microPublication BIOLOGY

11/3/2026 - *Open Access*

The Amplite® fluorometric assay does not reliably quantify H_2O_2 in *Arabidopsis* leaf extracts

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Abstract

Hydrogen peroxide (H_2O_2) is a key reactive oxygen species in plants. We evaluated a commercial Amplite® fluorometric H_2O_2 assay in *Arabidopsis thaliana* leaf extracts. The extracts produced strong Amplite® Red-dependent fluorescence without exogenous horseradish peroxidase (HRP), and fluorescence signals were higher following high-light treatment than following dark treatment. However, this difference could not be quantitatively attributed to H_2O_2: adding 5–15 µM H_2O_2 produced no concentration-dependent increase with or without HRP, whereas the same additions increased fluorescence in assay buffer. Thus, under the conditions examined, the fluorescence observed in *Arabidopsis* leaf extracts did not permit reliable H_2O_2 quantification.

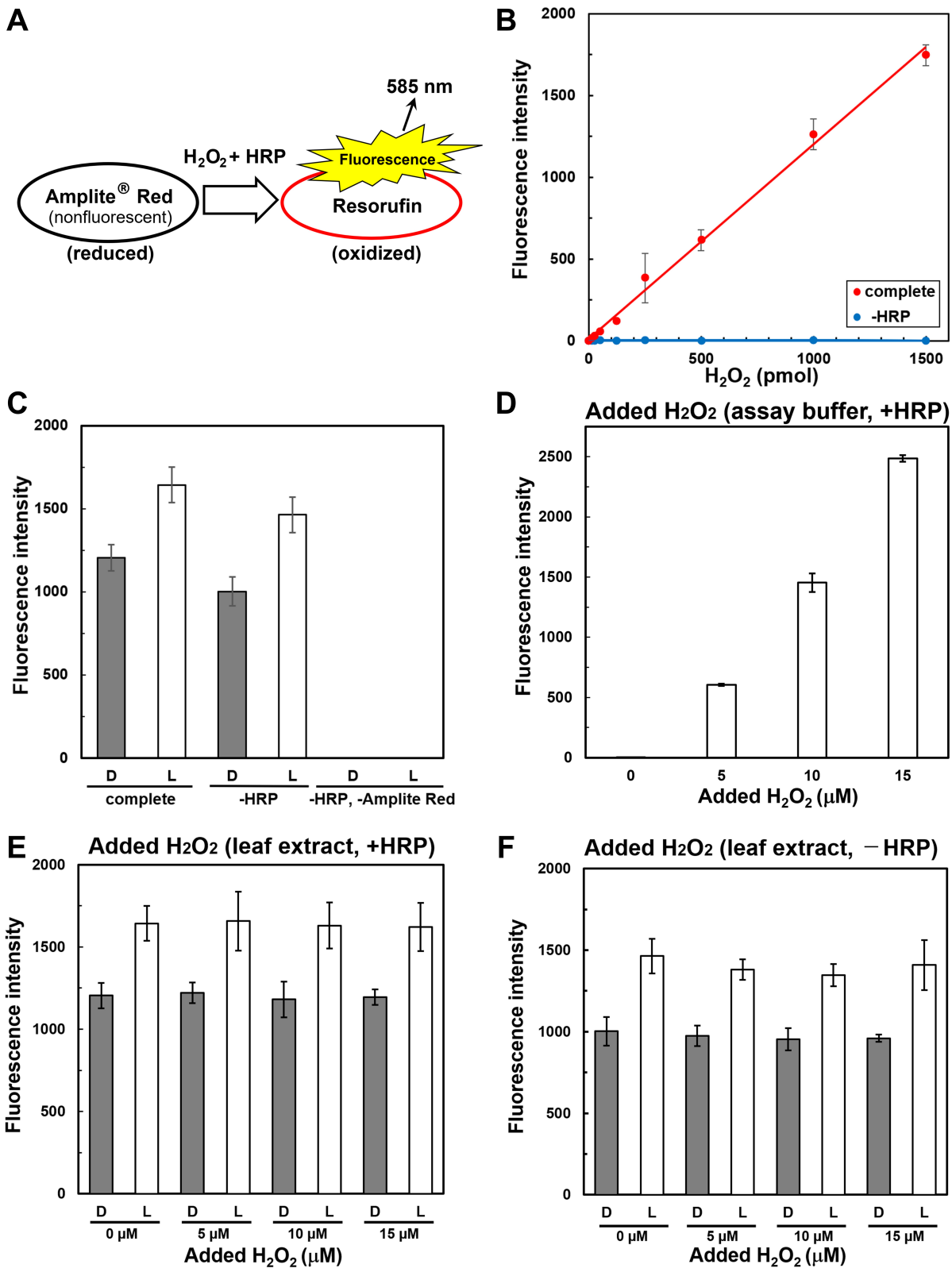


Figure 1. Fluorescence responses of the Amplite® assay to H2O2 in assay buffer and *Arabidopsis thaliana* leaf extracts:

(A) Schematic illustration of the detection mechanism of the Amplite® Fluorimetric Hydrogen Peroxide Assay Kit (Red fluorescence). **(B)** Standard curve generated using hydrogen peroxide standards (0, 12.5, 25, 50, 125, 250, 500, 1,000 and 1,500 pmol per 50 µL). **(C)** Fluorescence measurements of *Arabidopsis* leaf extracts prepared from approximately 40 mg fresh weight under the assay conditions defined below. **(D–F)** Fluorescence responses to added H_2O_2 in assay buffer and *Arabidopsis* leaf extracts. Exogenous H_2O_2 was added at final concentrations of 0, 5, 10, and 15 µM to assay buffer containing Amplite® Red and HRP but no leaf extract **(D)**, to *Arabidopsis* leaf extracts under complete assay conditions **(E)**, or to *Arabidopsis* leaf extracts under assay conditions lacking HRP **(F)**. D and L indicate extracts prepared from dark-treated and high-light-treated plants, respectively, in panels C, E and F. Data represent the mean ± SD. Data in panels B and D were obtained from three independent assay replicates, whereas data in panels C, E, and F were obtained from three biological replicates. “complete” indicates reactions containing Amplite® Red and HRP; “−HRP” indicates reactions without HRP; and “−HRP, −Amplite® Red” indicates reactions without either HRP or Amplite® Red. Panel B and panels D–F were obtained in separate assay runs; panels D–F were measured in parallel using the same instrument settings.

Description

Reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), are generated during the light reactions of photosynthesis. Excess ROS can damage photosynthetic components, including Photosystem I, thereby reducing the efficiency of photosynthetic electron transport (Vilyanen and Kozuleva, 2025). ROS are traditionally visualized in plant leaves using nitro blue tetrazolium staining for superoxide (Jabs et al., 1996; Lee et al., 2002; Okegawa and Motohashi, 2015) and 3,3′-diaminobenzidine (DAB) staining for hydrogen peroxide (Bindschedler et al., 2006; Rojas et al., 2012; Okegawa and Motohashi, 2015). However, although these methods enable spatial visualization in leaves, they require multiple procedural steps and are not readily quantitative. Fluorescence-based assays are widely used for hydrogen peroxide detection in non-plant systems (Mori et al., 1991; Von Rein and Hirschelmann, 1991; Zhang et al., 2021). Commercial kits are readily available for fluorescence-based H_2O_2 detection. Because H_2O_2 levels in leaves are relatively low, we selected a highly sensitive Amplite® fluorometric H_2O_2 assay. The assay is based on HRP-catalyzed oxidation of Amplite® Red in the presence of H_2O_2 (Zhou et al., 1997). Amplite® Red (CAS: 119171-73-2) is chemically identical to Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine). Amplex Red has been used without exogenous HRP for semiquantitative visualization of ROS in living *Arabidopsis* root hairs (Kubenova et al., 2023), indicating that probe fluorescence can be generated in intact plant tissues without added HRP. However, the observation of such fluorescence does not, by itself, establish either that the signal originates from H_2O_2 or that it quantitatively reflects H_2O_2 concentration. We therefore evaluated whether the fluorescence measured with the Amplite® assay in *Arabidopsis* leaf extracts could be used for H_2O_2 quantification by determining whether it increased in response to known additions of H_2O_2.

Amplite® Red undergoes horseradish peroxidase (HRP)-catalyzed oxidation in the presence of hydrogen peroxide, generating fluorescence with an emission maximum at ~585 nm (Figure 1A). We assessed whether this fluorescence could be used to quantify hydrogen peroxide using a fluorescence microplate reader. We first generated a standard curve in assay buffer using 0–1.5 nmol H_2O_2 standards (Figure 1B, complete). Omission of HRP from the assay solution eliminated the detectable fluorescence (Figure 1B, −HRP), indicating that H_2O_2 alone did not produce detectable fluorescence under the conditions tested.

We next examined the fluorescence response of *Arabidopsis* leaf extracts. In land plants, high-light conditions promote ROS production by the excess reducing power generated through photosynthetic electron transport in chloroplasts; high-light treatment was therefore used as a physiological treatment expected to increase ROS production relative to dark treatment (Okegawa and Motohashi, 2015). Leaf extracts were prepared from plants exposed to either dark or high-light conditions. Amplite® Red-dependent fluorescence was observed under both dark and high-light conditions; however, the high-light-treated samples exhibited approximately 1.4-fold higher intensity than the dark-treated samples (Figure 1C, dark and light (complete)). Furthermore, no fluorescence signals were detected in the leaf extracts in the absence of both HRP and Amplite® Red (Figure 1C, dark and light (−HRP, −Amplite® Red)). However, this treatment-associated difference in fluorescence did not establish a quantitative difference in H_2O_2 concentration.

Subsequently, we examined the fluorescence response of the leaf extracts in the absence of exogenous HRP (Figure 1C, −HRP). Although H_2O_2 alone did not generate detectable Amplite® Red fluorescence in assay buffer without HRP (Figure 1B), the leaf extracts produced strong fluorescence even when HRP was omitted. The fluorescence intensities were comparable to those obtained under the complete assay conditions. These results demonstrate that exogenous HRP was not required for the generation of Amplite® Red-dependent fluorescence in the leaf extracts. However, this finding does not establish that the observed fluorescence originated from H_2O_2 or quantitatively reflected H_2O_2 concentration, because endogenous peroxidases or other components of the leaf extract may support probe oxidation. We therefore tested whether the leaf-extract fluorescence increased in response to known additions of H_2O_2.

To determine whether the fluorescence observed in *Arabidopsis* leaf extracts quantitatively reflected H_2O_2, known amounts of H_2O_2 were added to the extracts. Although the same amounts of H_2O_2 produced the expected increases in

fluorescence in assay buffer (Figure 1D), no corresponding increase was observed in the leaf extracts either with or without exogenous HRP (Figures 1E and 1F). Thus, the relationship between H_2O_2 concentration and fluorescence established in assay buffer could not be applied to the leaf extracts. Moreover, the present results do not establish that the fluorescence observed in the leaf extracts originated from H_2O_2. Possible explanations include rapid removal of added H_2O_2 by endogenous H_2O_2-scavenging activities, inhibition or quenching of the Amplite® Red reaction by components of the leaf extract, and strong probe-derived fluorescence that is not quantitatively related to H_2O_2 concentration. The present experiments do not distinguish among these possibilities. An Amplex Red-based method has previously been used to quantify H_2O_2 in tissue extracts from several plant species, including *Arabidopsis*, after optimization of the extraction conditions and evaluation of tissue-dependent loss of added H_2O_2 (Chakraborty et al., 2016). Thus, the present results do not indicate that Amplex Red is intrinsically unsuitable for plant samples. Our conclusion is limited to the conditions tested here: fluorescence from *Arabidopsis* leaf extracts could not be reliably converted to H_2O_2 concentrations using a standard curve prepared in assay buffer.

In conclusion, *Arabidopsis* leaf extracts produced substantial Amplite® Red fluorescence both with and without exogenous HRP. However, fluorescence did not increase in proportion to the amount of H_2O_2 added to the extracts, whereas a clear concentration-dependent response was obtained in assay buffer. Thus, irrespective of the mechanism responsible for probe oxidation or loss of the added H_2O_2, the buffer-based standard curve could not be used to quantify H_2O_2 in the leaf extracts. Therefore, neither the observed fluorescence nor the difference between high-light- and dark-treated samples could be quantitatively attributed to H_2O_2. These results show that the Amplite® fluorometric assay does not provide reliable quantitative measurements of H_2O_2 in *Arabidopsis* leaf extracts under the conditions tested.

Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) seeds were surface-sterilized in 5% (v/v) sodium hypochlorite for 5 minutes, and sown on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1.2% (w/v) agar and 1% (w/v) sucrose. Plants were grown in Petri dishes under continuous light (50 µmol photons m^{-2} s^{-1}) at 23 °C for 21 days in a growth chamber.

Preparation of Arabidopsis leaf extracts

For dark treatment, plants were incubated in complete darkness for 12 hours. For high-light treatment, plants were exposed to high light intensity (1,000 µmol photons m^{-2} s^{-1}) for 1 hour as a physiological treatment expected to increase ROS production. After treatment, leaves (approximately 40 mg fresh weight) were harvested and immediately frozen in liquid nitrogen in a 2-mL screw-cap tube with a loop cap containing a 7-mm zirconia bead (1392-200-SS-LP-C, WATSON, Tokyo, Japan). Tissue samples were homogenized using a bead homogenizer (Fisherbrand Bead Mill24, Fisher Scientific, Waltham, MA, USA) at speed (S) = 2.10 m s^{-1} for one 15-s cycle.

Amplite® fluorometric assay of Arabidopsis leaf extracts

Fluorescence responses were measured using the Amplite® Fluorimetric Hydrogen Peroxide Assay Kit (Red fluorescence; AAT Bioquest, Pleasanton, CA, USA). Assay buffer (1,500 µL) was added to homogenized samples, followed by centrifugation at 20,000 × *g* for 10 minutes. Assay reactions were prepared using the manufacturer-recommended reaction volumes: 50 µL of each supernatant was mixed with 50 µL of working solution containing Amplite® Red with or without HRP, giving a final assay volume of 100 µL per well. After incubation for 30 min at room temperature while protected from light, fluorescence was measured in a 96-well black plate (#3915, Corning) using a fluorescence microplate reader (BioTek Synergy HTX, Agilent, Santa Clara, CA, USA). Excitation was set at 528 nm (20-nm bandwidth), and emission was detected at 590 nm (20-nm bandwidth).

To examine the fluorescence response to added H_2O_2, H_2O_2 was added to 50-µL aliquots of the leaf extracts to give final assay concentrations of 0, 5, 10, and 15 µM. These samples were then assayed with Amplite® Red in the presence or absence of HRP as described above. Control reactions contained assay buffer instead of leaf extract. The control and leaf-extract reactions shown in Figure 1D–F were analyzed in parallel under the same measurement conditions.

Use of artificial intelligence

OpenAI Codex (GPT-5.6 Sol) was used solely for English-language refinement and consistency checks during manuscript revision. It was not used to draft the original manuscript, generate experimental data, perform primary data analysis, or determine scientific conclusions. All AI-assisted changes were reviewed and approved by the authors.

Reagents

Hydrogen peroxide detection assay kit

The Amplite® Fluorimetric Hydrogen Peroxide Assay Kit (Red fluorescence; AAT Bioquest Cat. No. 11501) was used for fluorometric measurements in *Arabidopsis* leaf extracts. The kit includes Amplite® Red, 3% hydrogen peroxide, assay buffer, HRP, and DMSO.

Acknowledgements: We thank Dr. N. Sato for excellent technical assistance.

References

Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, Denoux C, Hayes T, Gerrish C, Davies DR, Ausubel FM, Bolwell GP. 2006. Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. The Plant Journal 47: 851-863. DOI: 10.1111/j.1365-313X.2006.02837.x

Chakraborty S, Hill AL, Shirsekar G, Afzal AJ, Wang GL, Mackey D, Bonello P. 2016. Quantification of hydrogen peroxide in plant tissues using Amplex Red. Methods 109: 105-113. PubMed ID: 27476009

Jabs T, Dietrich RA, Dangl JL. 1996. Initiation of Runaway Cell Death in an *Arabidopsis* Mutant by Extracellular Superoxide. Science 273: 1853-1856. DOI: 10.1126/science.273.5283.1853

Kuběnová L, Haberland J, Dvořák P, Šamaj J, Ovečka M. 2023. Spatiotemporal distribution of reactive oxygen species production, delivery, and use in Arabidopsis root hairs. Plant Physiology 193: 2337-2360. PubMed ID: 37666000

Lee BH, Lee H, Xiong L, Zhu JK. 2002. A Mitochondrial Complex I Defect Impairs Cold-Regulated Nuclear Gene Expression. The Plant Cell 14: 1235-1251. DOI: 10.1105/tpc.010433

Mori I, Fujita Y, Toyoda M, Kato K, Yoshida N, Akagi M. 1991. Fluorimetric determination of hydrogen peroxide by use of the fluorescence reaction between N-(4′-hydroxyphenyl)-N-(4-methylquinolinyl)amine and cobalt(II) in the presence of trimethylstearylammonium chloride. Talanta 38: 683-686. DOI: 10.1016/0039-9140(91)80157-U

Murashige T, Skoog F. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15: 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x

Okegawa Y, Motohashi K. 2015. Chloroplastic thioredoxin *m* functions as a major regulator of Calvin cycle enzymes during photosynthesis *in vivo*. The Plant Journal 84: 900-913. DOI: 10.1111/tpj.13049

Rojas CM, Senthil-Kumar M, Wang K, Ryu CM, Kaundal A, Mysore KS. 2012. Glycolate Oxidase Modulates Reactive Oxygen Species–Mediated Signal Transduction during Nonhost Resistance in *Nicotiana benthamiana* and *Arabidopsis*. The Plant Cell 24: 336-352. DOI: 10.1105/tpc.111.093245

Vilyanen DV, Kozuleva MA. 2025. Photosynthetic Control and Its Role in Protection of Photosystem I against Photoinhibition. Biochemistry (Moscow) 90: 840-859. DOI: 10.1134/S0006297925601121

Von Rein C, Hirschelmann R. 1991. Comparison of a fluorimetric and a colorimetric method for the determination of hydrogen peroxide production by rat pleural macrophages. Journal of Pharmacological Methods 26: 61-65. DOI: 10.1016/0160-5402(91)90054-9

Zhang GQ, Li YS, Liu WP, Gao XF. 2021. A fluorimetric and colorimetric dual-signal sensor for hydrogen peroxide and glucose based on the intrinsic peroxidase-like activity of cobalt and nitrogen co-doped carbon dots and inner filter effect. Analytical Methods 13: 3196-3204. DOI: 10.1039/D1AY00781E

Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. 1997. A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases. Analytical Biochemistry 253: 162-168. DOI: 10.1006/abio.1997.2391

Funding: This work was supported by JSPS KAKENHI Grant Number 16K07409 and by a grant from the Center for Plant Sciences, Kyoto Sangyo University.

Conflicts of Interest: The authors declare that there are no conflicts of interest present.

Author Contributions: Akira Fukuwatari: investigation, methodology, data curation, writing - review editing. Yuto Nawa: investigation, methodology, data curation, writing - review editing. Ken Motohashi: conceptualization, methodology, supervision, validation, writing - original draft, writing - review editing.

Reviewed By: Anonymous

Nomenclature Validated By: Anonymous

History: Received January 7, 2026 **Revision Received** September 7, 2026 **Accepted** October 27, 2026 **Published Online** November 3, 2026 **Indexed** November 17, 2026

11/3/2026 - *Open Access*

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Citation: Fukuwatari A, Nawa Y, Motohashi K. 2026. The Amplite® fluorometric assay does not reliably quantify H_2O_2 in *Arabidopsis* leaf extracts. microPublication Biology. 10.17912/micropub.biology.002016