Biochemical and Biophysical Research Communications 452 (2014) 503–508

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# RoGFP1 is a quantitative biosensor in maize cells for cellular redox changes caused by environmental and endogenous stimuli

Xiaoning Liu, Jiamei Wu, Hao Liu, Na Zong, Jun Zhao *

*Faculty of Maize Functional Genomics, Department of Crop Genome Research and Genetic Improvement, Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, People's Republic of China*
*National Key Facility for Crop Gene Resources and Genetic Improvement, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, People's Republic of China*

ARTICLE INFO

*Article history:*
Received 16 August 2014
Available online 27 August 2014

*Keywords:*
ROS
RoGFP1
Redox change
Maize
ZmAPX
RNAi

ABSTRACT

Reduction–oxidation-sensitive green fluorescent proteins (roGFPs) have been demonstrated to be valuable tools in sensing cellular redox changes in mammalian cells and model plants, yet have not been applied in crops such as maize. Here we report the characteristics of roGFP1 in transiently transformed maize mesophyll protoplasts in response to environmental stimuli and knocked-down expression of ROS-scavenging genes. We demonstrated that roGFP1 in maize cells ratiometrically responds to cellular redox changes caused by $H_2O_2$ and DTT, as it does in mammalian cells and model plants. Moreover, we found that roGFP1 is sensitive enough to cellular redox changes caused by genetic perturbation of single ROS genes, as exemplified by knocked-down expression of individual *ZmAPXs*, in maize protoplasts under controlled culture conditions and under stress conditions imposed by $H_2O_2$ addition. These data provide evidence that roGFP1 functions in maize cells as a biosensor for cellular redox changes triggered by genetic lesion of single ROS genes even under stress conditions, and suggest a potential application of roGFP1 in large-scale screening for maize mutants of ROS signaling involved in development and stress resistance.


## 1. Introduction

Reactive oxygen species (ROS), as signals and modulators for cellular redox changes caused by metabolic imbalance between energy production and consumption in response to developmental and environmental cues, regulate many different processes in plants such as growth and development [1,2], hormone signaling [3], abiotic stress responses [4,5], pathogen defense and systemic acquired resistance [6,7], and programmed cell death [8,9]. Due to their highly reactive nature, however, ROS accumulation and signaling are tightly controlled by a delicate balance between production and scavenging, which determines the chemical identity, location, intensity and duration of ROS signals. While numerous sources for ROS production including those in photosynthetic and respiratory electron transport chains, during photorespiration and NADPH oxidase, and the ROS-scavenging enzymes such as superoxide dismutases, catalases, ascorbate peroxidases, glutathione peroxidases and compounds including ascorbate, glutathione and tocopherol have been identified [10–12], the regulatory mechanisms of how the localized and temporal accumulation of ROS is achieved, sensed and transduced in plant cells remain largely unknown, due mainly to the limitations of detection methods for monitoring the spatial and temporal dynamics of different ROS.

Traditionally, the reduced to oxidized glutathione ratio [GSH/GSSG] is measured to reflect the accumulation of ROS, because electrons for ROS detoxification are drawn at least in part from the GSH pool leading to changes of thiol redox potential [13]. Recently, various ROS-sensitive fluorescent dyes, such as the most popular dihydrodichlorofluorescein ($H_2$DCF) have been widely used to monitor ROS levels in plant processes as light stress responses, tip growth and defense signaling [14–21]. However, these methods exhibit such more drawbacks as being destructive, integrated, irreversible, point-in-time, and providing only a static redox status [22]. Alternative probes for the imaging of thiol redox potential have been developed based on GFP-based biosensors. By introducing point mutations (C48S, S147C, and Q204C) into wild type GFP, redox-sensitive variants roGFP1 and roGFP2 (with an additional S65T mutation) were generated, which undergo significant conformational changes in an oxidizing environment by forming disulfide bonds between the two introduced Cys147 and Cys204 residues,

* Corresponding author at: Faculty of Maize Functional Genomics, Department of Crop Genome Research and Genetic Improvement, Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, People's Republic of China.
*E-mail address:* zhaojun01@caas.cn (J. Zhao).

http://dx.doi.org/10.1016/j.bbrc.2014.08.107
0006-291X/

resulting in the shifting of the GFP excitation peak from 400 to 490 nm [23], by which roGFPs have therefore been used to report spatial and temporal intracellular oxidation status by ratiometric analysis (400/490 nm excitation) [24–29], which is non-destructive, compartmental, reversible, real-time and providing dynamic variation trends compared to other methods for assessing intracellular redox status [30,31]. It was further demonstrated that roGFP2 expressed in the cytosol equilibrates with the cellular glutathione redox buffer via glutaredoxin (GRX) as a mediator of reversible electron flow between glutathione and roGFP [24].

Maize is one of the most important crops in the world, which is the major source of food, feed and industrial raw material. Dissection of ROS-mediated signaling network regulating growth, development and stress resistance is the prerequisite for genetic improvement of maize for higher yield. To achieve this goal, GFP-based biosensors are valuable to monitor the spatial and temporal production of ROS during maize development or response to stress. Although roGFPs have been showed ratiometrically responsive to cellular redox changes, their activity remains unclear in crops such as maize. In an attempt to use roGFP1 as an indicator for cellular redox changes to screen for ROS signaling mutants, we first examined the response of roGFP1 to redox changes in maize cells. Our results showed that roGFP1 expressed in maize mesophyll protoplasts, like in mammalian and *Arabidopsis* cells, responds ratiometrically to dynamic cellular redox changes caused by exogenous factors or genetic perturbation.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Seeds of maize inbred line Q319 were germinated in pots containing vermiculite and were grown under a 16-h light/8-h dark photoperiod at 26 °C with light intensity of 108 μmol/$m^2$/s. For cDNA cloning and expression profiling of stress- and/or ABA-responsive *APX* genes in maize, V3 seedlings were first transferred to water to adapt for 1 h and then treated with 20% PEG, 100 μM ABA, and 250 mM NaCl, respectively, for various times. Leaves were sampled at 0, 1.5, 3, 6, 12, and 24 h. For protoplast isolation, germinated seeds with 1-cm long shoots were moved into a dark chamber at 26 °C to grow until the second leaf was about 10–15 cm long, and the middle parts (6–8 cm) of the second leaves were used to isolate protoplasts.

### 2.2. Protoplast isolation and transient transformation

Maize mesophyll protoplasts were prepared according to a protocol from Jen Sheen's lab [32] with minor modifications. Briefly, healthy leaves were cut into 0.5–1 mm fine segments and digested in an enzyme solution in the dark with gentle shaking at room temperature for about 5 h. After incubation, protoplasts were harvested, washed, and suspended in W5 solution. For transient experiments, 10 μg of plasmids were added to 200 μl of protoplasts in MMG solution, to which an equal volume of 30% PEG-$Ca^{2+}$ solution was gradually added. The mixture was incubated for 15 min before W5 solution was added to stop the reaction. The transfected protoplasts were collected, resuspended in W5 solution, and incubated at room temperature under weak light for 16 h. For stress treatment, the cultures were treated with $H_2O_2$ or DTT for 5–10 min. For RNA isolation, the transformed protoplasts were collected and stored at −80 °C until further analysis.

### 2.3. Bioinformatic analysis of maize APX genes family

Based on sequence information on AtAPX family members [12], homologs sequences of AtAPXs in maize genome, namely *ZmAPX* genes, were identified using PLAZA2.5 (http://bioinformatics.psb.ugent.be/plaza/). A phylogenetic tree of *APX* genes between *Arabidopsis thaliana* and *Zea mays* was constructed using the CLUSTAL and MEGA programs. Conserved domains in deduced amino acid sequences of APXs were searched using SMART (http://smart.embl-heidelberg.de/help/smart_glossary.shtml).

### 2.4. Plasmid construction

To make a construct for roGFP1 expression driven by ubiquitin promoter in maize protoplasts, the roGFP1 coding region was isolated from c-roGFP1 plasmid [26] by *Nco* I and *Afl* II double digestion, Klenow filled, and inserted into the *Eco*R V site downstream of the *Ubiquitin* promoter in the Pubi-Tnos expression cassette of vector pGEM-9zf-Pubi-Tnos which was generated by cloning the Pubi-Tnos expression cassette from pGA1611 [33] into pGEM-9zf backbone, resulting in plasmid Pubi-roGFP1.

RNAi constructs of *ZmAPXs*, were made using the method as described previously [34] with minor modifications. Briefly, the cDNA fragments of *ZmAPXs* were respectively cloned by RT-PCR with gene-specific primers (Suppl. Table 1) using RNAs (see below) prepared from maize seedling. The 80-nucleotide (nt) intron-hairpin (ihp) sequence

(5′-AGGCGTCCACTCAGGTAAGTCGAGCCCGTACAGCTAACTGCTACTGTCATGCTCAGCAGTGCAGGGAGTGGACGCCTACG-3′), containing the second intron of maize RING zinc finger protein-like source intron (GRMZM2G044537), was synthesized and phosophorylated. *ZmAPX* cDNA was digested with *Sfi* I (New England Biolabs) and purified using a Qiagen PCR Purification Kit. The restriction enzymes left a 3′ CGT overhang to allow for subsequent ligation to the phosophorylated 80-nt ihp sequence, which contained a compatible 3′ ACG overhang. The digestion product was ligated with the 80-nt ihp sequence at 16 °C overnight. The resulting dumbbell DNA molecules were amplified with a TempliPhi 100 amplification kit according to the manufacturer's protocol (Amersham Biosciences, UK), except that the specific primers RCA1 (5′-TGCTGAGCATGACAG-3′) and RCA2 (5′-CCGTACAGCTAACTG-3′) were used instead of the random primers. After an overnight incubation at 30 °C, the amplified products were digested with *Asc*I (New England Biolabs), leaving the single-unit inverted repeats, which were purified on a 0.8% agarose gel. The purified fragment was then ligated to pGEM-9zf-Pubi-Tnos digested with *Asc* I, generating pGEM-9zf-*ZmAPX*RNAi vectors where the expression of *ZmAPX* inverted repeats was driven by the *Ubiquitin* promoter.

### 2.5. RNA isolation and real-time RT-PCR

Total RNAs were isolated using Trizol reagent according to the manufacturer's protocol (TRIzol® Reagent) from maize V3 leaves for *ZmAPXs* cDNA cloning and expression analysis of *ZmAPXs* in response to stress/ABA treatments, or from transiently transformed mesophyll protoplasts with *ZmAPX*RNAi constructs for expression analysis of endogenous *ZmAPXs*. For first strand cDNA synthesis, the removal of genomic DNA and reverse transcription were performed simultaneously using the TaKaRa PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time). To avoid random amplification, oligod (T) was used as the primer instead to replace the RT Primer Mix™ provided in the kit in the reverse transcription of protoplasts RNAs. For real-time RT-PCR, diluted cDNA was mixed with SYBR *Premix Ex Taq*™ (TaKaRa) in 20 μl reaction volume. Gene-specific primers used here were listed in Suppl. Tables 2 and 3. The reaction was done using the ABI 7500 system was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 34 s, and a final melting curve analysis. The cycle threshold (Ct) values, corresponding to the PCR cycle number at

which fluorescence emission reaches a threshold above baseline emission, were determined and the relative fold differences were calculated by $2^{-\Delta\Delta Ct}$ method using the *GAPDH* gene as an endogenous reference and the untreated leaves or mock-transfected protoplasts as a calibrator. Samples were run in triplicates on each 96-well plate and were repeated 3 times for each experiment.

### 2.6. Confocal imaging and ratiometric analysis

RoGFP1-expressing maize protoplasts treated with different concentration of $H_2O_2$ or DTT and/or co-expressing *ZmAPX*RNAi were imaged using a Zeiss LSM 700 confocal microscope equipped with lasers for 405- (track 1) and 488-nm (track 2) excitation. For excitation of track1 and track 2, fluorescence was collected with a bandpass filter of 405–520 nm and 488–520 nm, respectively. A total of 20 mesophyll cells each were imaged in 6-stacks scan mode and projected as maximum intensity projections using LSM software. A fluorescence ratiometric analysis of the images was performed using IMAGEJ software (http://rsb.info.nih.gov/ij/download.html). Quantitative statistic analysis of 405/488 nm ratios was calculated as the mean of 20 cells ± S.E determined in ratio images.

## 3. Results

### 3.1. RoGFP1 responds to redox changes caused by $H_2O_2$ and DTT

In mammal and model plants such as *Arabidopsis* and tobacco, roGFP1 is redox sensitive and its fluorescence ratio is dependent on the redox potential [23,26,27,29]. The more oxidized the redox status is, the higher the ratio goes. To determine whether roGFP1 is also responsive to redox changes and can be ratiometrically measured in crop cells, maize mesophyll were prepared and transformed with Pubi-roGFP1, and treated with $H_2O_2$ and DTT. As shown in Fig. 1A, strong fluorescence emitted at 520 nm was observed after excitation at 405 or 488 nm, indicating that roGFP1 was expressed in transformed protoplasts and has two excitation peaks. As the concentration of oxidizing $H_2O_2$ became increasingly higher in the ambient conditions, the 405/488 nm fluorescence ratios of GFP1 in maize protoplasts increases from 2.08 (control) to 3.95 (10 mM $H_2O_2$), whereas addition of reductive DTT in the media resulted in a decreasing 405/488 nm ratios from 2.08 (untreated) to 1.42 (2.5 mM DTT) (Fig. 1B). These data demonstrate that roGFP1 in maize cells responds, reflected in altered 405/

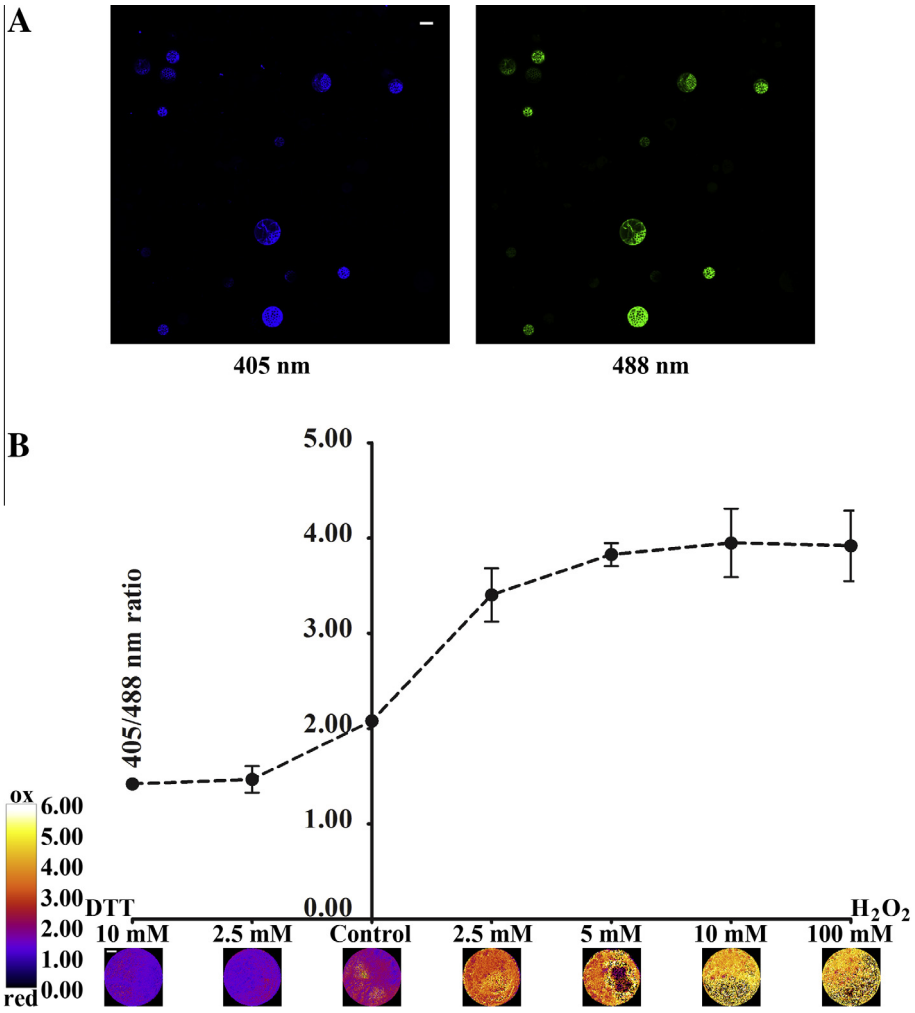


**Fig. 1.** RoGFP1 expression in maize mesophyll protoplasts and its responsiveness in 405/488 nm fluorescence ratio to redox changes caused by $H_2O_2$ and DTT. (A) Fluorescence images with excitation at 405 and 488 nm of protoplasts expressing roGFP1 taken with 10× lens. (B) 405/488 nm fluorescence ratios of roGFP1 in protoplasts treated with different concentrations of $H_2O_2$ or DTT. Stacks of images were taken with CLSM and their maximum projections were exported for the calculation of ratio images using IMAGEJ software. The threshold was set between 0 and 6 when calculating 405/488 nm fluorescence ratio and the resultant ratio images were mapped to a pseudo-color "Look Up Table", which was adjusted such that ratios indicative of reduced roGFP1 were manifested in blue and ratios indicative of oxidized roGFP1 were shown in yellow. Scale bar = 6 μm. The data are representative of 3 experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

488 nm excitation fluorescence ratio, to cellular redox changes caused by environmental stimuli $H_2O_2$ and DTT, as it does in mammalian cells and model plants.

### 3.2. Identification of stress/ABA-responsive APX genes in Z. mays

The ultimate goal of this research is to establish a roGFP1-based redox-sensing system in maize for mutant screening of ROS signaling in response to stress. To this end, a stress-responsive marker gene which is involved in ROS homeostasis/signaling is needed to test the sensing system. The glutathione–ascorbate (GSH–ASC) cycle plays an important role in the control of cellular levels of ROS [35]. To select a maker gene from this cycle for the test, we identified the maize homologs of *Arabidopsis* APXs and characterized their stress responsiveness.

Using PLAZA 2.5 platform with sequences of *Arabidopsis* APXs as queries, eighteen putative *ZmAPX* genes (Suppl. Table 4) were identified, among which eight have been characterized previously [36]. Phylogenetic analysis of APXs from maize and *Arabidopsis* (Suppl. Fig. 1) showed that ZmAPX1.1 (GRMZM2G137839) and

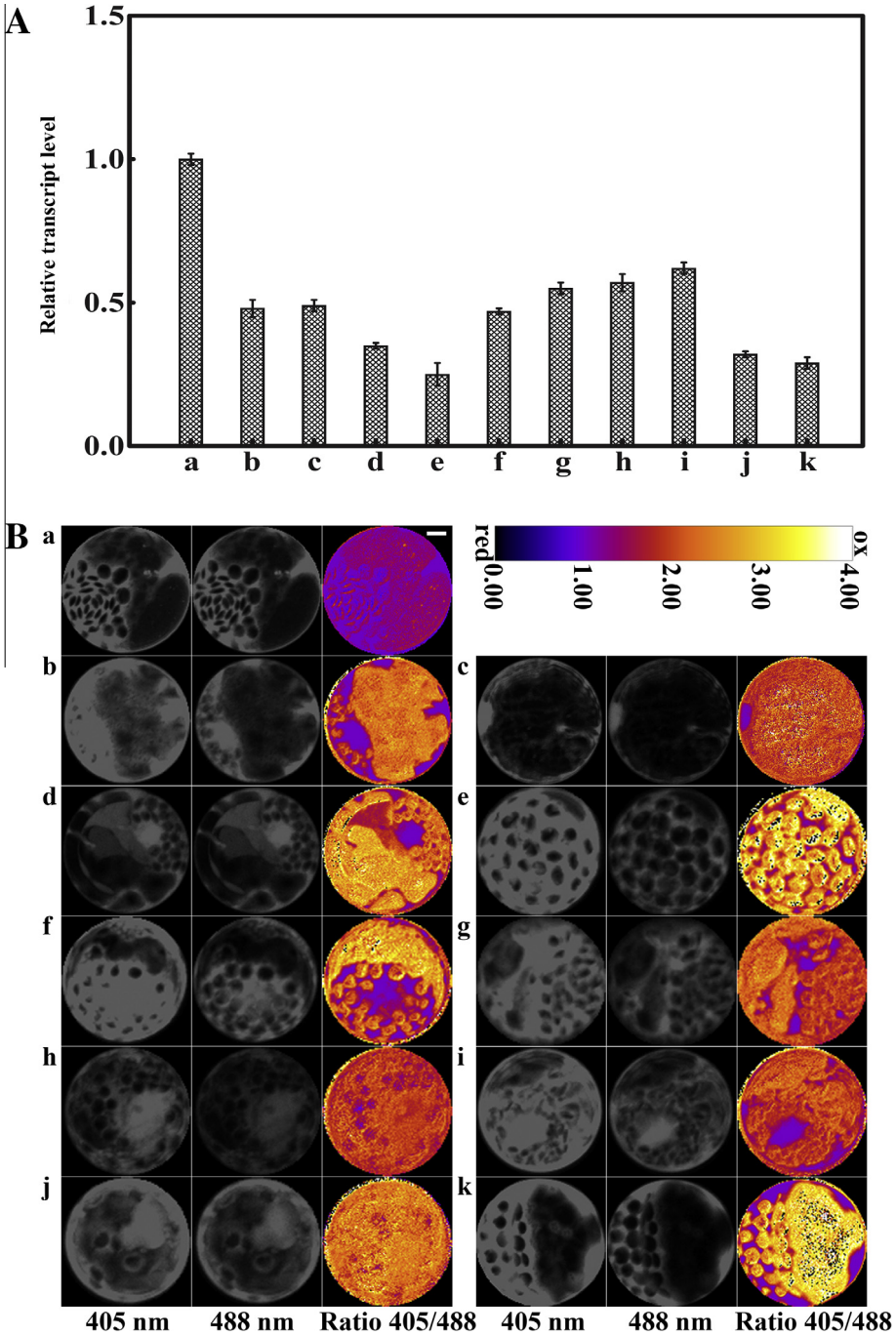


**Fig. 2.** RoGFP1 responds to cellular oxidation caused by knocked-down expression of individual *ZmAPXs* in maize mesophyll protoplasts. (A) Transcript levels of individual *ZmAPXs* in protoplasts transformed with corresponding RNAi constructs of the same genes relative to that in untransformed protoplasts by real-time RT-PCR. (B) 405/488 nm fluorescence ratio images of protoplasts transformed with individual *ZmAPX*RNAi constructs. The threshold of resultant ratio image was set between 0 and 4 when calculating 405/488 nm fluorescence ratio. Scale bar = 6 μm. a, untransformed control; b, *ZmAPX1.1*; c, *ZmAPX1.2*; d, *ZmAPX3*; e, *ZmAPX3.1*; f, *ZmAPX8*; g, *ZmAPX2*; h, *ZmAPX2.3*; i, *ZmAPX2.4*; j, *ZmAPX2.5*; and k, *ZmAPX3.2*. The data are representative of 3 experiments.

1.2 (GRMZM2G054300) are the closest homologs of *Arabidopsis* cytosolic APX 1 (At1g07890) which is the key ROS-scavenging mechanism in the cytosol and plays a central role in cross-protecting chloroplast from oxidative stress [37]. ZmAPX8 (GRMZM2G006791) and 8.1 (GRMZM2G120517) have the highest similarity with *Arabidopsis* stromal/mitochondrial isozyme of APX (At4g08390) [38]. ZmAPX3 (GRMZM2G004211) and 3.1 (GRMZM2G460406) are most similar to *Arabidopsis* peroxisome-targeted APX (At4g35000) [39].

To explore whether stress/ABA exerts influence on *ZmAPX* gene expression, the expression profiles of ten *ZmAPX*s were examined by real-time quantitative RT-PCR in maize leaves treated with PEG, NaCl or ABA. As shown in Suppl. Fig. 2, the transcript of all *ZmAPXs* tested was induced by at least one of the treatments. Notably, 10-fold increase in transcripts at its peak was observed for *ZmAPX3.2* in response to PEG, while the remaining ones except *ZmAPX2.3* showed 4- to 5-fold increases. In addition, the peak transcript level of *ZmAPX3.1* showed a 7-fold induction by ABA and that of *ZmAPX3* exhibited a 4-fold increase under salt stress.

Based on the data above, *ZmAPX1.1*, *1.2*, *3*, *3.1* and *8* were selected as stress-responsive ROS-scavenging marker genes for the test of roGFP1 response to redox changes caused by alteration of ROS gene expression.

### 3.3. RoGFP1 responds to redox changes caused by altered gene expression of ZmAPXs

To further explore whether roGFP1 responds to redox changes caused by genetic perturbation of genes involved in ROS homeostasis/signaling, Ubiquitin promoter-driven *ZmAPX* RNAi constructs for ten *ZmAPXs* were made (Suppl. Fig. 3), and each was co-transformed with Pubi-roGFP1 into maize mesophyll protoplasts before the expression of *ZmAPXs* and roGFP1 fluorescence were examined. As shown in Fig. 2, the transcript levels of *ZmAPXs* in transformed protoplasts measured by real time RT-PCR were lowered down to 25–62% of that in mock-treated protoplasts, indicating that the expression of *ZmAPXs* was reduced by RNA interference (Fig. 2A). Consequently, the 405/488 nm excitation ratio elevated higher in protoplasts expressing individual RNAi constructs of the marker *ZmAPXs* (2.05, 2.04, 2.32, 2.62 and 2.10 for RNAi of *ZmAPX1.1*, *1.2*, *3*, *3.1* and *8*, respectively) compared with the 1.22 ratio value in mock-transformed control (Fig. 2B), indicating that roGFP1 is able to sense the redox changes caused by knock-down expression of single marker *ZmAPXs*. Based on these results, the same tests were also done with RNAi constructs of the remaining five putative *ZmAPXs* (*ZmAPX2*, *2.3*, *2.4*, *2.5* and *3.2*) and similar results were obtained, i.e., the 405/488 ratio of roGFP1 increased in protoplasts transformed with RNAi constructs in comparison with that in mock-transformed protoplasts (Fig. 2B), suggesting that the five genes encode functional ZmAPXs involved in cellular thiol redox control.

### 3.4. RoGFP1 responds to redox changes caused by combination of genetic perturbation and environmental stimuli

For screening maize ROS signaling mutants in response to environmental stress using roGFP1 biosensor, it is important to know to what extent roGFP1 is sensitive to redox changes caused by genetic perturbation of ROS genes in maize cells under stress conditions which usually cause high level accumulation of ROS. To test this, *ZmAPX3.1*RNAi and *ZmAPX3.2*RNAi constructs each were co-transformed with Pubi-roGFP1 into maize mesophyll protoplasts before treatment with $H_2O_2$, and then roGFP1 fluorescence was examined. As shown in Fig. 3, roGFP1 showed a fluorescence ratio of 3.37 in $H_2O_2$-treated control protoplasts expressing roGFP1 only (Fig. 3A). By contrast, expression of individual RNAi constructs in protoplasts with the same stress-treatment resulted in a fluorescence ratio of 4.23 and 4.21 for *ZmAPX3.1*RNAi and *ZmAPX8.2*RNAi, respectively (Fig. 3B and C). These data indicate that roGFP1 is sensitive enough to detect redox changes caused by genetic perturbations of single ROS genes even under stress conditions, which usually cause high level accumulation of ROS in plant cells.

## 4. Discussion

The molecular mechanisms regulating ROS signaling in plants, particularly in crops, remain largely unknown due mainly to limitations in monitoring the complex dynamics of ROS. The recently developed GFP-based biosensors have been documented valuable tools in sensing cellular redox status thus ROS homeostasis/signaling in mammal and model plants, yet have not been applied in crops. In this study, we demonstrated that roGFP1 responds ratiometrically to redox changes caused by exogenously applied $H_2O_2$ and DTT in maize mesophyll protoplasts (Fig. 1), which is consistent with that observed previously in mammal and model plants [23,26,27,29]. In addition, we also found that roGFP1 is sensitive enough to cellular redox changes caused by genetic perturbation of single ROS genes, as exemplified by knocked-down expression of individual *ZmAPXs*, in maize protoplasts cultured in controlled conditions and even under stress conditions imposed by $H_2O_2$ addition in the media (Figs. 2 and 3), which echoed the observation that GRX1-roGFP2 was capable of detecting the cytosolic glutathione redox change caused by gr1 mutation in *Arabidopsis* [13].

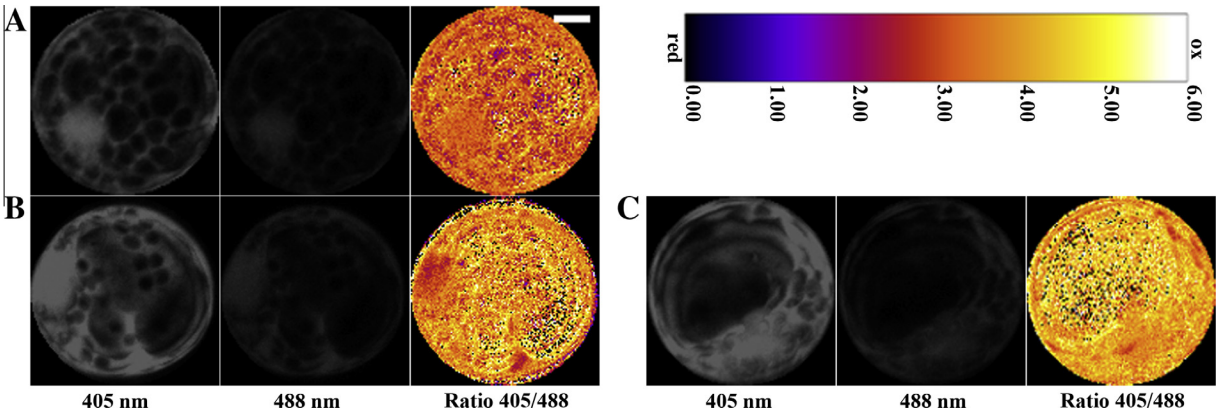


**Fig. 3.** RoGFP1 responds to cellular oxidation caused by knocked-down expression of single *ZmAPXs* in maize mesophyll protoplasts treated with 2.5 mM $H_2O_2$. 405/488 nm fluorescence ratio images of roGFP1 in $H_2O_2$-treated protoplasts untransformed (A) and that transformed with *APX3.1* RNAi (B) and *APX3.2* RNAi (C) are shown. The threshold of resultant ratio image was set between 0 and 6 when calculating 405/488 ratio. Scale bar = 6 μm. The data are representative of 3 experiments.

These data provide evidence that roGFP1 functions in maize cells as a biosensor for cellular redox changes evoked by genetic lesion of single ROS genes even under stress conditions, and can be used in large-scale screening for maize mutants of ROS signaling in growth and development and stress responses. For this effort, roGFP1-expressing transgenic maize plants are being generated and genome-wide RNAi libraries of different maize tissues have been constructed in our lab.

APXs of GSH–ASC cycle play an important role in the scavenging of cellular ROS and are encoded by nine genes in *Arabidopsis* genome [10,11,35]. In this study, eighteen putative *ZmAPXs* were identified based on sequence homology, conserved APX catalytic domain and responsiveness to stress/ABA (Suppl. Figs. 1 and 2, Suppl. Table 4). We examined the expression patterns of ten *ZmAPXs*, namely *ZmAPX1.1*, *1.2*, *2*, *2.3*, *2.4*, *2.5*, *3*, *3.1*, *3.2*, and *8*, and found that they were induced by at least one of the stress/ABA treatments (Suppl. Fig. 2), which is basically consistent with that observed previously [36]. Moreover, knocked-down expression of the ten individual *ZmAPXs* resulted in more oxidized cellular redox status under both unstressed and stressed conditions (Figs. 2 and 3). These data indicate that the ten *ZmAPXs* encode functional APX isozymes that play important roles in the control of cellular ROS levels under both normal growth and stress conditions

## Acknowledgments

The authors are grateful to Professor Lewis Feldman for kindly providing the c-roGFP1 plasmid. This work was supported by the National Basic Research Program of China (Grant Number 2014CB138205) and by the National Special Program for GMO Development of China (Grant Number 2014ZX08003-004).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.107.

## References

[1] C. Gapper, L. Dolan, Control of plant development by reactive oxygen species, Plant Physiol. 141 (2006) 341–345.
[2] M.J. Considine, C.H. Foyer, Redox regulation of plant development, Antioxid. Redox Signal. (2014), http://dx.doi.org/10.1089/ars.2013.5665.
[3] J.M. Kwak, V. Nguyen, J.I. Schroeder, The role of reactive oxygen species in hormonal responses, Plant Physiol. 141 (2006) 323–329.
[4] N. Suzuki, S. Koussevitzky, R. Mittler, et al., ROS and redox signalling in the response of plants to abiotic stress, Plant Cell Environ. 35 (2012) 259–270.
[5] N. Suzuki, G. Miller, C. Salazar, et al., Temporal-spatial interaction between reactive oxygen species and abscisic acid regulates rapid systemic acclimation in plants, Plant Cell 25 (2013) 3553–3569.
[6] M.A. Torres, J.D. Jones, J.L. Dangl, Reactive oxygen species signaling in response to pathogens, Plant Physiol. 141 (2006) 373–378.
[7] A. Baxter, R. Mittler, N. Suzuki, ROS as key players in plant stress signalling, J. Exp. Bot. 65 (2014) 1229–1240.
[8] J.L. Dangl, J.D. Jones, Plant pathogens and integrated defence responses to infection, Nature 411 (2001) 826–833.
[9] H.T. Xie, Z.Y. Wan, S. Li, et al., Spatiotemporal production of reactive oxygen species by NADPH oxidase is critical for tapetal programmed cell death and pollen development in Arabidopsis, Plant Cell 26 (2014) 2007–2023.
[10] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410.
[11] R. Mittler, S. Vanderauwera, M. Gollery, et al., Reactive oxygen gene network of plants, Trends Plant Sci. 9 (2004) 490–498.
[12] R. Mittler, S. Vanderauwera, N. Suzuki, et al., ROS signaling: the new wave?, Trends Plant Sci 16 (2011) 300–309.
[13] L. Marty, W. Siala, M. Schwarzlander, et al., The NADPH-dependent thioredoxin system constitutes a functional backup for cytosolic glutathione reductase in Arabidopsis, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 9109–9114.
[14] G. Agati, P. Matteini, A. Goti, et al., Chloroplast-located flavonoids can scavenge singlet oxygen, New Phytol. 174 (2007) 77–89.
[15] L. Cardenas, A. Martinez, F. Sanchez, et al., Fast, transient and specific intracellular ROS changes in living root hair cells responding to nod factors (NFs), Plant J. 56 (2008) 802–813.
[16] S.M. Coelho, C. Brownlee, J.H. Bothwell, Feedback control of reactive oxygen and Ca signaling during brown algal embryogenesis, Plant Signaling Behav. 3 (2008) 570–572.
[17] S.M. Driever, M.J. Fryer, P.M. Mullineaux, et al., Imaging of reactive oxygen species in vivo, Methods Mol. Biol. 479 (2009) 109–116.
[18] C. Flors, M.J. Fryer, J. Waring, et al., Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, singlet oxygen sensor green, J. Exp. Bot. 57 (2006) 1725–1734.
[19] J. Foreman, V. Demidchik, J.H. Bothwell, et al., Reactive oxygen species produced by NADPH oxidase regulate plant cell growth, Nature 422 (2003) 442–446.
[20] P. Liu, R.L. Li, L. Zhang, et al., Lipid microdomain polarization is required for NADPH oxidase-dependent ROS signaling in *Picea meyeri* pollen tube tip growth, Plant J. 60 (2009) 303–313.
[21] M. Orozco-Cardenas, C.A. Ryan, Hydrogen peroxide is generated systemically in plant leaves by wounding and system in via the octadecanoid pathway, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 6553–6557.
[22] X. Wang, H. Fang, Z. Huang, et al., Imaging ROS signaling in cells and animals, J. Mol. Med. (Berl) 91 (2013) 917–927.
[23] G.T. Hanson, R. Aggeler, D. Oglesbee, et al., Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators, J. Biol. Chem. 279 (2004) 13044–13053.
[24] A.J. Meyer, T. Brach, L. Marty, et al., Redox-sensitive GFP in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer, Plant J. 52 (2007) 973–986.
[25] S. Rosenwasser, I. Rot, A.J. Meyer, et al., A fluorometer-based method for monitoring oxidation of redox-sensitive GFP (roGFP) during development and extended dark stress, Physiol. Plant. 138 (2010) 493–502.
[26] K. Jiang, C. Schwarzer, E. Lally, et al., Expression and characterization of a redox-sensing green fluorescent protein (reduction–oxidation-sensitive green fluorescent protein) in Arabidopsis, Plant Physiol. 141 (2006) 397–403.
[27] M. Schwarzlander, M.D. Fricker, C. Muller, et al., Confocal imaging of glutathione redox potential in living plant cells, J. Microsc. 231 (2008) 299–316.
[28] M. Schwarzlander, M.D. Fricker, L.J. Sweetlove, Monitoring the in vivo redox state of plant mitochondria: effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge, Biochim. Biophys. Acta 1787 (2009) 468–475.
[29] C.T. Dooley, T.M. Dore, G.T. Hanson, et al., Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators, J. Biol. Chem. 279 (2004) 22284–22293.
[30] W.G. Choi, S.J. Swanson, S. Gilroy, High-resolution imaging of $Ca^{2+}$, redox status, ROS and pH using GFP biosensors, Plant J. 70 (2012) 118–128.
[31] S.J. Swanson, W.G. Choi, A. Chanoca, et al., In vivo imaging of $Ca^{2+}$, pH, and reactive oxygen species using fluorescent probes in plants, Annu. Rev. Plant Biol. 62 (2011) 273–297.
[32] S.D. Yoo, Y.H. Cho, J. Sheen, Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis, Nat. Protoc. 2 (2007) 1565–1572.
[33] J.S.J.S. Lee, K.H. Jung, G. An, Binary vectors for efficient transformation of rice, J. Plant Biol. 42 (1999) 310–316.
[34] G. Sen, T.S. Wehrman, J.W. Myers, et al., Restriction enzyme-generated siRNA (REGS) vectors and libraries, Nat. Genet. 36 (2004) 183–189.
[35] G. Noctor, C.H. Foyer, ASCORBATE AND GLUTATHIONE: keeping active oxygen under control, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 249–279.
[36] Y.J. Liu, Y. Yuan, Y.Y. Liu, et al., Gene families of maize glutathione-ascorbate redox cycle respond differently to abiotic stresses, J. Plant Physiol. 169 (2012) 183–192.
[37] S. Davletova, L. Rizhsky, H. Liang, et al., Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis, Plant Cell 17 (2005) 268–281.
[38] O. Chew, J. Whelan, A.H. Millar, Molecular definition of the ascorbate-glutathione cycle in Arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants, J. Biol. Chem. 278 (2003) 46869–46877.
[39] F.J. Corpas, J.B. Barroso, L.A. del Rio, Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells, Trends Plant Sci. 6 (2001) 145–150.