of 508 bp and 470 bp, giving rise to pMBS298 (PSAN) and pMBS641 (PSBO), respectively. The primers used are listed in Supplemental Table S1. All PCRs were conducted with Q5 High-Fidelity Polymerase (NEB) following the manufacturer’s instructions. Error-free cloning was verified by Sanger sequencing. These newly constructed level 0 parts were then complemented with level 0 parts (pCM) from the Chlamydomonas MoClo toolkit (Crozet et al., 2018) to fill the respective positions in level 1 modules as follows: A1-B1 – pCM0-015 (HSP70A-RBCS2 promoter + 5’ UTR); A1-B2 – pCM0-020 (HSP70A-RBCS2 promoter + 5’ UTR) or pCM0-016 (PSAD promoter + 5’ UTR); B2 – pCM0-053 (USPA chloroplast transit peptide), pMBS639 (HSP70B chloroplast transit peptide), pMBS640 (CDJ1 chloroplast transit peptide), pMBS298 (PSAN thylakoid lumen targeting peptide), pMBS641 (PSBO thylakoid lumen targeting peptide), pCM0-057 (HSP70C mitochondrial transit peptide), or pCM0-056 (BIP1 ER targeting signal); B3/4 – pMBS419 (roGFP2-Tsa2ΔC<sub>R</sub>), pMBS418 (roGFP2-Orp1) or pMBS467 (roGFP2); B5 – pCM0-100 (3xHA), pCM0-101 (MultiStop), pCM-111 (BIP ER retention sequence), or pCM-109 (SV40 nuclear localization signal); B6 – pCM0-119 (RPL23 3’ UTR). The HSP70A-RBCS2 fusion promoter used here contains 467 bp of HSP70A sequences upstream of the start codon in optimal spacing with respect to the RBCS2 promoter (Lodha et al., 2008; Strenkert et al., 2013). The respective level 0 parts and destination vector pICH47742 (Weber et al., 2011) were combined with BsaI and T4 DNA ligase and directionally assembled into the 11 level 1 modules shown in Figure 1B. The level 1 modules were then combined with pCM1-01 (level 1 module with the aadA gene conferring resistance to spectinomycin flanked by the PSAD promoter and terminator) from the Chlamydomonas MoClo kit, with plasmid pICH41744 containing the proper end-linker, and with destination vector pAGM4673 (Weber et al., 2011), digested with BpiI, and ligated to yield the 11 level 2 devices displayed in Figure 1B. All MoClo constructs employed and generated are
listed in Supplemental Table S2. All newly generated plasmids can be ordered from the Chlamydomonas Research Center (https://www.chlamycollection.org/).

**Protein analysis by SDS-PAGE**
Protein extraction (SDS–PAGE, semi-dry blotting and immunodetections were carried out as described previously (Hammel et al., 2020). Primary antibodies used for immunodetection were mouse anti-HA (Sigma H9658, 1:10,000) and rabbit anti-GFP (Roche, Cat. No. 11814460001, 1:5,000). The secondary antibody was m-IgGκBP-HRP (Santa Cruz Biotech sc-516102, 1:10,000). Densitometric band quantifications after immunodetections were done with the FUSIONCapt Advance program (PEQLAB).

**RoGFP2 fluorescence recordings**
RoGFP2 fluorescence was recorded using a CLARIOstar fluorescence plate reader (BMG-Labtech) as described previously for yeast (Morgan et al., 2016) and adapted to Chlamydomonas as follows: Chlamydomonas cells were grown in constant light to a density of ~3x10^6 cells/mL. 1x10^7 cells were used per well and were harvested by centrifugation at 3800 rpm for 2 min at 25°C. Cells were resuspended in 100 mM MES-Tris buffer (pH 7.0) to a cell density of 5x10^7 cells/mL; 200 μL of cell suspension was transferred to each well of a black, clear flat-bottomed 96-well imaging plate (BD Falcon 353219). For calibration and later data processing, control samples of fully oxidized and fully reduced sensors were prepared by adding N,N,N′,N′-tetramethylazodicarboxamide (diamide) to a final concentration of 20 mM and DTT to a final concentration of 100 mM to cells in two different wells, respectively. Additional wells to record roGFP2 fluorescence at steady-state, at increasing H_2O_2 concentrations, or after different treatments were prepared. Each biological replicate was measured as technical duplicates. Before starting the measurement, the 96-well imaging plate was centrifuged at 30 g for 5 min at 25°C. Chlamydomonas UVM4 recipient cells were always included as negative control. The measurement mode of the plate reader was based on the “Fluorescence Intensity” coupled with the “Bottom Optic” option, with a positioning delay of 0.2 sec, 40 flashes per well and the desired number of measurement cycles (about 200 cycles) and time per cycle (about 20 sec). The gain adjustment was set to 80% maximum and an
automatic gain adjustment for each wavelength was performed immediately before
the measurement. RoGFP2 exhibits two excitation maxima at 400 nm and 475–490
nm with fluorescence emission monitored at 510 nm. Therefore, we ensured that the
number of multichromatics was set to 2 and that the correct excitation (410 and
480 nm) and emission (510 nm) filters were selected. The measurement was started
and increasing H$_2$O$_2$ solutions were added after 10 cycles. After the fluorescence
measurement was done, MARS Data Analysis software was used to subtract the
auto-fluorescence of UVM4 control cells. The “blank corrected” values were taken
to calculate the degree of sensor oxidation (OxD) using the following equation (Meyer
and Dick, 2010):

$$
OxD\ RoGFP2 = \frac{I_{405}^{sample} \times I_{488}^{red} - I_{405}^{red} \times I_{488}^{sample}}{I_{405}^{sample} \times I_{488}^{red} - I_{405}^{sample} \times I_{488}^{ox} + I_{405}^{ox} \times I_{488}^{sample} - I_{405}^{red} \times I_{488}^{sample}}
$$

where $I$ is the fluorescence intensity at 510 nm after excitation at either 405 nm or
488 nm. The subscripts ‘ox’, ‘red’ and ‘sample’ indicate the fluorescence intensity
measured for the fully oxidized and fully reduced controls and the sample,
respectively.

**NEM-based alkylation for measuring H$_2$O$_2$ levels in cell cultures.**

The exact cell number of Chlamydomonas cells grown to 1-3x10$^6$ cells/mL was
determined and the volume to harvest 2.5x10$^7$ cells was calculated. For rapid
‘trapping’ of probe oxidation, 50-mL Falcon tubes were preloaded with one fourth
of the final volume with 100 mM MES-Tris (pH 7.0) buffer containing 40 mM NEM.
Chlamydomonas cells were harvested in prefilled Falcon tubes by centrifugation at
3,800 rpm for 3 min at room temperature and subsequently resuspended in 500 µL of
100 mM MES-Tris (pH 7.0) buffer containing 40 mM NEM to a final cell density of
5x10$^7$ cells/mL. 200 µL (corresponding to 10$^7$ cells) of cell suspension was
transferred to a 96-well plate. For calculating OxD, fully oxidized (with diamide) and
reduced (with DTT) samples were added and probe oxidation was measured
following the protocol described above with 15 measurements per biological sample
to reduce technical variability. To verify efficient trapping of the sensor oxidation state
with NEM, roGFP2 fluorescence recordings using 0.1 mM H$_2$O$_2$ was performed as
described above. 20 µL of NEM (200 mM stock) was added to a final concentration of 16.67 mM NEM per well at the indicated time differences.

Continuous monitoring of real-time changes in H₂O₂ levels during heat stress via connected tubing and a peristaltic pump
Chlamydomonas cells (~5x10⁶ cells/mL) were cultivated under agitation at a constant light intensity of ~40 µmol photons m⁻² s⁻¹ and continuously circulated through a quartz cuvette inserted into a spectrofluorometer (Jasco FP-8300) via connected tubing and a peristaltic pump (Watson Marlow 101U, flow rate: 1 mL/s). After 20 min, cells were exposed to 40°C for 30 min in a water bath, and shifted back to 23°C for 30 min. The ratio of the fluorescent light intensities emitted after excitation at 405 and 488 nm was monitored every 30 s. The measurement mode of the spectrofluorometer was based on the “Fixed wavelength measurement” with an excitation bandwidth of 5 nm and an emission bandwidth of 10 nm.

Confocal microscopy
All images were acquired using a Zeiss LSM880 AxioObserver confocal laser scanning microscope equipped with a Zeiss C-Apochromat 40x/1.2 W AutoCorr M27 water-immersion objective. Fluorescent signals of green fluorescent protein (GFP) (excitation/emission 488 nm/491-589 nm) and chlorophyll autofluorescence (excitation/emission 633 nm/647-721 nm) were processed using the Zeiss software ZEN 2.3 or ImageJ.

Supplemental Files.
Supplemental Figure S1. Screening of transformants accumulating the roGFP2 sensor by immunoblotting.
Supplemental Figure S2. Localization and fluorescence properties of a weakly accumulating, stroma-targeted roGFP2-Tsa2ΔCᵣ sensor.
Supplemental Figure S3. Fluorescence readout at excitation wavelengths 405 nm and 488 nm in the best accumulating ARpro and PSADpro transformants.
Supplemental Figure S4. Real-time monitoring of H₂O₂ levels in the cytosol and stroma under steady-state conditions at different cell densities.
Supplemental Figure S5. Establishment of a cytosolic roGFP2-Orp1 sensor.
Supplemental Figure S6. Effect of metronidazole and paraquat on growth.
Supplemental Figure S7. Analysis of fluorescence properties of the roGFP2-Tsa2ΔCR sensor at different temperatures.
Supplemental Figure S8. Analysis of the effects of NAD(P)H oxidase inhibitor DPI and heat on sensor oxidation.
Supplemental Table S1. Primers used for cloning.
Supplemental Table S2. MoClo constructs employed and generated.
Supplemental Table S3. Transgenic lines generated, number of transformants analyzed, and number of localization events observed in different cells.
Supplemental Data Set S1. Summary of statistical analyses.

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Author Contributions
J.N. generated all constructs and performed all experiments. D.S recorded the confocal microscopy images. J.O. helped with setting up the fluorescence recordings. M.S. and B.M. conceived and supervised the project and wrote the article with contributions from all authors.
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Figure 1. Targeting roGFP2 sensors to various compartments of a Chlamydomonas cell
(A) Schematic representation of the level 0 part of the roGFP2-Tsa2ΔC<sub>R</sub> H<sub>2</sub>O<sub>2</sub> sensor. The 2,132-bp coding region (exons shown as boxes), interrupted by the three RBCS2 introns (thin lines), was synthesized with optimal Chlamydomonas codon usage. RoGFP2 (light gray) is separated from Tsa2ΔC<sub>R</sub> (dark gray) by a linker (gray).

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(B) Using MoClo, the roGFP2-Tsa2ΔCₘ part or only the roGFP2 part was equipped with the HSP70A-RBCS2 promoter (ARpro) or the PSAD promoter (PSADpro) and the RPL23 terminator (RPL23-T) as well as various sequences adding N- or C-terminal targeting signals to target the sensors to the cytosol (cyt), the stroma, the thylakoid lumen (lumen), the nucleus (nuc), mitochondria (mito), or the endoplasmic reticulum (ER). The resulting level 1 modules were combined with another level 1 module comprising the aadA resistance marker flanked by the PSAD promoter and terminator (Specₚ) to yield the 11 level 2 devices shown.

(C) Comparison of roGFP2 accumulation levels in transformants generated with the level 2 devices shown in (B). The transformants shown represent those with highest protein accumulation levels among at least 13 independent transformants screened for each construct. Total cell protein extracts corresponding to 1.5 µg chlorophyll for each transformant and the UVM4 recipient strain (none) were separated by SDS-PAGE and analyzed by immunoblotting using an antibody against GFP. Signals retrieved from three independent experiments were quantified and normalized to the signal obtained with the transformant harboring a construct driving the expression of roGFP2-Tsa2ΔCₘ (encoding a cytosolic sensor) from the HSP70A-RBCS2 promotor (ARpro), which was set to 100%. Mean values are given below the panel (± standard deviation, n=3). A representative experiment is shown, with Ponceau staining demonstrating equal loading. The asterisk indicates a non-specific cross-reaction with the GFP antibody.

(D) Representative confocal microscopy images of individual cells of the untransformed UVM4 strain (none) and the transformants analyzed in (C). Shown are GFP fluorescence, chlorophyll autofluorescence (Chl) and both signals merged. 4-50 individual cells were analyzed per transformant strain, with consistent localization (Supplemental Table S3).
Figure 2. Real-time monitoring of H$_2$O$_2$ levels in different subcellular compartments under steady-state conditions and after the addition of H$_2$O$_2$.

(A-H) Fluorescence measurement of roGFP2-Tsa2ΔC$_R$ and roGFP2 in transformant strains shown in Figure 1C and 1D under steady-state conditions (no H$_2$O$_2$ added, blue) and after the addition of H$_2$O$_2$ at concentrations of 0.1 mM (green), 0.5 mM (magenta), and 1 mM (yellow). Values were calculated relative to those obtained for fully reduced (0) and fully oxidized (1) sensors. Shown are means of three independent experiments, error bars represent standard deviation. Sensors were targeted to the cytosol (A-C), the chloroplast stroma (D), the chloroplast lumen (E), the mitochondrial matrix (F), the nucleus (G), or the endoplasmic reticulum (H).
Figure 3. Verification of efficient trapping of the sensor oxidation state with N-ethylmaleimide (NEM).
Chlamydomonas cells accumulating roGFP2-Tsa2ΔCt in the cytosol were treated with 0.5 mM H₂O₂ (magenta) and 16.67 mM NEM was added with time differences of 0 s (black), 37 s (yellow), and 1850 s (green). No H₂O₂ was added in the control (blue). Fluorescence was recorded and values were calculated relative to those obtained for fully reduced (0) and fully oxidized (1) sensors. Shown are means of three independent experiments, error bars represent standard deviation.
Figure 4.Monitoring H$_2$O$_2$ levels in different subcellular compartments under high-light exposure and after the application of metronidazole or paraquat.

Chlamydomonas transformant cells, accumulating roGFP2-TsaΔCix in the stroma (A), cytosol (B), mitochondrial matrix (C), or nucleus (D) were kept in a low light intensity of 30 µmol photons m$^{-2}$ s$^{-1}$ (blue) or exposed to high light of 1,000 µmol photons m$^{-2}$ s$^{-1}$ in the absence (yellow) or presence (red) of 10 µM DCMU. The oxidation state of the sensor was trapped by the addition of NEM and roGFP2 fluorescence was measured in a plate reader. Shown are mean values from three independent experiments, error bars represent standard deviation. Asterisks indicate significant differences with respect to the low light control (two-tailed, unpaired t-test with Bonferroni-Holm correction, $P < 0.05$). The absence of an asterisk means that there were no significant differences.

(E) Transformant cells accumulating roGFP2-TsaΔCix in the indicated compartments were grown in low light of 30 µmol photons m$^{-2}$ s$^{-1}$ (0) and then exposed to 1,500 µmol photons m$^{-2}$ s$^{-1}$ for 30 min, or kept at low light for 30 min in the presence of 2 mM metronidazole (M) or 1 µM paraquat (P), alone or together with 10 µM DCMU (MD, PD). The oxidation state of the sensor was trapped by the addition of NEM and roGFP2 fluorescence was measured in a plate reader. Error bars represent standard deviation from three independent experiments. Asterisks indicate significant differences with respect to the low light control (two-tailed, unpaired t-test with Bonferroni-Holm correction, $P < 0.05$). The absence of an asterisk means that there were no significant differences.
Figure 5. Monitoring H$_2$O$_2$ levels during heat stress and recovery

(A) Chlamydomonas cells accumulating roGFP2-Tsa2ΔC$_R$ in the cytosol were grown in a low light intensity of 30 µmol photons m$^{-2}$ s$^{-1}$ for 20 min, exposed to 40°C for 30 min, and shifted back to 23°C for 30 min. The ratio of the fluorescence light emitted after excitation at 405 and 488 nm, respectively, was monitored in real time using a spectrofluorometer (black line). The temperature in the culture was monitored in parallel (gray circles). Shown are mean values from three independent experiments, error bars represent standard deviation.

(B) Transformant cells accumulating roGFP2-Tsa2ΔC$_R$ in the cytosol, stroma, matrix, or nucleus were grown in a low light intensity of 30 µmol photons m$^{-2}$ s$^{-1}$ at 23°C (0), exposed to 40°C for 30 min (HS), and shifted back to 23°C for another 30 min (R). If indicated, cultures were supplied with 10 µM DCMU before the experiment was started. The oxidation state of the sensor was trapped by the addition of NEM and roGFP2 fluorescence was measured in a plate reader. Error bars represent standard deviation from three independent experiments. Asterisks indicate significant differences with respect to the 23°C (0) control (two-tailed, unpaired t-test with Bonferroni-Holm correction, P < 0.01). The absence of an asterisk means that there were no significant differences (P ≥ 0.05).
