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Research article

Superoxide-responsive gene expression in *Arabidopsis thaliana* and *Zea mays*

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ABSTRACT

Superoxide (O_2^-) and other reactive oxygen species (ROS) are generated in response to numerous biotic and abiotic stresses. Different ROS have been reported to elicit different transcriptional responses in plants, and so ROS-responsive marker genes and promoter::reporter gene fusions have been proposed as indirect means of detecting ROS and discriminating among different species. However, further information about the specificity of transcriptional responses to O_2^- is needed in order to assess potential markers for this critical stress-responsive signaling molecule. Using qRT-PCR, the expression of 12 genes previously reported to be upregulated by O_2^- was measured in *Arabidopsis thaliana* plants exposed to elicitors of common stress-responsive ROS: methyl viologen (an inducer of O_2^-), rose bengal (an inducer of singlet oxygen, $^1\Delta O_2$), and exogenous hydrogen peroxide (H_2O_2). Surprisingly, *Zinc-Finger Protein 12* (*AtZAT12*), which had previously been used as a reporter for H_2O_2, responded more strongly to O_2^- than to H_2O_2; moreover, the expression of an *AtZAT12* promoter-reporter fusion (*AtZAT12::Luc*) was enhanced by diethyldithiocarbamate, which inhibits dismutation of O_2^- to H_2O_2. These results suggest that *AtZAT12* is transcriptionally upregulated in response to O_2^-, and that *AtZAT12::Luc* may be a useful biosensor for detecting O_2^- generation *in vivo*. In addition, transcripts encoding *uncoupling proteins* (*AtUCPs*) showed selectivity for O_2^- in Arabidopsis, and an *AtUCP* homolog upregulated by methyl viologen was also identified in maize (*Zea mays* L.), indicating that there are O_2^-–responsive members of this family in monocots. These results expand our limited knowledge of ROS-responsive gene expression in monocots, as well as O_2^--selective responses in dicots.

1. Introduction

Superoxide (O_2^-) is a reactive oxygen species (ROS) observed in many if not all plant responses to biotic and abiotic stress. In reaction to biotic attackers such as pathogens and herbivores or abiotic challenges such as drought, salinity, and other environmental extremes, O_2^- generation can increase through two avenues: perturbation of metabolic processes and activation of signaling pathways that lead to enzymatic ROS generation (Choudhury et al., 2016). In the course of normal metabolism, O_2^- is produced by

Abbreviations: AtACT2, actin-2; *AtCOBL5*, COBRA-like protein5 precursor; *AtERD7*, senescence/dehydration-associated protein; *AtGRP*, glycine-rich protein; *AtIGMT1*, O-methyltransferase; *AtKIN*, kinase-like protein; *AtMPK9*, mitogen-activated protein kinase 9; *AtOGO*, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein; *AtPL*, phospholipase-like (PEARLI 4) family protein; *AtTub-2*, tubulin beta-2/beta-3; *AtUCP*, uncoupling protein; *AtZAT12*, zinc-finger protein 12; DDC, diethyldithiocarbamate; H_2O_2, hydrogen peroxide; Hyper, a hydrogen peroxide sensor; MV, methyl viologen; NADPH oxidases, nicotinamide adenine dinucleotide phosphate-oxidase; NBT, nitrotetrazolium blue chloride; $^1\Delta O_2$, singlet oxygen; O_2^-, superoxide; PSI, photosystem I; RB, rose bengal; RLUs, relative light units; ROS, reactive oxygen species; RT-qPCR, reverse transcriptase quantitative PCR; *ZmAPX1*, cytosolic ascorbate peroxidase; *ZmCAT1*, *catalase 1*; *ZmGSR1*, glutathione reductase 1; *ZmMPK3*, mitogen-activated protein kinase; *ZmSOD4*, superoxide dismutase 4; *ZmUCE4*, ubiquitin conjugating enzyme 4; *ZmUCP*, mitochondrial uncoupling protein; *ZmUMC2618*, uncoupling protein homolog; *ZmZAT12/ZmZFP*, zinc finger protein 12 homolog.

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photosystem I (PSI) in the chloroplast and by other electron transport chains in the mitochondria and peroxisomes, as well as by xanthine dehydrogenase involved in nucleotide recycling in the cytosol (Vaahtera et al., 2014; Zarepour et al., 2010). Stresses that disrupt electron transport and other metabolic processes in these organelles can lead to increased O_2^- generation; for example, water deficit and other stresses that cause stomatal closure decrease the availability of carbon dioxide for photosynthesis, resulting in increased electron transfer to molecular oxygen and consequent increases in the rate of O_2^- generation in the chloroplast. Stresses can also perturb the antioxidant systems that normally scavenge intracellular ROS, promoting stress-responsive accumulation of O_2^- (You and Chan, 2015). In addition to being an unavoidable by-product of metabolism, O_2^- can also be actively generated as part of stress-responsive signaling. Indeed, the oxidative burst, a rapid and transient accumulation of ROS in the apoplast, is a hallmark of plant defense responses against pathogens, and relies on synthesis of O_2^- by plasma membrane-bound NADPH oxidases (Torres et al., 2006). This oxidative response is initiated at the site where stress is first sensed, but can spread within and between cells and can even be propagated to other organs in the plant. Although O_2^- is too reactive to diffuse far from its site of generation, it is rapidly converted by superoxide dismutase to hydrogen peroxide (H_2O_2), a somewhat more stable ROS that can diffuse across membranes via aquaporin channels and transmit signals between subcellular compartments or neighboring cells (Bienert and Chaumont, 2014). On a larger spatial scale, apoplastic ROS can also trigger activation of NADPH oxidases in neighboring cells via calcium signaling, resulting in a "ROS wave" that can travel through the plant at rates of more than 8 cm/min (Gilroy et al., 2016).

As might be expected from the strong association of local and systemic O_2^- accumulation with plant stress responses, this molecule exerts a major influence on how stress impacts plant health. In some cases, stress-responsive accumulation of O_2^- and other ROS contribute to the cellular injuries caused by stress. Superoxide can irreversibly inactivate certain enzymes by oxidizing their iron-sulfur centers, and can also give rise to hydroxyl radicals, which readily react with lipids, nucleotides, and amino acids (Sharma et al., 2012; Farmer and Mueller, 2013). Consequently, stresses that cause prolonged ROS accumulation also cause oxidative damage to membrane lipids, DNA, and proteins; furthermore, tolerance of drought, salinity, metal toxicity, and other stresses has been linked to enhanced antioxidant systems in many different plant species, suggesting that ROS mediate much of the damage caused by these stresses (Sharma et al., 2012). On the other hand, rapid, tightly-regulated accumulation of O_2^- and other ROS early in the stress response can also promote successful plant adaptation to stress. Local ROS accumulation at the site of infection can have direct antibiotic effects on pathogens, and ROS can also promote cell wall cross-linking and remodeling to strengthen the physical barriers to biotic attack (O'Brien et al., 2012). In addition, O_2^- and other ROS play important roles in stress-responsive signaling, modulating the activities of kinases, transcription factors, and other regulatory proteins through direct structural modifications of the proteins as well as through signaling cascades (Apel and Hirt, 2004; Spoel and Loake, 2011). In coordination with phytohormones, ROS regulate important defensive processes such as stomatal closure, programmed cell death, and transcriptional upregulation of genes involved in plant defense and stress adaptation (Sharma et al., 2012). Suppressing the expression or activity of NADPH oxidases render plants more susceptible to certain pathogens, herbivores, and abiotic stresses, illustrating the importance of O_2^- generation to plant stress management (Kadota et al., 2014; Li et al., 2015; Miller et al., 2009; Xie et al., 2011).

In short, stress-responsive ROS accumulation can be part of the disease or part of the cure. To understand the influence of O_2^- and other ROS on plant adaptation to diverse stresses, it is important to examine the timing and localization of ROS accumulation, which often determines whether ROS are "friend or foe" (Mittler et al., 2011; Willems et al., 2016). Identifying which ROS are present is also important because different species have different chemical reactivities, and consequently differ in their impacts on membrane lipids, DNA, proteins, and transcript profiles (Shulaev and Oliver, 2006). However, several obstacles complicate the observation of O_2^- in plants. The short half-life and low abundance of O_2^- and other ROS require highly sensitive detection methods, and specificity, or the ability to distinguish among multiple ROS, is another challenge. Nitrotetrazolium blue chloride (NBT), one of the most commonly-used stains for detecting O_2^-, can also cross-react with other agents such as the CO_2^- radical, reductases, and molecular oxygen to yield false positives; in addition, it requires destructive sampling, which limits investigators' ability to observe the temporal dynamics of O_2^- accumulation (Armstrong and Whiteman, 2007; Swanson et al., 2011). Because ROS induce characteristic transcript profiles, marker genes have been proposed as highly sensitive means for indirect detection of ROS accumulation (Gadjev et al., 2006; Mehterov et al., 2012). Moreover, ROS-responsive promoters can be used to express reporter genes whose activity can be visualized in living tissues; for example, transgenic Arabidopsis plants that generate luminescence in response to $^1\Delta O_2$ were developed by expressing luciferase under the control of the promoter region of the $^1\Delta O_2$ –responsive *AAA-ATPase* gene (Baruah et al., 2009). A variant of yellow fluorescent protein whose fluorescence properties change in response to H_2O_2 (Hyper) has also been used in transgenic plants for *in vivo* observation of ROS induction (Hernández-Barrera et al., 2013). To our knowledge, however, no visual molecular markers have been reported to have selectivity for O_2^-. Numerous O_2^- –inducible genes have been identified in Arabidopsis, but their level of selectivity for O_2^- is unclear; considerable overlap exists in transcriptional responses to different ROS, and previous studies on the specificity of these transcriptional responses have relied primarily on meta-analyses of different data sets rather than on side-by-side comparisons of plant responses to different ROS (Gadjev et al., 2006; Vaahtera et al., 2014; Willems et al., 2016). Another knowledge gap is the question of whether, when useful ROS-responsive genes are identified in Arabidopsis, homologous marker genes can serve similar functions in crop plants. Interestingly, although O_2^--responsive transcript profiles have been studied extensively in Arabidopsis, there is a relative shortage of information on responses in monocots, including important staple crops such as maize.

This study examined how the expression of Arabidopsis genes previously reported to be responsive to O_2^- were influenced by an O_2^- elicitor methyl viologen (MV), exogenous H_2O_2, and an inducer of $^1\Delta O_2$, rose bengal (RB). For the Arabidopsis genes that responded most selectively to O_2^-, homologs were also identified in maize. Our results suggest that genes encoding uncoupling proteins (*UCPs*) may be useful markers in both species. In addition, although *Zinc Finger protein 12* (*ZAT12*) in Arabidopsis has previously been described as a marker for H_2O_2 or for general ROS responses, our results suggests that transcription of this genes responds far more strongly to O_2^- than to H_2O_2 or $^1\Delta O_2$.

2. Materials & methods

2.1. Gene expression analysis in Arabidopsis

2.1.1. Tissue collection and RNA extraction

A. thaliana ecotype Columbia (Col-0) were grown in potting soil consisting of peat moss, vermiculite and perlite (4:3:2). At

developmental stage rosette (Boyes et al., 2001), approximately four weeks after germination, plants were sprayed with 10 ml of water (control), 10 μM methyl viologen (MV) (Sigma, St. Louis, MO, USA), 10 mM H_2O_2(Sigma), and 200 μM rose bengal (RB) (Alfa Aesar, Reston, VA, USA). When treated plants are exposed to sunlight, MV generates highly reactive, oxygen-centered free radicals and capture an electron from PSI in chloroplasts to produce monocationic MV radical, which in turn rapidly oxidized by oxygen and reduction of oxygen to O_2^- (Hartel et al., 1992). Foliage was collected at 2- and 24 h after treatment, flash-frozen in liquid N_2, and stored at −80 °C until RNA extraction. Tissue collection was conducted in growth chambers maintained at 23 °C, ~65% humidity, and ~125 μmol m^{-2} s^{-1} light intensity (16L:8D photoperiod). Total RNA was isolated using Trizol Reagent (Life Technologies/Ambion, Grand Island, NY, USA) according to the manufacturers' instructions. The concentration of total RNA was measured using a NanoDrop spectrophotometer (Thermo Scientific, Inc. Wilmington, DE, USA), and the quality was confirmed using an Agilent 2200 TapeStation System.

2.1.2. Relative gene expression by reverse transcriptase quantitative PCR (RT-qPCR)

Twelve genes that were previously reported to be upregulated by O_2^- in Arabidopsis were selected for analysis, and primers were designed using a modified version of Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm) (Rozen and Skaletsky, 2000). Candidate genes and their primers are described in Table 1. Synthesis of cDNA was performed using 100 ng of total RNA, dNTP primers, and SuperScript III Reverse Transcriptase (Life Technologies/Invitrogen, Grand Island, NY, USA) according to the manufacturers' instructions. The quantity of cDNA was measured using a NanoDrop spectrophotometer and diluted to 100 ng/μl prior to quantitative PCR (qPCR). Relative gene expression was measured by performing qPCR on 100 ng of cDNA per sample with a Step OnePlus Real Time PCR System (AB Applied Biosystems Inc, Foster City, CA, USA) and a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) using the following PCR conditions: 15 min of denaturation at 95 °C followed by 40 cycles of amplification (15 s at 94 °C; 1 min at 60 °C; 1 min of extension at 72 °C), measuring fluorescence from PCR products after every cycle. Melting curve analysis was performed on PCR products from 60 to 95 °C in sequential steps of 0.3 °C for 1 min. For each treatment and time point, 5 biological replicates and 2 technical replicates were assayed. Data (Ct values) were first normalized to the expression levels of two endogenous controls (*AtTUB2 and AtACT2*), and then gene expression levels in each treatment were calculated relative to the water treated control using the ΔΔCt method (Pfaffl, 2001):

Relative Expression $= (E_{\text{target}})^{\Delta Ct_{\text{target}}}$ $(control - sample)/(E_{\text{ref}})^{\Delta Ct_{\text{ref}}}$ $(control - sample)$.

2.1.3. Absolute gene expression by RT-qPCR

For the three genes selected for absolute quantification (*AtUCP2, AtGRP,* and *AtZAT12*), DNA templates (each ~120 bp length) for developing standard curves were synthesized by IDT (Coralville, Iowa, USA) as follows: (1) *AtUCP2*-AT5G58970 oligonucleotide: 5'cttcttcgctgtctgcatcggttctccaattgatgtggtgaaatctagaatgatgggagactctacttaccgaaacacagtcgattgcttcatcaaaacgatgaagaccgaggggattatggcattctac3'; (2) *AtGRP*-AT1G04800 oligonucleotide: 5'cctcaccctcatcctctcctccacaagaagggctttaaaaaagaattcggcgacttaggtggtggtggtggtatcagcggtggaggcggctttggagcaggtggcggttggattggaggcagtgttggag3'; (3) *AtZAT12* At5g59820 oligonucleotide: 5'accgtgcgagtcacaagaagcctaacaacgacgctttgt cgtctggattgatgaagaaggtgaaaacgtcgtcgcatccttgtcccatatgtggagtggagtttccgatgggacaagctttgggaggacacatgaggaga3'. RT-qPCR was performed on a 5-fold dilution series of these synthetic DNA templates (0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng, and 0.00001 ng). Three standard curves for each oligonucleotide were obtained, each with very high R^2 values (*AtUCP2*: $y = 3.6615x + 3.1831$, $R^2 = 0.9994$; *AtGRP*: $y = 3.4991x + 4.176$, $R^2 = 0.9996$; *AtZAT12*: $y = 3.4418x + 4.5045$, $R^2 = 0.9982$). In parallel, *AtUCP2, AtGRP, AtZAT12* and two housekeeping controls (*AtTUB2 and AtACT2*) were measured by qPCR in cDNA samples from the same treatment groups examined for relative expression (2 h time point only; 5 biological replicates/treatment and 2 technical replicates/biological replicate). PCR conditions were the same as described for relative expression analysis in section 2.1.2. All the data (Ct values) were first normalized using the endogenous controls *AtTUB2* and *AtACT2*, and then transformed to quantitative measures (ng/μl) by comparison with the standard curves generated from dilution series of synthetic DNA templates.

2.2. Gene expression analysis in maize

2.2.1. Tissue collection and RNA extraction

Zea mays cv. B73 was grown in LC-1 potting mix from SunGro Horticulture (Agawam, MA, USA), and at growth stage V2, 10 days after germination, the corn seedlings were sprayed with MV or water. Preliminary observations of symptom development and gene expression revealed that maize is less sensitive to MV than Arabidopsis, and so a higher concentration of MV (1 mM) was used for maize than for Arabidopsis. Treatments were conducted under greenhouse conditions (23 °C, 350–550 μmol m^{-2} s^{-1}, 16 L: 8 D photoperiod). Samples were collected by cutting off the apical half of the 2nd leaf from the apex 2 h after treatment, frozen in liquid N_2, and stored at −80 °C until RNA extraction. Total RNA was extracted using Trizol according to manufacturer's instructions and further purified using the RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA), and RNA quality was assessed by spectrophotometry and gel electrophoresis.

2.2.2. Selection of maize genes for expression analysis

To identify homologs in maize of the Arabidopsis O_2^-–responsive genes *AtGRP*, *AtUCP1* and *AtUCP2*, and *AtZAT12*, BLASTP searches were performed against sequenced genomes of *Arabidopsis thaliana* and maize (*Zea mays*) in Phytozome version 12 (http://www.phytozome.net/) and in NCBI (http://www.ncbi.nlm.nih.gov/) databases using full-length protein sequences. The protein sequences were aligned by MUSCLE (Edgar, 2004), and then the alignment results were refined by Gblocks (Talavera and Castresana, 2007). The unrooted phylogenetic trees was constructed using the maximum likelihood method in MEGA version 6 (Hall, 2013). The reliability of the trees was estimated with 500 bootstrap iterations. The trees were visualized by using TreeGraph version 2 (Stöver and Müller, 2010). In addition to the gene of interest identified through these sequence comparisons, we also selected 7 maize genes for analysis on the basis of previous reports that they were upregulated by MV: *AtUCP* genes (GRMZM2G165629, GRMZM2G000510, GRMZM5G8528770) (Considine et al., 2003; Murphy et al., 2003; Smith et al., 2004), and *AtZAT12* genes (AC206217.2_FGT006, GRMZM2G001205, GRMZM2G048154, GRMZM5G804618) (Han et al., 2014; Mehterov et al., 2012). Primers (described in Table 1) for all the maize genes tested were designed using QuantPrime software (Arvidsson et al., 2008) and were tested for specificity as described by Bihmidine et al. (2015). The primers used in this study are also listed bottom in Table 1.

2.2.3. Relative gene expression by RT-qPCR

The influence of MV on the relative expression of the 11 genes described in section 2.2.2 was studied by RT-qPCR, with 5 biological replicates/treatment and 3 technical replicates/biological replicates. For each sample, 1 μg of total RNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Hercules,

Table 1
Primers used for gene expression analysis in Arabidopsis.

Abbreviation	GenBank Gene ID	Putative Encoded Protein	Primer Sequences Forward/Reverse (5'to 3′)	Amplicon Length
AtACT2	At3g18780	Actin-2	F: gccatccaagctgttctctc R: accctcgtagattggcacag	102
AtCOBL5	At5g60950	COBRA-like protein5 precursor	F: ttccaggatggaaaatgagc R: ctgcgggatatttcctttga	116
AtERD7	At2g17840	Senescence/dehydration-associated protein	F: atggccgttgactaaggatg R: ctccatctccgtcttcttcg	119
AtFER2	At3g11050	Ferritin 2	F: ctcttgagctccggctacac R: ccggttaacgacttcgtgtt	137
AtGRP	At1g04800	glycine-rich protein	F: cctcatcctctcctccacaa R: aacactgcctccaatccaac	120
AtIGMT1	At1g21100	O-methyltransferase	F: cctgggcacagttgaaagat R: gattgtgaatccggtctggt	137
AtKIN	At3g46280	kinase-like protein	F: aggagcgaaacaagcaaaaa R: caaatagctgcaaacgctga	141
AtMPK9	At3G18040	mitogen-activated protein kinase 9	F: tttgcagaaatgctcactgg R: ctcctcgccttttcattacg	134
AtOGO	At3G11180	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	F: acggcgaagagctagtgaag R: cgagggcattttgggtaata	148
AtPL	At4g38550	phospholipase-like (PEARLI 4) family protein	F: caaagcttcacaatgcagga R: attcgcgacggatacgtaag	111
AtTUB2	At5g62690	Tubulin beta-2/beta-3	F: agcaaatgtgggactccaag R: cacattcagcatctgctcgt	128
AtUCP1/AtPUMP1	AT3G54110	uncoupling protein 1	F: ttgaagattccgggtttcac R: aatggtgcccttgtaagcac	147
AtUCP2	At5G58970	uncoupling protein 2	F: gtctgcatcggttctccaat R: cataatcccctcggtcttca	111
AtZAT12/AtRHL41	At5g59820	zinc finger protein 12	F: tgcgagtcacaagaagccta R: gtgtcctcccaaagcttgtc	127
ZmAPX1	GRMZM2G316256	cytosolic ascorbate peroxidase	F:gctctgtcttgcatggcactcc R:gatgggctctagcaacctgacg	145
ZmCAT1	GRMZM2G088212	Catalase 1	F:ccgatgatcttggaaagggtggac R:tctgtcagtgcgtcaacccatc	129
ZmGSR1	GRMZM2G172322	glutathione reductase 1	F:ccaatagggtcaacctgacaccag R:tccatactcttcaattgcctgctc	176
ZmMPK3/ZmGPM443	GRMZM2G017792	Mitogen-Activated Protein Kinase	F:acagcgacatgatgacggagta R:ccaatcacctcggttatgag	196
ZmSOD4	GRMZM2G169890	Superoxide Dismutase 4	F:cgtgttgcttgtgggatcatcg R:tgttattctagcctgctggtgacg	104
ZmUCE4	GRMZM2G102471	Ubiquitin conjugating enzyme 4	F:gttccattggcaagcgaccatc R:ttggtgcggaaagacacctttg	126
ZmUCP1	GRMZM5G852877	Mitochondrial uncoupling protein 1	F:tacagggacctggtgctttc R:gggcgtgcacattctgttac	112
ZmUCP2	GRMZM2G165629	Mitochondrial uncoupling protein 2	F:tcttgtccagttcagggttccg R:aaaggaatggtgcacacctctg	160
ZmUMC2618	GRMZM2G000510	Uncoupling protein homolog	F:cgatcactgtgtgtgaagtgc R:tgtgtttgttgtcgtgtgct	145
ZmZAT12a	AC206217.2_FGT006	Zinc finger protein 12 homolog	F:gcggaatctgtttggacttgagc R:gcagtccagtatcagcgatttcc	116
ZmZAT12b	GRMZM2G048154	Zinc finger protein 12 homolog	F:agctctcggtcctcgtctttgc R:ctggcgtgacctcttgccatac	100
ZmZFP16-1	GRMZM2G001205	Zinc finger protein 12 homolog	F:gcaccggtctcttcttcttcttc R:ccttcatgcacatcagccaagc	156
ZmZFP	GRMZM5G804618	Zinc finger protein 12 homolog	F:gccaccagcttgatcgacacac R:gacttccgagcgccgtactttg	115

CA, USA) according to the manufacturer's protocol. Quantitative PCR was performed on 10 ng of cDNA using SoFast EvaGreen Supermix with Low ROX (Bio-Rad, Hercules, CA, USA), and a CFX384 Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), with the following PCR conditions: 95 °C denaturation for 2 min followed by 40 cycles of 95 °C for 5 s, 63 °C for 30 s, and a final temperature increment of 0.5 °C for 5 s from 65 °C to 95 °C for melting curve analysis. The analysis of relative gene expression for maize was performed with Bio-Rad CFX manager 3.1 software (Bio-Rad, Hercules, CA, USA). Dissociation curves for each amplicon were analyzed to verify the specificity of each amplification reaction. Transcript levels were normalized against the average of the maize housekeeping genes, *UBCP* (GRMZM2G102471). To calculate the relative gene expression level, the ΔΔCt method was employed (Pfaffl, 2001).

2.3. Analysis of a AtZAT12 promoter::reporter gene fusion

2.3.1. Introduction of the AtZAT12 promoter:: reporter gene fusion into Arabidopsis by floral dip transformation

A construct (PB001 *ZAT12:: Luc*) consisting of the *AtZAT12* promoter fused with a reporter gene encoding luciferase (*Luc*) (Davletova et al., 2005) was obtained from Dr. Gad Miller (Bar Ilan University, Ramat-Gan, Israel). This construct was propagated in *Agrobacterium tumefaciens* strain GV3101 in LB medium with spectinomycin (100 mg/l) and gentamycin (50 mg/l) until reach stationary phase (OD600 of 2.0). Cells were harvested and resuspended in 5% sucrose to a final OD600 of approximately 0.80, and used for floral dip transformation of Arabidopsis as described by Clough and Bent (1998). In brief, floral tissues were dipped into a solution containing *A. tumefaciens*, 5% sucrose and 0.02%

surfactant Silwet L-77, and then seeds obtained from treated plants were surface sterilized and underwent selection on glufosinate-ammonium (BASTA 50mg2.3/L, Bayer, Whippany, NJ) + Carbenicillin (100 mg/L) selection plates.

2.3.2. Analysis of AtZAT12::Luc reporter gene activity

Arabidopsis plants with stable expression levels of the transgene were grown as described in section 2.1.1, along with untransformed controls (Col-0). To measure the influence of ROS elicitors on reporter gene activity, plants were sprayed with 10 ml of water (control), 10 μM MV, 10 mM H_2O_2, or 200 μM RB (10 replicates/treatment). Thirty minutes after elicitor application, the plants were sprayed with the substrate for luciferase, luciferin (150 μg/ml), and luminescence was measured 30 min after luciferin application. To assess whether the superoxide dismutase (SOD, rubredoxin:superoxide oxidoreductase, EC 1.15.1.1) inhibitor diethyldithiocarbamate (DDC) influences constitutive or MV-responsive reporter gene activity, additional plants were sprayed with either water (control) or 100 μM DDC, followed 16 h afterward (ie. after an overnight incubation at room temperature) by treatment with 10 ml of water or 10 μM MV. One hour after MV treatment, plants were sprayed with luciferin (150 μg/ml), and luminescence was measured 30 min after luciferin application. To measure luminescence, a leaf disc (0.85 cm in diameter) was collected from the middle of the seventh rosette leaf of each plant and placed in a 24-well plate. The luminescence was measured using Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek Winooski, VT, USA).

2.4. Data analysis

Analysis of variance (ANOVAs) were used to analyze the main effects and interactions among treatments, and Student's *t-tests* were carried out to compare treatments to a single control group. All data was statistically analyzed using Prism 3.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Comparison of the effects of methyl viologen (MV), H_2O_2, and rose bengal (RB) on expression of putative O_2^-–responsive transcripts in Arabidopis

Twelve genes that were previously reported to be upregulated by O_2^- in Arabidopsis were screened for responsiveness to an elicitor of O_2^- (methyl viologen, MV), H_2O_2, and an elicitor of $^1\Delta O_2$ (rose bengal, RB) at 2- and 24 h after foliar treatments with these elicitors (Table 2). Several candidate genes (*AtFER, AtMPK9, AtIGMT1, AtOGO, AtPL, AtKIN*) responded at least as strongly to H_2O_2 and/or RB than to MV, and were eliminated from further consideration because of their lack of selectivity (Table 2). Subsequent experiments focused

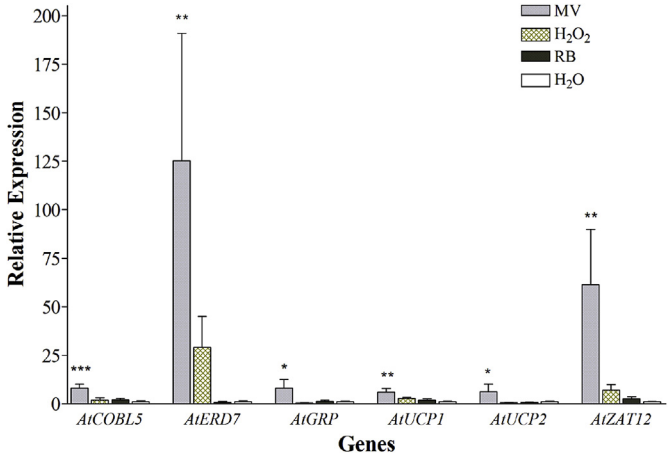

Fig. 1. Influence of ROS elicitors on relative expression of six superoxide-responsive genes in Arabidopsis. Gene expression was measured 2 h after treatment with an elicitor of O_2^- (10 μM methyl viologen, MV), H_2O_2 (10 mM) or an elicitor of $^1\Delta O_2$ (200 μM rose bengal, RB), as compared to water (H_2O). Expression was quantified relative to *AtTUB2* and *AtACT2*. Treatments that are significantly different from the water control according to Student's t-tests are labeled with asterisks (*P ≤ 0.1; **P ≤ 0.05; ***P ≤ 0.01; n = 5). Error bars represent the SEM.

Table 2
Expression patterns of 12 putative O_2^--responsive genes in Arabidopsis in response to ROS elicitors. For 12 genes that had previously been reported to be upregulated by O_2^- (columns 1–5), RT-qPCR was used to examine their expression levels in response to elicitors of O_2^- (10 μM methyl viologen, MV) or $^1\Delta O_2$ (200 μM rose bengal, RB), as well as to exogenous H_2O_2 (10 mM). Foliage for RNA extraction was collected 2- and 24 h after foliar treatments (n = 5). "++++" indicates ≥50 fold upregulation compared to water-treated controls; "+++" indicates 20–50 fold upregulation; "++" indicates 10–20 fold upregulation; "+", 4–10 fold upregulation; "±", 0–4 fold upregulation; "−", ≤1, down regulation; "nr", not reported.

Gene	Ref	Previous Reports			MV		H_2O_2		RB	
		O_2^-	H_2O_2	$^1\Delta O_2$	2 h	24 h	2 h	24 h	2 h	24 h
AtFER	1, 2	up	nr	nr	+	±	+++	+++	+	±
AtGRP	1, 4	+	−	nr	+	−	−	±	±	±
AtMPK9	1, 4	+	−	nr	−	+	+	±	±	−
AtIGMT1	3, 4	+	−	±	+	+	+	−	±	−
AtOGO	4	+	nr	nr	++++	±	+++	±	++++	±
AtPL	3, 4	+	−	±	±	−	++	±	+++	±
AtCOBL5	3	+	±	±	+	±	±	−	±	−
AtERD7	3	++	±	+	++++	++++	+++	++	+	+
AtKIN	1, 3, 4	+	−	−	−	−	++	±	+++	±
AtUCP1/ *AtPUMP1*		up	nr	nr	+	+	±	±	±	±
AtUCP2		up	nr	nr	+	++++	−	+	−	−
AtZAT12/ RHL41	1, 4	+++	±	nr	++++	−	+	±	±	±

1. Mehterov et al., 2012 (examined transcript profiles in in response to methyl viologen and a catalase inhibitor (aminotriazole) in two Arabidopsis mutants (*loh2* and *atr7*) with modified redox status).
2. Soós et al., 2006 (ferritin2 genes were upregulated by methyl viologen).
3. Gadjev et al., 2006 (presented a meta-analysis of publically available microarray studies).
4. Han et al., 2014 (examined transcript profiles in response to methyl viologen).

on the 2 h time point because genes that are upregulated early in the response to O_2^- would be more useful in detecting the timing of O_2^- accumulation than genes with a delayed response. *AtGRP*, *AtCOBL5*, *AtERD7*, *AtUCP1*, *AtUCP2*, and *AtZAT12* each showed at least a 6-fold increase in relative expression levels in response to MV at 2 h after treatment, with a stronger response to MV than to H_2O_2 or RB (Fig. 1).

Of these genes, *AtERD7* showed the greatest increase in transcript levels in response to MV at 2 h (94.8-fold upregulation); however, it also showed considerable variability within treatments, and had 29.0-fold higher transcript levels in H_2O_2-treated plants than in water-treated controls, although the difference between H_2O_2- and water-treated plants was not statistically significant ($p = 0.059$, Fig. 1). In contrast to *AtERD7*, *AtGRP* and *AtUCP2* showed more modest upregulation in response to MV (8.5- and 6.1-fold induction, respectively), but appeared unresponsive to H_2O_2 and RB (Fig. 1). Potentially, genes like *AtGRP* and *AtUCP2* that have a smaller dynamic range of expression could be harder to detect, but would offer less background noise when used as marker genes than genes with large dynamic ranges like *AtERD7*. Compared to *AtERD7*, *AtGRP*, and *AtUCP2*, *AtZAT12* appeared to be intermediate in responsiveness and selectivity, with a 61.4-fold increase in transcript levels in response to MV, and expression levels in the H_2O_2-treated and RB-treated plants that were within 10-fold of the water-treated controls (Fig. 1).

3.2. The absolute gene expression of three superoxide-responsive candidate genes

Whether genes with a modest dynamic range of expression would be appropriate for use as markers would depend in part upon whether their absolute expression levels were high enough to allow robust detection. The absolute expression levels of *AtGRP*, *AtUCP2*, and *AtZAT12* were measured by RT-qPCR (Fig. 2). For all 3 genes, the highest expression levels were observed in the MV-

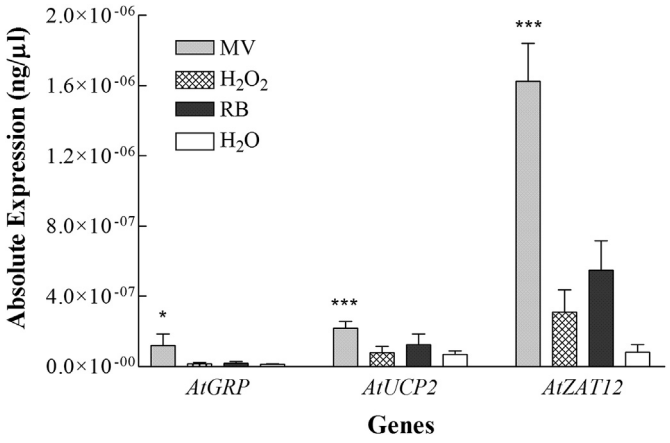

Fig. 2. Absolute expression of three superoxide-response candidate genes in Arabidopsis. Absolute gene expression was measured by standard curves 2 h after treatment with methyl viologen (MV, 10 µM), H_2O_2 (10 mM), or rose bengal (RB, 200 µM). All data (Ct values) were normalized using two reference genes: *AtTUB2* and *AtACT2*. Treatments that are significantly different from the water control according to Student's t-tests are labeled with asterisks (*$P \leq 0.1$; **$P \leq 0.05$; ***$P \leq 0.01$; $n = 5$). Error bars represent the SEM.

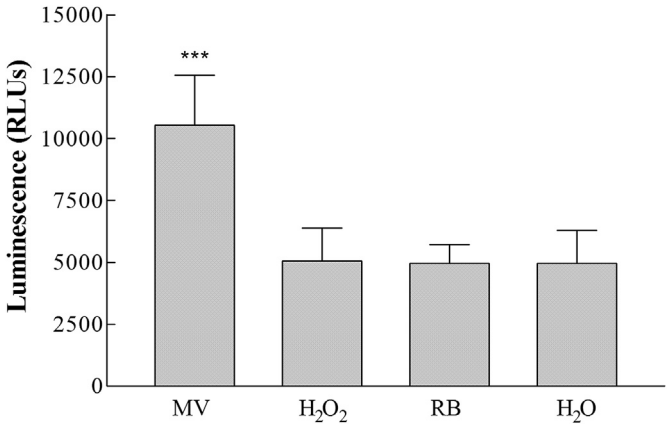

Fig. 3. The activity of the *AtZAT12::Luc* reporter in Arabidopsis in response to ROS elicitors. Plants were treated with methyl viologen (MV, 10 µM), H_2O_2 (10 mM), rose bengal (RB, 200 µM) or water 1.5 h before measurements were taken. A one-way ANOVA revealed significant differences among treatments ($p = 0.0422$). Treatments labeled with asterisks differ significantly from the water-treated control according to Student's t-tests (***$P \leq 0.01$; $n = 10$). RLUs (Relative Light Units).

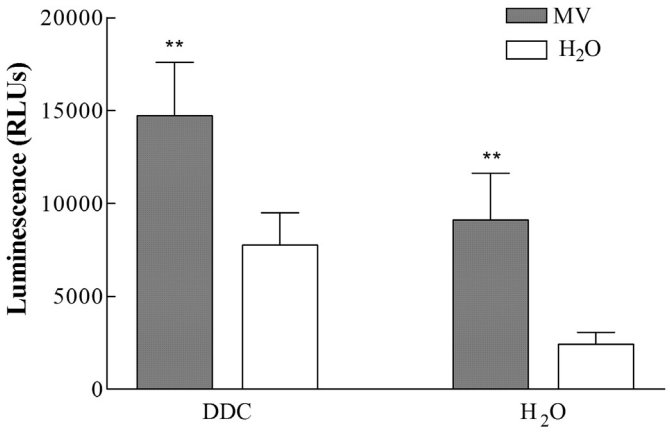

Fig. 4. The activity of the *AtZAT12::Luc* reporter in Arabidopsis in response to a superoxide elicitor and an inhibitor of superoxide dismutase. Plants were pretreated with water or diethyldithiocarbamate (DDC, 100 µM), which inhibits conversion of O_2^- to H_2O_2, and then (16 h after DDC treatment) were treated again with water or methyl viologen (MV, 10 µM) to induce O_2^- generation. Measurements were taken 1.5 h after MV treatment. A two-way ANOVA revealed significant effects of both DDC ($p = 0.013$) and MV ($p = 0.0025$), but no significant interaction between the two ($p = 0.95$). Treatments labeled with asterisks differ significantly from the water-treated control according to Student's t-tests (**$P \leq 0.05$; $n = 12$).

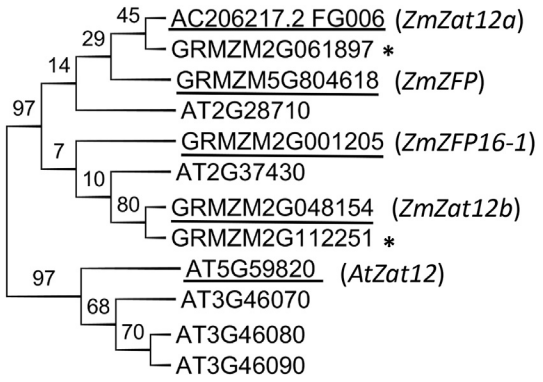

Fig. 5. Homologs of *AtZAT12* in maize. The relatedness of maize homologs (identified by MaizeGDB gene records beginning in GRM or AC) to *AtZAT12* and other related zinc finger protein genes in Arabidopsis (identified by Genbank accession numbers beginning with AT) was assessed by comparison of the putative protein sequences using the maximum likelihood method in MEGA version 6. Genes whose expression was analyzed in this study are underlined. Asterisks denote maize transcripts that are not expressed in foliage.

treated samples compared to water-treated controls or plants treated with H_2O_2 or RB. Of the three genes, *AtZAT12* had the highest absolute expression in response to MV, with 10^{-6} ng of cDNA template per μl of reaction volume. This translates to approximately 19 *AtZAT12* transcripts per ng of total RNA, and ~589,000 transcripts per leaf (~83 mg) of starting material. If a typical mature Arabidopsis leaf has 3000 or fewer cells (Autran et al., 2002), this would translate to an average expression level of ~200 transcripts/cell. Expression levels were an order of magnitude lower for *AtUCP2* (2×10^{-7} ng/μl; ,~4 transcripts per ng of total RNA and ~117,800 transcripts/leaf) and *AtGRP* (10^{-7} ng/ml, or ~2 transcripts per ng of total RNA and ~58,900 transcripts/leaf).

Fluorescent or luminescent reporter gene fusions could potentially be used to track expression of *AtUCP2* or *AtGRP*, because a single mRNA can be translated hundreds of times, and the enzymatic or fluorescent activity of reporter proteins such as luciferase or GFP lead to even further signal amplification. Recently, for example, molecular beacons have successfully been deployed for single-molecule detection of RNA transcripts in mammalian cells (Zhao et al., 2016). However, the higher expression levels observed for *ZAT12* make this gene more amenable for use in *in vivo* detection of plant responses to ROS.

3.3. The influence of ROS elicitors on the activity of reporter gene driven by the AtZAT12 promoter (AtZAT12::Luc) in Arabidopsis

To follow up on observations that MV induced *AtZAT12* expression more strongly than H_2O_2 or RB (Fig. 1), Arabidopsis plants were transformed with a luciferase reporter gene driven by the *AtZAT12* promoter (*AtZAT12::Luc*), and reporter gene activity was assessed by measuring luminescence in plants treated with 10 μM MV, 10 mM H_2O_2 and 200 μM RB. In the presence of the substrate luciferin, foliage previously treated with MV generated significantly more luminescence than plants treated with water, H_2O_2, or RB; moreover, H_2O_2 or RB treatments did not cause significant increases in luminescence compared to water (Fig. 3). Luminescence was not detected in plants that lacked the luciferin substrate or the reporter gene (*AtZAT12::Luc*) (data not shown).

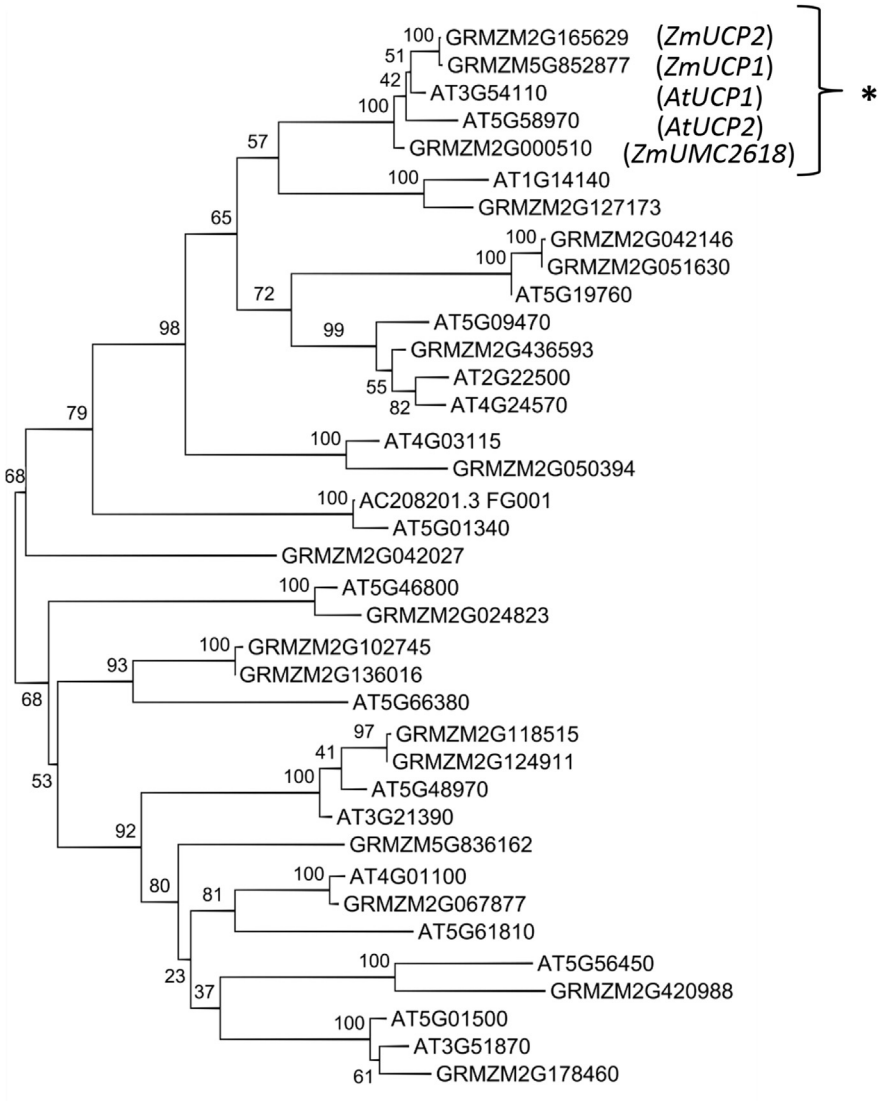

Fig. 6. Homologs of *AtUCP1 and AtUCP2* in maize. The relatedness of maize homologs (identified by MaizeGDB gene records beginning in GRM or AC) to *AtUCP1*, *AtUCP2*, and other related uncoupling protein genes in Arabidopsis (identified by Genbank accession numbers beginning with AT) was assessed by comparison of the putative protein sequences using the maximum likelihood method in MEGA version 6. Genes whose expression was analyzed in this study are identified with an asterisk.

These results indicate that the *AtZAT12* promoter is more responsive to an elicitor of O_2^- (MV) than to exogenous H_2O_2 or a $^1\Delta O_2$ elicitor (RB).

AtZAT12 encodes a zinc-finger protein that plays a key role in regulating transcriptional responses to environmental stresses that trigger ROS production in Arabidopsis. It is transcriptionally induced by multiple stresses, including temperature extremes, drought, salt, wounding, light and dark stress, and insect herbivory (Davletova et al., 2005; Miller et al., 2009; Schweizer et al., 2013); it in turn upregulates antioxidants such as ascorbate peroxidase and downregulates the uptake of iron, a mineral that enables the production of damaging hydroxyl radicals (Rizhsky et al., 2004). Loss-of-function of the *ZAT12* gene increases susceptibility to salinity and osmotic stress (Davletova et al., 2005), and strong constitutive expression of *ZAT12* in transgenic plants promotes tolerance of chilling, osmotic stress, light stress, and MV in Arabidopsis (Davletova et al., 2005; Vogel et al., 2005), and tolerance of drought and heat shock-induced oxidative stress in tomato (Shah et al., 2013; Rai et al., 2012). In short, *ZAT12* plays a critical role in managing environmentally-induced oxidative stress, and our results suggest that O_2^- may be a critical cue in regulating its transcription.

3.4. The influence of a superoxide dismutase (SOD) inhibitor on the activity of (AtZAT12::Luc) in Arabidopsis

Although MV leads to generation of O_2^-, this ROS is quickly converted to the less toxic, more stable ROS H_2O_2 by SOD. Therefore, it is possible that MV could activate *AtZAT12* expression by promoting endogenous H_2O_2 accumulation, rather than through a direct effect of O_2^-. To test this possibility, the effects of a pharmacological inhibitor of SOD, diethyldithiocarbamate (DDC), on *AtZAT12::Luc* reporter activity was examined singly and in combination with 10 μM MV. Both DDC (Two-way ANOVA, $p = 0.0133$), and MV ($p = 0.0025$) caused significant increases in reporter gene activity as measured by luminescence, and there was no significant interaction between the two treatments ($P = 0.951$) (Fig. 4).

The fact that DDC promoted rather than inhibited *AtZAT12::Luc* activity strongly suggests that the *AtZAT12* promoter is more responsive to O_2^- than to H_2O_2. This finding is consistent with a recent meta-analysis that re-analyzed publically available microarray data, which reported that in these public data sets, *AtZAT12* is upregulated by MV but not by H_2O_2 (Vaahtera et al., 2014). While earlier studies had observed transcriptional activation of *ZAT12* in response to H_2O_2, and had concluded that *AtZAT12::Luc* could be used as a measure of plant responses to H_2O_2 (Desikan et al., 2001; Davletova et al., 2005), the induction of *ZAT12* observed in these prior studies could potentially be due to the ability of H_2O_2 to induce of O_2^-–generating NADPH oxidases (Gilroy et al., 2016), rather than to a direct effect of H_2O_2 on *ZAT12* expression. Interestingly, H_2O_2 has recently been reported to regulate degradation of the *ZAT12* protein (Brumbarova et al., 2016; Le et al., 2016), and so O_2^- and H_2O_2 may regulate *ZAT12* protein accumulation at the transcriptional and post-translational levels, respectively.

3.5. Superoxide-responsive candidate genes expressed in maize

Although numerous studies have examined transcriptional responses to ROS accumulation in Arabidopsis, far less is known about transcriptional responses to ROS in crop plants, and particularly in important monocots such as maize. Here, the maize genome was searched for homologs of Arabidopsis genes encoding *AtUCPs*, *AtZAT12*, and *AtGRP*. Putative homologs for *AtUCP* (2G165629, 2G000510, 5G852877) and *AtZAT12* (AC206217, 2G001205, 2G048154, 5G804618) were identified (Figs. 5 and 6), but no genes with close homology to *AtGRP* were found. Expression

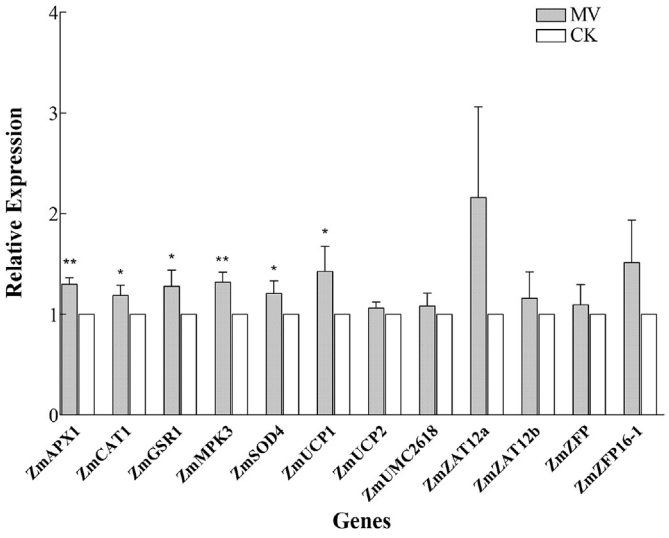

Fig. 7. Influence of methyl viologen on expression of maize homologs of *AtUCPs* and *AtZAT12*. Gene expression was measured 2 h after treatment with methyl viologen (MV, 1 mM) or water. Treatments that differ significantly from the water control according to Student's t-tests are labeled with asterisks (*$P \leq 0.1$; **$P \leq 0.05$; $n = 5$). Error bars represent the SEM.

of these seven genes was compared to that of five genes previously reported to be upregulated by MV in maize: *ZmAPX1* (*cytosolic ascorbate peroxidase*), *ZmCAT1 (catalase 1)*, *ZmGSR1(glutathione reductase1)*, *ZmMPK3(mitogen-activated protein kinase)*, and *ZmSOD4(superoxide dismutase 4)* (Fig. 7). Only one homolog (*AtUCP* homolog 5G852877) was upregulated in maize 2 h after treatment with 1 mg/ml MV, although MV increased expression of all five genes used as positive controls.

These results identify a putative uncoupling protein in maize (5G852877) that may be of use in studying responses to O_2^- in this crop. Uncoupling proteins (*UCPs*) are present in the inner membrane of mitochondria to dissipate the proton gradient formed through respiration, and they have been found to be activated by O_2^- in both plants and animals (Considine et al., 2003; Echtay et al., 2002; Begcy et al., 2011). Interestingly, expression of the *AtZAT12* homologs identified in maize did not show statistically significant differences between MV-treated plants and water-treated controls, although salt stress-responsive homologs of *ZAT12* have been identified in another monocot, rice (Imran et al., 2016), and transgenic constitutive expression of *BcZAT12* from *Brassica carinata* in rice has been reported to improve drought tolerance (Devi et al., 2016). Further work on the function and regulation of *ZAT12* homologs in monocots is warranted.

4. Conclusions

Of a panel of 12 genes previously reported to be upregulated by O_2^- in Arabidopsis, half of these genes were as or more responsive to other ROS (H_2O_2 and/or $^1\Delta O_2$) than to O_2^-, indicating that they would not be adequately selective markers for O_2^-. The remaining 6 genes showed varying degrees of selectivity for O_2^-, with *AtUCP2* and *AtGRP* showing the lowest responsiveness to H_2O_2 and $^1\Delta O_2$. A O_2^- responsive homolog of *AtUCP2* was also identified in maize. *AtZAT12*, which encodes a key regulator of transcriptional responses to oxidative stress in Arabidopsis, also proved to be more strongly upregulated by O_2^- than by H_2O_2 and $^1\Delta O_2$; moreover, inhibiting conversion of O_2^- to H_2O_2 with a pharmacological inhibitor of SOD enhanced *ZAT12* promoter activity as measured with a *AtZAT12::Luc* reporter gene. These results suggest that although

AtZAT12 was previously reported to be a marker for H_2O_2 accumulation, this gene may respond preferentially with higher levels to O_2^-, and *AtZAT12::Luc* may therefore be a useful biosensor for O_2^-.

Contributions

JX conducted gene expression analysis and reporter gene activity assays in Arabidopsis, TT assayed gene expression in maize, CPM developed Arabidopsis lines transformed with the *AtZAT12::Luc* reporter, FG and DB were responsible for project design, and all authors contributed to writing the manuscript.

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