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### ► To cite this version:

Alexis Porcher, Emmanuel Duffour, Frederic Perisse, Sébastien Menecier, Vincent Guérin, et al.. Rapid changes in stress-related gene expression after short exposure of Arabidopsis leaves to cold plasma. Journal of Plant Physiology, 2025, 304, pp.154397. 10.1016/j.jplph.2024.154397 . hal-04820129

**HAL Id: hal-04820129**

**<https://hal.science/hal-04820129v1>**

Submitted on 11 Dec 2024

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## Rapid changes in stress-related gene expression after short exposure of Arabidopsis leaves to cold plasma

Alexis Porcher<sup>a,1</sup>, Emmanuel Duffour<sup>a</sup>, Frédéric Perisse<sup>a</sup>, Sébastien Menecier<sup>a</sup>, Vincent Guérin<sup>b</sup>, Maxime Moreau<sup>a</sup>, Chloé Davranche<sup>b</sup>, Françoise Paladian<sup>a</sup>, Pierre Bonnet<sup>a</sup>, Alain Vian<sup>b,\*</sup>

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### ARTICLE INFO

#### Keywords:

Cold-atmospheric plasma  
Stress-related genes  
Arabidopsis  
*RSH* genes  
Hydrogen peroxide

### ABSTRACT

Cold Atmospheric Plasma (CAP) technology has emerged as a promising tool in various biological applications due to its ability to generate a composite signal comprising reactive oxygen and nitrogen species, ultraviolet radiation, and electromagnetic fields, all while maintaining a stable temperature. Although CAP treatments have demonstrated significant effects on seed germination and plant growth, the direct molecular responses of plants to CAP exposure remain poorly understood. In this study, young *Arabidopsis thaliana* leaves were exposed to a brief 5- or 30-s localized CAP treatment, resulting in rapid and localized tissue damage without causing lethal effects on the entire plant. Molecular analyses conducted on the entire plant rosette revealed a notable increase in hydrogen peroxide levels, along with the upregulation of stress-related genes, akin to a wound response. Of particular interest, the activation of RelA/SpoT Homolog (*RSH*) genes encoding proteins that regulate the synthesis of the stress marker (p)ppGpp, also known as alarmone, and playing a major role in the energetic regulation of photosynthesis, occurred shortly after CAP exposure. The expression of *RSH* genes was up-regulated after 5s CAP exposure, while the wound stress marker *ZAT12* remained unaffected, highlighting a specific signalling pathway to activate *RSH* genes. This finding suggests the potential involvement of the alarmone signalling pathway in the plant's response to CAP exposure, thereby opening avenues for further exploration of metabolic pathways and signalling cascades induced by CAP treatment.

### 1. Introduction

Cold Atmospheric Plasmas (CAP) are created through a high voltage, high frequency discharge established between two electrodes separated by a dielectric to prevent arc transition discharge. This leads to a plasma in a non-equilibrium state, where the kinetic energy of electrons is very high, while that of heavy particles (ions, atoms, molecules) remains low, because of their very inelastic collisions. Most of the time, the plasma gases used in biology applications are helium, argon, air or a mix of them (Varilla et al., 2020; Min et al., 2022; Nwabor et al., 2022; Xi et al., 2024). The gas temperature, which mainly relies on the energy of heavy particles, is then consequently close to room temperature. Nevertheless, inelastic electron/gas collisions enable the excitation of the internal energy of the gas and the moving of ionized molecules within the gas

flux generates an electromagnetic field so that a CAP actually appears as a composite signal with three different elements: i) a chemical component, mainly made up of RONS (Reactive Oxygen and Nitrogen Species), such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>), hydroxide radical (HO<sup>•</sup>), oxygen singlet <sup>1</sup>O<sub>2</sub> and different forms of nitrogen oxides ii) a UV component, more or less important, depending on the device and parameter settings and iii) a high frequency electromagnetic field (within the range of kHz to MHz) that results from the electric supply and the movement of charged particles. Cold atmospheric plasma can therefore be applied directly onto biological matter with minimal heating (Starić et al., 2020).

Exposure of biological material to CAP is nowadays increasingly considered a high value procedure to clean and sanitize surfaces (Katsigiannis et al., 2022), cure plant diseases (Zhang et al., 2014;

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Adhikari et al., 2020a; Laroussi, 2020) and increase plants' resistance against pests (Ikmal Misnal et al., 2021; Ziuzina et al., 2021). Most of the research work about CAP effects on plants has focused on the exposure of seeds and the subsequent (delayed) consequences on germination, seedling development, and plant growth. It is worth noting that seed exposure to CAP weakens the cuticle (Dauwe et al., 2021) and the seed testa (Adhikari et al., 2020b), favouring seed imbibition which in turn improves seed germination (Ling et al., 2015; Mildaziene et al., 2019; Será et al., 2021; Perea-Brenes et al., 2023). Likewise, germinations from CAP-treated seeds grow faster than those from unexposed control seeds (Kitazaki et al., 2014; Ghasempour et al., 2020), giving rise to more vigorous plants (Mildaziene et al., 2019). Cui et al. (2021) showed a significant increase in GA1, GA3 and *trans*-zeatin riboside concentrations in plasma treated samples that could favour seedling growth. An increased gibberellin content was also noticed after CAP treatment in sunflowers (Mildaziene et al., 2019). Also, the RONS concentration increases in the vicinity of the embryo (Shelar et al., 2022) after exposing seeds to CAP, causing an increase in the scavenging activity (Ling et al., 2015; Cui et al., 2022). A higher level of hydrogen peroxide production and scavenging activity was also noticed in adult plants issued from CAP-treated seeds, thus relying on the plant's ROS production. Li et al. (2021) found a higher level of *RBOH1* gene expression (an initial step in hydrogen peroxide production), RBOH activity being associated with an increased level of hydrogen peroxide in four-leaf stage tomatoes (aged about 3 weeks) issued from CAP-treated seeds. These observations suggest that changes (up- or down-regulation) in the genome expression can persist over time (*i.e.* on the developing plant) in the plant after seed exposure to CAP. This was clearly shown by authors who investigated the transcriptome/proteome after the application of CAP. Cui et al. (2021) and Waskow et al. (2022a, 2022b) noticed that most genes were downregulated while the expression of only a few was stimulated. They showed that the response to CAP treatment was quite similar to plant wounding or plant pathogen responses, with a strong impact on several key-metabolic pathways involved in oxidative metabolism, phenylpropanoids and glucosinolate pathways. In contrast, Lyu et al. (2023) found in broccoli sprouts an almost similar number of up- and down-regulated genes (3803 and 3657, respectively) after exposing seeds to CAP. The differential gene expression analysis on this dataset showed an enrichment of genes involved in glutathione and ascorbic acid metabolisms and plant pathogen response, which are often associated with oxidative stress physiological pathways. Darigh et al. (2023) showed that stress-related transcription factors (*WRKY1* and *ERF1B*) were up-regulated after CAP treatment, along with secondary metabolism genes.

The exposure of embryogenic callus culture cells to CAP caused an immediate increase in superoxide dismutase and catalase activities (also higher after 2 weeks), suggesting the production of ROS after CAP exposure (Puač et al., 2014). Zhou et al. (2023) exposed mature blueberries to CAP and found an increase in the phenolic metabolism, indicating an elevation of the antioxidant activity. In parallel, direct very short exposures (1–6 s) of *Arabidopsis* leaves to CAP caused damages that remained limited to the epidermis cells but caused an important increase of ROS, resulting in cell death (Seol et al., 2017). The impact of this treatment on different actors of plant stress metabolism was however not investigated. Similarly, exposure of *Arabidopsis* seedlings to CAP caused increases in oxygen and nitrogen reactive species, intracellular calcium and, for the strongest exposures, increased lipid peroxidation (Cui et al. 2021, 2022). Likewise, Venus flytrap leaf closing or mimosa leaf movements (Volkov et al., 2017) were noticed after CAP exposure, denoting that the plant responds systematically to the stimulus in a similar way to mechanical stimulation.

However, there is to our knowledge no report that exposure to CAP on seeds or plants activates the expression of the RelA/SpoT Homolog (*RSH*) genes that control the synthesis and degradation of the stress marker (p)ppGpp - also known as the "stringent response" or the "alarmone" - that repress chloroplastic transcription and translation,

and affects nuclear genome expression through chloroplast retrograde signalling to adapt new environmental constraints (Boniecka et al., 2017). *RSH* genes are however activated after a wide range of environmental signals including wounding (Takahashi et al., 2004) and ABA application (Mizusawa et al., 2008), salt and osmotic stress (Wang et al., 2023), methyl jasmonate and hydrogen peroxide (Kim et al., 2009). The ppGpp and *RSH* genes, originally identified as energy stress markers in bacteria (Cashel and Kalbacher, 1970), play a crucial role in regulating chloroplast activity in plants under stress. Since CAP is a composite treatment involving UV light, electromagnetic fields (EMF), and reactive oxygen species (ROS)—all of which can impact the photosynthetic machinery—we hypothesized that analyzing *RSH* levels could offer valuable insights into the plant's response to CAP exposure. The aim of the present work was therefore to explore the ability of the CAP stimulus to trigger rapid molecular responses in *Arabidopsis* plants after a short exposure, especially changes in *RSH* gene expression. Here we bring evidence demonstrating that a short exposure to CAP caused an increase in hydrogen peroxide metabolism and changes in the expression pattern of different stress-related genes, including the alarmone *RSH* proteins pathway that potentially strongly impacts subsequent plant behaviour.

## 2. Material and methods

### 2.1. Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) were sown in a substrate for young plants (TraySubstrat U44-551, Klasmann-Deilmann, Bourgoin Jallieu, France), stratified for 2 day at 4 °C, and then grown for six days in environmentally controlled conditions: temperature 21 ± 1 °C, L:D 16h:8h, PAR 180–200 μmol m<sup>-2</sup> s<sup>-1</sup> provided by LED-tubes (TLED 4265, Agromoon sprl, Manage, Belgium). At the cotyledon stage, the seedlings were transplanted individually into 5 cm pots containing the same substrate as previously and grown for ten more days under identical environmental conditions.

### 2.2. Experimental setup

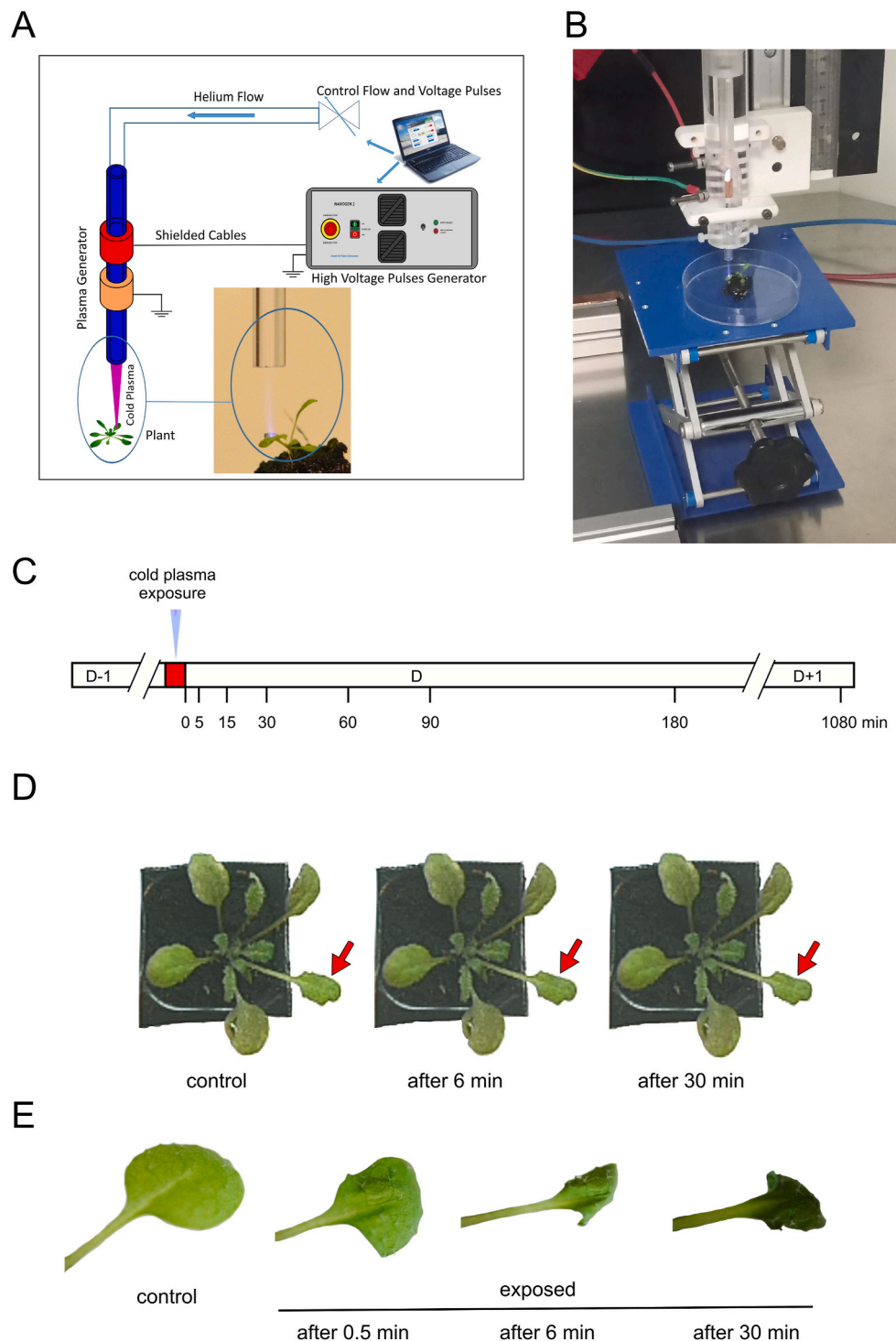
Fig. 1 depicts a schematic view of a plasma jet based on a high voltage dielectric barrier discharge configuration. Two electrodes are placed on a Pyrex® capillary, 6 mm outer diameter, 1 mm thick. These two electrodes have a length of 10 mm and are spaced 10 mm apart. Helium is injected into the tube with a flow rate of 3 L/min (purity 4.5) and electrodes are powered by a mono-polar square pulse high voltage generator (Voltage 7 kV, 20 kHz, Duty cycle 20%). The plasma obtained at the outlet of the tube is a helium plasma interacting with air which is applied to the plant.

### 2.3. Exposure to cold plasma and sample collection

The plants were moved the day before the exposure to a culture chamber in the immediate vicinity of the exposure device to avoid stress responses relative to plant moving. They were individually exposed to the extremity of the CAP dart that was applied for 5 or 30 s to a developed leaf. The plant was then immediately returned to the culture chamber and individually collected in liquid nitrogen after different times of kinetic (Fig. 1). Controls (*i.e.* non exposed plants) were handled similarly and collected throughout the length of the kinetic and pooled together.

### 2.4. RNA isolation and gene expression determination

RNA was isolated from the whole rosette using the Nucleospin RNA plant Mini Kit including DNase treatment (Macherey Nagel, Hoerd, France), according to the manufacturer's instructions. Total RNA was quantified, checked for quality and 500 ng was converted to cDNA using the iScript Reverse transcription supermix for RT-qPCR (Bio-Rad,



**Fig. 1.** Experimental set-up.

**A.** General scheme of the experimental set-up. A generator delivers a high voltage signal through shielded cables to a custom-made electrode unit which continuously circulates a constant flux of helium where it is ionized and emitted as a cold plasma dart. **B.** Illustration of the device. Note the electrode and the plant being treated under the plasma dart. The whole system is placed under a fume hood, as ozone is released during CAP production. **C.** Time course of the experiment. The plants were moved to a culture chamber in the immediate vicinity of the experimental set-up the day before the exposure (D-1) to avoid transport stress. The exposure to CAP (5 or 30 s) was carried out 2 h after lights were on, and the plants were returned to the growth chamber up to the harvesting point (5, 15, 30, 60, 90, 180 and 1080 min later). **D.** Typical Arabidopsis plant aspect before (control) and 6 and 30 min after a 5s CAP exposure. Note that the red arrows point to the targeted leaf which shows a slight wilting after treatment. **E.** Leaf appearance before (control) and 0.5, 6 and 30 min after a 30s CAP exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



Marnes-la-Coquette, France) following the manufacturer procedure. Relative mRNA accumulations were assayed using real time quantitative PCR (RT-qPCR) in a final volume of 15  $\mu$ l containing 3  $\mu$ l cDNA (50-fold diluted), 1  $\mu$ l of primers pairs (10  $\mu$ M), 4  $\mu$ l iQ SYBR Green supermix (Bio-Rad, Marnes-la-Coquette, France) and 7  $\mu$ l of ultrapure water. The PCR amplifications were performed in a CFX96 Real Time System (Bio-Rad) using the cycling program previously described (Porcher et al. 2021, 2023). Relative transcript abundances were expressed relative to the control condition (unexposed samples harvested along the kinetic) after  $2^{-\Delta\Delta C_t}$  calculation (Livak and Schmittgen, 2001) along with two house-keeping genes (Aldehyde dehydrogenase, *ALDH* and Glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*), taken as internal references (Jin et al., 2019). The primer sequences used in this study are available in the Supplementary Table 1.

## 2.5. Hydrogen peroxide quantification

Hydrogen peroxide was determined using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen). Briefly, plants (about 20 mg) were ground in liquid nitrogen to a fine powder and resuspended in 100  $\mu$ l of 1X reaction buffer, centrifuged (5min, 14,000 $\times$ g) and the supernatant diluted 10 times with reaction buffer, and mixed with an equal volume of reaction mix in a black MicroWell plate, half being completed with catalase. After 30 min of incubation at room temperature, the H<sub>2</sub>O<sub>2</sub> quantity was determined by fluorescence measurement (excitation 570 nm, emission 590 nm) using FLUOstar Omega microplate reader (BMG Labtech, Champigny s/Marne, France). The quantity of H<sub>2</sub>O<sub>2</sub> was calculated from a standard curve obtained with known concentrations of commercial H<sub>2</sub>O<sub>2</sub> and corrected with the value of the sample containing only catalase and expressed as nmol of H<sub>2</sub>O<sub>2</sub> g<sup>-1</sup> of fresh tissue (Noctor et al., 2016).

## 2.6. Statistical analysis

Statistical analysis was conducted using the R statistical software (R Core Team, 2020) using the statistical tests described in the figure captions. Graphics were produced using Veusz 3.6.2 (<https://veusz.github.io/>) software.

## 3. Results

### 3.1. Morphological effects of cold plasma leaf exposure

Short CAP treatments caused rapid and permanent damage to the exposed leaf, with the severity proportional to the duration of exposure. A 5 s (5 s) treatment caused minor effects, such as mild wilting (Fig. 1D, red arrows), whereas a 30 s (30 s) treatment resulted in rapid destruction of the targeted leaves (Fig. 1E). After the longest treatment, the leaves began wilting by the end of exposure and were fully wilted within 30 min (Fig. 1E). Neither the leaves exposed to 5 s nor those exposed to 30 s of CAP ever recovered their original appearance, the damages were permanent and severely injurious in the case of the 30 s treatment. In contrast, the rest of the plants remained unaffected (Fig. 1D). Molecular and biochemical analyses were conducted on the remaining, unexposed parts of the rosette, suggesting a systemic response from the plant after localised exposure to a single leaf.

### 3.2. Stress-related gene transcript accumulation

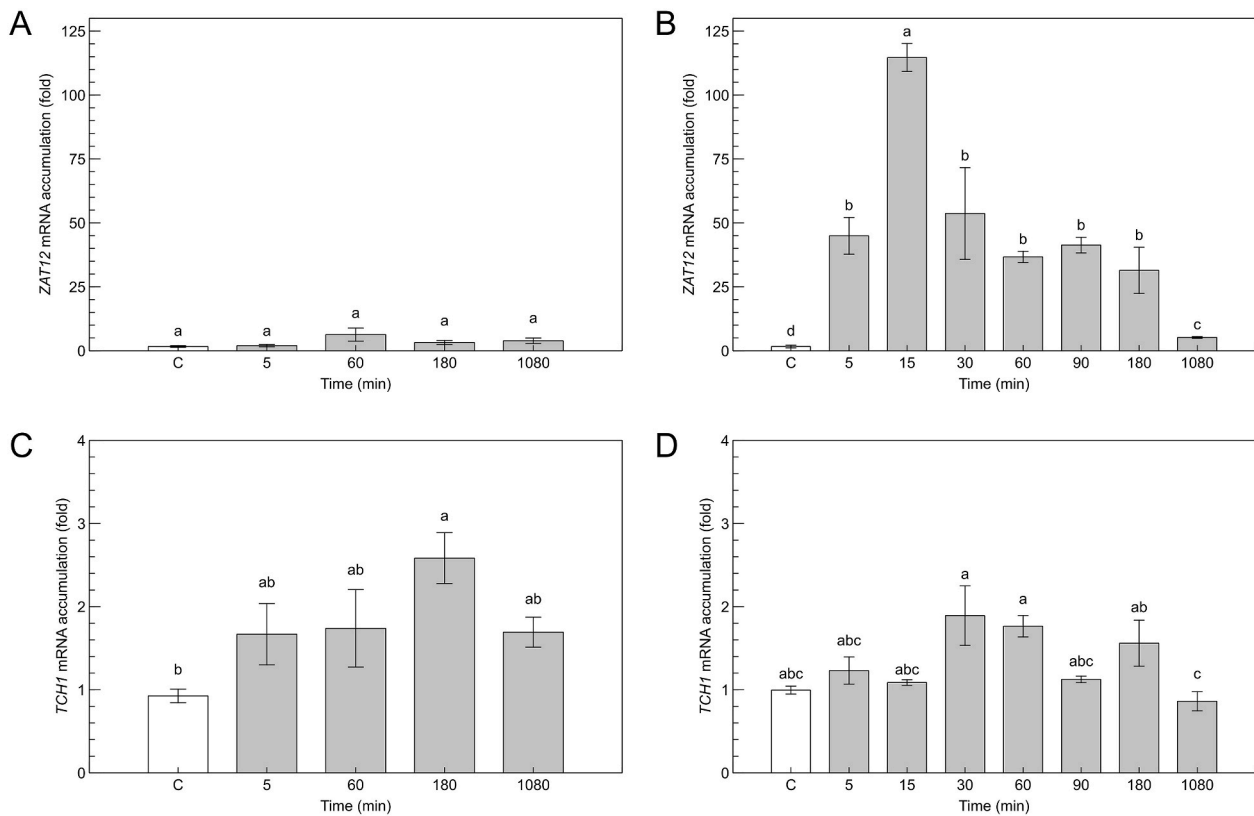
We first evaluated the plant stress response to the 5 and 30 s CAP treatments by following the expression of two characterized gene markers, namely *ZAT12* and *TCH1*. The expression of the *Zinc-Finger Protein ZAT12* transcription factor is upregulated by diverse environmental stresses and serves as a valuable gene marker for stress induction across many environmental cues, including electromagnetic fields (Davletova et al., 2005; Rai et al., 2014; Porcher et al., 2023). Here we

found that the 5 s and 30 s CAP treatment triggered a different *ZAT12* expression pattern (Fig. 2A and B). Indeed, while the accumulation of *ZAT12* transcripts increased dramatically (more than 100-fold) within 15 min (Fig. 2B) and remained higher than that measured in unexposed plants up to 180 min after a 30 s exposure to CAP, it remained essentially unchanged after a 5 s treatment (Fig. 2A). In contrast, the expression level of *TOUCH 1 (TCH1)*, a calmodulin-encoding gene known to be upregulated in response to various environmental stimuli, such as mechanical stress, heat shock, and light stress (Braam and Davis, 1990; Braam et al., 1997), increased after both 5- and 30 s CAP treatments, but with different timing (Fig. 2C and D). The 5 s treatment evoked an increase in *TCH1* transcript levels 180 min post-exposure (2.2-fold, Fig. 2C) whereas the 30 s treatment induced an earlier response, becoming detectable 30 min post-exposure (1.8-fold, Fig. 2D).

The *RelA/Spot Homolog (RSH)* genes, which regulate (p)ppGpp metabolism in plants—a stress messenger also referred to as the "alarmone" (Boniecka et al., 2017)—were impacted by exposure to CAP treatments (Figs. 3 and 4). Although the 5 s CAP treatment did not induce an increase in *ZAT* transcript levels, it was sufficient to significantly upregulate all *RSHs* gene expressions (Fig. 3). Specifically, the expression levels of *RSH2* and *RSH3*, major (p)ppGpp synthases (though also possessing hydrolase activity, Avilan et al., 2019), were both upregulated: *RSH2* mRNA level began rising within 5 min and peaked (approximately 10-fold) between 60- and 180 min post-exposure (Fig. 3B), whereas *RSH3* expression increased and reached its maximum (~4-fold) at 180 min (Fig. 3C). The expression of *RSH1*, which functions exclusively as a hydrolase (Sugliani et al., 2016; Avilan et al., 2019), showed a modest but significant increase (1.6-fold) starting at 180 min post-treatment (Fig. 3A). In contrast, the *cRSH* isoform, that exhibits a C-terminal EF-hand Ca<sup>2+</sup>-binding domain and that operates as a Ca<sup>2+</sup>-dependent (p)ppGpp synthase (Masuda et al., 2008; Sugliani et al., 2016), showed an mRNA accumulation that initially decreased 5 min after exposure but gradually increased, reaching a maximum at 1080 min (Fig. 3D). A 30 s CAP treatment also induced *RSH*-related mRNA expression, though with distinct dynamics: *RSH2* mRNA levels rose sharply, increasing 4-fold within 15–30 min post-exposure, followed by a decline to about 1.5-fold, before showing a second, weaker accumulation between 90 and 180 min (Fig. 4B). In contrast, *RSH3* exhibited only a late response, with its expression firstly remaining close to that of unexposed plants, until increasing 1.8-fold between 90 and 180 min after treatment (Fig. 4C). Meanwhile, the expression levels of *RSH1* and *cRSH* remained essentially unchanged throughout the observed time period (Fig. 4A and D).

### 3.3. Hydrogen peroxide metabolism

Since *ZAT12* is highly implicated in the regulation of reactive oxygen species transduction pathway after the application of abiotic stress (Davletova et al., 2005; Khedia et al., 2019), we decided to explore the ROS metabolism along the plant treated with 30s of CAP, the only condition that showed a huge increase of *ZAT12* expression level (Fig. 2B). Accordingly, we first measured the accumulation of hydrogen peroxide after exposure to CAP (Fig. 5). The amount of H<sub>2</sub>O<sub>2</sub> present in the control plants (about 190 nmol g<sup>-1</sup> FW) increased rapidly to 371 nmol g<sup>-1</sup> FW (about 94%) 15 min after the exposure (Fig. 5). The level of H<sub>2</sub>O<sub>2</sub> remained at a high level (240–260 nmol g<sup>-1</sup> FW) in the exposed plants, even 18h after the exposure (Fig. 5). We therefore monitored the expression level of the NADPH oxidase/respiratory burst oxidase homolog (*RBOH*), especially the isoform D that is implicated in plant responses to stress (Miller et al., 2009). The expression level of *RBOHD* increased highly and very rapidly (6.5-fold in 15 min) after the 30 s exposure to the CAP (Fig. 6A). This increase was rather transient, since it rapidly returned to expression levels close to that measured in the control plants: 60–180 min after the exposure, the accumulation was about 2-fold higher than that measured in the controls and was similar 1080 min later. In contrast, the expression level of *RBOHF*, mostly



**Fig. 2.** Effect of cold plasma exposure on *ZAT12* and *TCH1* gene expression.

Relative transcript accumulation of *ZAT12* transcription factor gene in unexposed (control, white bars) and exposed (grey bars) plants at different harvesting points after the end of 5s (A) or 30s (B) exposure to CAP. Relative transcript accumulation of *TOUCH1* (*TCH1*) gene in unexposed (control, white bars) and exposed (grey bars) at different harvesting points after the end of 5s (C) or 30s (D) exposure to CAP. Data are means of  $n = 3$  biological independent replicates  $\pm$  se. Different letters indicate significant differences ( $p$ -value  $< 0.05$ ) after Kruskal–Wallis nonparametric rank tests followed by a two-by-two comparison matrix by Mann–Whitney nonparametric tests.

involved in plant development and stomatal movement (Arnaud et al., 2023), slightly increased after 15–30 min and 90–180 min (Fig. 6B). It is worth noting that the maximum accumulation of *RBOHD* transcripts (15 min, Fig. 6A) also corresponded to higher amounts of  $H_2O_2$  in plants (Fig. 5). We then monitored the transcript levels of *CATALASE 2* and *ASCORBATE PEROXIDASE 1* genes (*CAT2* and *APX1*, respectively), whose corresponding proteins are involved in  $H_2O_2$  scavenging. We observed that the expression pattern of *CAT2* was not statistically affected by the plant exposure to CAP (Fig. 7A). In contrast, *APX1* gene expression showed a significant increase (about 1.6–1.8-fold, Fig. 7B) 60–90 min after the exposure, at a compatible timescale for detoxifying the  $H_2O_2$  produced as early as 15 min after exposure (Fig. 5). The return to standard expression level was effective for *APX1* 180 min after the exposure.

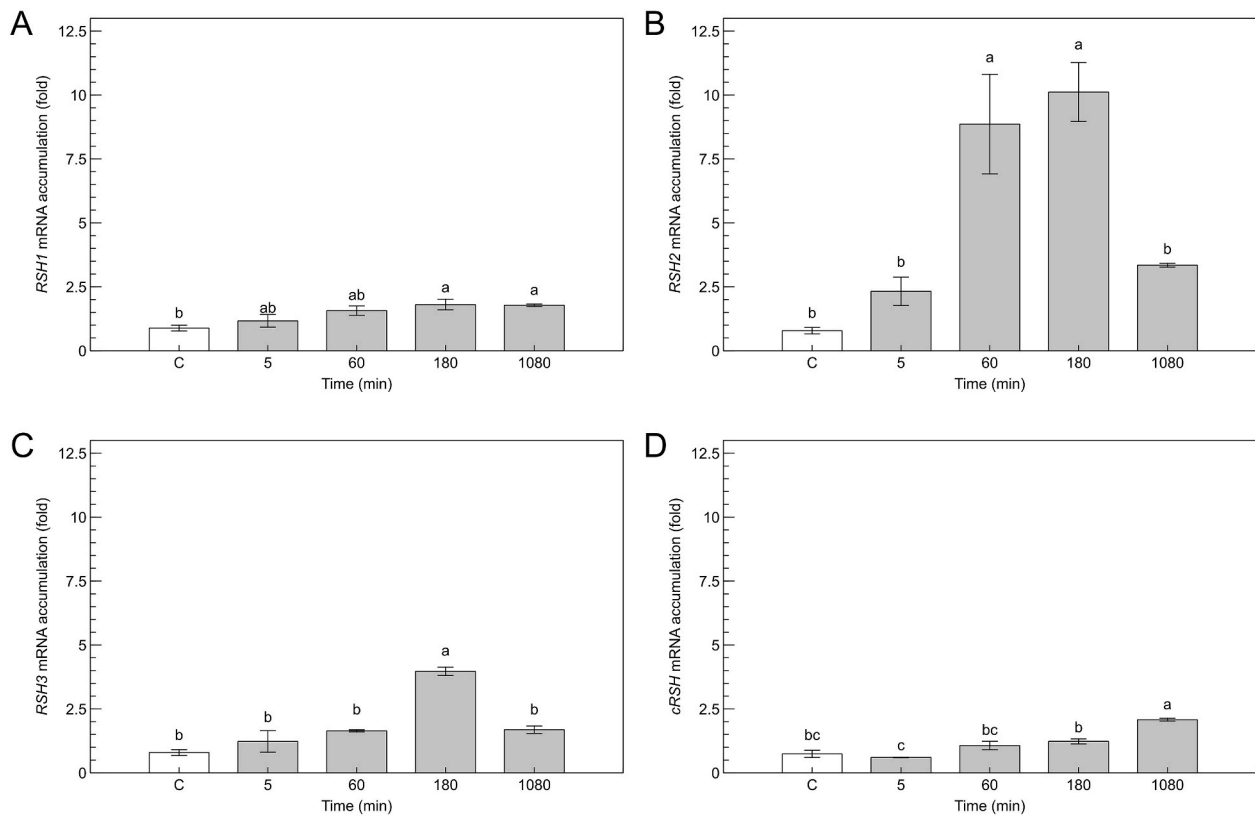
#### 4. Discussion

Most experiments on the biological effects of exposure to CAP have been carried out on seeds, and thus applied to the integumentary envelope that was weakened by the treatment. The changes observed in seed germination and the subsequent development of the embryo into a seedling are therefore indirect actions of CAP exposure (Waskow et al., 2022a). Relatively few studies have investigated the direct effects of CAP application on green plant tissue which, however, proved to be very responsive to this treatment (Puač et al., 2014; Seol et al., 2017).

In the present work, we aimed to submit young *Arabidopsis* plants to a 5 or 30 s exposure to CAP to evaluate the immediate consequences in terms of biological effects and metabolic changes. While adverse effects of plasma irradiation on plant leaves have previously been reported after

a rather drastic treatment (Seol et al., 2017), the exposure condition used here caused severe damage only to the 30 s exposed leaf, which gradually irreversibly withers within minutes following the exposure. In most cases, however, this wilting remained confined to the treated leaf, suggesting that this treatment is not lethal to the plant while causing irreversible tissue damage to the exposed leaf. Our observations were in accordance with previous work underlining the fact that CAP treatment of plant tissue causes alterations but not the death of the plant (Seol et al., 2017; Cui et al., 2022, Fig. 1D and E). It is worth noting that our analysis performed on the whole rosette, including the exposed leaf, did not allow us to formally discriminate between local responses and those occurring at a distance.

Here we showed that short exposure to CAP (5 s), which caused minor damages to the targeted leaf (Fig. 1D) can trigger *TCH1*, *RSH2*, *RSH3* and *cRSH* transcript accumulation (Figs. 2C and 3B–D) but fails to activate *ZAT12* gene expression that remained constant (Fig. 2A). In contrast, a longer exposure (30 s) that caused severe damage to the targeted leaf (Fig. 1E) led to a faster accumulation of *TCH1* transcripts (Fig. 2D), a massive accumulation of *ZAT12* transcripts (over 100-fold, Fig. 2B), alongside the accumulation of *RSH2* and *RSH3* transcripts (Fig. 4B and C). These observations suggest that *RSH* gene accumulation could occur systematically after short (5 s) CAP exposure in the absence of severe tissue damage. Longer CAP exposure (30 s) evokes a strong increase (about 94%) in hydrogen peroxide 15 min after the exposure (Fig. 5). This result is consistent with former observation after exposing green plants to CAP reporting an increase in hydrogen peroxide (Puač et al., 2014; Cui et al., 2022). It was reported an increase in *RBOH1* (the equivalent of *RBOHD* in tomato) gene expression and RBOH activity, a NADPH oxidase implicated in the very first but indirect step of hydrogen



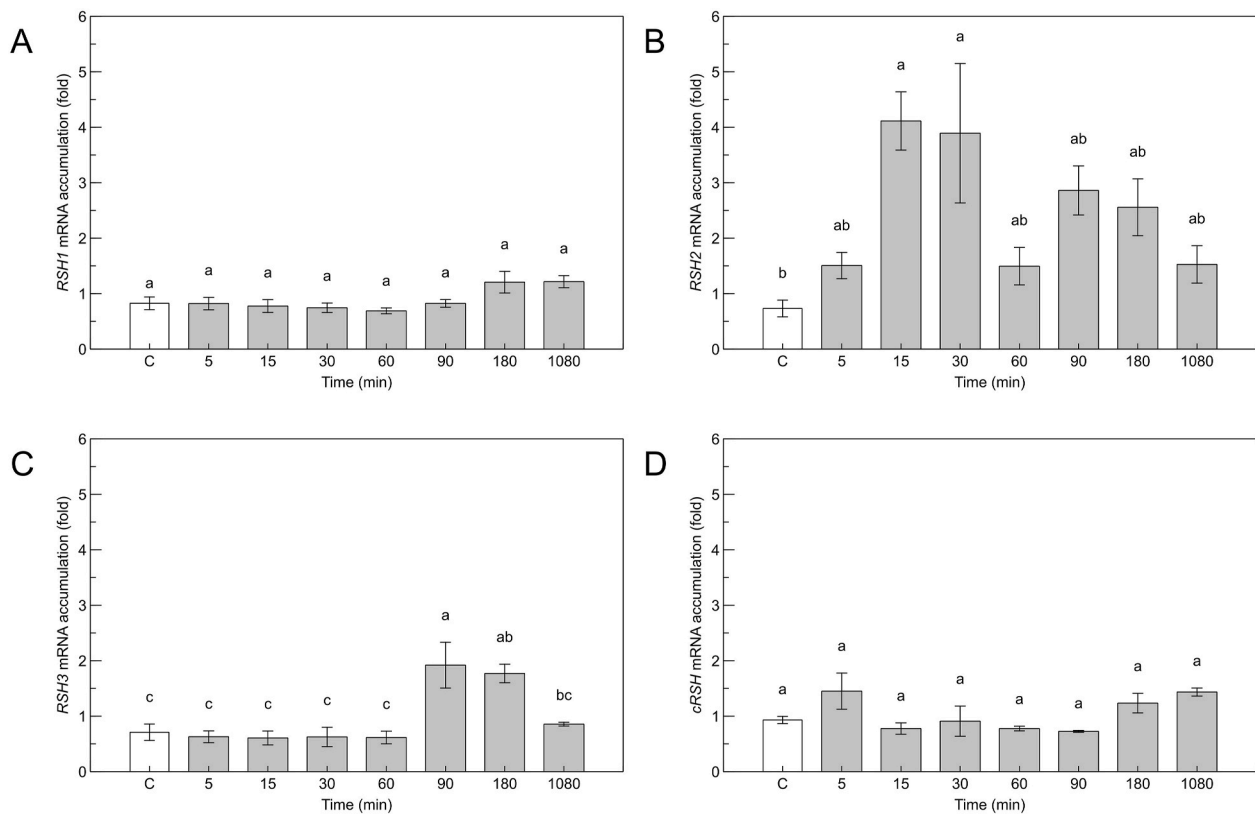
**Fig. 3.** Effect of 5s-cold plasma exposure on *RSH* gene expressions.

Relative transcript accumulation of RelA/Spot Homolog proteins genes *RSH1* (A), *RSH2* (B), *RSH3* (C) and *cRSH* (D) genes in unexposed (control, white bars) and exposed plants (5, 60, 180, 1080 min after a 5 s exposure to CAP, grey bars). Data are means of  $n = 3$  biological independent replicates  $\pm$  se. Different letters indicate significant differences ( $p$ -value  $< 0.05$ ) after Kruskal–Wallis nonparametric rank tests followed by a two-by-two comparison matrix by Mann–Whitney non-parametric tests.

peroxide production in tomato seedlings grown from seeds treated with CAP (Li et al., 2021). We noticed the same increase in *RBOH* gene expression (Fig. 6): *RBOHD* was sharply increased (5-fold) 15 min after the exposure while *RBOHF* remained nearly constant. Previous works have highlighted the synergistic role of *RBOHD* and *RBOHF* in plant responses to pathogen attacks and environmental cues (Torres et al., 2002). Interestingly, *RBOH1* gene expression was increased after short exposure (1min) to CAP in tomato (Adhikari et al., 2020b). Here, moderate *RBOHF* gene expression changes (Fig. 6B) after CAP exposure indicates that the increase in hydrogen peroxide relies mainly on the *RBOHD* isoform, while plant exposure to high frequency (2.45 GHz) electromagnetic field, which also causes hydrogen peroxide increase, only affects *RBOHF* gene expression (Porcher et al., 2023). The ROS scavenging activity noticed after CAP exposure involved ascorbate peroxidase whose corresponding transcript accumulation (about 2-fold) occurred 90 min after CAP exposure (Fig. 7B), while that of catalase remained essentially unchanged (Fig. 7A). This result suggests a possible involvement of the ascorbic acid/glutathione detoxification pathway, implicated in the fine tuning of hydrogen peroxide level, while catalase gene expression (*CAT2*), responsible for bulk, low affinity hydrogen peroxide detoxification (Gill and Tuteja, 2010) was not affected after CAP exposure. It is worth noting that our observations are in agreement with those of Adhikari et al. (2020b) who did not report a change in *CAT* gene expression after 1 min exposure to CAP, while Cui et al. (2022) however found a significant increase in *CAT* activity after 1 min CAP treatment, indicating that the enzymatic activity of pre-existing catalase may be stimulated by short CAP exposure and contribute to hydrogen peroxide degradation. However, a longer exposure (10 min) evoked a strong increase in the expression of several ROS-related genes such as peroxidase and catalase (Adhikari et al., 2020b). One also could note

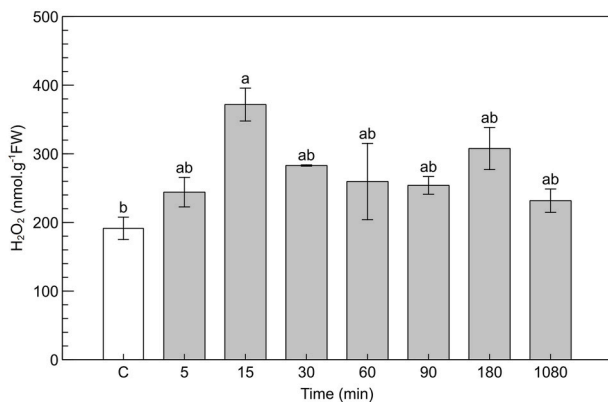
that APX1 activity is dependent upon ZAT12 (Rizhsky et al., 2004), whose expression is dramatically stimulated after CAP exposure. The activation of ROS metabolism, and particularly of hydrogen peroxide, is a major cellular event capable of triggering signalling pathways leading to the activation of specific metabolic pathways and changes in genome expression (Waszczak et al., 2018) in relation to calcium metabolism (Marcec et al., 2019) and TOR (Target Of Rapamycin) signalling pathway (Qureshi et al., 2022; Ma et al., 2023). Indeed, CAP seed exposure (40–80 s) induced the expression of *WRKY1* transcription factor (x9.9–13.3-fold) that is implicated in various cellular processes, including plant hormone metabolism, and is required in ROS signalling (Jiang et al., 2017). Here we showed that *ZAT12*, a transcription factor implicated in many stress-related response pathways in plants (Liu et al., 2022), was drastically up-regulated shortly after the 30 s CAP treatment (114-fold after 15 min). This result fits well with the increase in ROS production since *ZAT12* transduction pathway is closely related to ROS (Davletova et al., 2005), and may be part of the mechanism (Gilroy et al., 2016; Fichman and Mittler, 2020) ensuring the systemic nature of the plant response to local exposure to CAP.

Aside from the ROS triggered transduction signal, our results aimed to point out the contribution of the stress marker alarmone, also called the stringent response, that has been initially characterised in bacteria and subsequently found in eukaryotes, including plants. Its effector, the hyperphosphorylated nucleotide guanosine-3',5'-tetraphosphate and pentaphosphate (p)ppGpp, accumulates after various kinds of stress and regulates the transcription of many genes and cellular activities inhibiting growth-related processes and inducing stress responses (Takahashi et al., 2004; Maekawa et al., 2015). The metabolism of (p)ppGpp is controlled by the genes *RSH* that are capable of synthesis and degradation of (p)ppGpp. Here we found an induction of *RSH* genes



**Fig. 4.** Effect of 30s-cold plasma exposure on *RSH* gene expressions.

Relative transcript accumulation of RelA/SpoT Homolog proteins genes *RSH1* (A), *RSH2* (B), *RSH3* (C) and *cRSH* (D) genes in unexposed (control, white bars) and exposed plants (5, 15, 30, 60, 90, 180, 1080 min after a 30 s exposure to CAP, grey bars). Data are means of  $n = 3$  biological independent replicates  $\pm$  se. Different letters indicate significant differences ( $p$ -value  $< 0.05$ ) after Kruskal–Wallis nonparametric rank tests followed by a two-by-two comparison matrix by Mann–Whitney non-parametric tests.



**Fig. 5.** Effect of 30s-cold plasma exposure on H<sub>2</sub>O<sub>2</sub> production.

Amounts of hydrogen peroxide were measured in unexposed (control, white bar) and exposed plants (5, 15, 30, 60, 90, 180 and 1080 min after 30 s exposure to CAP, grey bars). Data are means of  $n = 3$  biological independent replicates  $\pm$  se. Different letters indicate significant differences ( $p$ -value  $< 0.05$ ) after Kruskal–Wallis nonparametric rank tests followed by a two-by-two comparison matrix by Mann–Whitney non-parametric tests.

responsible for (p)ppGpp production after two different durations of CAP treatments (Figs. 3 and 4): in both, *RSH2* and *RSH3* accumulated while *RSH1*, that acts as a hydrolase toward (p)ppGpp, slightly increased (5 s, Fig. 3) or remained essentially constant (30s, Fig. 4). It is important to note that in the 5 s CAP treatment, the *RSHs* expression levels were modified, while the wounding marker gene *ZAT12* remains constant (Fig. 2A). Taken together, these results strongly suggest the implication

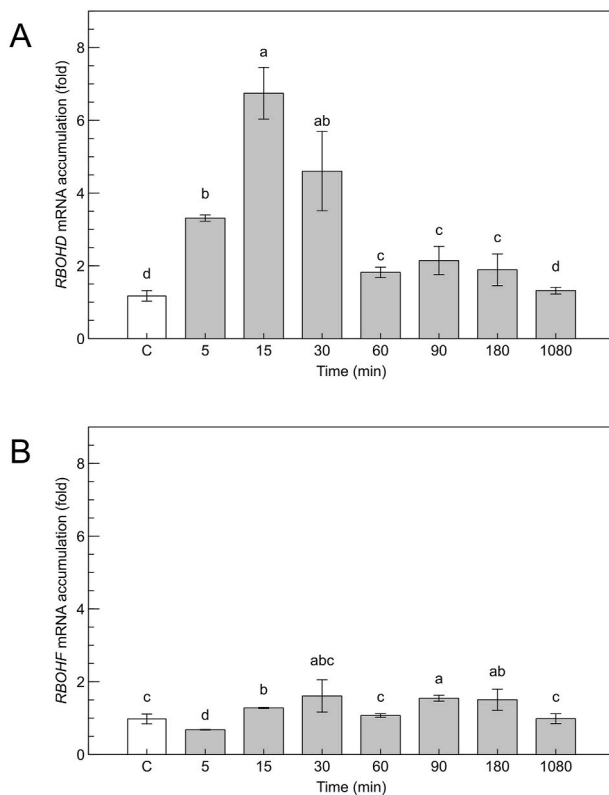
of the alarmone signalling pathway in the plant response to CAP exposure, regardless of the severity of the damage resulting from the application of CAPs. The target of this signalling pathway is mainly chloroplastic (Romand et al., 2022; Boniecka et al., 2017), but also affects the nuclear expression of chloroplastic genes (Romand et al., 2022). The expression level of *cRSH*, a calcium-regulated (p)ppGpp synthase (Turkan et al., 2023), only showed an increase in the 5s CAP treatment while remaining affected by the longest one. This could be explained by the fact that a short CAP treatment could be perceived by the plants as a soft stimulus, closer to a gentle mechanical stimulus (also involving calcium signalling) than a real wounding-like response induced by injurious treatment that physically damages the plant tissue. The potential activation of energy metabolism genes after CAP treatment (Zhang et al., 2017) makes also a strong case for the involvement of *RSH* genes in the plant's response to CAP exposure since TOR kinase (an essential component of cell energy state regulation) is able to stimulate *RSH* gene expression (Imamura et al., 2018; D'Alessandro, 2022).

Surprisingly, none of the whole genome expression (Cui et al., 2021; Waskow et al. 2022a, 2022b; Darigh et al., 2023; Lyu et al., 2023) and metabolomic (Lyu et al., 2023) analysis after CAP exposure of seedlings reported changes in *RSH* expression. This could be the consequence of a too late (several days) analysis point, as the expression level of *RSH* genes varies very early (15–30min) after exposure to CAP or be related to the technology used to generate the CAP (Dielectric Barrier Discharge vs atmospheric pressure cold plasma jet).

## 5. Conclusions

The potential activation of the alarmone signalling pathway after





**Fig. 6. Effect of 30s-cold plasma exposure on *RBOH* genes expressions.** Relative transcript accumulation of the NADPH oxidase/respiratory burst oxidase homolog (*RBOH*) gene isoforms *-D* (A) and *-F* (B) in unexposed (control, white bars) and exposed plants (5, 15, 30, 60, 90, 180, 1080 min after a 30 s exposure to CAP, grey bars). Data are means of  $n = 3$  biological independent replicates  $\pm$  se. Different letters indicate significant differences ( $p$ -value  $< 0.05$ ) after Kruskal–Wallis nonparametric rank tests followed by a two-by-two comparison matrix by Mann–Whitney non-parametric tests.

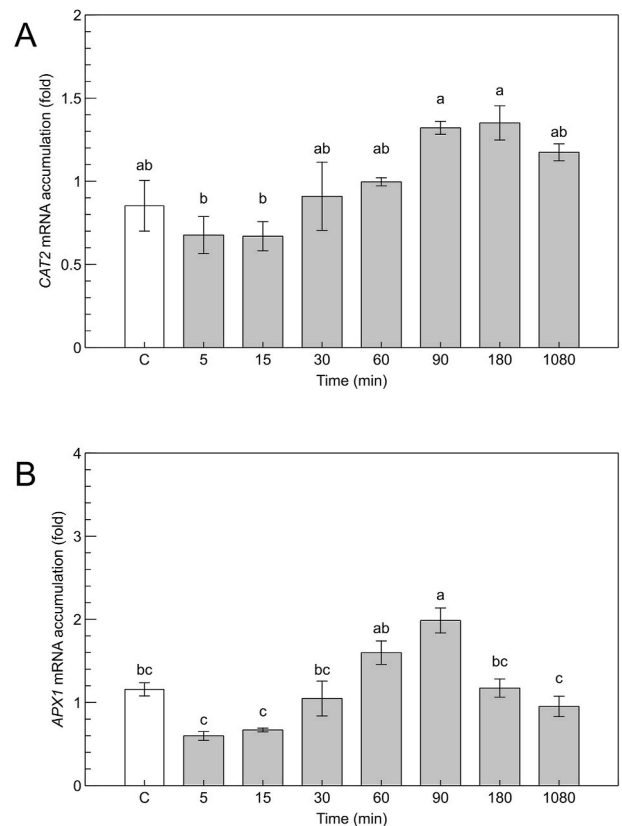
CAP exposure opens exciting new insights to understand the diversity of metabolic pathways changes and cross talks evoked by CAP exposure. Thus, we proposed the alarmone signalling pathway to constitute an important component of the plant response to CAP exposure and suggest that future large-scale genome expression or metabolomic analysis after CAP exposure should focus on alarmone signal transduction.

#### CRedit authorship contribution statement

**Alexis Porcher:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Emmanuel Duffour:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Frédéric Perisse:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Sébastien Menecier:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Vincent Guérin:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Maxime Moreau:** Investigation. **Chloé Davranche:** Writing – review & editing, Investigation, Formal analysis. **Françoise Paladian:** Writing – review & editing, Conceptualization. **Pierre Bonnet:** Writing – review & editing, Conceptualization. **Alain Vian:** Writing – original draft, Validation, Supervision, Investigation, Formal analysis, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



**Fig. 7. Effect of 30s-cold plasma exposure on *CAT2* and *APX1* genes expressions.** Relative transcript accumulation of the catalase *CAT2* (A) and *APX1* (B) genes in unexposed (control, white bars) and exposed plants (5, 15, 30, 60, 90, 180, 1080 min after a 30 s exposure to CAP, grey bars). Data are means of  $n = 3$  biological independent replicates  $\pm$  se. Different letters indicate significant differences ( $p$ -value  $< 0.05$ ) after Kruskal–Wallis nonparametric rank tests followed by a two-by-two comparison matrix by Mann–Whitney non-parametric tests.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jplph.2024.154397>.

#### Data availability

Data will be made available on request.

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