

# Zooming into sub-organellar localization of reactive oxygen species in guard cell chloroplasts during abscisic acid and methyl jasmonate treatments

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Regulation of stomata movements is crucial for plants ability to cope with their changing environment. Guard cell's (GC) water potential directs water flux inside/outside this cell, which eventually is causing the stoma to open or close, respectively. Some of the osmolytes which accumulates in the GC cytoplasm and are known to play a role in stomata opening are sugars, arising from chloroplast starch degradation. During stomata closure, the accumulated osmolytes are removed from the GC cytoplasm. Surprisingly little is known about prevention of starch degradation and forming additional sugars which may interfere with osmotic changes that are necessary for correct closure of stomata.

One of the early events leading to stomata closure is production of reactive oxygen species (ROS) in various sub-cellular sites and organelles of the stoma. Here we report that ROS production during abscisic acid (ABA) and methyl jasmonate (MJ) stimuli in *Arabidopsis* GC chloroplasts were more than tripled. Moreover, ROS were detected on the sub-organellar level in compartments that are typically occupied by starch grains. This observation leads us to suspect that ROS function in that particular location is necessary for stomata closure. We therefore hypothesize that these ROS are involved in redox control that lead to the inactivation of starch degradation that takes place in these compartments, thus contributing to the stoma closure in an additional way.

## Introduction

### Starch breakdown and closure of stomata

Stomatal movements have been the subject of extensive investigation for their role in plant-environment interactions, especially during periods of drought.<sup>1,2</sup> The opening and closing of the stomata pore is controlled by water fluxes that enter or exit the guard cells, resulting in cell swelling or shrinking, respectively. The water potential of guard cells, which determines the direction of the water flow is governed by osmoregulation,<sup>3</sup> mainly using K<sup>+</sup> and Cl<sup>-</sup> ions or malate and sucrose (SUC) molecules.<sup>4</sup> The opening of stomata in response to blue light was shown to be coupled to Starch degradation in guard cells' chloroplasts, and the sugars that originate from that process were determined as an additional source of osmoticum.<sup>5</sup> Moreover, starch degradation occurs also under natural illumination conditions, resulting in induction of stomatal opening.<sup>6</sup>

The closure of the stomata, however, must involve exclusion of osmolyte molecules from the cytoplasm of the opened guard-cells

to the apoplast or to its vacuole. In addition, the formation of new additional osmoticum in the same interior has to be prevented. Therefore, starch degradation that sustains stomatal opening should be inactivated during that time. Surprisingly, to the best of our knowledge very little is known about the latter event and how is it regulated.

The guard cell chloroplasts (GCC) are different from the mesophyll cell chloroplasts (MCC) by several features. In many species the typical ultra-structure of GCC include small grana stacks and large starch grains, which occupy most of the plastid interior and the size of GCC is much larger than the size of MCC.<sup>7-9</sup> Moreover, there are much fewer GCC per stomatal cell as compared with the high number of MCC in the mesophyll cells.

Recent studies showed that the mechanism of starch degradation in *Arabidopsis* leaves is very different from the well characterized starch breakdown in cereal endosperm, as well as in legume seeds.<sup>10,11</sup> The role of the key enzyme,  $\alpha$ -amylase, has been questioned,<sup>12</sup> and the enzyme that breaks down the starch

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granule into glucans has not been identified.<sup>10</sup> Analysis of starch degradation in *Arabidopsis* leaves was shown to be regulated by the cellular redox state.<sup>10,13,14</sup> It is initiated by  $\alpha$ -amylase, which catalyzes the cleavage of  $\alpha$ -1,4-glucosidic bond of amylose and amylopectin. The enzyme is found in nearly all plants, animals and microorganisms. Accumulation of hydrogen peroxide in plant cells causes inactivation of the  $\alpha$ -amylase.<sup>15,16</sup> Osmotic stress was shown to impair the rate of diurnal starch accumulation in leaves of wild-type plants, but had no effect on starch accumulation in mutants of thioredoxin that cannot reduce  $\beta$ -amylase. Moreover, the mutants were impaired in stomatal opening, suggesting involvement of  $\beta$ -amylase activity in sustaining stomatal opening during the day.<sup>17</sup> The thioredoxin was shown to activate starch degradation pathway in illuminated mesophyll cells upon osmotic stress, similar to the diurnal pathway of starch degradation in guard cells, which is also dependent on thioredoxin-regulated  $\beta$ -amylase. Furthermore, oxidized thioredoxin inactivated starch degradation by  $\beta$ -amylase in illuminated mesophyll cells.<sup>17</sup>

In this paper we studied the production of ROS in GCC during stomata closure in response to treatment with ABA or MJ. Our results show that ROS production was more than tripled during these stimuli. Moreover, ROS accumulated in compartments that are typically occupied by starch grains. That intriguing finding leads us to suspect that ROS has a role in that location necessary for stomata closure. We suggest that ROS control starch degradation by regulating organellar redox, thus preventing formation of additional sugars which may interfere with osmotic changes during stomata closure.

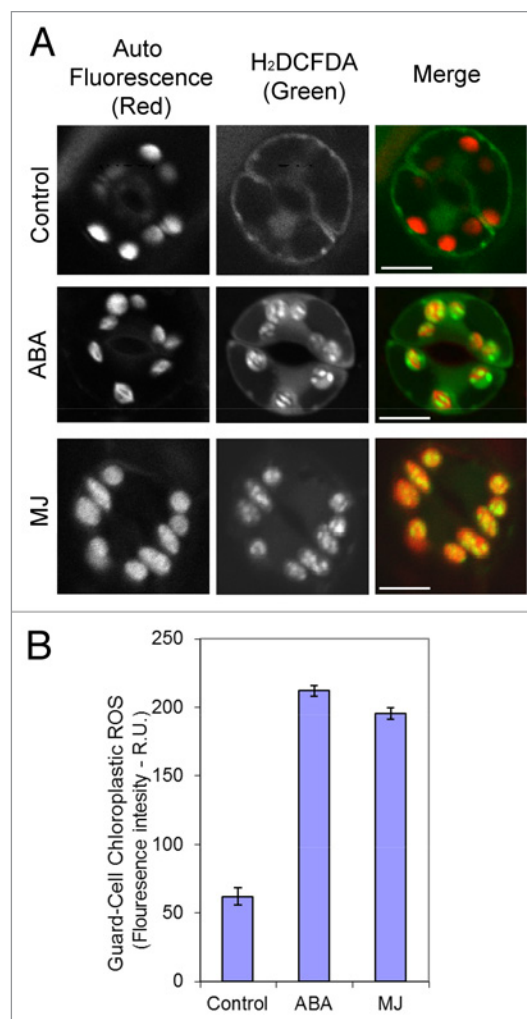
## Results and Discussion

### ROS formation in guard cell chloroplasts during stomatal closure

Recent studies showed that plant treatment by several phytohormones increased the production of Reactive Oxygen Species (ROS) in guard cells, resulting in stomatal closure.<sup>18,19</sup> However, the exact localization of ROS in the cell remained obscure. We showed that the production of ROS in the *Arabidopsis* stomata is localized in many different sub-cellular organelles, such as nucleus, cytoplasm, endosomes and chloroplasts.<sup>18</sup> Accumulation of  $H_2O_2$  in GCC during ABA treatment was also studied in guard cells of *Vicia-faba*.<sup>20,21</sup> However, those studies lacked high resolution of the sub-organellar ROS localization during induction in the chloroplasts.

### Sub-organellar localization of ROS in GCC during treatment with ABA or MJ

Here we show highly localized ROS production in GCC (Fig. 1A). Measurements of  $H_2O_2$  levels showed that they were more than tripled in *Arabidopsis* GCC during ABA and MJ treatments (Fig. 1B). Moreover, the induction of ROS in GCC was not spread evenly but appeared restricted to some inner compartments of the chloroplast (Fig. 1A). High resolution study that included endomembrane staining with MitoFluor™ Red 589 in addition to  $H_2DCFDA$  showed that the accumulation of ROS in GCC occurred in large spaces, typically occupied by starch grains (Fig. 2, full complete Z stacks are presented in Figs. S1, S2



**Figure 1.** Induction of ROS production in *Arabidopsis* guard cells' chloroplasts by ABA and MJ. (A) Leaf disks were prepared from wild-type plants and treated with ABA and MJ. After incubation of leaf disks in solution with the phytohormones, cells were loaded with  $H_2DCFDA$  as described in methods. Shown are representative images of single section confocal microscopy (1  $\mu$ m thick) of guard cells from the different treatments. Scale bar = 10  $\mu$ m. (B) Quantification of the  $H_2DCFDA$ -dependent fluorescence of individual chloroplasts shown in (A). Quantification was done using ImagePRO-Plus program from projected images of 2–3 confocal Z stack sections of individual chloroplasts, as described in methods. Shown is a representative one of three replicate experiments, n = 30 chloroplasts from 10 guard cells sampled from three different young rosette leaves ( $\pm$  SE). R.U. relative unites.

and S3). It is notable, that the signal was not detected on the inner membranes of chloroplasts that represent the small grana stacks, where the PSI and PSII are located.

### Concluding Remarks

In summary, we found ROS accumulation in GCC in compartments typically occupied by starch grains, following treatments with ABA and MJ. We suspect that the function of ROS in this location is to block the source of osmoticum formation by inactivating starch degradation. This suggestion is in line with

previous in vitro studies in *Arabidopsis* chloroplasts that showed regulation of starch degradation by redox potential, which controlled the activity of amylases in chloroplasts.<sup>16</sup> Additional effects of ROS production in GCC might influence other activities, such as gene expression<sup>22</sup> and transport of molecules between cytoplasm and chloroplasts.

### Experimental procedures

#### Plant material

WT *Arabidopsis* plants (COL) were grown in soil pots located in growth chamber (light 120  $\mu$ E in short day conditions, 8 h light). Leaf epidermal strips or leaf discs were harvested and treated as described in Leshem et al. (2010). MJ and ABA were applied at 20  $\mu$ M for 2 h.

#### ROS detection and Confocal microscopy

Treated leaf epidermal strips or discs were floated in 20 mM KCl, 1 mM CaCl<sub>2</sub>, 5 mM MES-KOH, pH 6.15, at 20 °C and loaded with 10  $\mu$ M H<sub>2</sub>DCFDA (Molecular Probes-Invitrogen, Eugene OR) and 6  $\mu$ M MitoFluor™ Red 589 (Molecular Probes-Invitrogen) and the fluorescent signal was detected using confocal microscope as described by Leshem et al. (2010). All confocal images were processed by ImagePro Plus software package (Media Cybernetics, Bethesda, MD). Quantification of the H<sub>2</sub>DCFDA-dependent signal in GCC was performed by projecting Z-stack images of a single chloroplast (2–3 sections, 1  $\mu$ m thick) of the separate H<sub>2</sub>DCFDA filter and analyzing the mean signal intensity of the integrated image by ImagePro Plus analysis.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

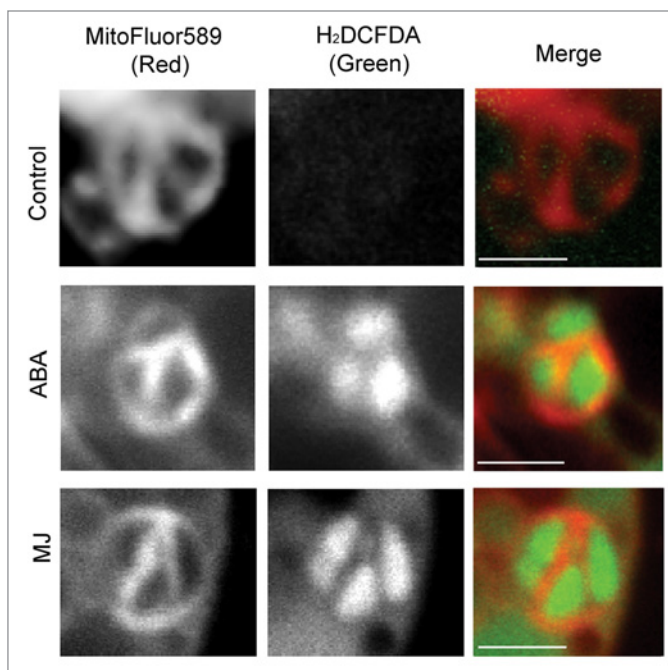
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### Supplementary Material

Supplementary material may be found here:  
<http://www.landesbioscience.com/journals/psb/article/25689/>

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**Figure 2.** Sub-organellar localization of ROS in guard cell chloroplasts during ABA and MJ stimuli. Simultaneous staining of ROS by H<sub>2</sub>DCFDA and the intracellular membrane dye MitoFluor589 combined with red auto fluorescence, in guard cell chloroplasts of wild type epidermal peals. ABA and MJ were applied as in **Figure 1**. Loading of dyes and fluorescence detection were as described by Leshem et al. (2010). Shown are representative single section (1  $\mu$ m thick) confocal images of individual chloroplasts from the different treatments. For the complete Z stack image series see Supplemental Figures 1,2 and 3 for control, ABA and MJ treatments, respectively. Scale bar = 2  $\mu$ m.

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