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Article

A Chimeric Peptide for Shielding Plant Photosynthetic Systems against Excess Light Stress via Chloroplast-Targeted ROS Quenching

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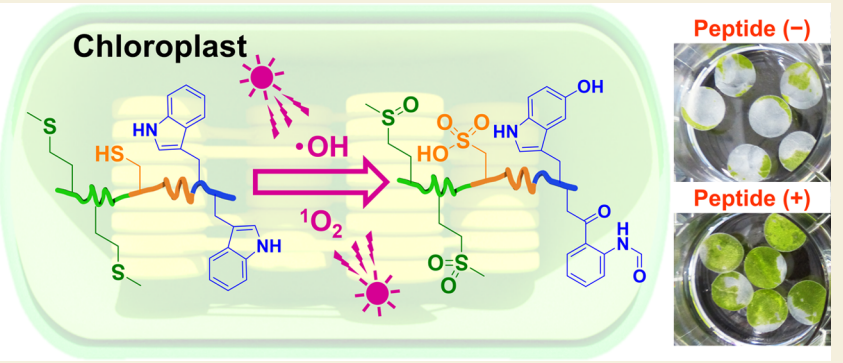

ABSTRACT: The ability to quench reactive oxygen species (ROS) overproduced in plant chloroplasts under light stress conditions is essential for securing plant photosynthetic performance and agricultural yield. Although genetic engineering can enhance plant stress resistance, its widespread application faces limitations due to challenges in successful transformation across plant species and public acceptance concerns. This study proposes a nontransgenic chemical approach using a designed chimeric peptide that scavenges ROS within plant chloroplasts for managing light stress. The chimeric peptide was strategically designed by combining cell-penetrating and chloroplast-targeting sequences, each with antioxidant ability against destructive ROS such as hydroxyl radical (•OH) and singlet oxygen ($^1O_2$). Our analyses involving various cell-penetrating peptides and a chloroplast-targeting peptide revealed that the •OH-scavenging ability predominantly relied on side chain oxidation in tryptophan residues, while the $^1O_2$-quenching capacity was attributed to the oxidation of cysteine and methionine side chains. We further demonstrated that the chimeric peptide could traverse the cell wall and membranes to reach chloroplasts, where it scavenged •OH and $^1O_2$ and alleviated light-stress-induced chlorophyll degradation in leaves. Foliar spraying of the peptide successfully protected photosynthetic activity in leaves exposed to excessive light, highlighting its potential for practical agricultural applications. This work can offer a promising approach for managing abiotic stress without genetic modifications and provide valuable insights into the design of effective peptide-based ROS quenchers specifically targeting plant chloroplasts.

KEYWORDS: *Chimeric peptides, ROS scavenging, Chloroplast, Photosynthesis protection, Amino acid oxidation*

INTRODUCTION

Safeguarding plants from adverse environmental conditions is essential for ensuring global food security as the global population grows and agricultural products is adversely impacted by climate change.[1] Light energy is a vital source for plant growth through photosynthetic carbon assimilation, but excessive light, which surpasses photosynthetic capacity, can serve as a major environmental stressor, negatively affecting plant growth and productivity.[2] Under conditions of excessive light, plants overproduce reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radical (•OH), as byproducts of photosynthesis in chloroplasts.[3] Light-dependent ROS overproduction is known as photooxidative stress,[4] which causes damage to various cellular components, including pigments, proteins, nucleic acids, and lipids, ultimately resulting in cell death. Excessive ROS accumulation in chloroplasts leads to reduced photosynthetic efficiency through the inactivation of the photosynthetic machinery, specifically photosystem II (PSII), in a process termed photoinhibition.[5] To protect plants from photooxidative stresses and photoinhibition, researchers have employed a transgenic approach in which enzymatic and nonenzymatic antioxidants are efficiently synthesized in transgenic plants.[6,7] However, genetic trans-

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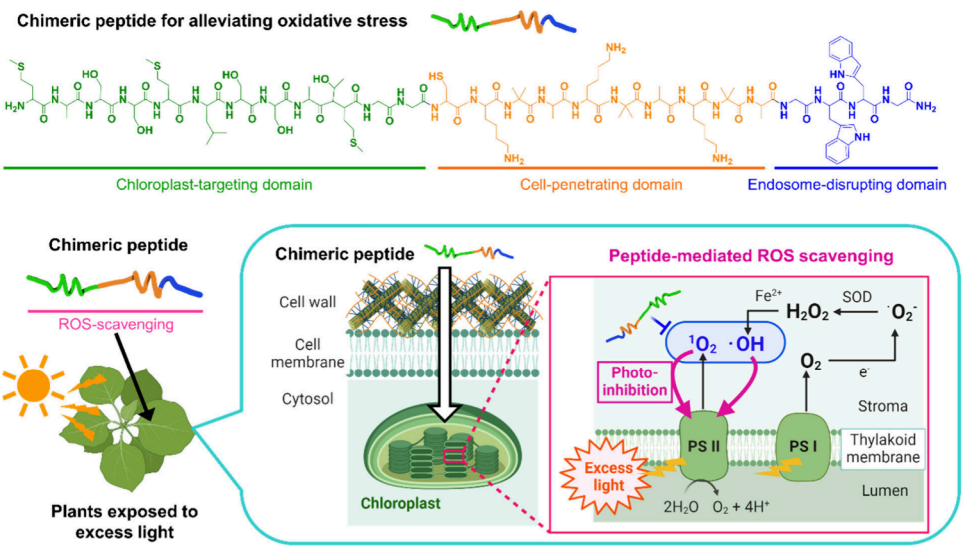

Figure 1. Schematic overview of chimeric peptide-mediated ROS scavenging in plant chloroplasts for alleviating photooxidative stress under conditions of excessive light. The chimeric peptide consists of chloroplast-targeting, cell-penetrating, and endosome-disrupting domains with antioxidant activity. Abbreviations: photosystem I (PSI), superoxide dismutase (SOD). Created with BioRender.com.

formation remains challenging for many plant species due to difficulties in gene transfer and/or regeneration.[8] Additionally, transgenic plants often experience unexpected growth inhibition and are strictly regulated in many countries and regions due to biosafety concerns,[9,10] which may offset the benefits of the transgenic approach. To overcome these limitations, a nontransgenic approach for alleviating photooxidative stresses in plants is desirable.

The targeted delivery of antioxidants to chloroplasts is a promising strategy for protecting plants from photooxidative stress by eliminating excess ROS.[11,12] This process requires a multifunctional tool that can efficiently enter plant cells, target chloroplasts, and scavenge ROS, especially $^1O_2$ and •OH, which are more reactive than H_2O_2 and O^{2-} and resistant to elimination by plant antioxidant enzymes.[13] Limited systems have been reported to quench harmful ROS like •OH in plant chloroplasts.[14−16] Cerium oxide nanoparticles have a unique ability to reduce •OH in a catalytic manner.

However, their large-scale application could be limited by the absence of a scalable delivery method like foliar spraying. Moreover, the mechanisms underlying their targeting and entering chloroplasts remains elusive. One of the key challenges is integrating antioxidant, cell-permeation, and chloroplast-targeting capabilities into an ROS-scavenging system. We focused on peptides as a versatile platform because of their diverse functions.[17] For instance, cell-penetrating peptides (CPPs) facilitate cargo transport into plant cells,[18,19] while chloroplast-targeting peptides (CTPs) enable the transfer of foreign DNA and nanomaterials into plant chloroplasts.[16,20,21] Antioxidant peptides can scavenge ROS in mammalian cells,[22] but their application in plants, correlation between amino acid sequence and antioxidant capacity, and design for cell permeation and chloroplast targeting remain unexplored.

The goal of this study was to develop a versatile molecular tool to manage photooxidative stress in plants by combining cell-penetrating, chloroplast-targeting, and ROS-scavenging capabilities into a single peptide (Figure 1). Initially, we screened various CPPs for their ability to eliminate •OH and $^1O_2$, identifying a promising CPP with tryptophan (Trp) and cysteine (Cys) residues that contribute to scavenging •OH and $^1O_2$, respectively. To confer chloroplast-targeting capability, this selected CPP was fused with a CTP sequence, in which methionine (Met) residues served as additional antioxidants effective against $^1O_2$. This process generated an all-in-one chimeric peptide for chloroplast-targeted ROS scavenging. When applied to plant leaves, this chimeric peptide exhibited efficient •OH- and $^1O_2$-scavenging capacities within plant chloroplasts. The *in vivo* ROS-scavenging ability of the peptide significantly suppressed chlorophyll degradation in leaf discs under photooxidative stress induced by an ROS-generating herbicide or excessive light exposure. Furthermore, we demonstrated that foliar application of the peptide through both spraying and syringe infiltration effectively alleviated photoinhibition in a model plant under excessive light conditions. The multifunctional nature of this chimeric peptide offers a nontransgenic and scalable approach to mitigate photooxidative stress and photoinhibition in plants, showing promise for various agronomic applications.

RESULTS AND DISCUSSION

Screening of CPPs for ROS Scavenging

We previously explored the cellular uptake efficiency of 55 CPPs in various model plant systems.[23] Recently, we developed diverse CPP types, including peptides with unnatural amino acids for improved enzymatic stability,[24] endosomal escaping peptides for enhanced cytosolic translocation,[21,25] and macropinocytosis-inducing peptides.[19,26] We selected 21 CPPs with diverse amino acid sequences to investigate their ROS-scavenging ability (Table S1). The selected CPPs encompass amphiphilic peptides with multiple lysine (MG2d, BP100, $(KL)_5$, A_5K_5, $(AK)_5$), histidine (LAH4L1), glutamic acid (GALA), or α-aminoisobutyric acid residues (KAibA); arginine-rich cationic peptides (Tat, R9, dTat); and lysine-histidine alternating cationic peptides ($(KH)_9$). We also included proline-containing CPP derivatives ($(P_2KH_3)_3$, $(P_3KH)_3$, $(P_2K)_9$, $(P_2K_2)_7$) and multidomain CPPs with cationic and endosomal escape domains. The latter comprises macropinocytosis-inducing peptides with a sarcosine

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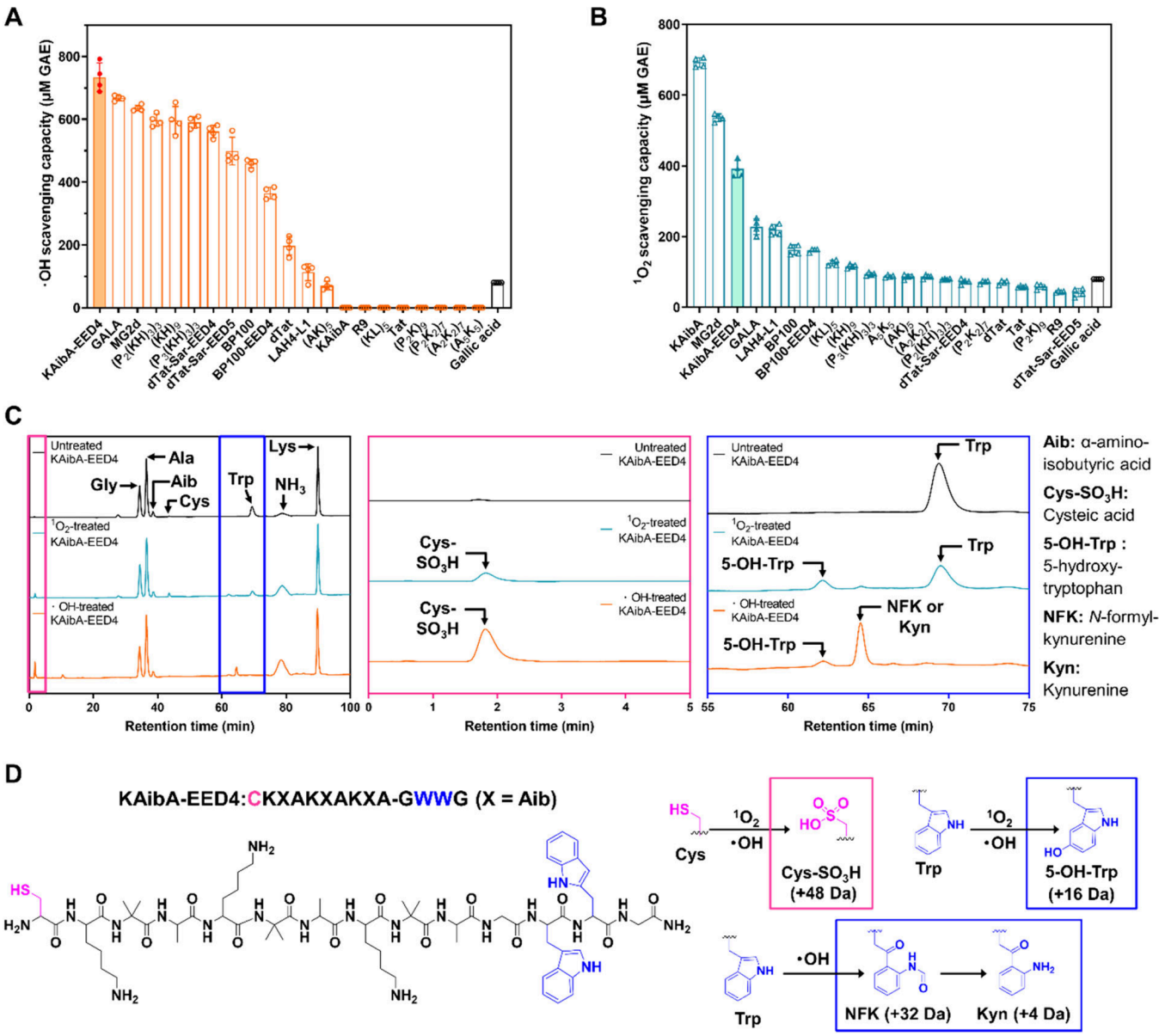

Figure 2. Screening of •OH- and $^1O_2$-scavenging CPPs and identification of oxidation sites in the selected CPPs. (A, B) Comparison of the (A) •OH- and (B) $^1O_2$-scavenging capacities of various CPPs at a concentration of 80 μM obtained from fluorescence-based assays (shown in Figures S1−3). Gallic acid (80 μM) served as a reference antioxidant. The data are presented as the mean ± standard error (n = 4). (C) Ion exchange chromatograms obtained from amino acid analyses of untreated, $^1O_2$-treated, and •OH-treated KAibA-EED4 after vapor-phase hydrolysis by 6 M hydrochloric acid (HCl). After hydrolysis, *N*-formylkynurenine (NFK) and kynurenine (Kyn) eluted at the same retention time (∼64.3 min) according to ion exchange chromatography (Figure S7). (D) Oxidation products of Cys and Trp residues in KAibA-EED4 after •OH or $^1O_2$ treatment.

linker to mitigate cytotoxicity (dTat-Sar-EED4, dTat-Sar-EED5) and endosome-escaping peptides without a linker (KAibA-EED4, BP100-EED4). The 21 peptides were tested for their antioxidant capacity against •OH and $^1O_2$ because cellular components are more severely damaged by these ROS than by other ROS.[27] The •OH-scavenging ability of the CPPs was assessed by monitoring the fluorescence quenching of a fluorescence dye caused by •OH-mediated oxidation (Figures S1, S2). In this assay, fluorescein (1 μM) served as the dye and •OH was generated via the Fenton-like reaction in the presence of H_2O_2 (1.6 mM) and cobalt(II) ions (115 μM). Additionally, the $^1O_2$-scavenging capacity was evaluated using a fluorogenic probe exhibiting fluorescence in response to $^1O_2$ (Figure S3). We employed singlet oxygen sensor green (SOSG, 1 μM) and an endoperoxide (3-(1,4-Epidioxy-4-methyl-1,4-dihydro-1-naphthyl)propionic acid, 250 μM) as the fluorogenic probe and the $^1O_2$ source, respectively.[28,29] Notably, in the •OH-scavenging capacity assay, both H_2O_2 and •OH could react with peptides, potentially affecting the results. In contrast, $^1O_2$ was generated exclusively from the endoperoxide, ensuring higher specificity in that assay. Our screening revealed that CPPs containing aromatic amino acid residues (Phe, Tyr, or Trp) tended to exhibit high •OH-scavenging capacity, while those containing Cys were effective scavengers of $^1O_2$ (Figure 2A,B, Table S1). Among the tested CPPs, KAibA-EED4 (CKXAKXAKXAGWWG, where X is α-aminoisobutyric acid, Aib)[21] was selected for further investigation due to its short length (14 amino acid residues);

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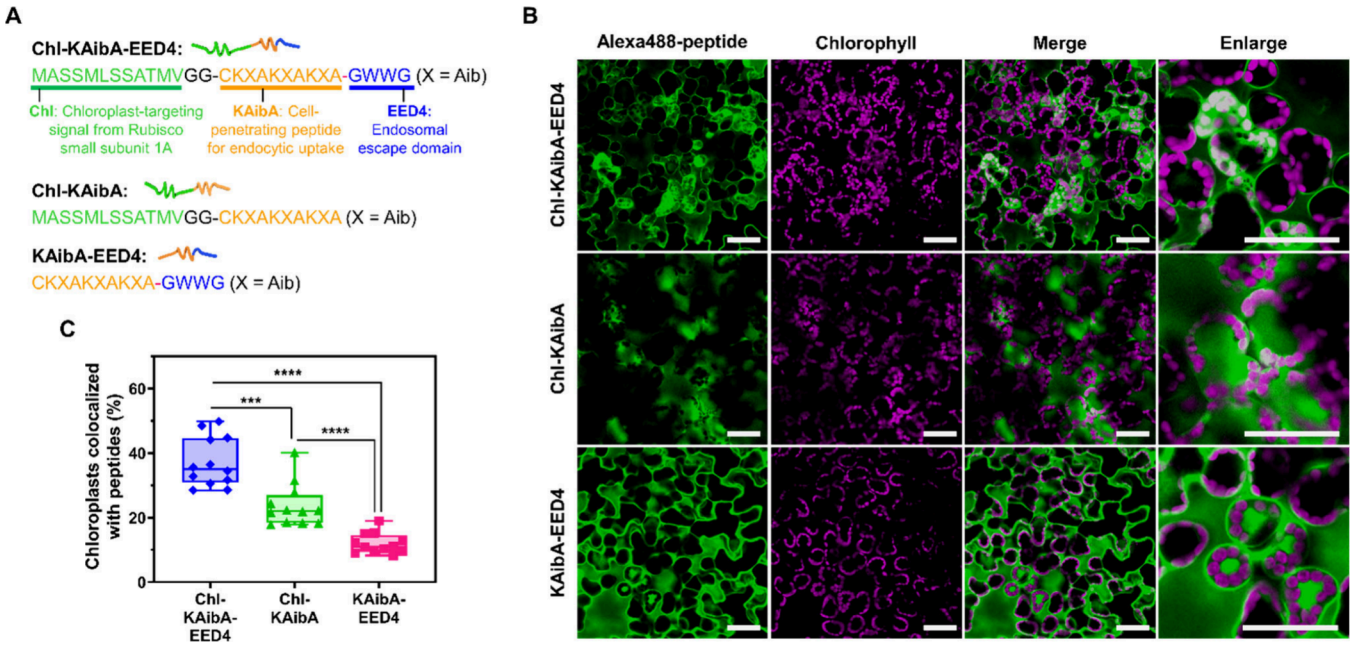

Figure 3. Chloroplast-targeting ability of the designed chimeric peptides. (A) Amino acid sequences and functional domains of Chl-KAibA-EED4, Chl-KAibA, and KAibA-EED4. (B) Confocal images showing the subcellular localization of fluorescently (Alexa488)-labeled peptides in leaves of *N. benthamiana* (30 days after germination (DAG)) 1 h postinfiltration. Scale bar, 50 μm. (C) Box plot representation of the percentage of chloroplasts colocalized with peptides: the boxes represent the interquartile range, the lines within the boxes represent the median values, and the upper and lower whiskers represent the highest and lowest values, respectively. Statistical significance: ***$P < 0.001$, ****$P < 0.0001$ based on Tukey's multiple comparisons test ($n = 12$ biological replicates).

due to this property, KAibA-EED4 is amenable for fusion with the chloroplast-targeting sequence and possesses the highest •OH-scavenging and third-highest $^1O_2$-scavenging capacity (Figure 2A,B).

Upon treatment with •OH or $^1O_2$, we observed several oxidation products of KAibA-EED4 in the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS spectra (Figure S4). MALDI-TOF MS/MS analyses of both •OH- and $^1O_2$-treated peptides indicated that the N-terminal Cys residue was oxidized (+48 Da, Figures S5, S6). This Cys oxidation product was identified as cysteic acid containing a sulfonic acid group, as confirmed by amino acid analyses of the •OH- and $^1O_2$-treated peptide hydrolysates (Figures 2C,D, S7). We confirmed that the cysteic acid formation did not occur during laser irradiation in the MALDI-TOF MS analyses (Figures S5, S6, S8, S9). Additionally, a Trp oxidation product (+16 Da) was detected in the MS/MS analyses of both •OH- and $^1O_2$-treated peptides (Figures S10, S11), with additional oxidation products (+4 Da and +32 Da) observed for the •OH-treated peptide (Figure S12). Amino acid analyses suggested that these oxidation products most likely corresponded to kynurenine (+4 Da), 5-hydroxy tryptophan (+16 Da), and *N*-formylkynurenine (+32 Da, Figure 2C,D), although other oxidation products may occur (Figure S8). These findings highlight the importance of the Cys and Trp residues in KAibA-EED4 as major oxidation sites contributing to •OH and $^1O_2$ scavenging.

Chloroplast-Targeting Abilities of Designed Chimeric Peptides

We aimed to confer chloroplast-targeting ability to the antioxidant CPP KAibA-EED4 by attaching a CTP. CTPs, found in various plants and photosynthetic organisms, have diverse sequences with most being 50−70 amino acids long.[30] Since shorter peptides are generally more amenable to chemical synthesis than longer ones, we chose a minimal CTP derived from *Arabidopsis thaliana* Rubisco small subunit 1A (Chl: MASSMLSSATMVGG) with sufficient chloroplast-targeting ability and good water solubility.[20,21] The resulting chimeric peptide, termed Chl-KAibA-EED4 (Figures 3A, S13), was meticulously designed with three crucial domains. These domains include a cationic domain (KAibA) facilitating cellular uptake through endocytosis,[25] an endosomal escape domain (EED4) promoting cytosolic translocation after endocytosis,[25,26,31] and a chloroplast-targeting domain (Chl) for specific recognition by translocon complexes at the outer and inner envelope membranes (TOC and TIC).[32] Despite their short lengths, the KAibA and EED4 domains were demonstrated to be functional in plant cells.[24,25] To assess the role of each domain, we compared the subcellular localization of Chl-KAibA-EED4 with that of control peptides (KAibA-EED4 and Chl-KAibA, Figure 3A) in the leaves of *Nicotiana benthamiana*, a model plant species. These peptides were fluorescently labeled, infiltrated into the leaves, and observed using confocal laser microscopy (CLSM). CLSM revealed that Chl-KAibA-EED4 exhibited the most efficient translocation to chloroplasts (Figure 3B,C). KAibA-EED4, lacking the Chl domain, entered plant cells but did not colocalize with chloroplasts; in comparison, Chl-KAibA translocated to chloroplasts with less efficiency (Figure 3B,C). The concentration of the peptides affected their chloroplast colocalization rate (Figure S14). Specifically, Chl-KAibA-EED4 was more efficiently colocalized with chloroplasts at 5 μM than at lower concentrations (0.5 and 1 μM, Figure S14A), and formed large aggregates at 10 μM (Figure S14B). Notably, the superior colocalization rate of Chl-KAibA-EED4 with chloroplasts, as opposed to that of Chl-KAibA, highlighted the crucial role of the EED4 domain (which is responsible for cytosolic

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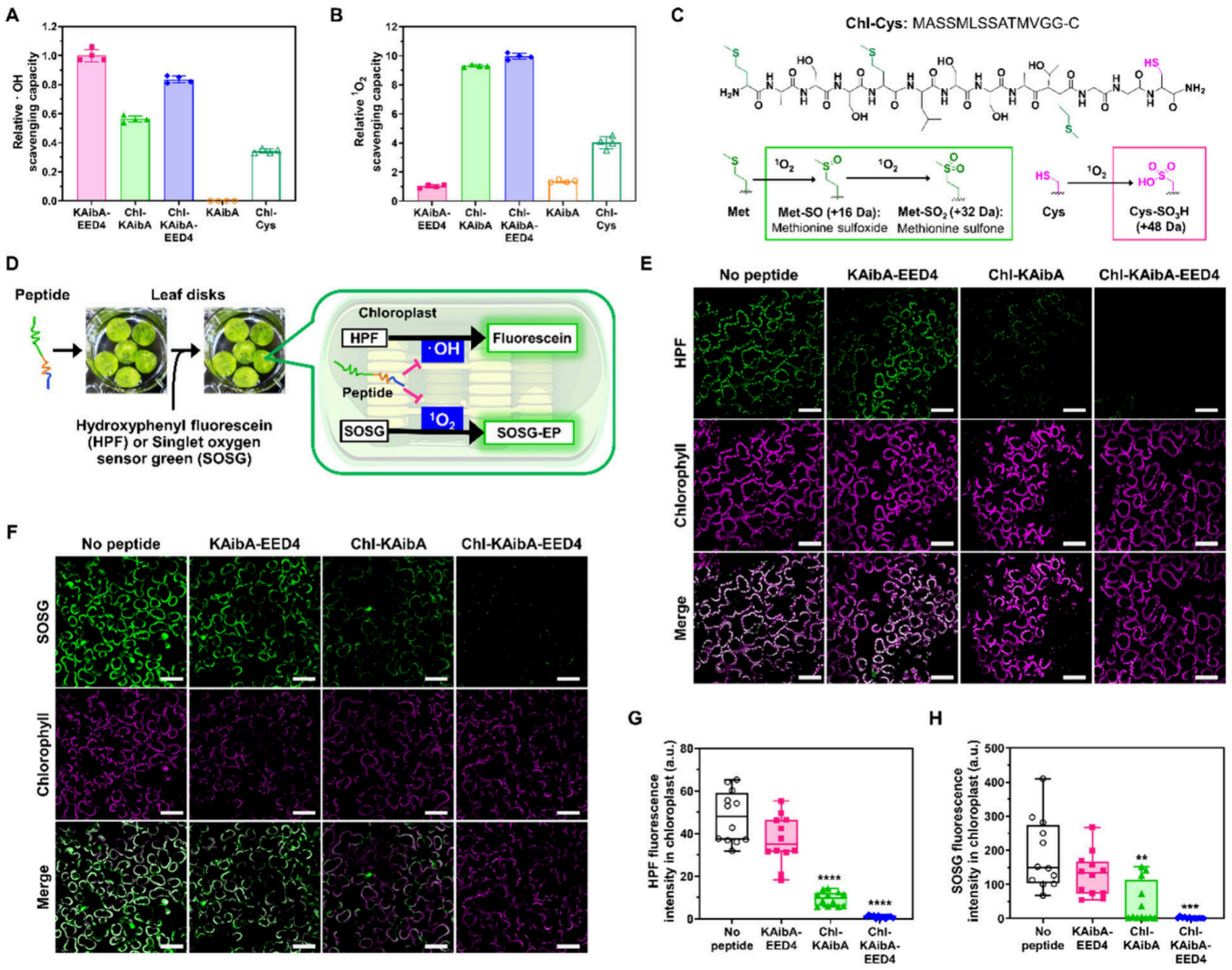

Figure 4. *In vitro* and *in vivo* ROS-scavenging ability of chimeric peptides. (A, B) Relative (A) •OH- and (B) $^1O_2$-scavenging capacities of chimeric peptides and their functional domains obtained from fluorescence-based assays (shown in Figure S15). The data are presented as the mean ± standard error (n = 4). (C) Oxidation sites in Chl-Cys after $^1O_2$ treatment. (D) Schematic illustration of peptide-mediated •OH and $^1O_2$ scavenging in chloroplasts. The production of •OH and $^1O_2$ in chloroplasts was detected by the fluorogenic probes hydroxyphenyl fluorescein (HPF) and singlet oxygen sensor green (SOSG), respectively. (E, F) Confocal images indicating the cellular (E) •OH and (F) $^1O_2$ levels detected by HPF and SOSG fluorescence, respectively, in *N. benthamiana* (30 DAG) leaf discs treated with peptide solutions (25 μM) or water (no peptide) for 30 min, followed by incubation with HPF (10 μM) or SOSG (50 μM) for 1 h in the dark. Scale bar, 50 μm. (G, H) Box plot representation of the relative (G) HPF and (H) SOSG fluorescence intensities. Statistical significance compared to the control (no peptide): $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ based on Dunnett's T3 test (n = 12 biological replicates).

translocation[21,25]) in chloroplast targeting (Figure 3B,C). These findings substantiate the rationale behind our chimeric peptide design, which integrates the three functional domains.

Chimeric Peptide-Mediated ROS Scavenging *In Vitro* and *In Vivo*

To compare the *in vitro* ROS-scavenging abilities of Chl-KAibA-EED4, Chl-KAibA, and KAibA-EED4, we evaluated their •OH and $^1O_2$ scavenging efficiencies using the same fluorescence-based assays employed for the initial CPP screening (Figure S15). The •OH scavenging ability of Chl-KAibA-EED4 was comparable to that of KAibA-EED4 and almost double that of Chl-KAibA (Figures 4A, S15A), highlighting the contribution of the Trp residues in the EED4 domain to •OH scavenging. Compared to the KAibA domain, Chl-KAibA scavenged •OH more efficiently due to the •OH-scavenging capacity of the Chl domain (Figures 4A, S15A), in which Met residues would quench •OH or possibly H_2O_2.[33] Notably, Chl-KAibA-EED4 and Chl-KAibA exhibited approximately 10 times greater $^1O_2$ scavenging capacities than that of KAibA-EED4 (Figures 4B, S15B). This was most likely due to the high $^1O_2$ scavenging capacity of the Chl domain (Figures 4B, S15B), in which Met residues were oxidized by $^1O_2$ to produce methionine sulfoxide and methionine sulfone, as shown by MALDI-TOF MS/MS and amino acid analyses (Figures 4C, S16, S17). As the Cys, Met, and Trp residues in Chl-KAibA-EED4 are major oxidation sites, their antioxidant capacities were compared. We found that Trp exhibited the highest •OH-scavenging capacity, while Cys and Met served as more effective scavengers for $^1O_2$ than Trp (Figure S18). These results underscore the importance of combining Cys, Met, and Trp in the design of ROS-scavenging peptides.

To evaluate the *in vivo* ROS-scavenging potential of Chl-KAibA-EED4, Chl-KAibA, and KAibA-EED4, we employed

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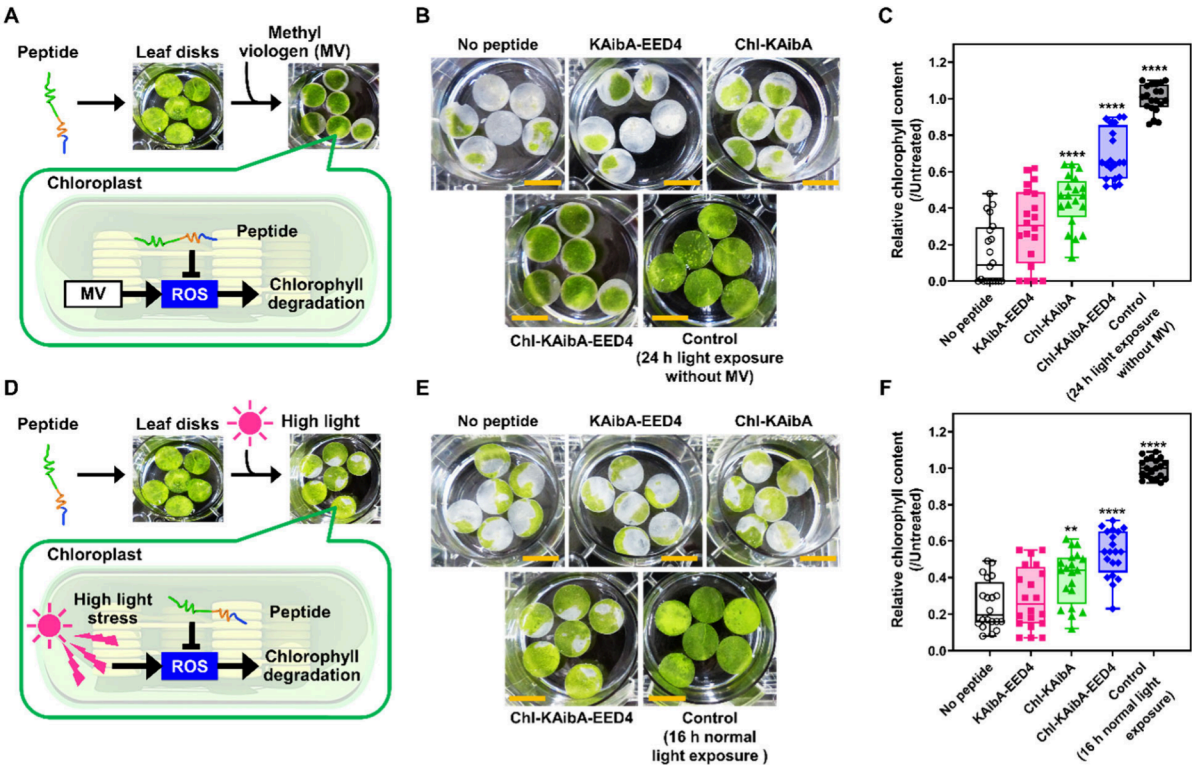

Figure 5. Chimeric peptide-mediated leaf protection from destructive ROS in chloroplasts. (A, D) Schematic illustration of ROS scavenging by chimeric peptides in chloroplasts suppressing chlorophyll degradation. Chloroplast ROS were produced by (A) MV application or (D) excess light exposure. (B, E) Representative images showing phenotypic differences in *N. benthamiana* (30 DAG) leaf discs treated with peptide solutions (25 μM) or water (no peptide) under the following stress conditions: (B) 24 h exposure to normal light (200 μmol m^{-2} s^{-1}) with MV (0.35 μM or 0 μM (control)) or (E) 16 h exposure to excess (1200 μmol m^{-2} s^{-1}) or normal (200 μmol m^{-2} s^{-1}) light (control). Scale bar, 10 mm. (C, F) Box plot representation of the relative chlorophyll content of leaf discs after (C) the MV treatment described in (B) or (F) the excess light exposure described in (E). Statistical significance compared to the control (no peptide): $^{**}P < 0.01$ and $^{****}P < 0.0001$ based on Dunnett's T3 test ($n = 30$ biological replicates).

ROS-responsive fluorogenic probes, such as hydroxyphenyl fluorescein (HPF) and SOSG,[29,34] which generate fluorescent products in response to •OH and $^1O_2$, respectively (Figure 4D). CLSM images of *N. benthamiana* leaves not treated with the peptides revealed intense fluorescence signals colocalized with chlorophyll (Figure 4E−H), indicating that •OH and $^1O_2$ were generated in chloroplasts. We treated leaves with each peptide at a concentration of 25 μM. At this concentration, chimeric peptides exhibited no cytotoxicity (Figure S19), unlike previously reported Trp-containing cationic peptides.[19]

Chimeric peptides formed large aggregates above 50 μM (Figure S20). These factors led to the selection of 25 μM as the optimal concentration for experiments. Treatment with Chl-KAibA or Chl-KAibA-EED4 notably reduced the fluorescence signals from both HPF and SOSG, whereas this reduction was not observed after KAibA-EED4 treatment (Figure 4E−H). These results suggest that Chl-KAibA and Chl-KAibA-EED4 can mitigate •OH and $^1O_2$ in chloroplasts without causing cytotoxicity in plants. Notably, Chl-KAibA-EED4 was a more effective scavenger of •OH and $^1O_2$ than Chl-KAibA (Figure 4E−H), likely due to its superior chloroplast-targeting ability (Figure 3B,C).

Protective Effects of Chimeric Peptides against ROS-Induced Chlorophyll Degradation in Plant Leaves

We utilized the ROS-generating herbicide methyl viologen (MV)[35] to treat leaf discs from *N. benthamiana*, aiming to investigate whether the designed chimeric peptides can mitigate ROS-induced chlorophyll degradation in chloroplasts (Figure 5A). The leaf discs were treated for 1.5 h with KAibA-EED4, Chl-KAibA, or Chl-KAibA-EED4, followed by a 24 h incubation with MV under continuous light. We validated that normal light (200 μmol m^{-2} s^{-1}) exposure without MV treatment for 24 h did not induce chlorophyll loss in leaf discs (Figure S21). MV treatment was previously shown to result in $O_2^{\bullet-}$ production in plant cells.[35] Our CLSM observation using HPF and SOSG revealed that treatment with 0.35 μM MV elevated the cellular levels of •OH but did not affect those of $^1O_2$ (Figure S22).

In the absence of peptide treatment, the leaf discs turned almost white due to the loss of chlorophyll resulting from the MV treatment (Figure 5B,C). Conversely, treatment with Chl-KAibA and Chl-KAibA-EED4 significantly improved the chlorophyll content of the leaf discs, indicating that plant leaves were successfully protected from ROS-induced oxidative stress. Additionally, we assessed the effects of peptide treatment on ROS-induced chlorophyll degradation in leaf discs under excessive light conditions (Figure 5D). In the absence of peptides, ROS overproduced under excessive light conditions caused substantial chlorophyll degradation (Figure 5E,F). This chlorophyll degradation was significantly alleviated by treatment with Chl-KAibA and Chl-KAibA-EED4 but not by treatment with KAibA-EED4. Among the chimeric peptides, Chl-KAibA-EED4 was the most efficient at reducing ROS-mediated chlorophyll degradation induced by excessive light exposure, as well as by MV treatment. The superior

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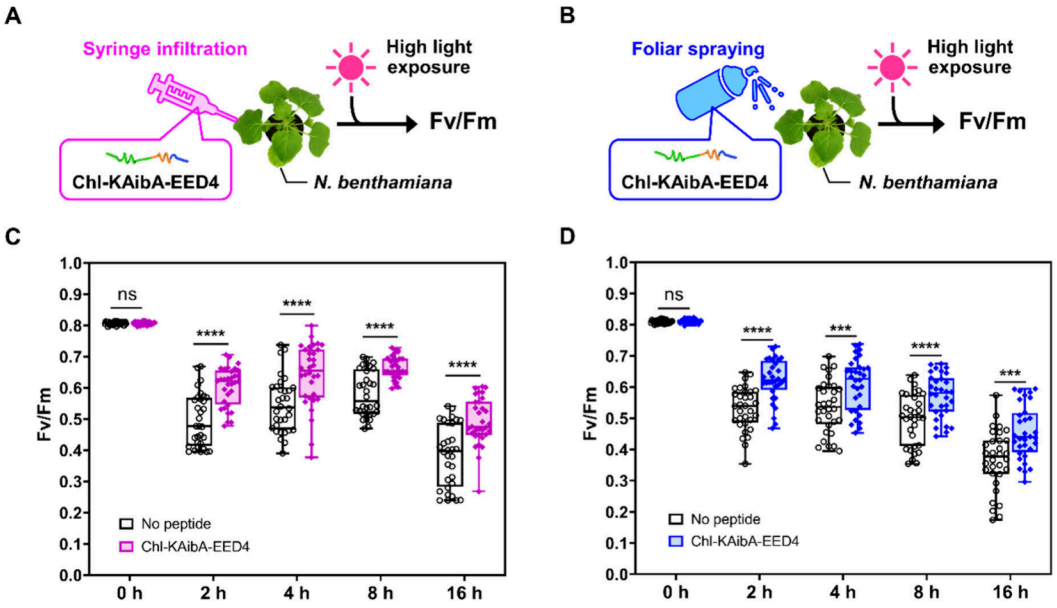

Figure 6. Chimeric peptide-mediated protection of plants from photoinhibition. (A, B) Schematic illustration of (A) syringe infiltration and (B) foliar spraying of Chl-KAibA-EED4 (25 μM) to alleviate photoinhibition in *N. benthamiana* (30 DAG) leaves exposed to excess light (1200 μmol m^{-2} s^{-1}) at 25 °C for up to 16 h. The degree of photoinhibition was monitored by measuring the photosynthetic activity (Fv/Fm) in dark-adapted (10 min) leaves. (C, D) Box plot representation of changes in the Fv/Fm of control (no peptide) or peptide-introduced (Chl-KAibA-EED4) leaves by (C) syringe infiltration or (D) foliar spraying under excess light conditions. Statistical significance between "No peptide" and "Chl-KAibA-EED4" at each time point: ***$P < 0.001$ and ****$P < 0.0001$ based on an unpaired t test with Welch correction ($n = 32$ biological replicates). "ns" represents no significance.

performance of Chl-KAibA-EED4 was supported by its enhanced chloroplast-targeting ability (Figure 3B,C) and in vivo ROS-scavenging capacity (Figure 4E−H). Notably, our results cannot rule out the possibility that the peptides scavenge ROS other than •OH and $^1O_2$ (i.e., $O_2^{\bullet-}$ and H_2O_2) due to the complex effects of low-dose MV treatment and excessive light exposure on ROS production in plant cells.

Protective Effects of Chl-KAibA-EED4 Foliar Spraying against Photoinhibition under Excess Light Conditions

The overproduction of reactive oxygen species (ROS) in chloroplasts under excessive light is known to contribute to photoinhibition by causing oxidative damage to the D1 and D2 reaction center proteins of PSII and inhibiting the repair of damaged PSII.[36−38] We aimed to mitigate photoinhibition by utilizing the ROS-scavenging properties of Chl-KAibA-EED4 during intense light exposure. Chl-KAibA-EED4 was introduced into the leaves of *N. benthamiana* plants through syringe infiltration, which has been often used to deliver various substances inside plant leaves,[39] followed by an incubation period of up to 16 h under excessive light conditions (Figure 6A). This light exposure period was justified based on typical daylight hours in a single day for practical agricultural applications. We assessed the extent of photoinhibition by measuring the Fv/Fm ratio, which reflects PSII activity,[40] at various time points. During the 2 to 16 h period of intense light exposure, there was a noticeable decrease in the Fv/Fm ratio (Figure 6C). However, this reduction was significantly alleviated by Chl-KAibA-EED4 infiltration, revealing the protective effects of the peptide against photoinhibition (Figure 6C). Additionally, we explored the potential of foliar spraying with Chl-KAibA-EED4 to protect plants from photoinhibition (Figure 6B). CLSM confirmed that the foliar-sprayed peptide were efficiently delivered to chloroplasts in the presence of a surfactant (0.025% (w/v) Silwet L-77, Figure S23), which improved the wetting of the plant surfaces.[41] Silwet L-77 exhibited cytotoxicity to plant leaves at the concentration of 0.1% (w/v) in combination with Chl-KAibA-EED4 (25 μM), but not at 0.025% (w/v, Figure S24). Similar to peptide infiltration, foliar spraying significantly mitigated the reduction in Fv/Fm caused by excessive light exposure (Figure 6D). We further examined whether repeated administration of Chl-KAibA-EED4 via foliar spraying led to more effective protection of plants from photoinhibition. Compared to a single application, repeated (two-time) peptide spraying achieved significantly improved protection against photoinhibition (Figure S25). Although the protective effects of our peptide against photoinhibition appeared to be transient, this system can offer a significant advantage in its applicability to foliar spraying, a crucial feature for large-scale application that has not been achieved by previously developed ROS quenchers for plant protection.[14,15,34] Furthermore, as repeated peptide spray enhanced the protective effect (Figure S25), limitations related to the transient nature of the peptide could potentially be addressed. These results highlight the efficacy of foliar peptide spraying as a strategy for managing photoinhibition in plants.

CONCLUSIONS

In this study, we developed a multifunctional chimeric peptide that effectively alleviates photooxidative stress and photoinhibition by scavenging destructive ROS, including $^1O_2$ and •OH, within plant chloroplasts. Our design combined CPPs and CTPs, each with antioxidant activity against $^1O_2$ and •OH. The Trp residue in the peptide predominantly scavenged •OH, while the Cys and Met residues effectively served as scavengers for $^1O_2$. The designed chimeric peptide, with a Cys- and Trp-containing CPP and a Met-containing CTP, successfully penetrated plant cells, reaching chloroplasts to

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scavenge $^1O_2$ and •OH. This process reduces chlorophyll degradation caused by a herbicide or excessive light exposure. Our results further demonstrated that foliar spraying of the chimeric peptide alleviated photoinhibition in mature plant leaves under excessive light conditions.

Genetic transformation, a mainstream approach to enhancing plant resistance against abiotic stresses, is limited by the difficulty of transformation across diverse plant species and the complex nature of plant stress responses.[8] Despite possessing stronger resistance to stress, genetically engineered plants often exhibit reduced growth.[9] Our nontransgenic strategy utilizing chimeric peptides may offer a solution to these challenges. Further optimization will involve refining the peptide design for enhanced cell-penetrating, chloroplast-targeting, and ROS-scavenging efficiency. As peptide abilities vary among plant species,[23] species-independent chimeric peptide are needed or the peptide sequence must be customized. Our research underscores the importance of incorporating Cys, Trp, and Met residues into peptides for effective $^1O_2$ and •OH elimination, guiding potent antioxidant peptide design. Balancing subcellular localization and antioxidant capabilities maximizes the benefits of large-scale peptide application via foliar spraying in agricultural systems by reducing the amount of peptide needed.

The chimeric peptide in this study provides transient ROS quenching on demand, minimizing disruptions to vital ROS signaling in cellular processes.[42] However, achieving prolonged protection requires a substantial amount of peptide, increasing overall costs. For sustained protection, codelivery of the peptide with catalytic ROS scavengers, such as cerium oxide nanoparticles,[14,34] may be useful. While our focus is on photooxidative stress, this method may be applied to protect plants from other stressors (e.g., drought, heat, and salinity) that trigger ROS overproduction.[27] In essence, our chimeric peptide can serve as a nontransgenic approach for managing plant abiotic stresses in various agricultural applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00478.

Experimental details as well as supporting figures and tables (PDF)

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Author Contributions

T.M. and K.N. conceived the project and designed the experiments. T.M. performed all the experiments and analyzed all the experimental data. S.R.M.-Y. contributed to the Fv/Fm measurements. T.M. and K.N. wrote and edited the manuscript. All authors discussed and commented on the manuscript. CRediT: **Takaaki Miyamoto** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, resources, validation, visualization, writing - original draft, writing - review & editing; **Shamitha Rao Morey-Yagi** data curation, formal analysis, investigation, methodology, writing - original draft; **Keiji Numata** conceptualization, funding acquisition, investigation, project administration, supervision, validation, visualization, writing - review & editing.

Notes

The authors declare no competing financial interest.

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