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Role of mitochondria and chloroplasts during stomatal closure: Subcellular location of superoxide and H_2O_2 production in guard cells of *Arabidopsis thaliana*

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Stomatal guard cells are unique in that they have more mitochondria than chloroplasts. Several reports emphasized the importance of mitochondria as the major energy source during stomatal opening. We re-examined their role during stomatal closure. The marked sensitivity of stomata to both menadione (MD) and methyl viologen (MV) demonstrated that both mitochondria and chloroplasts helped to promote stomatal closure in *Arabidopsis*. As in the case of abscisic acid (ABA), a plant stress hormone, MD and MV induced stomatal closure at micromolar concentration. All three compounds generated superoxide and H_2O_2, as indicated by fluorescence probes, BES-So-AM and CM-H_2DCFDA, respectively. Results from tiron (a superoxide scavenger) and catalase (an H_2O_2 scavenger) confirmed that both the superoxide and H_2O_2 were requisites for stomatal closure. Co-localization of the superoxide and H_2O_2 in mitochondria and chloroplasts using fluorescent probes revealed that exposure to MV initially triggered higher superoxide and H_2O_2 generation in mitochondria. In contrast, MD elevated superoxide/H_2O_2 levels in chloroplasts. However, with prolonged exposure, MD and MV induced ROS production in other organelles. We conclude that ROS production in mitochondria and chloroplasts leads to stomatal closure. We propose that stomatal guard cells can be good models for examining inter-organellar interactions.

Keywords. Co-localization; H_2O_2; organelles; reactive oxygen species; superoxide; signaling components

1. Introduction

Plants deploy diverse mechanisms to meet the challenges of abiotic or biotic stress (Ashapkin *et al.* 2020; Saharan *et al.* 2022; Munns and Millar 2023). Stomatal closure is one such step to limit transpiration and conserve water, besides preventing microbial pathogen entry into leaves. Guard cells sense and respond to environmental cues (Qi *et al.* 2018; Yang *et al.* 2020). Stomata open as guard cells become turgid, and close when they are flaccid. The influx of ions followed by water entry into the guard cells makes them turgid, whereas the efflux of ions followed by water makes guard cells flaccid (Daloso *et al.* 2017; Agurla *et al.* 2018).

Because of its importance, stomatal closure was extensively studied *in situ* and *ex situ*, using plant hormones or microbial elicitors. Abscisic acid (ABA, a plant hormone) induced stomatal closure, and its closure mechanisms were extensively studied (Agurla *et al.* 2018; Hedrich and Shabala 2018; Liu *et al.* 2022). Guard cell signaling components, such as protein phosphatase 2C (PP2C), SnRK 2.6 (OST1) kinase, reactive oxygen species (ROS), nitric oxide (NO), and Ca^{2+}, played crucial roles in ABA-induced stomatal closure (Bharath *et al.* 2021; Lim *et al.* 2022a; Liu *et al.* 2022).

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Guard cells have bio-energetically active chloroplasts and mitochondria to supply sufficient energy. The relatively high number of mitochondria over chloroplasts suggests the dominance of oxidative phosphorylation as the energy source for stomatal opening (Parvathi and Raghavendra 1995; Willmer and Fricker 1996). In contrast, the photosynthetic capacity of guard cells is limited as Rubisco and photosystem II components are quite low. Although the importance of mitochondria or chloroplasts for stomatal opening was emphasized, the role of these organelles during closure was not clear (Raghavendra 1981; Vani and Raghavendra 1989; Vavasseur and Raghavendra 2005; Lim *et al.* 2022b). Stress conditions such as heat, light, and drought often target both mitochondria and chloroplasts, impair their redox balance, and produce excess ROS inside the cells. Altered redox status and elevated ROS could activate a series of events leading to plant adaptation (Suzuki *et al.* 2012; Li and Kim 2021; Suzuki 2023). Thus, modulation of mitochondrial or chloroplast function could be significant for stomatal closure, but evidence is lacking.

Menadione (MD) and methyl viologen (MV) modulated mitochondrial and chloroplastic electron transport. MD blocked electron transport between complex I and II and caused superoxide generation in mitochondria (Obata *et al.* 2011; Aswani *et al.* 2019). On the other hand, MV produced superoxide in chloroplasts in a light-dependent manner (Aswani *et al.* 2019; Cui *et al.* 2019). Both MD and MV were used to create oxidative stress in tobacco protoplasts (Sun *et al.* 1999), *Triticum turgidum* roots (Livanos *et al.* 2012), and *Arabidopsis* leaves (Wang *et al.* 2019; Sipari *et al.* 2020). In a combined study, MD and MV helped induce oxidative stress in mitochondria or chloroplasts in pea and *Arabidopsis* leaves (Aswani *et al.* 2019; Bapatla *et al.* 2021). Unlike the extensive studies on other plant tissues, studies on stomatal response to MV or MD are very few (McAinsh *et al.* 1996).

The importance of mitochondria and chloroplasts during stomatal movement was expected, but the detailed mechanism during closure was not studied. Within guard cells, like in other plant cells, ROS production could occur in mitochondria, chloroplasts, and peroxisomes (Sierla *et al.* 2016; Postiglione and Muday 2020, 2023). We assessed the importance of mitochondria and chloroplasts during ABA-induced stomatal closure. Since stomatal closure invariably involved ROS generation as an early step, we chose to examine the levels of ROS on exposure to MD and MV. Both superoxide and H_2O_2 could contribute to cellular ROS. Our studies, therefore, paid particular attention to superoxide and H_2O_2 levels in guard cells.

We employed MD and MV to (i) study the essentiality of mitochondria and chloroplasts of guard cells to drive stomatal closure; (ii) examine the involvement of superoxide and H_2O_2, and finally (iii) monitor the real-time production and quantification of the superoxide and H_2O_2 in subcellular compartments of guard cells. These signaling events, including ROS (superoxide and H_2O_2) generation in guard cells, were compared with the effects of ABA, a plant hormone that typically induced stomatal closure. Exposure to MD or MV (which target mitochondria or chloroplasts, respectively) made the stomata close. The fluorescent probes demonstrated a rise in the levels of superoxide and then H_2O_2 of guard cells. However, ROS (the superoxide and subsequently H_2O_2) were not limited to a single organelle but spread to other organelles. MD stimulated ROS production in mitochondria and chloroplasts, whereas MV targeted chloroplasts and then mitochondria in guard cells of *Arabidopsis*. Our results emphasized the importance of both mitochondria and chloroplasts in increasing guard cell ROS levels and inducing stomatal closure.

2. Materials and methods

2.1 *Plant material*

Arabidopsis thaliana wild-type (Col-0) or mutants seeds were soaked in NaOCl-Tween20 solution, germinated in pots containing pre-mixed soilrite (Keltech Energies, Bangalore), and left in the dark for 3 days at 4°C. Seedlings were transplanted into individual pots and were grown in an environment-regulated growth room with 8 h light/16 h dark photoperiod at 22±1°C. Plants were supplied with a medium containing Murashige and Skoog plant salt mixture with $CaCl_2$ (4.3 g/L) once in 3 days, alternating with tap water once in 3 days. Mutants *csd1/3* (CS2103503), *fsd3* (CS886860), and *msd1* (SALK_203190C) were obtained from ABRC, USA.

2.2 *Chemicals*

Menadione (analytical standard), methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dichloride), ABA, tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid), diphenyleneiodonium chloride (DPI), salicyl hydroxamic acid (SHAM), and catalase were from Sigma-Aldrich (St Louis, MO, USA). Among the fluorescent probes, BES-So-AM was from Wako Pure Chemicals

Industries (Osaka, Japan), whereas MitoSOX™, MitoTracker™ Red CMXRos, and 2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) were from Invitrogen (USA). All other chemicals were from within India and were of analytical grade.

2.3 *Stomatal bioassay*

Stomatal closure in the abaxial epidermis was determined as earlier (Munemasa *et al.* 2007; Agurla *et al.* 2017). Leaves from 5- to 6-week-old plants were kept on 10 mM MES-KOH, pH 6.15, with 50 mM KCl and 50 μM $CaCl_2$, in 3 h light (200–250 μE m^{-2} s^{-1}) and at 25±1°C to open the stomata. Leaves were treated with test chemicals or modulators and left under light for 2.5 h. The modulators were applied 20 min before the test compounds. At the end of treatment, the epidermis was attached to a coverslip with Telesis 8 (Premiere Products Inc., Pacoima, CA, USA). The mesophyll was removed (by forceps), and the epidermis was flushed with water. Epidermal images were captured under a research microscope (Olympus CX21). The width of apertures was determined by a pre-calibrated image analysis system ProgRes® CapturePro 2.7.

2.4 *Monitoring superoxide and H_2O_2*

Guard cell superoxide levels were monitored by BES-So-AM (Gémes *et al.* 2016) with minor modifications, while H_2O_2 levels were monitored by CM-H_2DCFDA (Munemasa *et al.* 2007). After the stomata were open, the epidermis (ahering to the cover slip) was incubated with 30 μM BES-So-AM for 1 h or with 20 μM CM-H_2-DCFDA (20 min). The epidermis was rinsed twice with an incubation buffer to remove the external dye. The strips with fluorescent dye were treated with test compounds or modulators for 20 min. They were examined under a confocal laser microscope (Carl-Zeiss LSM880) or fluorescence microscope Nikon TE200 (excitation 488 nm/emission 510–530 nm). For quantification of superoxide/H_2O_2 levels, the mean fluorescence of at least 30 guard cells was measured using ImageJ software (*imagej.nih.gov*) for each treatment. The fluorescence was first normalized with the background, and the fluorescence intensity (in pixels) of the treated samples was compared with that of the control.

2.5 *Co-localization of superoxide or H_2O_2 in subcellular compartments*

Superoxide levels in subcellular organelles were evaluated by treating the epidermis with 30 μM BES-So-AM for 1 h in the dark, followed by washing and re-incubation with 5 μM MitoSOX™ mitochondrial superoxide indicator for 20 min with or without test compounds. For co-localization of H_2O_2, strips were first incubated with 20 μM CM-H_2DCFDA for 20 min followed by 1 μM MitoTracker™ Red CMXRos for 20 min. The best emission spectra for each probe were ensured before co-localization studies. Later each signal was unmixed to evaluate the particular site and also shown as a superimposed image. BES-So-AM or CM-H_2DCFDA signals were evaluated for co-localization

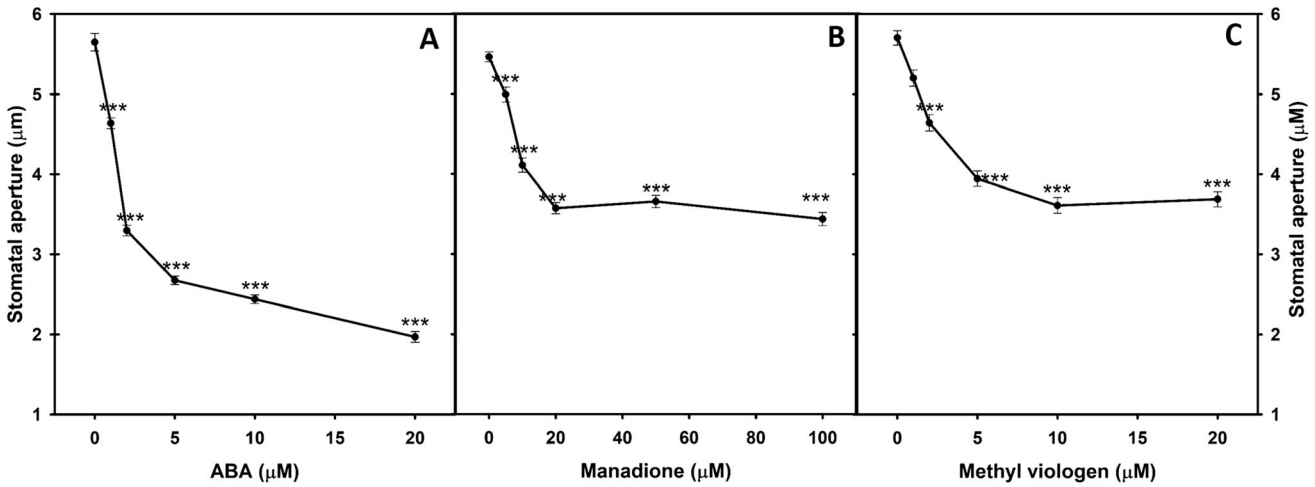

Figure 1. ABA or menadione (MD) or methyl viologen (MV) induced significant stomatal closure (***$p<0.001$) in the abaxial epidermis of *Arabidopsis thaliana* in a concentration-dependent manner. Concentrations of 10 μM were chosen for ABA and MD and 5 μM for MV for further experiments.

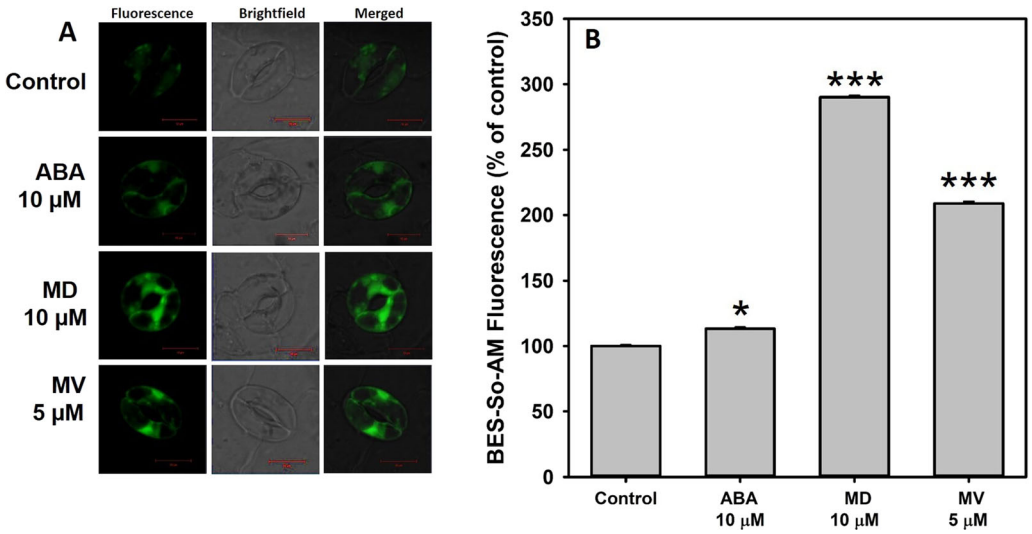

Figure 2. The patterns of superoxide levels in guard cells of *Arabidopsis thaliana* during stomatal closure by 10 µM ABA, 10 µM MD, and 5 µM MV, as revealed by confocal images of BES-So-AM (a superoxide specific fluorescence dye) (**A**). Compared with the control, maximum fluorescence intensity was with MD (***p<0.001) followed by MV (***p<0.001) but it was less significant in the case of ABA (*p<0.05) (**B**). Scale bar = 5 µm.

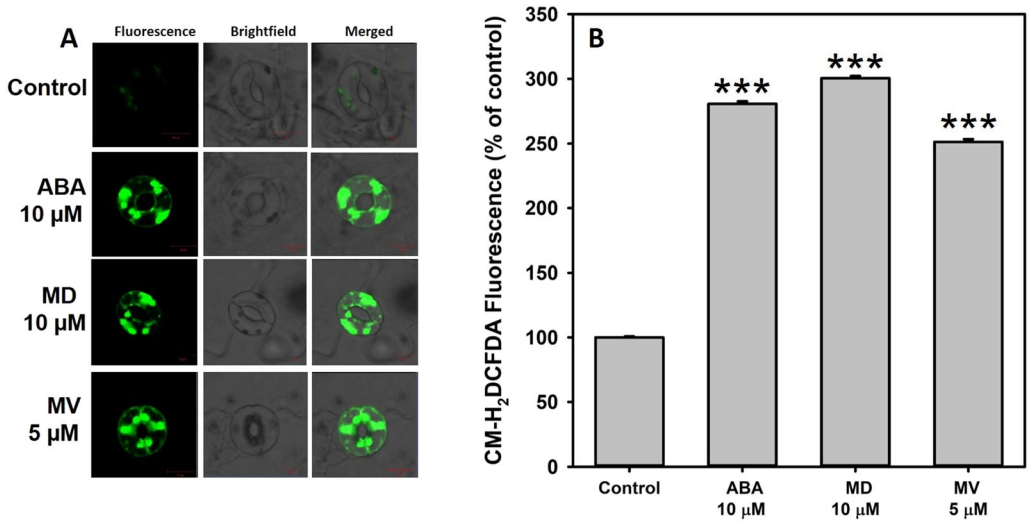

Figure 3. Patterns of H_2O_2 in guard cells of *Arabidopsis thaliana* during stomatal closure by 10 µM ABA, 10 µM MD, and 5 µM MV, as revealed by confocal images of CM-H_2DCFDA (a H_2O_2-specific fluorescence dye) (**A**). Marked increase in the level of H_2O_2 in guard cells was observed in ABA-, MD-, or MV-treated guard cells (***p<0.001) (**B**). Scale bar = 5 µm.

with chlorophyll autofluorescence or that of MitoSOX^{TM}/MitoTracker^{TM}. Samples were analyzed using the Zeiss Zen co-localization module to derive the Pearson correlation coefficient based on co-localization scatter plots. Pearson correlation values were considered one of the best quantifying co-localization patterns (Zinchuk *et al.* 2007; Adler and Parmryd 2010). Values above 0 and up to 1 were taken as positive correlations.

2.6 *Superoxide dismutase (SOD) activity*

About 100 mg of untreated (control) leaf samples were ground in liquid N_2 with 50 mM sodium phosphate buffer (pH 7.8). The homogenate was cleared by centrifugation at 12000g for 10 min at 4°C. SOD activity was determined in the supernatant (Beyer and Fridovich 1987; Bapatla *et al.* 2021).

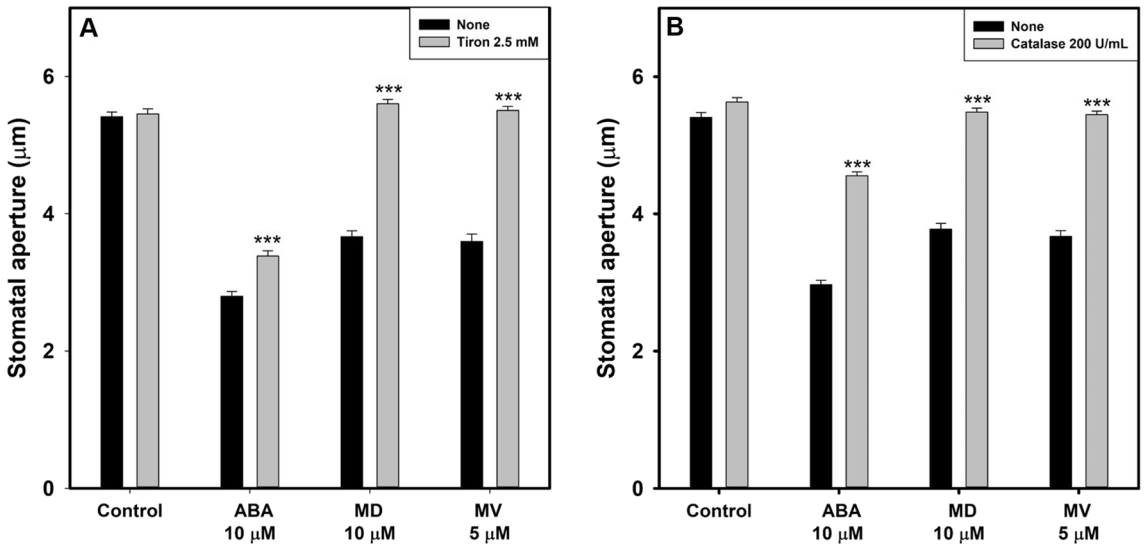

Figure 4. Reversal of ABA-, MD-, or MV-induced stomatal closure by tiron, a superoxide scavenger (**A**) or catalase, a H_2O_2 scavenger (**B**). Both tiron and catalase significantly reversed stomatal closure by MD or MV or ABA (***p<0.001), but the extent of reversal was partial in the presence of ABA.

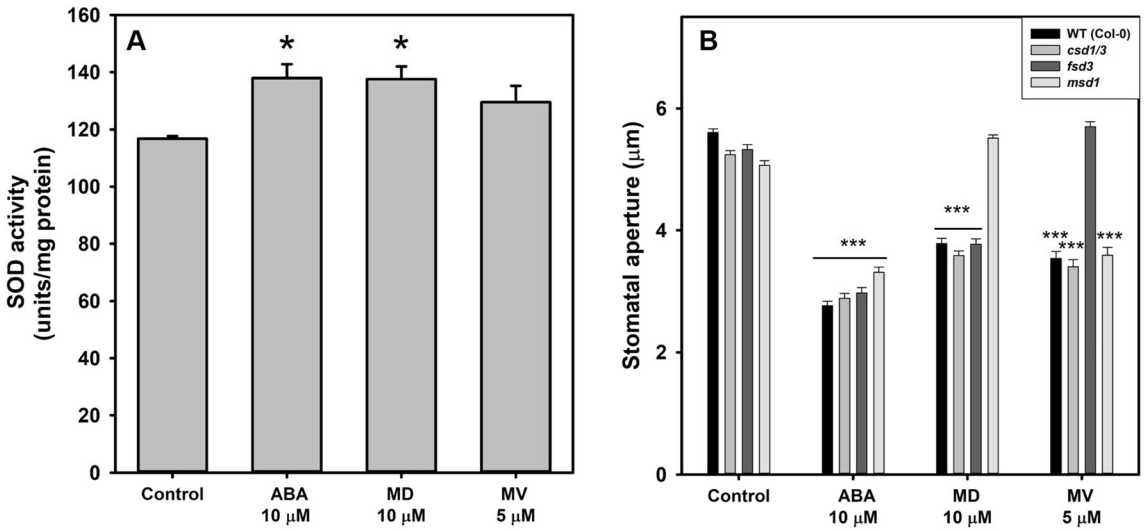

Figure 5. The activity of SOD in leaves of *Arabidopsis* on treatment with of ABA or MD or MV which increased marginally in response to ABA/MD (*p<0.05) or MV (**A**). Stomatal closure in SOD-deficient mutants in response to ABA or MD or MV (**B**). The mutants used were *csd1/3* (deficient in cytosolic Cu/Zn SOD1/extracellular Cu/Zn SOD3), *fsd3* (deficient in chloroplastic Fe SOD3), and *msd1* (deficient in mitochondrial Mn SOD1). Closure by ABA was significant (***p<0.001) in all three mutant, but closures by MD and MV were not significant in *msd1* and *fsd3*, respectively.

2.7 *Replications and statistics*

Stomatal bioassay and fluorescence measurements were from at least 30 guard cells each time. Averages of three to four independent experiments on separate days were considered for analysis. The significance was checked by one-way ANOVA. The p-value of <0.05 or less indicated significance. Graphs and statistical analysis were made using SigmaPlot 12.0.

3. Results

3.1 *MD- and MV-induced stomatal closure compared to ABA*

The effect of MD and MV on stomatal closure was assessed at varying concentrations. The plant stress hormone ABA was included as a positive control for comparison. All three compounds, ABA, MD, and MV,

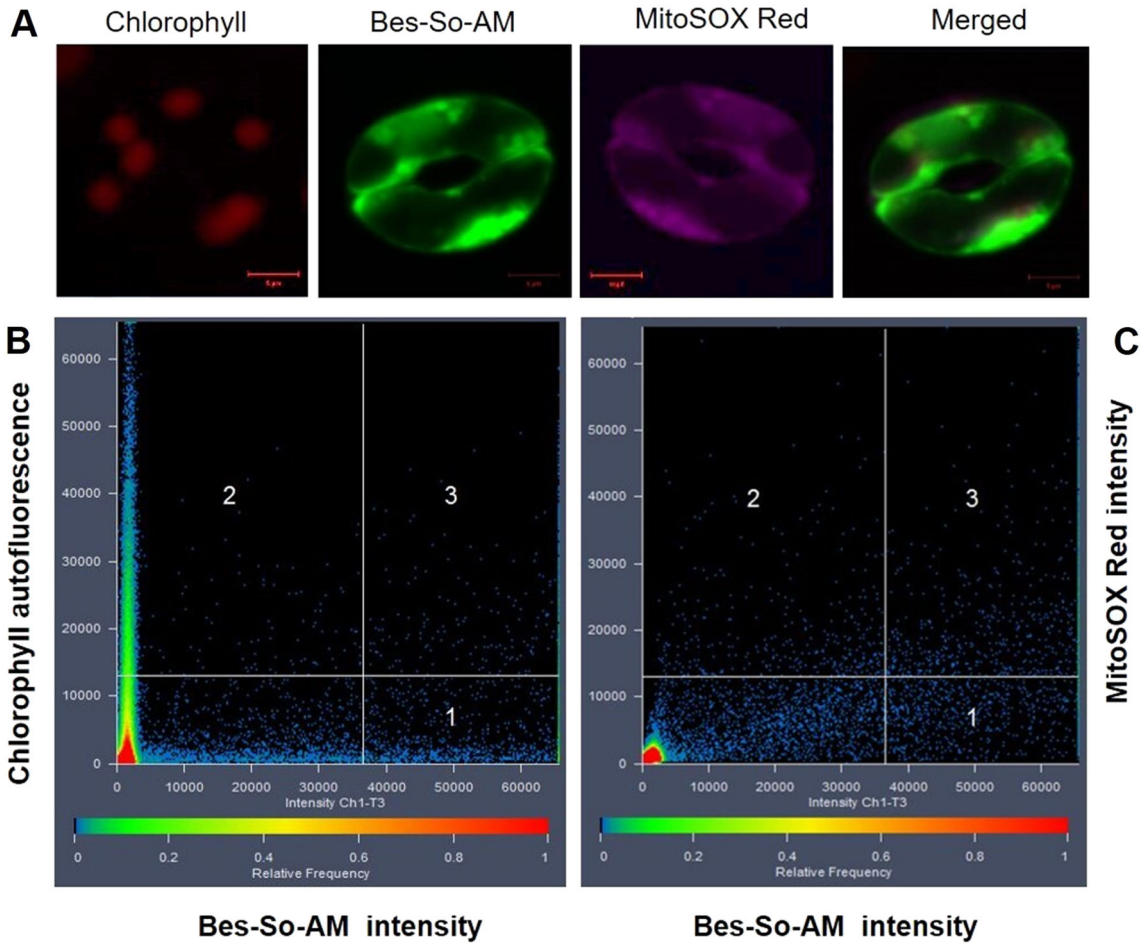

Figure 6. Co-localization of superoxide in mitochondria and chloroplasts of guard cells in response to 10 μM ABA (**A**–**C**). (**A**) Confocal Z-stack images of chlorophyll autofluorescence (red), superoxide specific BES-So-AM fluorescence (green), MitoSOX Red fluorescence (magenta) targeting mitochondrial superoxide, and the merged image. The lower panel represents scatterplots obtained from ZEN colocalization module. BES-So-AM green fluorescence against chlorophyll autofluorescence reflected colocalization of superoxide in chloroplasts (**B**); and BES-So-AM green fluorescence against MitoSOX Red fluorescence (magenta) showed co-localization of superoxide in mitochondria (**C**).

induced stomatal closure at micromolar concentrations (figure 1). However, the extent of closure by MD or MV was not as pronounced as with ABA. Concentrations of 10 μM ABA, 10 μM MD, and 5 μM MV were used for further experiments.

3.2 *Superoxide and H_2O_2 of guard cells increased during stomatal closure by MD or MV*

There was a marked increase in ROS (superoxide and H_2O_2) levels of guard cells when treated with ABA, MD or MV as visualized by superoxide or H_2O_2-specific fluorescent probes, BES-So-AM or CM-H_2-DCFDA, respectively (figures 2A and 3A). The extent of superoxide generation indicated by BES-So-AM was 2- to 3-fold higher over the control in response to MD/MV/ABA (figure 2B). Similarly, H_2O_2 levels, as indicated by CM-H_2DCFDA, were raised by 2.5- to 3-fold over the control when exposed to MD/MV/ABA (figure 3B). The use of scavengers confirmed the essentiality of superoxide and H_2O_2. Stomatal closure by either MD or MV was prevented by tiron (a superoxide scavenger), whereas the closure by ABA was not attenuated much (figure 4A). Catalase, a well-known scavenger of H_2O_2, reversed the stomatal closure induced by MD, MV, or ABA (figure 4B).

The superoxide gets converted to H_2O_2 by endogenous superoxide dismutase (SOD). ABA or MD enhanced SOD activity in leaves, much more potently than MV (figure 5A). Mutants that lacked SOD in each of these compartments were used to understand the role of SODs. Stomatal closure by MD or MV was disabled in *msd1* (deficient in mitochondrial Mn SOD1) or *fsd3*

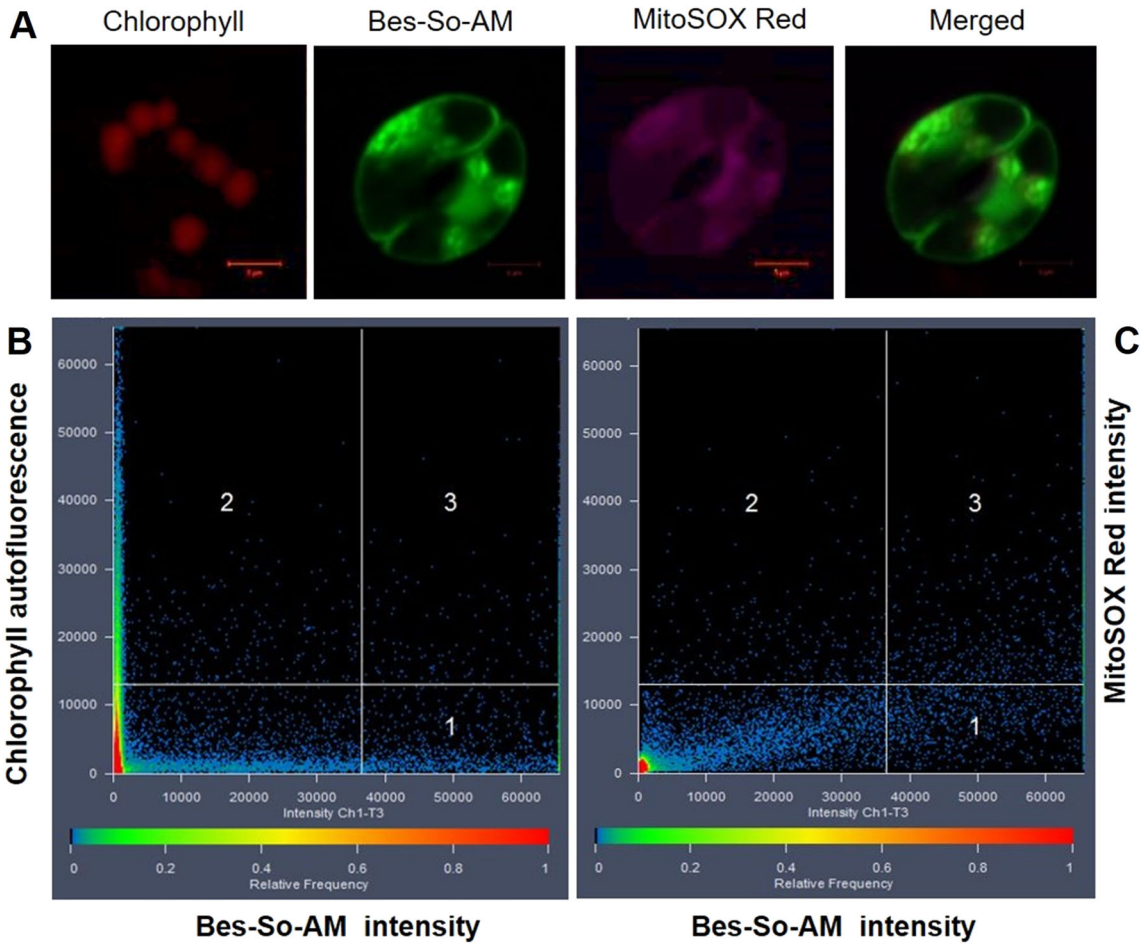

Figure 7. Co-localization of superoxide in mitochondria and chloroplasts of guard cells in response to 10 μM Menadione (**A**–**C**). Further details related to Z-stack images of guard cells and scatter plots are as those in figure 6.

(deficient in chloroplastic Fe SOD3) mutants, respectively (figure 5B). In contrast, there was no change in the closure response in the *csd1/3* double mutant (deficient in cytosolic Cu/Zn SOD1 and extracellular Cu/Zn SOD3). However, stomatal closure by ABA was partially recovered in *msd1* mutants.

3.3 *Intracellular location of superoxide and H_2O_2 in guard cells*

We attempted to assess the localization of superoxide and H_2O_2 in mitochondria and chloroplasts on exposure to MD/MV/ABA. The relative superoxide/H_2O_2 generation was expressed as Pearson correlation coefficients. The value of 1 reflects complete (100%) co-localization, while 0 indicates no co-localization (Zinchuk *et al.* 2007; Adler and Parmryd 2010). When the superoxide was monitored in the presence of ABA, the correlation coefficients were 0.1 for mitochondria and 0.02 for chloroplasts (figure 6). When treated with MD, the correlation coefficient for the superoxide location was 0.13 for mitochondria and 0.1 for chloroplasts (figure 7). However, in the case of MV, the superoxide production in chloroplasts in terms of correlation coefficient was 0.19, whereas in mitochondria, it was 0.12 (figure 8). The levels of H_2O_2 were much higher than those of superoxide, as indicated by the correlation coefficients. H_2O_2 levels were all greater in mitochondria than in chloroplasts, irrespective of exposure to ABA, MD, or MV. The correlation coefficients of H_2O_2 production by ABA were 0.28 for mitochondria and 0.02 for chloroplasts (figure 9). In the case of MD, the values for H_2O_2 generated in mitochondria and chloroplasts were 0.41 and 0.16, respectively (figure 10). However, the H_2O_2 level in response to MV for mitochondria was 0.26, and for chloroplasts, 0.2 (figure 11). In summary, correlation coefficient values indicated the predominant localization of superoxide or H_2O_2 in mitochondria, compared with chloroplasts (table 1).

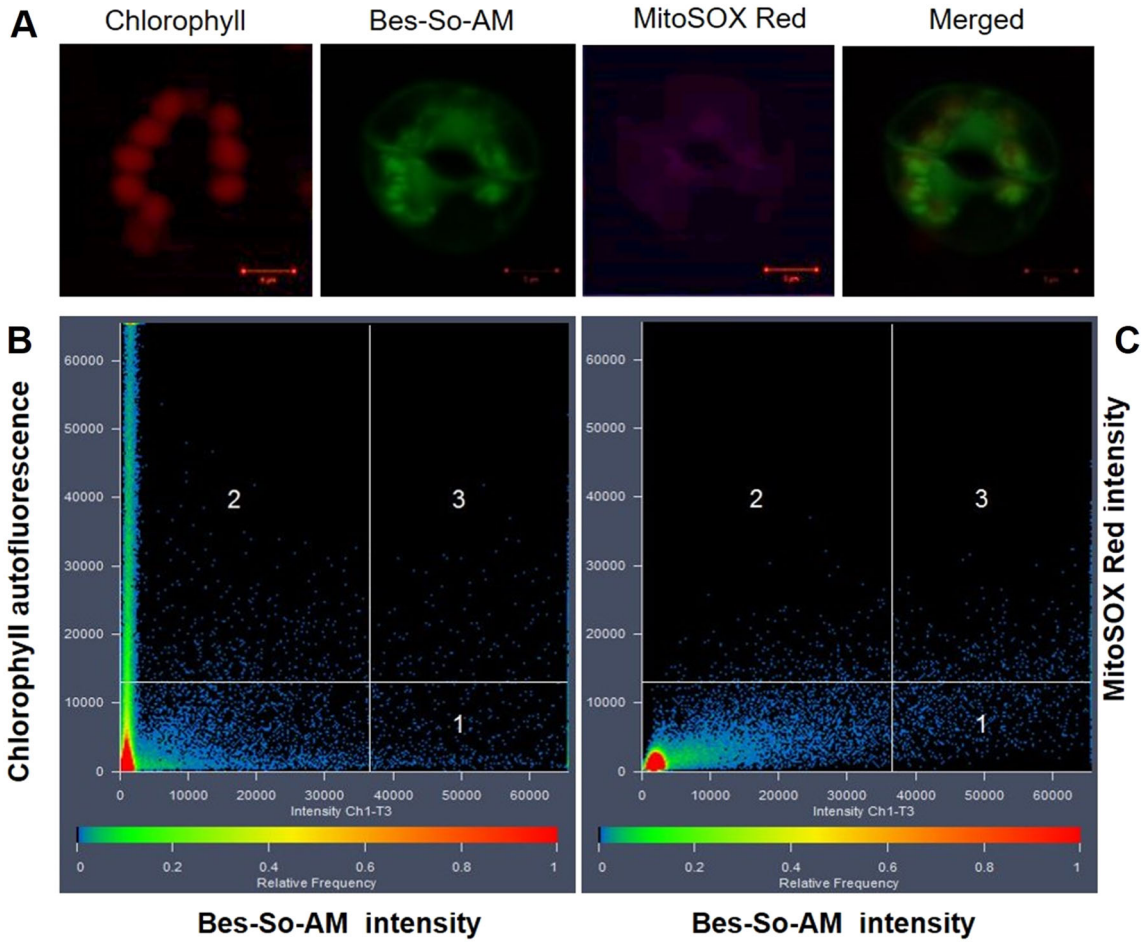

Figure 8. Co-localization of superoxide in mitochondria and chloroplasts of guard cells in response to 5 μM methyl viologen (**A**–**C**). Further details related to Z-stack images of guard cells and scatter plots are as those in figure 6.

4. Discussion

4.1 *Disruption of either mitochondrial or chloroplastic function led to stomatal closure*

Two significant sources of energy in plant cells are mitochondria and chloroplasts. Compared with leaf mesophyll cells, guard cells had a greater number of mitochondria than chloroplasts (Parvathi and Raghavendra 1995; Willmer and Fricker 1996; Santelia and Lawson 2016; Daloso *et al.* 2017). Similarly, guard cell chloroplasts differed from mesophyll in their PSI richness, while PSII and Rubisco were deficient (Reckmann *et al.* 1990; Lawson 2009; Lawson *et al.* 2014). Unfavorable conditions created an imbalance in the electron transport within these organelles, raised ROS levels, and caused oxidative damage (Suzuki *et al.* 2012; Li and Kim 2021). There had been an emphasis on the role of mitochondria and chloroplasts in guard cells, mainly concerning energetic or metabolite needs for stomatal opening (Vavasseur and Raghavendra 2005). The present study emphasized the importance of mitochondria and chloroplasts to sustain stomatal closure employing MD and MV. These two bioenergetic inhibitors interfere with electron transport in these two organelles.

Earlier reports on MV effects on stomata were ambiguous, since MV induced stomatal closure while inhibiting stomatal opening (McAinsh *et al.* 1996). Similarly, MV stimulated superoxide production in guard cells of *Alocasia macrorhiza* and pea (Samuilov *et al.* 2006; Lin *et al.* 2009). Menadione too accelerated H_2O_2 production and suppressed guard cell apoptosis (Samuilov *et al.* 2006). We could not find any report of using MD as the oxidant to modulate stomatal closure. However, our work illustrated that MD and MV could induce stomatal closure at very low (micromolar) concentrations, similar to ABA. Further, the closure by MD and MV was due to the significant increase in superoxide and H_2O_2 levels of guard cells, a phenomenon quite similar to the effects of ABA.

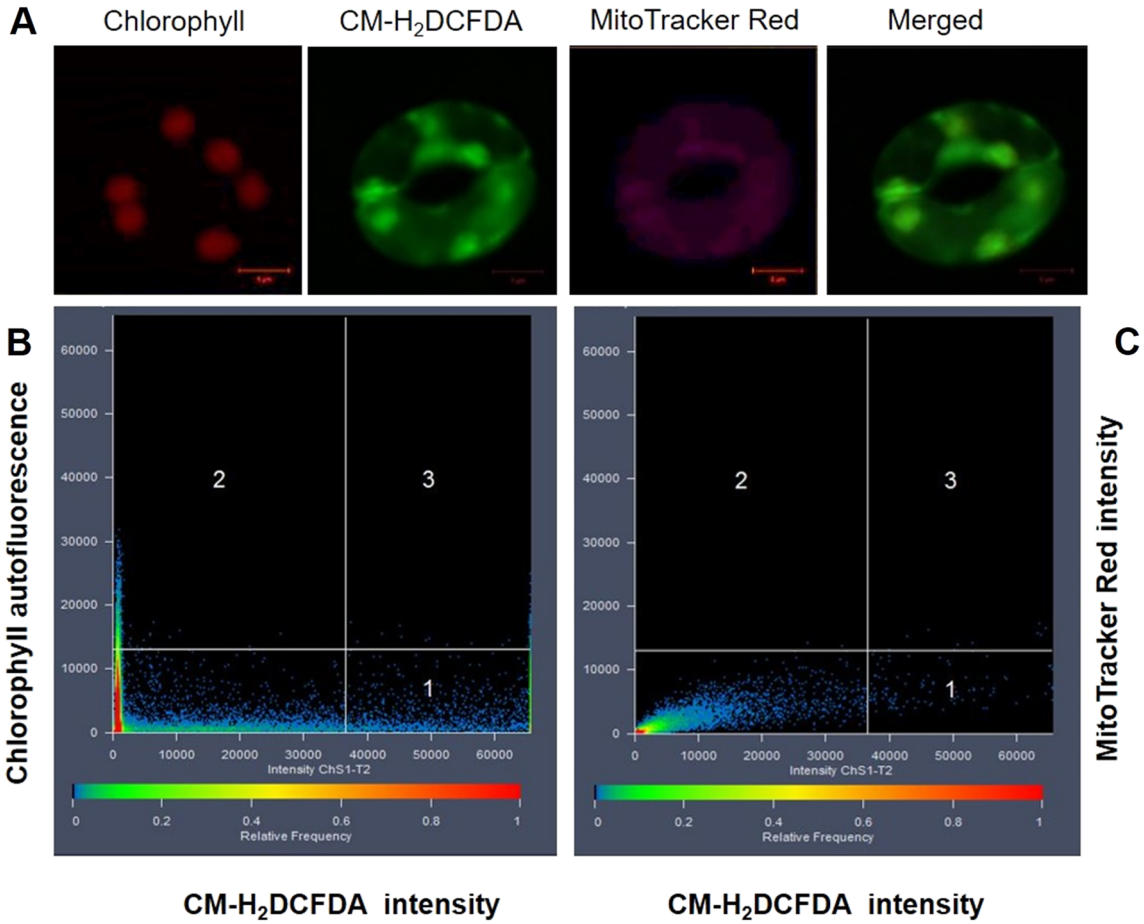

Figure 9. Co-localization of H_2O_2 in mitochondria and chloroplasts of guard cells in response to 10 μM ABA (**A**–**C**). (**A**) Confocal Z-stack images of chlorophyll autofluorescence (red), H_2O_2-specific CM-H_2DCFDA fluorescence (green), MitoTracker Red fluorescence (magenta) targeting mitochondrial H_2O and the merged image, (**B**) scatterplots obtained from ZEN colocalization module of CM-H_2DCFDA green fluorescence against chlorophyll autofluorescence reflected co-localization of H_2O_2 in chloroplasts, and (**C**) CM-H_2DCFDA green fluorescence against MitoSOX Red fluorescence (magenta) showed co-localization of H_2O_2 in mitochondria.

4.2 *Both superoxide and H_2O_2 of guard cells were essential for stomatal closure*

The rise in ROS was invariably a pivotal component of stomatal closure induced by ABA and other hormones such as methyl jasmonate (MJ) and salicylic acid (SA). Elevated ROS triggered in response to stress factors, such as drought and pathogen attack, activated other downstream components, all leading to closure of stomata. The events during stomatal closure have been periodically reviewed (Arnaud and Hwang 2015; Liu *et al.* 2022; Reis *et al.* 2022; Rodrigues and Shan, 2022). The involvement of superoxide or H_2O_2 could be confirmed using scavengers, tiron, or catalase. Superoxide radicals were produced when stomatal closure was induced in *Vicia faba*. The presence of tiron (a scavenger) suppressed the superoxide-based chemiluminescence caused by SA (Mori *et al.* 2001). Similar to their results, the ineffectiveness of MD or MV in inducing stomatal closure when tiron was present in the system indicated the essentiality of the superoxide. Additionally, applying tiron effectively limited superoxide production by MD or MV in *Pisum sativum* leaves (Bapatla *et al.* 2021).

Interestingly, on exposure, MD-mediated ROS production was initiated in mitochondria and involved chloroplasts, while MV enhanced ROS levels initiated in both mitochondria and chloroplasts (Lehmann *et al.* 2009; Cui *et al.* 2019; Ugalde *et al.* 2021). Earlier works on oxidative stress induced in plant systems by MD or MV were mostly with cell cultures and leaves (Sweetlove *et al.* 2002; Samuilov *et al.* 2006; Schwarzländer *et al.* 2009; Aswani *et al.* 2019). As redox-sensitive GFP showed MD initially disturbed redox status in mitochondria, but later the effect appeared in other compartments, such as cytoplast and plastids of roots (Lehmann

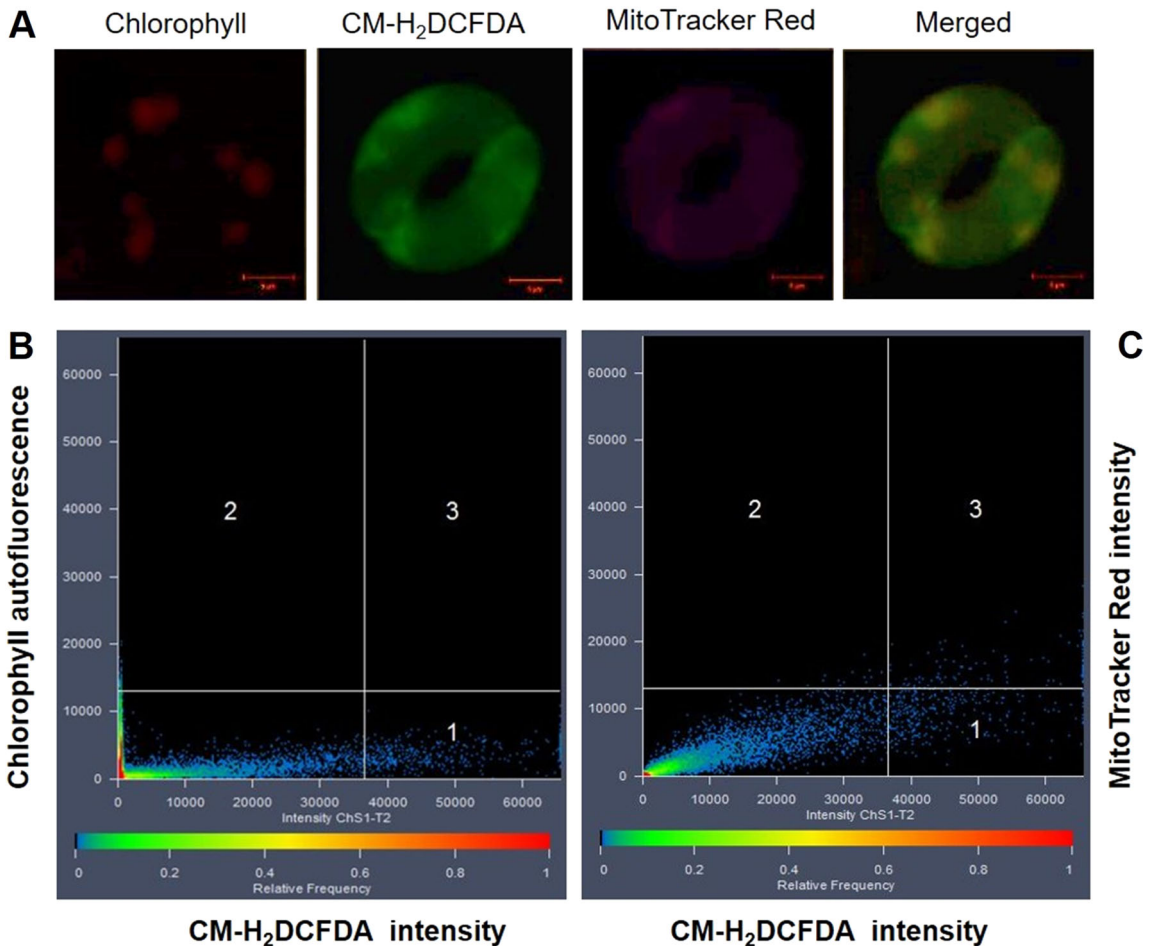

Figure 10. Co-localization of H_2O_2 in mitochondria and chloroplasts of guard cells in response to 10 μM menadione (**A**–**C**). Further details related to Z-stack images of guard cells and scatter plots are as in figure 9.

et al. 2009). In a similar study, redox-based sensors confirmed that MV primarily targeted chloroplasts for H_2O_2 generation, followed by the cytosol and mitochondria (Cui *et al.* 2019; Ugalde *et al.* 2021).

4.3 *MD and MV generated ROS (superoxide and H_2O_2) in both mitochondria and chloroplasts*

It is intriguing but not surprising that metabolic inhibitors that target either mitochondria or chloroplasts generate superoxide and H_2O_2 in both organelles. Like superoxide, the production of H_2O_2 by MD or MV started in mitochondria or chloroplasts and spread to other compartments. Such mobility complemented the importance of ROS as signaling molecules. The mobility of redox components across cellular organelles to regulate photorespiratory metabolism was reported in chloroplasts, mitochondria, and peroxisomes (Lehmann *et al.* 2009; Aswani *et al.* 2019; Cui *et al.* 2019; Bapatla *et al.* 2021; Ugalde *et al.* 2021).

4.4 *Chloroplastic and mitochondrial ROS (superoxide and H_2O_2) contributed to stomatal closure*

The evidence from the co-localization of superoxide/H_2O_2 and organelle-specific SOD mutants highlighted the significant sites of ROS generation by MD and MV. Reversal of stomatal closure by MD in the *msd1* mutant highlighted the importance of superoxide generation in mitochondria (figure 5B). According to an earlier study, the enhanced sensitivity to MV-induced ROS in mitochondrial *msd1*-deficient RNA*i* lines highlighted the importance of ROS production in the mitochondria of *Arabidopsis* leaves (Cui *et al.* 2019). Similarly, the ineffectiveness of MV in inducing stomatal closure in the *fsd3* mutant reflected the importance of superoxide production in chloroplasts (figure 5B). The mitochondrial or chloroplastic SOD-deficient mutants were more sensitive to stress conditions due to their altered cellular redox state. The mutant deficient in mitochondrial SOD [*msd1* or *oiwa*

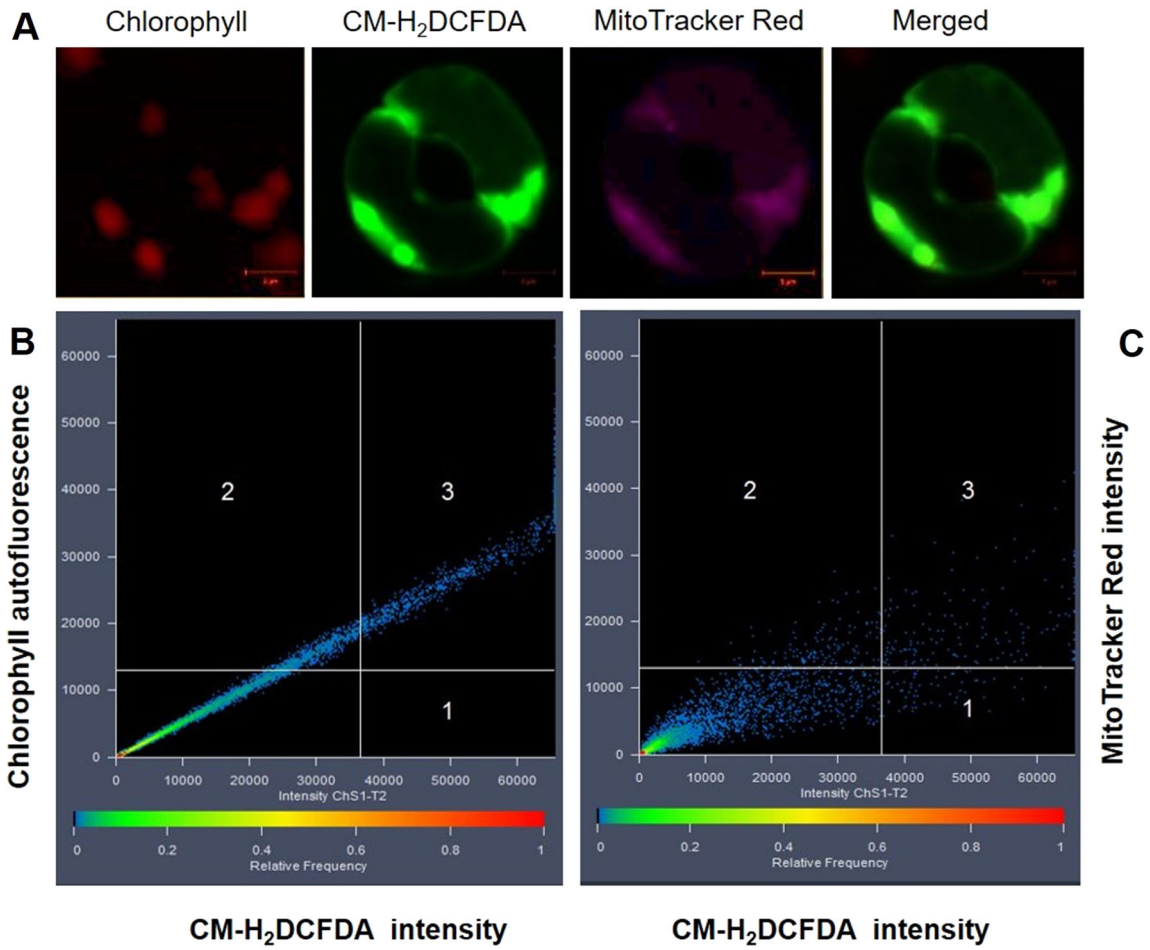

Figure 11. Co-localization of H_2O_2 in mitochondria and chloroplasts of guard cells in response to 5 µM methyl viologen (**A**–**C**). Further details related to Z-stack images of guard cells and scatter plots are as in figure 9.

(female gametophytic mutant impaired in Mn SOD1), named so due to their phenotype] exhibited reduced ROS levels and altered redox state of mitochondria (Morgan *et al.* 2008; Martin *et al.* 2013; Hu and Jinn 2022). Similarly, the *fsd3* mutant deficient in chloroplastic SOD exhibited higher superoxide and lower

Table 1. Representative colocalization coefficient values of superoxide (A) and H_2O_2 (B) production in mitochondria and chloroplasts in guard cells of *Arabidopsis thaliana*

	(A) Superoxide	
Treatment	BES-So-AM vs. chlorophyll autofluorescence Chloroplasts	BES-So-AM vs. MitoSOX Red Mitochondria
ABA 10 µM	0.02	0.10
Menadione 10 µM	0.10	0.13
Methyl viologen 5 µM	0.19	0.12
	(B) H_2O_2	
Treatment	CM-H_2DCFDA vs. chlorophyll autofluorescence Chloroplasts	CM-H_2DCFDA vs. Mito Tracker Red Mitochondria
ABA 10 µM	0.02	0.28
Menadione 10 µM	0.16	0.41
Methyl viologen 5 µM	0.20	0.26

H_2O_2 levels in chloroplasts, decreasing their photosynthetic efficiency (Myouga *et al.* 2008; Gallie and Chen 2019).

H_2O_2 was produced in chloroplasts, mitochondria, cytosol, peroxisomes, and plasma membranes during stomatal closure by ABA (Postiglione and Muday 2020, 2023). Thus, ROS (superoxide/H_2O_2) from both mitochondria and chloroplasts contributed to stomatal closure by ABA, MD, or MV.

4.5 *Events downstream of ROS during MD/MV action similar to those of ABA*

The rise in guard cell ROS was essential for stomatal closure by MD or MV, as in the case of ABA. The elevated ROS increased NO and Ca^{2+}, which activated ion-efflux making stomata close (Bharath *et al.* 2021; Lim *et al.* 2022a; Liu *et al.* 2022). In a few earlier reports, MD and MV promoted the rise in NO and Ca^{2+} in guard cells (McAinsh *et al.* 1996; Samuilov *et al.* 2006). Our observations emphasized the essentiality of ROS in stomatal closure by ABA, MD, or MV. Signaling components downstream of ROS during ABA-triggered stomatal closure included a plethora of intermediates such as nitric oxide, Ca^{2+}, Ca^{2+}-dependent kinases, MAP kinases, lipids, and ion channels. These downstream components also took part in MD- and MV-mediated stomatal closure. The modulation of signaling components downstream of ROS during closure by MD or MV needs to be examined further.

5. Concluding remarks

Ours is the first attempt to examine in detail the importance of mitochondria or chloroplasts of stomatal guard cells using MD and MV, which interfered with electron transport systems in mitochondria and chloroplasts, respectively. MD or MV promoted significant stomatal closure even at very low concentrations, pointing out the crucial role of mitochondria and chloroplasts during stomatal closure. An increase in ROS levels of mitochondria and chloroplasts was an essential event when closure was initiated. Redox imbalance in mitochondria affected the status of chloroplasts and *vice versa*. We suggest that guard cells can be an excellent experimental system for studying inter-organellar interactions involving mitochondria, chloroplasts, and the cytoplasm.

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Author contributions

ASR designed the work. SG, PB, and DS performed the experiments. ASR and GP supervised the experiments and analyzed the results. SG and ASR wrote the first draft. All the authors read and finalized the manuscript.

Declarations

Conflict of interest Authors declare no conflict of interest

References

Adler J and Parmryd I 2010 Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry A* **77** 733–742

Agurla S, Gayatri G and Raghavendra AS 2017 Signal transduction components in guard cells during stomatal closure by plant hormones and microbial elicitors; in *Mechanism of plant hormone signaling under stress* 1st edition (Ed.) G Pandey (USA: John Wiley & Sons) pp. 353–387

Agurla S, Gahir S, Munemasa S, *et al*. 2018 Mechanism of stomatal closure in plants exposed to drought and cold stress. *Adv. Exp. Med. Biol.* **1081** 215–232

Arnaud D and Hwang I 2015 A sophisticated network of signaling pathways regulates stomatal defenses to bacterial pathogens. *Mol. Plant.* **8** 566–581

Ashapkin VV, Kutueva LI, Aleksandrushkina NI, *et al*. 2020 Epigenetic mechanisms of plant adaptation to biotic and abiotic stresses. *Int. J. Mol. Sci.* **21** 7457

Aswani V, Rajsheel P, Bapatla RB, *et al*. 2019 Oxidative stress induced in chloroplasts or mitochondria promotes proline accumulation in leaves of pea (*Pisum sativum*): another example of chloroplast-mitochondria interactions. *Protoplasma* **256** 449–457

Bapatla RB, Saini D, Aswani V, *et al*. 2021 modulation of photorespiratory enzymes by oxidative and photo-oxidative stress induced by menadione in leaves of pea (*Pisum sativum*). *Plants* **10** 987

Beyer WF Jr and Fridovich I 1987 Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* **161** 559–566

Bharath P, Gahir S and Raghavendra AS 2021 Abscisic acid-induced stomatal closure: An important component of plant defense against abiotic and biotic stress. *Front. Plant Sci.* **12** 615114

Cui F, Brosché M, Shapiguzov A, He XQ, *et al*. 2019 Interaction of methyl viologen-induced chloroplast and mitochondrial signalling in *Arabidopsis*. *Free Radic. Biol. Med.* **134** 555–566

Daloso DM, Medeiros DB, Dos Anjos L, *et al*. 2017 Metabolism within the specialized guard cells of plants. *New Phytol.* **216** 1018–1033

Gallie DR and Chen Z 2019 Chloroplast-localized iron superoxide dismutases FSD2 and FSD3 are functionally distinct in *Arabidopsis*. *PLoS One* **14** e0220078

Gémes K, Kim YJ, Park KY, *et al*. 2016 An NADPH-oxidase/polyamine oxidase feedback loop controls oxidative burst under salinity. *Plant Physiol.* **172** 1418–1431

Hedrich R and Shabala S 2018 Stomata in a saline world. *Curr. Opin. Plant Biol.* **46** 87–95

Hu SH and Jinn TL 2022 Impacts of Mn, Fe, and oxidative stressors on MnSOD activation by AtMTM1 and AtMTM2 in *Arabidopsis*. *Plants* **11** 619

Lawson T 2009 Guard cell photosynthesis and stomatal function. *New Phytol.* **181** 13–34

Lawson T, Simkin AJ, Kelly G, *et al*. 2014 Mesophyll photosynthesis and guard cell metabolism impacts on stomatal behaviour. *New Phytol.* **203** 1064–1081

Lehmann M, Schwarzländer M, Obata T, *et al*. 2009 The metabolic response of *Arabidopsis* roots to oxidative stress is distinct from that of heterotrophic cells in culture and highlights a complex relationship between the levels of transcripts, metabolites, and flux. *Mol. Plant* **2** 390–406

Li M and Kim C 2021 Chloroplast ROS and stress signaling. *Plant Commun.* **3** 100264

Lim J, Lim CW and Lee SC 2022a Core components of abscisic acid signaling and their post-translational modification. *Front. Plant Sci.* **13** 895698

Lim SL, Flütsch S, Liu J, *et al*. 2022b Arabidopsis guard cell chloroplasts import cytosolic ATP for starch turnover and stomatal opening. *Nat. Commun.* **13** 652

Lin ZF, Liu N, Lin GZ, *et al*. 2009 *In situ* localisation of superoxide generated in leaves of *Alocasia macrorrhiza* (L.) Shott under various stresses. *J. Plant Biol.* **52** 340–347

Liu H, Song S, Zhang H, *et al*. 2022 Signaling transduction of ABA, ROS, and Ca^{2+} in plant stomatal closure in response to drought. *Int. J. Mol. Sci.* **23** 14824

Livanos P, Galatis B, Quader H, *et al*. 2012 Disturbance of reactive oxygen species homeostasis induces atypical tubulin polymer formation and affects mitosis in root-tip cells of *Triticum turgidum* and *Arabidopsis thaliana*. *Cytoskeleton* **69** 1–21

Martin MV, Fiol DF, Sundaresan V, *et al*. 2013 *oiwa*, a female gametophytic mutant impaired in a mitochondrial manganese-superoxide dismutase, reveals crucial roles for reactive oxygen species during embryo sac development and fertilization in *Arabidopsis*. *Plant Cell* **25** 1573–1591

McAinsh MR, Clayton H, Mansfield TA, *et al*. 1996 Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. *Plant Physiol.* **111** 1031–1042

Morgan MJ, Lehmann M, Schwarzländer M, *et al*. 2008 Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. *Plant Physiol.* **147** 101–114

Mori IC, Pinontoan R, Kawano T, *et al*. 2001 Involvement of superoxide generation in salicylic acid-induced stomatal closure in *Vicia faba*. *Plant Cell Physiol.* **42** 1383–1388

Munemasa S, Oda K, Watanabe-Sugimoto M, *et al*. 2007 The coronatine-insensitive 1 mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in *Arabidopsis* guard cells. Specific impairment of ion channel activation and second messenger production. *Plant Physiol.* **143** 1398–1407

Munns R and Millar AH 2023 Seven plant capacities to adapt to abiotic stress. *J. Exp. Bot.* **74** 4308–4323

Myouga F, Hosoda C, Umezawa T, *et al*. 2008 A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in *Arabidopsis*. *Plant Cell* **20** 3148–3162

Obata T, Matthes A, Koszior S, *et al*. 2011 Alteration of mitochondrial protein complexes in relation to metabolic regulation under short-term oxidative stress in *Arabidopsis* seedlings. *Phytochemistry* **72** 1081–1091

Parvathi K and Raghavendra AS 1995 Bioenergetic processes in guard cells related to stomatal function. *Physiol. Plant.* **93** 146–154

Postiglione AE and Muday GK 2020 The role of ROS homeostasis in aba-induced guard cell signaling. *Front. Plant Sci.* **11** 968

Postiglione AE and Muday GK 2023 Abscisic acid increases hydrogen peroxide in mitochondria to facilitate stomatal closure. *Plant Physiol.* **192** 469–487

Qi J, Song CP, Wang B, Zhou J, *et al*. 2018 Reactive oxygen species signaling and stomatal movement in plant responses to drought stress and pathogen attack. *J. Integr. Plant Biol.* **60** 805–826

Raghavendra AS 1981 Energy supply for stomatal opening in epidermal strips of *Commelina benghalensis*. *Plant Physiol.* **67** 385–387

Reckmann U, Scheibe R and Raschke K 1990 Rubisco activity in guard cells compared with the solute requirement for stomatal opening. *Plant Physiol.* **92** 246–253

Reis ADP, Carvalho RF, Costa IB, *et al*. 2022 Hydrogen peroxide is involved in drought stress long-distance signaling controlling early stomatal closure in tomato plants. *Braz. J. Biol.* **82** e267343

Rodrigues O and Shan L 2022 Stomata in a state of emergency: H_2O_2 is the target locked. *Trends Plant Sci.* **27** 274–286

Saharan BS, Brar B, Duhan JS, *et al*. 2022 Molecular and physiological mechanisms to mitigate abiotic stress conditions in plants. *Life* **12** 1634

Samuilov VD, Kiselevsky DB, Sinitsyn SV, *et al*. 2006 H_2O_2 intensifies CN-induced apoptosis in pea leaves. *Biochemistry* **71** 384–394

Santelia D and Lawson T 2016 Rethinking guard cell metabolism. *Plant Physiol.* **172** 1371–1392

Schwarzländer M, Fricker MD and Sweetlove LJ 2009 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** 468–475

Sierla M, Waszczak C, Vahisalu T, *et al*. 2016 Reactive oxygen species in the regulation of stomatal movements. *Plant Physiol.* **171** 1569–1580

Sipari N, Lihavainen J, Shapiguzov A, *et al*. 2020 Primary metabolite responses to oxidative stress in early-senescing and paraquat resistant *Arabidopsis thaliana rcd1* (radical-induced cell death1). *Front. Plant Sci.* **11** 194

Sun YL, Zhu HZ, Zhou J, *et al*. 1999 Menadione-induced apoptosis and the degradation of lamin-like proteins in tobacco protoplasts. *Cell Mol. Life Sci.* **55** 310–316

Suzuki N 2023 Fine tuning of ROS, redox and energy regulatory systems associated with the functions of chloroplasts and mitochondria in plants under heat stress. *Int. J. Mol. Sci.* **24** 1356

Suzuki N, Koussevitzky S, Mittler R, *et al*. 2012 ROS and redox signalling in the response of plants to abiotic stress. *Plant Cell Environ.* **35** 259–270

Sweetlove LJ, Heazlewood JL, Herald V, *et al*. 2002 The impact of oxidative stress on *Arabidopsis* mitochondria. *Plant J.* **32** 891–904

Ugalde JM, Fuchs P, Nietzel T, *et al*. 2021 Chloroplast-derived photo-oxidative stress causes changes in H_2O_2 and EGSH in other subcellular compartments. *Plant Physiol.* **186** 125–141

Vani T and Raghavendra AS 1989 Tetrazolium reduction by guard cells in abaxial epidermis of *Vicia faba*: Blue light stimulation of a plasmalemma redox system. *Plant Physiol.* **90** 59–62

Vavasseur A and Raghavendra AS 2005 Guard cell metabolism and CO_2 sensing. *New Phytol.* **165** 665–682

Wang B, Ding H, Chen Q, *et al*. 2019 Enhanced tolerance to methyl viologen-mediated oxidative stress via atgr2 expression from chloroplast genome. *Front. Plant Sci.* **10** 1178

Willmer CM and Fricker M 1996 *Stomata* (London. UK: Chapman and Hall)

Yang J, Li C, Kong D, *et al*. 2020 Light-mediated signaling and metabolic changes coordinate stomatal opening and closure. *Front. Plant Sci.* **11** 601478

Zinchuk V, Zinchuk O and Okada T 2007 Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. *Acta Histochem. Cytochem.* **40** 101–111

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