Short title: Whole-plant redox imaging

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Sensing stress responses in potato with whole-plant redox imaging

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One-sentence summary: Whole-plant imaging of potato plants expressing a genetically encoded biosensor allows for spatially resolved and nondestructive mapping of stress-induced redox perturbations.

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Abstract

Environmental stresses are among the major factors that limit crop productivity and plant growth. Various nondestructive approaches for monitoring plant stress states have been developed. However, early sensing of the initial biochemical events during stress responses remains a significant challenge. In this work, we established whole-plant redox imaging using potato (*Solanum tuberosum*) plants expressing a chloroplast-targeted redox-sensitive green fluorescence protein 2 (roGFP2), which reports the glutathione redox potential (E_{GSH}). Ratiometric imaging analysis demonstrated the probe response to redox perturbations induced by H_2O_2, DTT, or a GSH biosynthesis inhibitor. We mapped alteration in the chloroplast E_{GSH} under several stress conditions including, high-light, cold and drought. An extremely high increase in chloroplast E_{GSH} was observed under the combination of high-light and low temperatures, conditions that specifically induce PSI photoinhibition. Intriguingly, we noted a higher reduced state in newly developed compared to mature leaves under steady-state and stress conditions, suggesting a graded stress sensitivity as part of the plant strategies for coping with stress. The presented observations suggest that whole-plant redox imaging can serve as a powerful tool for the basic understanding of plant stress responses and applied agricultural research, such as toward improving phenotyping capabilities in breeding programs and early detection of stress responses in the field.

Introduction

Crop plants live in highly dynamic environments, in which abiotic stresses, such as salinity, drought, high temperature and high light, are thought to be the major constraints of crop production and ultimately of food security (Fahad et al., 2017). Continuous exposure to moderate stress levels or even to suboptimal growth conditions interrupts plant homeostasis and results in constant energy loss due to resource diversion towards the activation of defense and acclimation mechanisms (Zhu, 2016).

As a third major food crop, potato (*Solanum tuberosum)* productivity is crucial for worldwide food security (Devaux et al., 2014). Over the past 20 years, there has been a dramatic increase in potato production and demand in Asia, Africa, and Latin America (da FAOSTAT, 2014). The potato tubers are rich sources of carbohydrates and provide essential nutrients, such as dietary fiber, vitamins, minerals, protein, and antioxidants (Burlingame et al., 2009; Bach et al., 2012). Despite the wide distribution and adaptability of the potato plant to various environmental and climatic conditions, potato productivity is highly affected by environmental conditions (Bohnert, 2007). Even exposure to mild abiotic stresses such as drought and heat, as prevalent in potato-growing regions, reduces photosynthesis efficiency, which significantly impacts potato production and quality (Deblonde et al., 2001; George et al., 2017).

In the past few decades, there has been a growing demand for biosensing technologies that allow for dynamic monitoring of crop growth and stress status to support 'on-line' decisions ensuring the long-term sustainability of crop productivity. A range of technologies has been successfully implemented to nondestructively perceive information regarding the physiological status of plants, such as chlorophyll fluorescence imaging to detect photosynthetic activity (Wang et al., 2018), thermal imaging to estimate stomatal conductance and transpiration (Costa et al., 2013), multispectral imaging to evaluate crop water and nutrient status (Wang et al., 2018) and terahertz spectroscopy to detect plant drought stress responses (Born et al., 2014). However, technologies for early detection of the cellular biochemical signals involved in sensing and processing of environmental information regarding abiotic and biotic stress, are still lacking, mainly due to the destructive nature of common biochemical methods.

Stress-induced alterations in plant metabolism are typically accompanied by modifications in the levels of reactive oxygen species (ROS), which can differ in their chemical identity and subcellular localization (Foyer and Noctor, 2003; Gadjev et al., 2006; Miller et al., 2010; Møller and Sweetlove, 2010). Thus, increased ROS or oxidized metabolite levels, as well as the ROS-induced gene products, are common biomarkers for stress responses. Imaging and detection of ROS in live cells have been achieved using various fluorescent or dyes, such as 3,3'- diaminobenzidine (DAB), Amplex Red, and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Halliwell and Whiteman, 2004; Van Breusegem et al., 2008; Gao and Zhang, 2008; Zhang et al., 2009; Fichman et al., 2019; Fichman et al., 2020). A new type of nano-sensor probes, based on single-walled carbon nanotubes, was shown to enable real-time spatiotemporal monitoring of H_2O_2 within plants (Lew et al., 2020; Wu et al., 2020). While these probes can be easily implemented in a wide range of plants, and provide valuable information on alterations in ROS metabolism on the whole-plant level, they require the incorporation of the probes to plant tissues, making it challenging to investigate redox alterations on the whole-plant level over long, physiologically relevant time periods.

Genetically encoded redox-sensitive green fluorescent protein (roGFP) probes have been developed and used as biosensors in various model systems. *In vitro* characterization of roGFP activity pointed to its predominant interaction with the cellular GSH pool through the mediation of glutaredoxin activities (GRXs, Meyer et al., 2007). These *in vitro* observations were corroborated by the substantial *in vivo* oxidation of cytosol-targeted roGFP recorded upon external application of L-buthionine (S,R)-sulfoximine (BSO), a glutathione biosynthesis inhibitor that specifically inhibits γ-glutamylcysteine synthetase (GSH1, Meyer et al., 2007). In agreement, a higher roGFP oxidation state was measured in Arabidopsis (*Arabidopsis thaliana*) plant with aberrant GSH biosynthesis and reduction pathways (Meyer et al., 2007; Rosenwasser et al., 2010). The linkage between roGFP and the GSH pool suggests that endogenous GRXs mediate the interaction between GSH and roGFP and point to the possibility of *in vivo* GSH redox potential (E_{GSH}) probing by roGFP. Its mechanism of action is based on two engineered surface-exposed cysteine residues, which form an intramolecular disulfide bridge that impacts its fluorescence under oxidizing conditions. Due to their ratiometric nature, high sensitivity, reversibility, and insensitivity to pH alterations in the physiological range, roGFP-based redox sensors are powerful tools for investigating redox dynamics in subcellular compartments at high spatiotemporal resolution (Dooley et al., 2004; Hanson et al., 2004; Jiang et al., 2006b; Gutscher et al., 2008; Meyer and Dick, 2010; Schwarzländer et al., 2016; Nietzel et al., 2019; Albrecht et al., 2011; Bratt et al., 2016; Ugalde et al., 2020). Their reversibility enables the monitoring of their redox state at multiple points over time without damaging the tissue. Notably, as the roGFP redox state is regulated by counteracting oxidative and reductive reactions, it reflects the transmission of redox signals, leading to modulation of the redox state of native redox-sensitive proteins as part of plant stress acclimation (Meyer, 2008; Rosenwasser et al., 2014).

Despite the great potential in using genetically encoded biosensors to detect stress responses in plants, current approaches for measuring roGFP-based signals are mainly based on confocal microscopy and plate readers, which are suitable for plant pieces or small model plants, but not for intact plants grown in soil. Only recently, whole-plant fluorescence imaging has been demonstrated in *Arabidopsis* plants expressing genetically-encoded biosensors (Haber et al., 2021; Fichman and Mittler, 2021) but has not been applied in crop plants grown in soil. The global importance of potato crop and the availability of highly efficient transformation protocols renders potato an ideal platform for investigating genetically encoded biosensor performance in crop plants.

In this work, potato plants expressing chloroplast-targeted roGFP2 were generated and subjected to whole-plant roGFP ratiometric imaging analysis using a highly sensitive *in vivo* imaging system. The experimental setup enabled detection of *in planta* redox modification in response to several abiotic stress conditions that mimicked natural field conditions. The presented data demonstrate that whole-plant imaging of roGFP-expressing plants allowed for spatially resolved mapping of stress-induced redox perturbations over long periods. These results may have important implications on phenotyping capacities in large-scale breeding projects and on capabilities to detect stress conditions in crop plants under field conditions.

Results and Discussion

Generation of potato plants expressing a genetically encoded redox probe

The roGFP probe allows for quantitative, real-time readout of E_{GSH} in living cells (Meyer et al., 2007; Albrecht et al., 2011). To enable the monitoring of temporal alterations in the chloroplastic E_{GSH} (chl-E_{GSH}) on the whole-plant level, *Agrobacterium*-mediated genetic transformation was performed to obtain potato plants cv. *Desiree* expressing the roGFP2 probe in the chloroplast (chl-roGFP2, Supplemental Fig S1). Chloroplast targeting was achieved by using the *Arabidopsis* 2-Cys peroxiredoxin A signal peptide, which is targeted to the chloroplast stroma (König et al., 2002), as verified by the overlap of the chl-roGFP2 and the chlorophyll fluorescence signals (Fig. 1A). No phenotypic differences between several independent lines expressing the roGFP2 probe and wild type were observed; the line with the highest fluorescence intensity and lowest variability in roGFP2 signals between individual plants was selected for subsequent experiments (Supplemental Fig. S2). roGFP signal silencing, a phenomenon frequently reported for plant protein-based biosensors and which has been observed to increase over generations (Schwarzländer et al., 2016; Exposito-Rodriguez et al., 2017), was not detected, presumably since lines were vegetatively propagated, either from cuttings or tubers. Confocal microscopy analysis was used to validate the *in* vivo probe redox sensitivity, which was determined via ratiometric images derived from the division of the emitted fluorescence due to excitation at 405 nm by the emitted fluorescence following 488 nm excitation ($R_{405/488}$). These ratios, which indicate the roGFP2 oxidation state (Dooley et al., 2004; Hanson et al., 2004), were calculated for plants in a steady-state and following treatment with H_2O_2 or DTT. As shown in Fig. 1B&C, an increase and decrease in excitation ratios were observed following treatment with H_2O_2 and DTT, respectively. Relatively low ratio values were recorded under steady state conditions, indicating a highly reduced chl-roGFP2 state. These results are consistent with probe characteristics previously observed in plant cells (Schwarzländer et al., 2008).

In vivo redox imaging of potato biosensor plants

To establish *in vivo* quantitative mapping of the chl-E_{GSH} on a whole-plant level, roGFP2 probe emission at 515 nm was recorded from intact plants grown in soil using a highly sensitive *in vivo* imaging system (See Methods), following excitation at 405 nm and 465nm with light-emitting diodes (LEDs). To explore the ability of this setup to provide reliable information on *in planta* chloroplast-specific redox alterations without tissue autofluorescence perturbations, the fluorescent signal measured in plants expressing the roGFP2 probe was compared to that measured in wild type plants. As shown in Figure 1D, a clear separation between the pixel intensity histograms of roGFP2-expressing versus wild-type lines was observed following excitation at 405 nm and 465 nm, demonstrating an intense roGFP2 signal in the former lines. The measured autofluorescence signals were 2.2% and 9.6% of the total fluorescence signals, emitted from roGFP2-expressing plants, following excitation at 465nm and 405nm, respectively (Fig.1D, Supplemental Fig. S3). In all subsequent experiments, autofluorescence values obtained from wild-type plants exposed to the same conditions as roGFP-expressing lines were used for background correction (See Methods). Correction for background values is also highly important under specific stress conditions that increase autofluorescence, especially in the 405 nm- excitation range (Rosenwasser et al., 2010).

To monitor the *in planta* response to redox changes, 2.5-week-old chl-roGFP2-expressing plants grown from cuttings, were imaged under steady state conditions and following the application of H_2O_2 or DTT. While some variability in absolute roGFP fluorescence intensities was observed across different leaves, pixel-by-pixel $R_{405/465}$ images provided informative false-color images (Fig. 1E). Upon H_2O_2 treatment, an increase in 405 nm-excited fluorescence and a decrease in 465 nm-excited fluorescence were observed, demonstrating probe oxidation. Conversely, DTT treatment resulted in probe reduction, as demonstrated by the less intense signal following 405 nm excitation and increased brightness following 465 nm excitation. Ratiometric images of untreated plants showed the highly reduced chl-E_{GSH} throughout the whole plant. The temporal response of 4-5-week-old plants to oxidative conditions was further assessed by watering of soil-grown chl-roGFP2-expressing plants with 50 ml 1M H_2O_2. Ratiometric images demonstrated an increase in the chl-roGFP2 oxidation state, mainly in mature leaves, which became saturated after 12 min of exposure, and was then followed by reduction, reaching a steady-state level after 27 min (Fig. 1F, Supp Movie 1 and Supplemental Fig. S4).

Previous experiments using confocal imaging, showed that the application of BSO to *Arabidopsis* roots led to substantial oxidation of cytosol-targeted roGFP (Meyer et al., 2007). Hence, to further demonstrate that the whole-plant imaging approach allows for spatial resolution of redox alterations, we monitored chl-roGFP2 fluorescence signals following depletion of the cellular GSH pool by BSO. Images were obtained daily from four-week-old plants grown in soil and watered with 2.5mM

BSO. A significant increase in $R_{405/465}$ was observed, starting one day after BSO treatment and reaching maximum values ($R_{405/465}$ of 0.3±0.052 compared to 0.1±0.012 recorded from control plants) on the third day of treatment (Fig. 2A-D and Supplemental Fig. S5). These observations were further validated using confocal microscopy imaging analysis, which confirmed chloroplast-specific E_{GSH} oxidation (Fig 2E&F).

Interestingly, under steady-state conditions, newly developed leaves on the higher part of the plant exhibited lower $R_{405/465}$ values as compared to lower and mature leaves, suggesting a more reduced state of the E_{GSH} in young leaves (Fig 2A). Spatial differences were also observed following BSO application, where a higher chl-roGFP2 oxidation state was measured in young leaves (Fig 2A). However, these observations, which imply the higher sensitivity of young leaves to the inhibition of the *de novo* synthesis of GSH, can also result from an unequal distribution of BSO among the plant tissue.

To further calibrate the probe's dynamic range by defining $R_{405/465}$ values for the fully oxidized and reduced states, detached leaves were soaked in various H_2O_2 solutions and the redox state was monitored. Treatment of leaves with increasing H_2O_2 concentrations (10 mM to 1500 mM) resulted in increased $R_{405/465}$ (Fig 3 A&B, Supplemental Fig. S6A-C). At relatively high H_2O_2 concentrations (750-1000 mM), chl-roGFP2 fluorescence reached saturation ($R_{405/465}$=0.49) within 12 min. Notably, these values do not reflect the endogenous H_2O_2 concentrations, which are affected by penetration rates and detoxification activity. Treatment of leaves with 100 mM DTT resulted in a decrease in $R_{405/465}$ (0.09), demonstrating a dynamic range of 5.4 ($R_{405/465}$ for fully oxidized state divided by $R_{405/465}$ for fully reduced state), which is comparable to values determined using confocal microscopy and a plate reader (Schwarzländer et al., 2008; Rosenwasser et al., 2010). Markedly, $R_{405/465}$ values of the fully oxidized or fully reduced chl-roGP2 states in newly developed leaves were similar to those observed in mature leaves (Fig. 3C&D), suggesting that the observed differences in steady-state $R_{405/465}$ values indeed reflected differences in chl-roGFP2 oxidation state.

The oxidation degree (OxD) of chl-roGFP2 and chl-E_{GSH} in whole plants under steady-state conditions was then calculated using reference ratio values for fully oxidized and reduced states according to Meyer et al. (2007). The average OxD values were approximately 25%. Considering a stromal pH of 8, this OxD value would reflect an E_{GSH}= -346mV, which aligns with previous calculations of chloroplastic E_{GSH} under steady-state conditions in *Arabidopsis* plants (Schwarzländer et al., 2008; Rosenwasser et al., 2010). Interestingly, newly developed and mature leaves exhibited an average chl-roGFP2 OxD of 14% and 28%, respectively, demonstrating a deviation of 11 mV between different leaves on the same plant (Fig. 3C).

Daily measurements of chl-E_{GSH} under high light

Potato biosensor plants offer the opportunity to examine the *in vivo* influence of environmental stress conditions on the chl-E_{GSH} and to examine possible redox differences throughout plants. As the chl-E_{GSH} dynamically responds to changes in light intensities (Haber et al., 2021), we sought to apply the chl-roGFP2 probe to investigate potato plant response to changes in light intensities that mimic the kinetics and light intensities of field conditions. To this end, four-week-old plants were exposed to the following 16 h light conditions: Constant light)CL)- 200 µmol photons m^{-2} s^{-1}, Medium-light (ML) - light intensity was gradually increased, reaching a maximum value of 720 µmol photons m^{-2} s^{-1}, followed by a gradual decrease toward the end of the day, or High-light (HL) – gradual increases in light intensity, up to a maximum value of 1250 µmol photons m^{-2} s^{-1} light (Fig. 4). roGFP fluorescence images were taken every two hours, starting from light onset. As shown in Figure 4A&B and Supplemental Fig. S7, plant exposure to increasing light intensities (ML and HL) resulted in an increased whole-plant chl-roGFP2 oxidation state, reaching maximum values in the middle of the day. Similar oxidation dynamics were observed in plants exposed to ML and HL, with slightly higher oxidation values under the HL treatment. For example, at 8 h from light onset, OxD values of 47±6% and 43±6% were recorded in plants exposed to HL and ML, respectively. Relatively constant chl-roGFP2 OxD, of approximately 30%, was measured throughout the day in plants exposed to CL. The difference in the roGFP2 oxidation state between HL and CL-exposed plants is equivalent to a 10mV increase in chl-E_{GSH}, in agreement with values obtained in *Arabidopsis* plants under high-light conditions (Haber et al., 2021). Return to steady state values was detected 14 h after light onset, when light intensity was reduced to 200 µmol photons m^{-2} s^{-1}. The inspection of ratiometric images revealed that although a light-induced oxidation response was detected in all plant leaves, mature leaves responded stronger to increasing light intensities than young leaves, with differences of 28% and 10% in chl-roGFP2 OxD between HL and CL conditions 8 hr from light onset in old versus young leaves, respectively (Fig. 4A&C). Taken together, these observations demonstrate that spatial heterogeneity and physiological responses of chl-E_{GSH} to light intensity can be nondestructively monitored in crop plants grown in soil. As high light induces H_2O_2 production (Exposito-Rodriguez et al., 2017), the increase in chl-E_{GSH} is likely a reflection of a new balance point between photosynthesis-dependent ROS production and NADPH-dependent glutathione reductase (GR) activity. Notably, as roGFP2 oxidation-reduction dynamics may reflect similar patterns occurring in many redox-regulated metabolic proteins, specifically those regulated by native GRXs (Meyer, 2008; Rosenwasser et al., 2014), it may provide an important readout of stress-induced metabolic alterations.

Profound chl-E_{GSH} oxidation under high light and cold temperatures

Various environmental conditions lead to an imbalance between photosynthesis light absorption and downstream carbon assimilation reactions, resulting in increased plant sensitivity to excess light energy. Specifically, photosystem I (PSI) photo-inactivation was observed in chilled potato leaves exposed to high light, likely due to ROS accumulation (Havaux and Davaud, 1994). Such stress combinations are of interest as they result in crop yield reductions and may pose a risk to plant tissues.

To examine the effect of chilling stress on the chl-E_{GSH}, chl-roGFP2-expressing plants were exposed to a low temperature (3°C) and to each of the three daily light treatments mentioned above (Fig. 4); whole-plant oxidation patterns were measured every two hours during the light period. In plants exposed to 3°C + CL, chl-roGFP2 OxD exhibited a stable state of approximately 50%, with a slight reduction after two hours in the light period. In contrast, marked oxidation levels were observed in plants exposed to the combination of cold temperature and high light, reaching chl-roGFP2 OxD values of 60% and 80% in plants exposed to 3°C+ ML and HL, respectively, after 10 hours in the light (Figure 5A&B and Supplemental Fig. S8). No decrease in OxD levels in 3°C+ HL plants was observed when the light was dimmed at the end of the day. Remarkably, despite the harsh conditions, a more reduced chl-roGFP2 state was observed in newly developed leaves on the upper part of the plants, particularly near the meristem, as compared to mature leaves, throughout the day, demonstrating heterogeneous responses between old and young leaves (Fig. 5A&C). Taken together, low temperature and increasing light conditions induced extensive chl-E_{GSH} oxidation, reaching levels comparable to those measured after application of extremely high concentrations of exogenous H_2O_2 or to those observed following depletion of the GSH pool by BSO, implying a correlation between PSI photoinhibition and chl-E_{GSH} oxidation.

The demand for reduced GSH under chilling stress was also observed in transgenic tomato plants with suppressed glutathione reductase activity, which demonstrated aggravated PSI photoinhibition and delayed PSI recovery (Shu et al., 2011). The extremely high roGFP2 oxidation state and the fact that no reduction was observed after returning plants to normal light conditions suggest that plants failed to acclimate to such intensive stress. The significant changes in chl-E_{GSH} (~32 mV) may indicate the induction of cell death, aligning with previously reported associations between a similar range of changes in the chloroplast and mitochondrial E_{GSH} and cell death in *Arabidopsis* and diatoms (Rosenwasser et al., 2014; Van Creveld et al., 2015; Bratt et al., 2016; Volpert et al., 2018).

Higher activity of several photoprotective mechanisms, including upregulation of energy dissipation via heat and photorespiration, in young compared to mature leaves was observed in crop plants (Bertamini and Nedunchezhian, 2003; Jiang et al., 2006). The higher reduced state of the chl-E_{GSH} in young leaves may result from their decreased photosynthetic activity, which raises the threshold at which light causes increased ROS production and subsequent chl-E_{GSH} oxidation. While the induction of these photoprotective pathways results in suboptimal photosynthetic efficiency, it may protect the photosynthetic machinery from severe destruction. Thus, the co-existence of leaves with differential chl-E_{GSH} values can be viewed as an evolutionary compromise between photosynthetic efficiency and photo-protection, enabling young leaves to better withstand unpredictable increases in light intensities and older leaves to photosynthesize more efficiently.

Drought stress initiates early oxidation of chl-E_{GSH}

Stomata closure in response to water stress restricts CO_2 diffusion and reduces Calvin-Benson cycle (CBC) reactions, resulting in higher ROS production due to channeling excessive light energy toward molecular oxygen (Suzuki et al., 2012). An increase in glutathione reductase (GR) activity was reported under HL and water stress conditions, suggesting the increased activity of the ascorbate-GSH cycle (Yang et al., 2008; Gill and Tuteja, 2010). To explore *in vivo* drought-induced redox alterations in chloroplasts, chl-roGFP2 oxidation dynamics were followed during drought stress. Watering of four-week-old plants was stopped, and roGFP imaging was acquired from the third day of irrigation termination and onward. Starting from six days after water was withheld (Fig. 6), a gradual increase in chl-roGFP2 OxD levels was noted, with oxidation initiating in the peripheral leaves, and later spreading to all plant leaves (Fig. 6A, Supplemental Fig S9-10). No significant changes in chl-roGFP2 OxD was detected in well-watered plants during the experiment.

The rise in chl-roGFP2 OxD in water-stressed plants perfectly paralleled the decrease in carbon assimilation rates (Fig. 6B&C), pointing to the consistent relationship between photosynthetic activity and chl-E_{GSH} and suggesting that the increase in chl-E_{GSH} reflects an imbalance between light absorption and the ability to utilize it in the CBC. The decline in stomatal conductance observed one day before the increase in chl-roGFP2 OxD (Fig 6B&D) further supports the notion that lower CBC activity due to a decrease in intracellular CO_2 levels resulted in an increase in chl-E_{GSH}. Steady state redox levels and photosynthetic parameters were restored upon re-watering of water-stressed plants (Day 12), demonstrating the probe's ability to sense the reversibility of stress response. The observed increase in chl-E_{GSH} in water-stressed plants may reflect a broader increase in cellular ROS production, as suggested by the oxidation of the cytosolic and mitochondrial E_{GSH} reported for *Arabidopsis* plants (Jubany-Mari et al., 2010; Bratt et al., 2016). Future evaluations of organelle-specific changes in E_{GSH} using plant lines expressing the roGFP2 probe in various subcellular compartments will enable dissection of the exact microenvironment in which redox alterations are initiated under water stress conditions.

The GSH redox state is widely used as a marker of oxidative stress (Noctor et al., 1998; Schafer and Buettner, 2001; Dietz, 2003; Kranner et al., 2006; Meyer, 2008). Specifically, monitoring redox changes in photosynthesizing chloroplasts can provide valuable information regarding the response of leaf photosynthesis to environmental stresses. This is of great interest in crop plants in which plant productivity is greatly affected by the level of stress imposed on the photosynthetic machinery. The presented data suggest that crop plants expressing genetically encoded fluorescent sensors that report the chloroplast E_{GSH} can be a powerful tool to evaluate plant stress responses.

The presented methodology can be expanded to plant lines expressing the roGFP2 in other cellular compartments (e.g., mitochondria and peroxisomes) or other biosensors such as the roGFP2-Orp1 or iNAP, which monitor dynamic changes in H_2O_2 and NADPH, respectively (Nietzel et al., 2019; Lim et al., 2020). Markedly, a sufficient signal-to-noise ratio is crucial to obtain reliable quantitative ratiometric images. While the bright fluorescence of the chl-roGFP2 resulted in relatively minor autofluorescence values and large dynamic range (Fig. 1D and Supplemental Fig S7-9), working with biosensor lines exhibiting less intense fluorescence signals would require particular caution. Reasonably, a more rigorous quantitative approach for autofluorescence correction will be needed. As demonstrated for confocal microscopy analysis, assessing the structured autofluorescence, typically observed in plant tissues in response to 405 nm excitation, can be achieved by recording emissions at 435–485 nm. Then, correction for the autofluorescence bleed-through into one of the biosensor channels can be achieved by subtracting a scaled version of the autofluorescence image from the biosensor image (Fricker, 2016). Similar corrections may provide high-quality whole-plant ratiometric images, even for plant lines exhibiting dim fluorescence signals.

The applications of the presented whole-plant redox imaging methodology can be broad, including, for example, in testing the performance of various chemicals in improving plant tolerance under stress conditions. Plant lines expressing genetically encoded biosensors can also be applied to improve high-throughput plant phenotyping in plant breeding programs and ultimately serve as highly sensitive tools for early detection of stress responses in the field. Notably, the need for highly sensitive fluorescence cameras may limit the accessibility of this technology, raising the need to develop portable and sensitive instruments to monitor fluorescence signals in the field. Taken together, given the significant role of redox metabolism in plant acclimation to stress conditions, crop plants expressing redox sensors can expand the basic understanding of plant stress physiology and extend the arsenal of early noninvasive tools for detection of stress-induced physiological changes in crops.

Materials and Methods

Plant material, growth conditions, and experimental setup

Wild type and chl-roGFP2-expressing potato (*Solanum tuberosum*) plants were planted in moist soil in 26.82 x 53.49 cm pots and placed in a controlled-environment greenhouse. Plants were vegetatively propagated from cuttings. All experiments were performed on 3-4-week-old plants in a FytoScope FS-RI 1600 plant growth chamber (Photon Systems Instruments). Plants were moved to the chamber several days before the experiments to allow acclimation to the chamber environment. In all experiments, plants were incubated in 60-70% relative humidity (RH) and ambient CO_2.

Production of transgenic plants

Potato leaves (cv. Désirée) were used for *Agrobacterium*-mediated infection, as previously described (Ooms et al., 1987; Teper-Bamnolker et al., 2017). Leaves were taken from clean culture and infected by *Agrobacterium tumefaciens* strain LBA 4404 harboring the pART27 plasmid, which contains the chl-roGFP2 construct (Haber et al., 2021). After the inoculation, cultures were transferred to cali induction medium containing kanamycin 50 (mg/L), cefotaxime 500 (mg/L), 6-benzylaminopurine (BAP) 0.1 mg/L and 1-naphthaleneacetic acid (NAA) 5mg/L, for 10 days. Then, plants were transferred to shot induction medium (regeneration medium) containing zeatin-riboside (ZR) 2mg/L, NAA 0.02 mg/L gibberellic acid 3 GA3 0.02mg/L and appropriate antibiotics. Medium was refreshed every 14 days until shoots appeared. Transgenic explants from cultures that were fully regenerated with no roots were transferred to moist soil for rooting and sorting. Appropriate lines were selected by evaluating the chl-roGFP2 fluorescence signal (Supplemental Fig S1).

Confocal microscopy

Images were acquired with Leica DMI4000 confocal system (Leica Microsystems) and the LAS X Life Science Software, while using HCX APO U-V-I 40x/0.75 DRY UV objective. Images were acquired at a 4096×4096-pixel resolution, with a 507-534 nm emission bandpass and PMT gain of -628.2(V) following excitation at 488nm or 405nm for chl-roGFP2 fluorescence detection. For those images, 15% and 30% of the maximum laser intensity were used for 488nm and 405nm excitation, respectively. A 652nm - 692nm emission bandpass and PMT gain of -571.4(V) following excitation at 488nm were used to image chlorophyll fluorescence. Autofluorescence following excitation at 405 nm was recorded at 431–469 nm. Merged images were generated using Fiji software. Background subtraction of confocal images was conducted by subtracting the mean value of a user-defined region of interest (ROI) that did not include chloroplasts and pixel-by-pixel subtraction of autofluorescence values from the 405 nm image. The ratiometric images were created by dividing, pixel by pixel, the 405 nm image by the 465 nm image, and displaying the result in false colors using Matlab.

Gas exchange measurements

Carbon assimilation measurements were made using the portable gas analyzer Li-Cor-6800 gas exchange (LICOR, Lincoln, NE, USA). The leaf chamber was set to maintain a constant CO_2 level of 400 ppm. Light intensities were similar to those applied in the growing chamber. The temperature and humidity were set at 25°C and 70%. Net photosynthesis (A) and stomatal conductance to water vapor (Gs) were measured. Between 6-9 plants were randomly selected for each treatment and measurements were taken at 12 PM each day.

Chl-roGFP2 fluorescence measurements and image analysis

Whole-plant chl-roGFP2 fluorescence was detected using an Advanced Molecular Imager HT (Spectral Ami-HT, Spectral Instruments Imaging, LLC., USA), and images were acquired using the AMIview software. For chl-roGFP2 fluorescence detection, excitation was performed with 405 nm±10 or 465 nm±10 LED light sources and a 515 nm±10 emission filter was used. For chlorophyll detection, a 405 nm±10 LED light source and 670 nm±10 emission filter were used. All images were taken under the following settings: exposure time = 1s, pixel binning =2, field of view (FOV) = 25cm, LED excitation power 40% and 60%, for 405nm, 465nm excitations, respectively. Excitation power for chlorophyll detection was 5%. Chlorophyll autofluorescence was measured to generate a chlorophyll mask, which was then used to select pixels that returned a positive chlorophyll fluorescence signal. Only those pixels were subsequently considered for the roGFP analysis. For background correction, the average signal of wild type plants without chl-roGFP2 was determined and subtracted from the values detected in the chl-roGFP2 fluorescence signals. Ratiometric images were created by dividing, pixel by pixel, the 405 nm image by the 465 nm image, and displaying the result in false colors. Images were preprocessed using a custom-written Matlab script.

For calibration of the probe response, detached, fully expanded leaves were immersed in 1 M H_2O_2 or 100 mM DTT, and ratiometric images for fully oxidized and fully reduced states, respectively, were then acquired. roGFP2 OxD (the relative quantity of oxidized roGFP proteins) was calculated for individual plants based on the whole-plant fluorescence signal, according to Equation 1 (Meyer et al., 2007).

Equation 1:

$$OxD_{roGFP2} = \frac{R - R_{\mathrm{red}}}{(I_{465\mathrm{min}}/I_{465\mathrm{max}})(R_{\mathrm{ox}} - R) + (R - R_{\mathrm{red}})}$$

where R represents the 405/465 fluorescence ratio at the indicated time and treatment, R_{red} the 405/465 fluorescence ratio under fully reduced conditions, R_{ox}- the 405/465 fluorescence ratio under fully oxidized conditions, $I465_{ox}$- the fluorescence emitted at 515 nm when excited at 465 nm under fully oxidized conditions and $I480_{red}$- the fluorescence emitted at 515nm when excited at 465nm under fully reduced conditions. E_{GSH} values were calculated according to Schwarzlander et al. (2008). Box plots were created using the BoxPlotR tool (Spitzer et al., 2014)

Supplemental Data

Supplemental Figure S1. Schematic steps of *Solanum tuberosum* 'Desiree' transformation.

Supplementary Figure S2. Comparison of chl-roGFP2 ratio values under steady-state conditions in three independent lines expressing chl-roGFP2 in the chloroplast stroma

Supplementary Figure S3. Whole-plant imaging of WT and transgenic plant expressing roGFP2 in the chloroplast.

Supplementary Figure S4. Pixel distribution analysis of plants watered with H_2O_2.

Supplementary Figure S5. Pixel distribution analysis of plants watered with Buthionine sulphoximine (BSO).

Supplementary Figure S6. Pixel distribution analysis of leaves treated with various H_2O_2 concentrations.

Supplementary Figure S7. Fluorescence intensity of chl-roGFP2 and WT plants under high light.

Supplementary Figure S8. Fluorescence intensity of chl-roGFP2 and WT plants under high light and cold stress (3°).

Supplementary Figure S9. Fluorescence intensity of chl-roGFP2 and WT plants under different.

Supplementary Figure S10. Comparison between chl-roGFP2 OxD in young versus old leaves on Day 10 of the drought experiments.

Supplementary Movie S1. Whole-plant redox imaging of a potato plant watered with H_2O_2

Acknowledgments: We thank Andreas J. Meyer for his critical comments on the manuscript. We thank Dani Eshel and Paula Teper-Bemnolker for providing the starting materials and protocol for potato transformation.

Figure legends

Figure 1: Live imaging of potato plants expressing roGFP2 in chloroplasts: A, Confocal images of chl-roGFP2-expressing potato plants showing (chl-roGFP2 excitation: 488 nm). Red lines indicate 10 µm length B, Ratiometric confocal images of chloroplasts during rest, fully oxidized (1000 mM H_2O_2) and fully reduced (100mM DTT) states are shown. Red lines indicate 10 µm length. C, Quantification of ratiometric images in B presented as box plot (n=3). Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. D, Comparison between the emission intensities detected by whole-plant imaging at 515 nm in wild type (WT) and roGFP2-expressing plants, following excitation with 465 nm or 405 nm. The data are summarized as a violin plot reflecting the pixel distribution of a representative plant. E, Fluorescence whole-plant images and ratiometric analysis of chl-roGFP2 signals during rest and under fully oxidized (1000mM H_2O_2) and fully reduced (100mM DTT) conditions. Individual plant images were digitally combined for comparison. F, Whole-plant ratiometric images of potato plant watered with 1M H_2O_2 and monitored over 42 minutes. The numbers at the top represent the time from H_2O_2 application. The yellow arrows highlight the oxidation changes of a specific leaf during H_2O_2 treatment. Individual plant images were digitally combined for comparison. A movie visualization is depicted as Supp. Movie S1.

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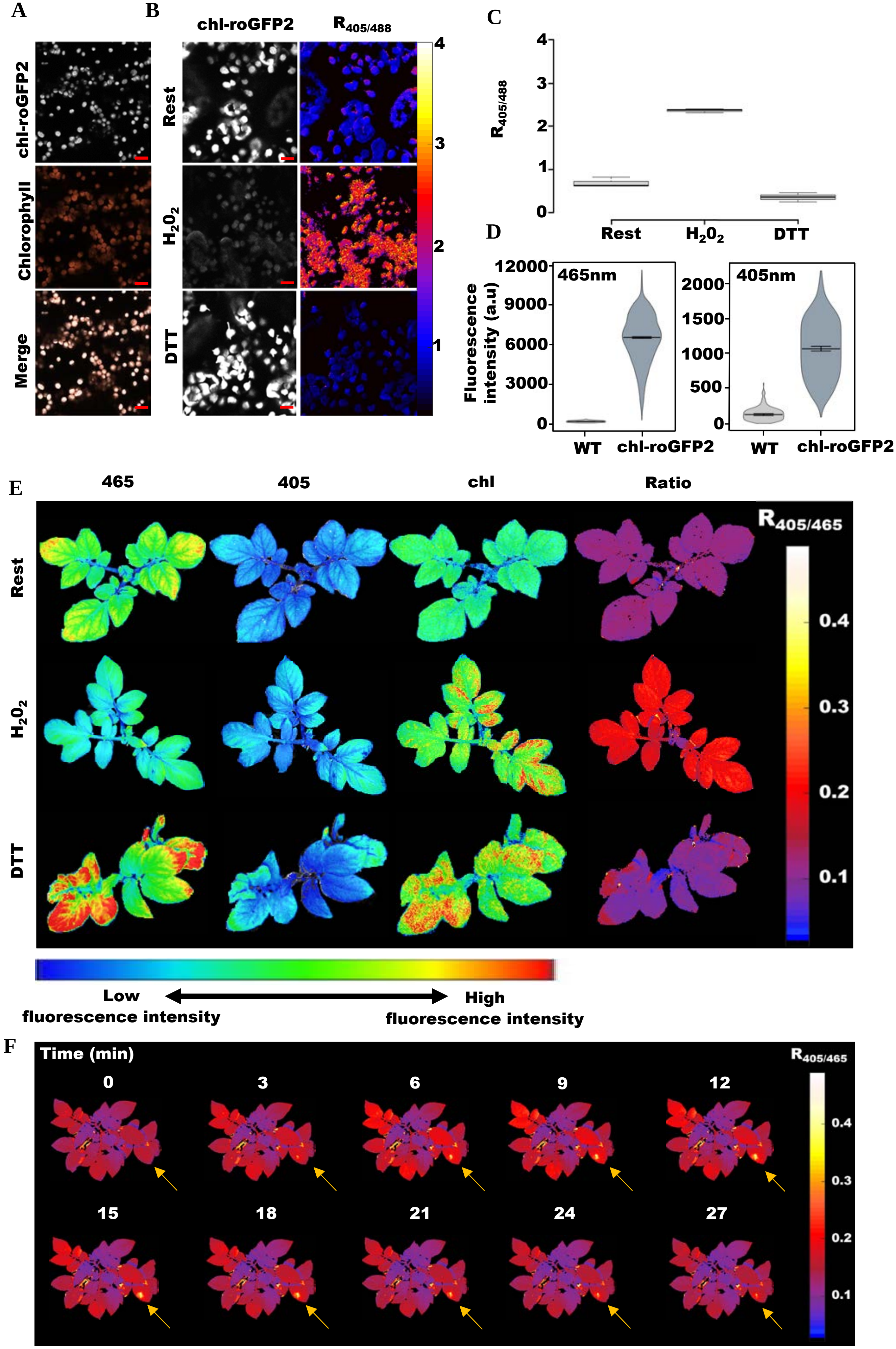
A
B
chl-roGFP2
Chlorophyll
Merge
chl-roGFP2
$R_{405/488}$
Rest
H_2O_2
DTT
4
3
2
1
C
$R_{405/488}$
Rest
H_2O_2
DTT
D
465nm
405nm
Fluorescence intensity (a.u)
WT
chl-roGFP2
E
465
405
chl
Ratio
$R_{405/465}$
Low fluorescence intensity
High fluorescence intensity
F
Time (min)
0
3
6
9
12
15
18
21
24
27
$R_{405/465}$

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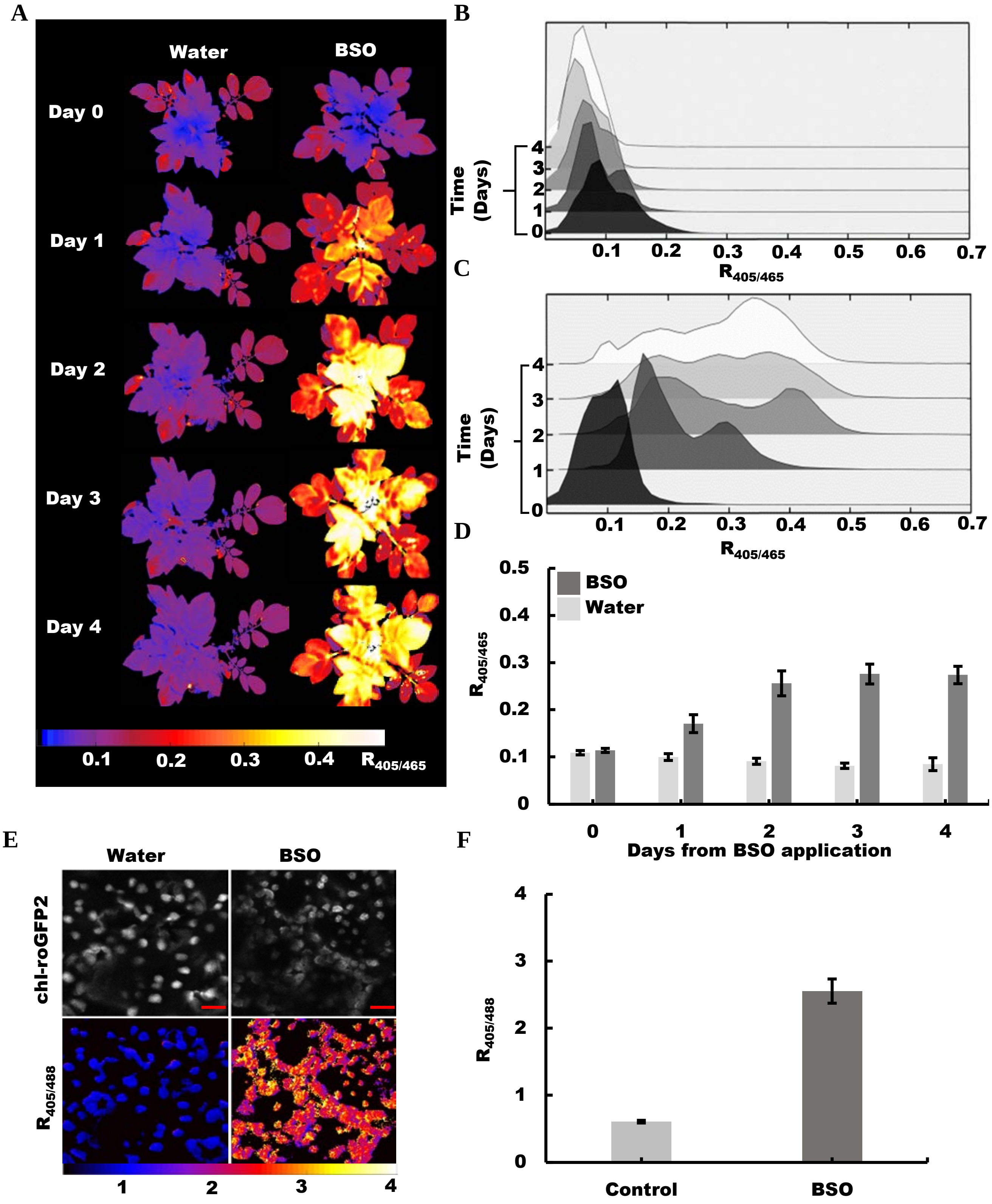

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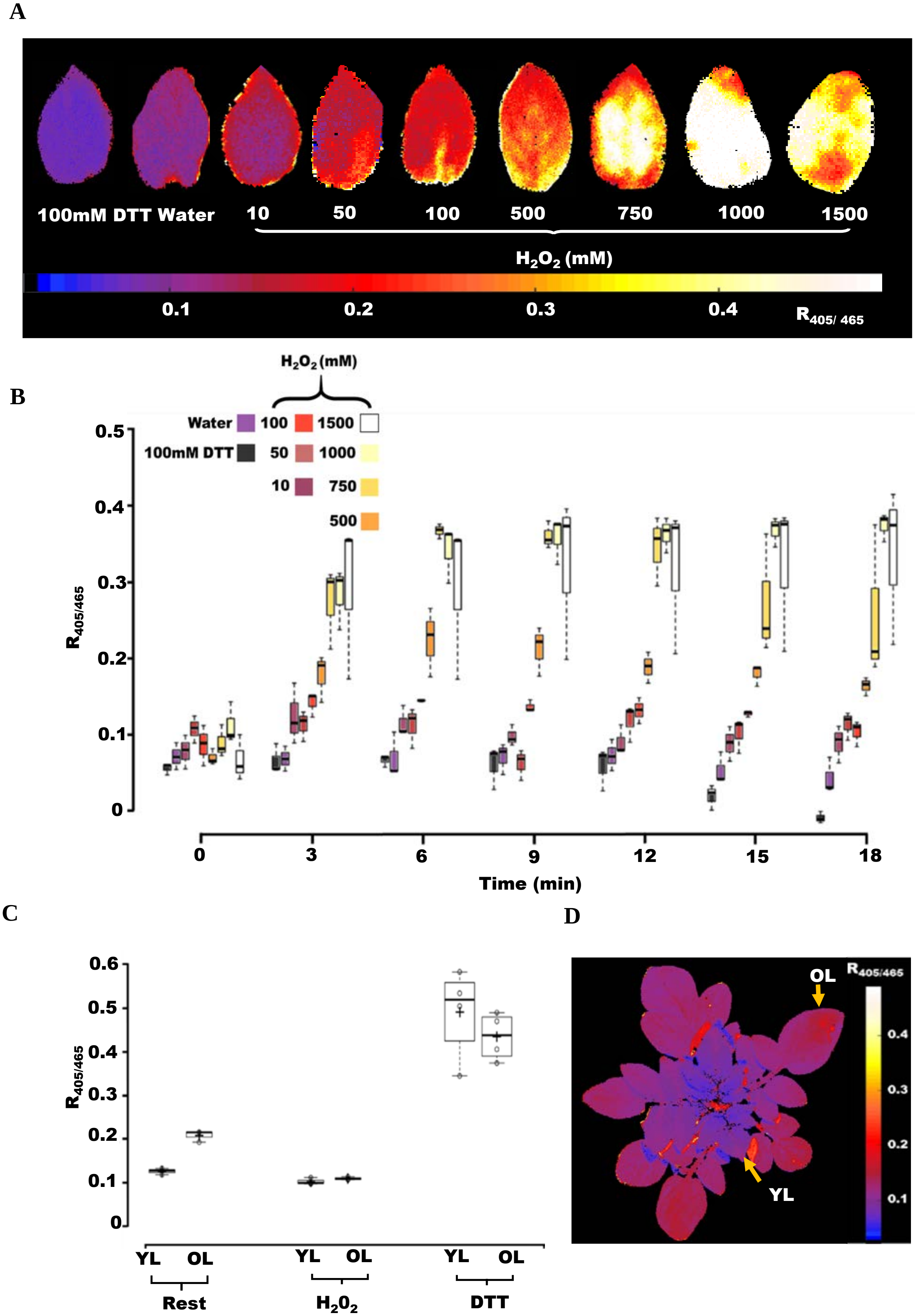
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100mM DTT Water
10
50
100
500
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1000
1500
H2O2 (mM)
0.1
0.2
0.3
0.4
R405/ 465
B
H2O2 (mM)
Water
100mM DTT
100
50
10
1500
1000
750
500
R405/465
0.5
0.4
0.3
0.2
0.1
0
0
3
6
9
12
15
18
Time (min)
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R405/465
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0.5
0.4
0.3
0.2
0.1
0
YL OL
Rest
YL OL
H202
YL OL
DTT
D
R405/465
OL
YL
0.4
0.3
0.2
0.1

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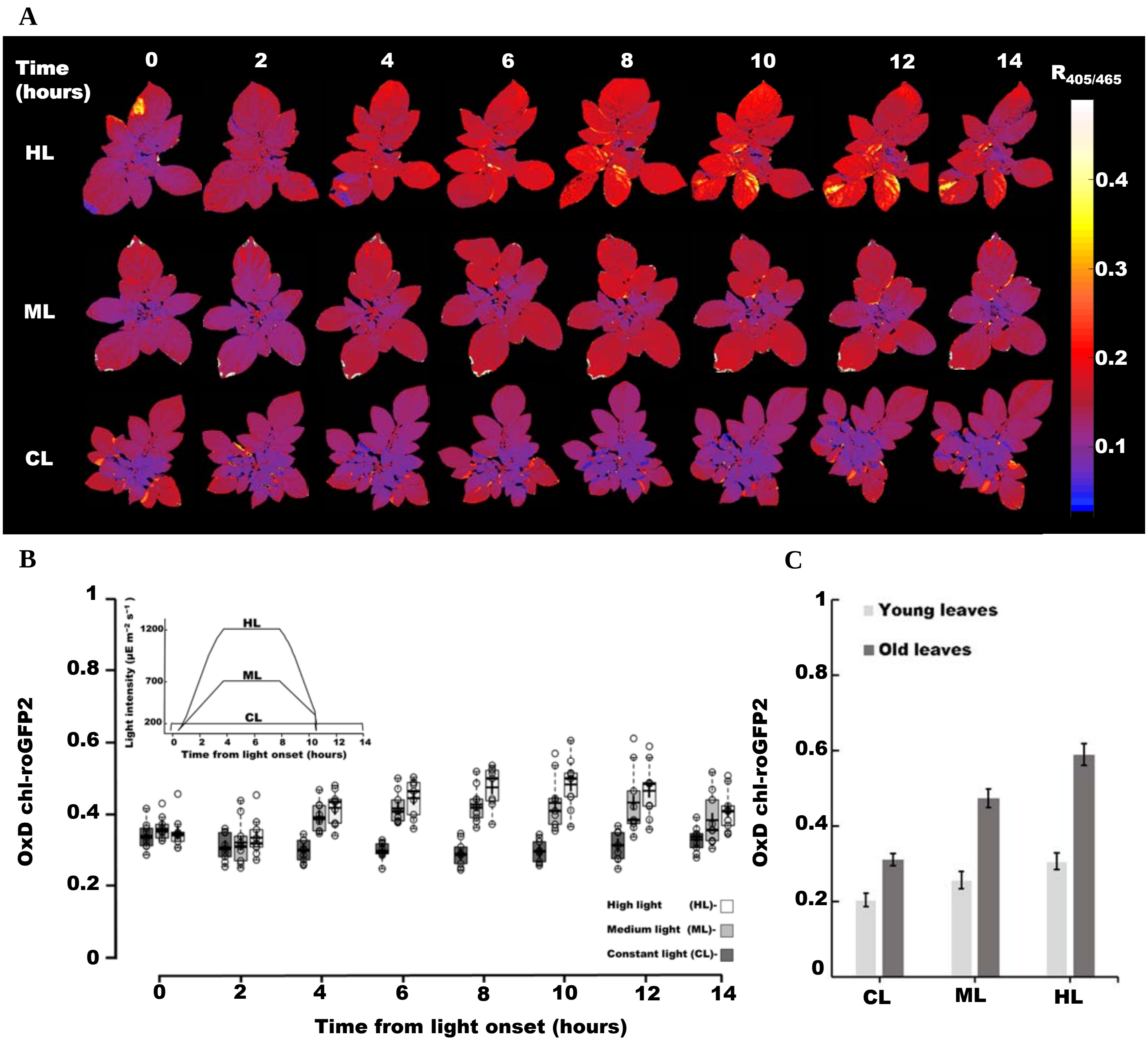

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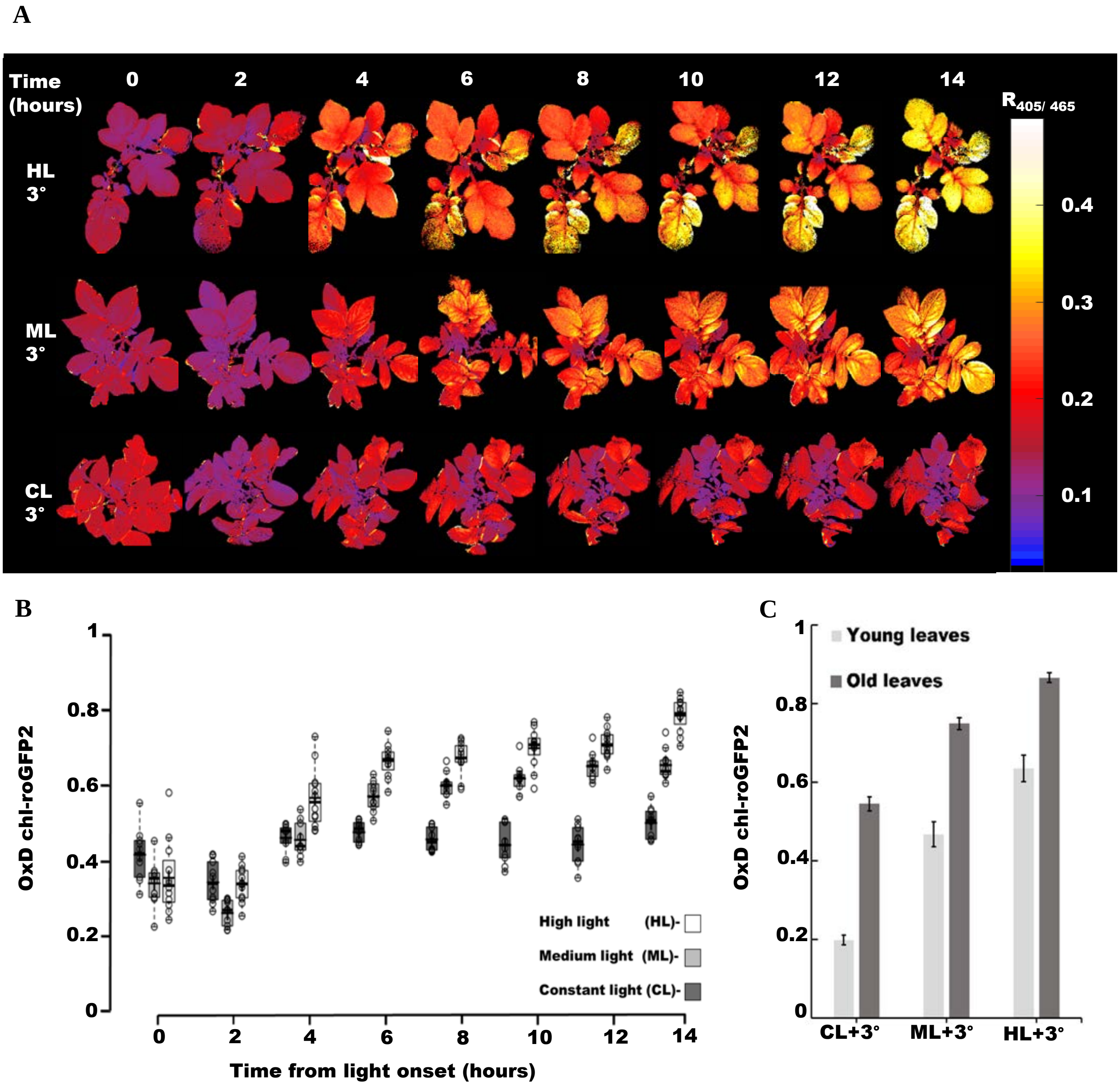

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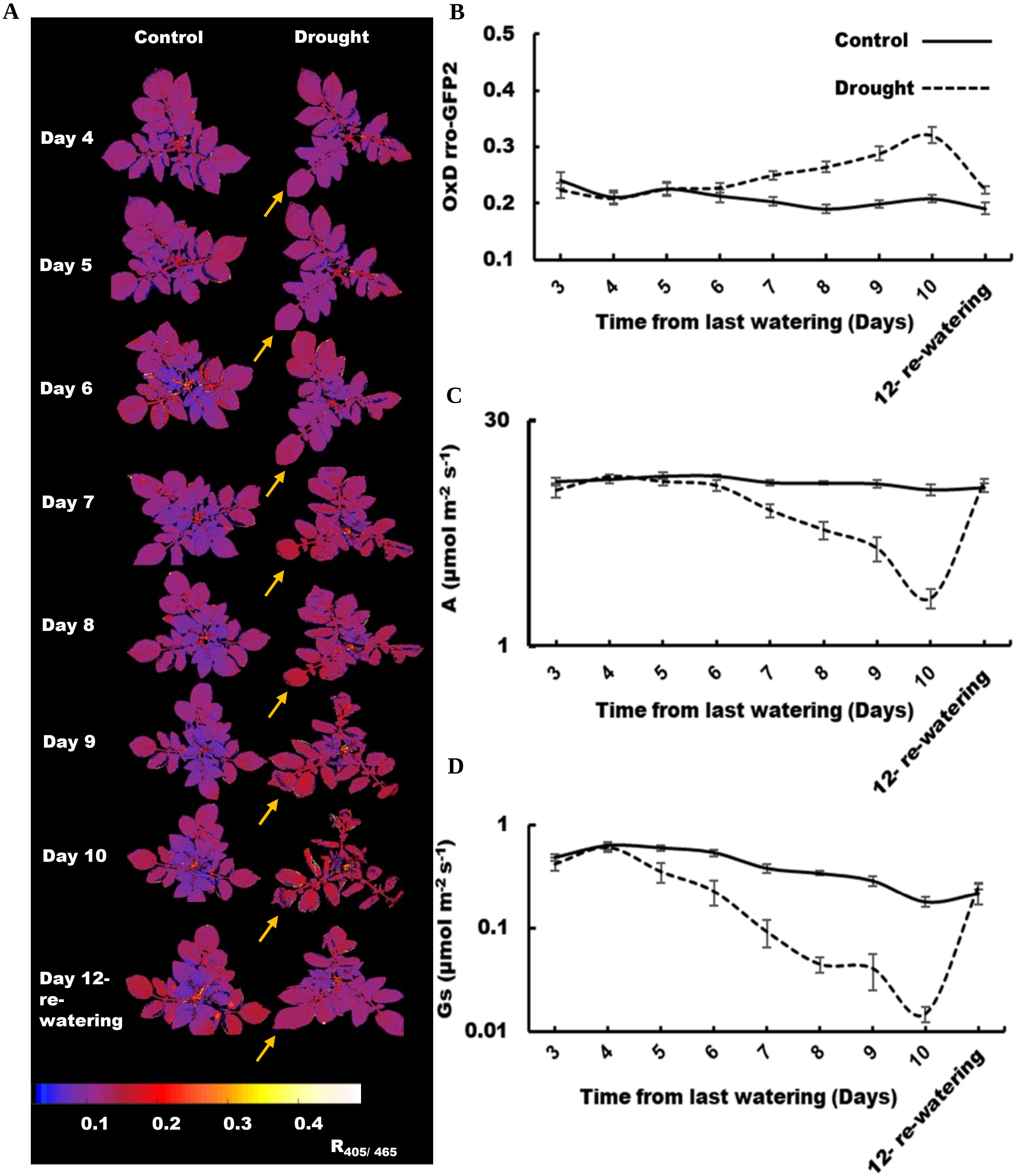

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