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Anion channel activity is necessary to induce ethylene synthesis and Programmed Cell Death in response to oxalic acid.

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Abstract

Oxalic acid is thought to be a key factor of the early pathogenicity stage in a wide range of necrotrophic fungi. Studies were conducted to determine whether oxalate could induce programmed cell death in *Arabidopsis thaliana* suspension cells and to detail the transduction of the signalling pathway induced by oxalate. *A. thaliana* cells were treated with millimolar concentrations of oxalate. Cell death was quantified and ion flux variations were analysed from electrophysiological measurements. Involvement of anion channel and ethylene in the signal transduction leading to programmed cell death were determined by using specific inhibitor. Oxalic acid induced a programmed cell death displaying cell shrinkage and fragmentation of DNA into internucleosomal fragments with requirement for active gene expression and *de novo* protein synthesis, characteristic hallmarks of programmed cell death. Other responses generally associated with plant cell death, such as anion effluxes leading to plasma membrane depolarization, mitochondrial depolarization and ethylene synthesis, were also observed following addition of oxalate. Regarding our results, we propose a model in which oxalic acid activates an early anionic efflux which is a necessary prerequisite for the synthesis of ethylene and for the programmed cell death observed in *A. thaliana* cells.

Key words: anion channel, *Arabidopsis thaliana*, ethylene, oxalic acid, programmed cell death.

Abbreviations: 9-AC, 9-anthracen carboxylic acid; AIB, alpha-aminoisobutyric acid; AD, Actinomycin D; AOA, aminooxiacetic acid; Chx, cycloheximide; CsA, cyclosporin A; ET, ethylene; FDA, fluorecein diacetate; $\Delta\Psi_m$, mitochondrial membrane potential; gli, glibenclamide; NA, niflumic acid; PM, plasma membrane; PCD, programmed cell death;

PTP, permeability transition pore; ROS, reactive oxygen species; V_m, Plasma membrane potential.

Introduction

The phytotoxin oxalic acid is found to be produced by many necrotrophic plant pathogens. The early stage of infection by these fungi involves the production and the accumulation of large amount of oxalic acid which appear to be an essential determinant of pathogenicity (Noyes & Hancock, 1981; Dutton & Evans, 1996; Guimaraes & Stotz, 2004). Accumulation of oxalate often reaches millimolar concentrations in infected tissues (Bateman & Beer, 1965; Marciano *et al.*, 1983). Once produced and accumulated, oxalate plays a key role provoking some disease-like symptoms independent of pathogen presence (Bateman & Beer, 1965; Noyes & Hancock, 1981). Moreover, *Sclerotinia sclerotium* mutants deficient in oxalate synthesis are no longer pathogenic (Godoy *et al.*, 1990) and transgenic plant expressing oxalate-decarboxylase show enhanced resistance to phytopathogenic fungus that utilize oxalic acid during infection (Kesarwani et al. 2000, Livingstone et al. 2005). Acidification of plant tissues enhanced by oxalate accumulation drives the activation of various fungal enzymes including specific isoforms of endo-polygalacturonase (Manteau *et al.*, 2003; Favaron *et al.*, 2004; Kars *et al.*, 2005) and proteinases (Manteau et al., 2003; ten Have et al., 2004). Oxalate was shown to block a signaling event in the oxidative burst pathway which could compromise the defense responses of the host plant independently of both its acidity and its affinity for calcium (Cessna et al., 2000).

A wide variety of phytotoxins has also been shown to induce PCD in plant cells, among them AAL-toxin (Gechev *et al*., 2004), FB1 (Asai *et al*., 2000), fusicoccin (Malerba *et al*.,

2003) and victorin (Curtis and Wolpert 2004). These toxins are able to induce defense signaling pathways which are dependent on reactive oxygen species (ROS), jasmonic acid (JA) and ethylene (ET), and which lead to PCD. Recently, detailed analyses carried out with the phytotoxin fusaric acid demonstrated the induction of early defense-related responses, such as an increase in $[Ca^{2+}]_{cyt}$, plasma membrane (PM) depolarization, an increase in anion current, an extracellular alkalization and a production of ROS, followed by accumulation of phytoalexin (Bouizgarne *et al*., 2006) and PCD (Samadi & Shahsavan Behboodi, 2006). Anion channel-mediated anion effluxes were also shown to be an essential component of cryptogein-induced cell shrinkage during PCD (Wendehenne *et al.*, 2002; Gauthier, 2007). However, transduction of the signals that are involved in PCD activation seems dependent on the stimuli. We are still far from fully understanding the phytotoxin-specific cell death mechanisms even if DNA fragmentation and cell shrinkage, mediated by a net efflux of water caused by the release of ions, seem to be major hallmarks of the PCD process in plant and in animal cells (Maeno *et al.*, 2000; Lam, 2004; Okada *et al.*, 2006).

There is a lack of information concerning the induction of PCD by oxalate in plant cells. The purpose of this work was 1) to investigate whether oxalate could induce PCD and 2) to detail the transduction of the PCD signal induced by oxalic acid. Our work unambiguously shows that oxalate can induce PCD in *A. thaliana* plant cells and that this PCD is regulated by the activation of an anion channel and by the synthesis of ET. A model summarising the different pathways leading to PCD in *A. thaliana* cells subjected to oxalic acid is also proposed.

Materials and methods

Chemicals

The pH of oxalic acid (Ethanedioic acid) solution was systematically adjusted to 5.8 with KOH before addition to the culture medium.

Cell culture conditions

For this study, *Arabidopsis thaliana* L. (ecotype Columbia) suspension-cultures were used. Suspension cells have been shown to be a convenient system for identifying early physiological events induced by pathogens or their derived elicitors (Cessna *et al.*, 2000; Wendehenne *et al.*, 2002; Bouizgarne *et al.*, 2006; Samadi & Shahsavan Behboodi, 2006; Gauthier, 2007; Reboutier *et al.*, 2007). They show physiological responses to various stimuli, similarly to the autonomous cellular responses in intact tissues, especially the morphological features of dying cells during PCD (van Doorn & Woltering, 2005), and thus allow the observation of events in each single cell or the real time behavioral monitoring of large populations of cells. *A. thaliana* suspension cells were grown in Gamborg medium (pH 5.8). They were maintained at 22 ± 2°C, under continuous white light (40 µE m^{-2} s^{-1}) and continuous shaking (gyratory shaker) at 120 rpm. Cell suspensions were sub-cultured weekly using a 1:10 dilution. All experiments were performed at 22 ± 2°C using log-phase cells (4 days after sub-culture).

Cell viability assays

Cell viability was assayed using the vital dye neutral red. Cells (50 µl) were incubated for 5 minutes in 1 ml phosphate buffer pH 7 with neutral red to a final concentration of 0.01%. Cells that did not accumulate neutral red were considered dead. Cell viability was also assayed using vital dye, Evans blue in the presence of oxalate alone or with the appropriate pharmacological effectors. Cells (50 µl) were incubated for 5 minutes in 1 ml phosphate buffer pH 7 supplemented with Evans blue to a final concentration of 0.005%.

Cells that accumulated Evans blue were considered dead. At least 1000 cells were counted for each independent treatment.

Cell death was also quantified using the fluorescein diacetate (FDA) spectrofluorimetric method (Reboutier *et al.*, 2007). Briefly, 4 days old *A. thaliana* suspension cells were collected and washed by filtration in a suspension buffer containing 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 10 mM HEPES (H10 medium) adjusted to pH 5.8 (with KOH). One ml of cell suspension was incubated in the presence of oxalate. At each incubation time, 500 µl of the suspension was diluted in 1.5 ml of H10 medium in a quartz cuvette. Then, FDA was added at a final concentration of 12 µM and the fluorescence increase was monitored over a 2 minutes period using a Hitachi F-2000 spectrofluorimeter. The slope of fluorescence increase, representing cell viability, was calculated for each treatment, and directly compared with non-treated cells. Cell death was calculated as follows: % of cell death = 100 × (slope of treated cells / slope of non-treated cells). The experiment was repeated at least 4 times for each condition.

DNA extraction and analysis

Frozen cells were ground in liquid nitrogen and genomic DNA was extracted according to the CTAB method of Haymes *et al.* (Haymes *et al.*, 2004). DNA electrophoresis was performed to assess DNA fragmentation. DNA samples (5 µg/lane) were loaded on a 1.8% agarose gel, stained with 0.2 µg/ml ethidium bromide

Electrophysiology

Individual cells were impaled and voltage-clamped in the culture medium using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) for discontinuous single electrode voltage clamp experiments as previously described (El-Maarouf *et al.*, 2001; Reboutier *et al.*, 2002; Brault *et al.*, 2004). Voltage and current were digitized with a

personal computer fitted with a Digidata 1320A acquisition board (Axon Instruments). The electrometer was driven by pClamp software (pCLAMP8, Axon Instruments). The experiments were conducted on 4 days old cultures (main ions in the medium after 4 days of culture: 9 mM K^+, 11 mM NO_3^-) (Reboutier et al., 2002). Experiments were performed at 22 ± 2°C.

Mitochondrial membrane potential measurement

A. thaliana cells were prepared as described for FDA measurement (0.1g fresh weight ml^{-1}) in a medium containing 50 mM HEPES, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 10 mM Glc (pH 7.0). Before treatment, cells were first stained with the mitochondrial membrane potential probe JC-1 by incubating 2 ml of cell suspensions for 15 minutes (24°C in the dark) with 2 µg/ml JC-1 (3 µM). JC-1 from Molecular Probes Inc. (Eugene, OR) was dissolved and stored according to the manufacturer's instructions. Treated cells without prior washing were subjected to analysis using a Hitachi F-2000 spectrofluorimeter. The excitation wavelength used was 500 nm. Fluorescence signals were collected using a band pass filter centered at 530 and 590 nm.

Ethylene measurement

Four ml of cells were sub-cultured in 10 ml flasks tightly closed with serum caps, maintained at 22°C under constant shacking. After 2 hours, a 2 ml gas sample was taken from each flask and injected into a gas chromatograph (Hewlet Packard 5890 series II) equipped with a flame ionization detector and an activated alumina column (6 mm in internal diameter, 50 cm long, 50-80 mesh) for ethylene determination. Results are presented as means of 4 measurements ± s.d. and are expressed as picomole of ethylene produced per 1 g of fresh matter.

Statistics

Significant differences between treatments were determined by the Mann and Whitney test and P values <0.05 were considered significant.

Results

Oxalate induces programmed cell death

Millimolar concentrations of oxalate (up to 10 mM), close to those found in infected tissues (Bateman & Beer, 1965; Marciano *et al.*, 1983), were tested on host cell viability using *A. thaliana* suspension-cultured cells. Three different methods were used in parallel to analyze cell death in order to obtain reliable measures (Rizhsky *et al*., 2004): the live/dead staining methods with combinations of fluorescein diacetate (FDA), neutral red staining, and Evans blue staining. Albeit these detection methods lead to small variability in oxalate-induced cell death, a 24 hours treatment with increasing concentrations of oxalate resulted in a dose-dependent cell death (Figure 1c, d). In order to discriminate between the effects of oxalic acid itself as an inducer of PCD and a putative cytosol acidification due to diffusion of oxalic acid into the cytosol we tested the impact of acetic acid on *A. thaliana* cell death. Acetic acid up to 12 mM only slightly affects the cell viability when compared to oxalic acid (Fig. 1e). Percentage of dead cells, quantified with FDA, reached a plateau after 8 hours of treatment with 6 mM oxalate (Figure 1f). Oxalate induced vacuole shrinkage (Figure 1a, right hand picture) led to a complete collapse of the dead cells (Figure 1b, right hand picture). In order to check whether oxalate-induced cell death is an active mechanism requiring active gene expression and cellular metabolism, *A. thaliana* cell suspensions were treated with actinomycin D (AD), an inhibitor of RNA

synthesis, or with cycloheximide (Chx), an inhibitor of protein synthesis, 15 minutes prior to 6 mM oxalate addition. Although pretreatments of *A. thaliana* cells with these inhibitors resulted in a slight increase of cell death (Figure 1f), AD and Chx significantly reduced the oxalate-induced cell death (24 hours after oxalate treatment) from 97% to 54% and 46%, respectively (Figure 1g). These results indicated that the oxalate-induced cell death required active cell metabolism, namely gene transcription and de novo protein synthesis. In order to check whether this active cell death displays other apoptotic features we further looked for a putative nuclear DNA cleavage in a ladder of internucleosomal fragments. Gel analysis of DNA extracted from cell suspensions after a 12 hours treatment with 6 mM oxalate showed a typical DNA laddering (Figure 1h). This specific DNA cleavage induced by 6 mM oxalate has been shown to be dependent on active gene expression and *de novo* protein synthesis since it was not detected after addition of AD or Chx to the suspension cell cultures (Figure 1h). Taken together, these data clearly indicate that millimolar concentrations of oxalate induce PCD in *A. thaliana* cells.

Activation of anion channels is a crucial early event for oxalate-induced cell death

Cell shrinkage is a major hallmark of PCD. This process may be mediated by a net efflux of water resulting from the release of anions and K^+ (Maeno *et al*., 2000). Indeed, anion efflux, detectable as a current increase, has been reported to be a necessary event to achieve cell death in tobacco suspension cells subjected to a treatment with cryptogein (Wendehenne *et al.*, 2002; Gauthier, 2007). We undertook an electrophysiological approach in order to determine the role of oxalate on cell membrane potential and on anion currents. The value of the resting membrane potential (V_m) of control cells in their culture medium was -47 ± 5 mV (n=96), similar to those found in previous studies (Reboutier *et al.*, 2002; Bouizgarne *et al*., 2006; Reboutier *et al.*, 2007). Oxalate induced a rapid depolarization of the cell PM (Figure 2a) reaching a maximal value within 2 ± 1 minutes

(n=10). This PM depolarization was concentration-dependent (Figure 2b). Previous electrophysiological studies and pharmacological analyses had identified an anion current which displays the main characteristics of slow anion channels (Reboutier *et al.*, 2002) in the PM of *A. thaliana* cells. This current was shown to be sensitive to structurally unrelated anion channel inhibitors, 9-anthracen carboxylic acid (9-AC), glibenclamide (gli) and niflumic acid (NA) (Reboutier *et al.*, 2002; Brault *et al.*, 2004; Reboutier *et al.*, 2007). Oxalate also induced a concentration dependent increase of anion current (not shown) reaching 580% at 6 mM (Figure 2c, d, e) from a mean control value of -0.28 ± 0.08 nA (n=45). The increase in anion current might explain the depolarization due to oxalate since pretreatment of cells with gli or 9-AC (200 µM) reduced it to almost zero (Figure 2e). The effects of anion channel blockers on the induction of cell death by oxalate were also tested. Oxalate (6 mM) induced around 90% of cell death within 24 hours (Figure 1c, d). When treated with anion channel blockers, only a slight cell death was induced (Figure 2f, g), thus significantly lowering the level of oxalate-induced cell death (Figure 2f, g). These results suggested that the anion current increase was a required upstream event in the signaling pathway leading to oxalate-induced cell death.

Mitochondrial depolarization is involved in oxalate-induced cell death

Since alterations of mitochondrial function, notably a $\Delta\Psi_m$ loss, might also play a role in the early stages of PCD induction in both plants and animals (Vianello *et al.*, 2007), we checked whether oxalate could lead to a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$). The K^+ ionophore valinomycin (1 µM, Figure 3a) was used as a positive control of $\Delta\Psi_m$ decrease. In untreated cells, the JC-1 fluorescence ratio of mitochondria displaying a high $\Delta\Psi_m$ versus mitochondria presenting a low $\Delta\Psi_m$ was largely superior to 1 (Figure 3a). This ratio decreased with a time- and a concentration-dependent manner upon addition of oxalate, reaching a value below 1 indicating that oxalate induced a significant decrease of

$\Delta\Psi_m$ in most mitochondria (Figure 3a). Since mitochondrial $\Delta\Psi_m$ decrease during cell death was reported to be due to the formation of the mitochondrial permeability transition pore (PTP) (Vianello *et al*., 2007), we tested the effect of cyclosporine A (CsA), a well-known inhibitor of the PTP, on oxalate-induced mitochondrial depolarization and oxalate-induced cell death. A pre-treatment with CsA significantly reduced the oxalate-induced mitochondrial depolarization after 2 hours (Figure 3b), indicating that mitochondrial PTP was involved in oxalate-induced mitochondrial depolarization. Although treatment with CsA only increased cell death to a certain level (35% of oxalate-induced cell death), pre-treatment with CsA significantly inhibited the oxalate-induced cell death by 20% (Figure 3c) after 24 hours. Interestingly, the oxalate-induced decrease of $\Delta\Psi_m$ was not inhibited by 9-AC or gli (Figure 3d) indicating that the early anion current increase was not involved in the signalling pathway leading to the oxalate-induced mitochondrial formation of the PTP and further suggesting that at least two different pathways could be involved in the oxalate-induced cell death.

Ethylene synthesis is involved in oxalate-induced cell death

In *A. thaliana*, resistance to the oxalate-producing pathogen *Botrytis cinerea* depends on ET signaling (Glazebrook, 2005). As ET is also known to modulate cell death (Overmyer *et al*., 2003; Lam, 2004), we checked whether inhibitor of ET synthesis could act on oxalate-induced cell death. Treatment of suspension cells by aminooxyacétique acid (AOA), an inhibitor of ACC synthase (Yu *et al*., 1979), or by alpha-aminoisobutyric acid (AIB), an inhibitor of ACC oxidase (Satoh & Esashi, 1980) at 200 µM for 24 hours induced a 25% increase in cell death (Figure 4a). However, both inhibitors significantly reduced the level of oxalate-induced cell death, especially AIB which reduced cell death by 30% (Figure 4a). We thus further checked for ET synthesis. Oxalic acid at 6 mM did not induce ET production after 1 hour of treatment (not shown). However, after 2 hours a

100% increase in ET amount was observed in the cell culture flask (Figure 4b). The effects of anion channel blockers on ET synthesis by oxalate were also tested. Treatment with anion channel blockers inhibited ET synthesis by oxalate and led to a decrease of the control ET level (Figure 4b). These results suggest that an anion current-dependent ET synthesis is involved in the pathway leading to oxalate-induced cell death.

Discussion

Oxalate is essential for the development of disease symptoms and pathogenicity of various necrotrophics among the most damaging plant pathogens (Dutton & Evans, 1996). Pathogenecity of fungal oxalate was previously ascribed to its acidity, which is believed to aid fungal invasion by direct cellular toxicity or by the establishment of a more suitable apoplastic pH for cell wall-degrading enzymes (or both) (Dutton & Evans, 1996). Although oxalate-induced cell death develops more rapidly without adjusting the pH of the oxalate solution (data not shown), the activity remained even when acidification was prevented. This suggests that acidification is not the only mode of oxalic acid action bringing about deleterious effects. Indeed, oxalate-induced cell death has been shown to be achieved and completed at concentrations measured in leaf materials infected with pathogenic strains of *Sclerotinia* (Marciano *et al*., 1983). Here we demonstrate that oxalate induces drastic cell death which fulfils the criteria for PCD in cultured *A. thaliana* cells, in a fashion similar to that initiated by other phytotoxins. First, we have shown that oxalate induced a genetically controlled PCD in *A. thaliana* cultured cells that required active gene expression and de novo protein synthesis, and that this was associated with apoptosis-like features, such as cleavage of nuclear DNA. These data fulfill the widely accepted criteria for PCD, described as the genetically controlled and ordered processes that require active metabolism. The

oxalate-induced cell shrinkage, another hallmark of PCD process in both plant and animal cells (Maeno *et al.*, 2000; Lam, 2004), was probably due to a large activation of anion channels by oxalate. This activation of anion release appears important since various anion channel blockers, previously shown to be effective in *A. thaliana* suspension cells (Reboutier *et al.*, 2002; Brault *et al.*, 2004; Reboutier *et al*., 2007), decreased the oxalate-induced cell depolarization, anion channel increase and finally cell death. Such an efflux of anions would drive water efflux leading to cell shrinking. In various mammalian cell types, apoptotic volume decrease , which is mediated by water loss caused by activation of anion channels and the ensuing K^+ efflux, is an early prerequisite to apoptotic events including cell shrinkage, cytochrome c release, activation of proteases (including caspase) and nucleases, and ultimately leading to PCD (Okada *et al*., 2006). Involvement of ion release via anion flux modulation is considered to be most essential among the earliest responses of plant cells to avirulent pathogens or elicitors capable of inducing PCD (Lam, 2004). Recently we have shown that the involvement of anion channels, a critical component of the cell death process in cryptogein-induced cell shrinkage during PCD of tobacco suspension cells, promoted the accumulation of vacuolar processing enzymes showing caspase-1 activity involved in the disruption of vacuole integrity observed during this cell death (Gauthier, 2007). As a whole, these data suggest that the oxalate-induced anion channel activation is not a passive secondary aspect of PCD, but an event that inevitably drives the whole process. These data are reminiscent of those observed in response to cryptogein (Wendehenne *et al.*, 2002; Gauthier, 2007) and pointed out a critical role for anion channels in the signaling response to pathogens. Anion channels are now becoming recognized as important players in signaling pathways associated with adaptation of plant cells to abiotic and biotic environmental stresses (de Angeli *et al*., 2007), and here we further strengthen this evidence. In addition, we show that anion current increase is a

necessary upstream event in oxalate-induced ET synthesis. Moreover, this event is certainly linked to the oxalate-induced PCD since it involves ET synthesis as evidenced by using blockers of ACC synthase and ACC oxidase, two key enzymes in ET synthesis pathway (Wang et al., 2002). Interestingly, anion effluxes are also necessary for the oxalate related induction of ET-dependent defense related genes (eg. *PDF1-2*, not shown) in seedlings. Our data are in agreement with previous reports indicating that ET synthesis is involved in elicitor-induced cell death (Quin & Lan, 2004), victorin-induced PCD (Curtis and Wolpert 2004) and developmentally induced PCD (Overmyer *et al*., 2003), and more generally during the interaction between plants and necrotrophic pathogens, especially the oxalate-producing pathogens (Glazebrook, 2005).

We further show that oxalate-induced cell death processes could involve an alteration of mitochondrial functions caused by the activation of the PTP that results in the dissipation of mitochondrial electrical potential ($\Delta\Psi m$), since oxalate-induced mitochondrial depolarization and cell death were attenuated in the presence of CsA. Literally, PTP can be defined as a voltage-dependent, high-conductance mitochondrial membrane channel (Vianello *et al*., 2007). These data are consistent with our increasing knowledge that support the importance of plant mitochondria in the control of PCD (Lam et al., 2004 ; Vianello *et al*., 2007), where a mitochondrial permeability transition precedes PCD (Curtis and Wolpert 2004, Vianello *et al*., 2007) by allowing the release of proteins from the mitochondrial inter-membrane space. However, at this time the role of PTP in apoptosis is quite controversial, since recent studies show that sustained PTP opening is predominantly involved in necrosis (Nakagawa, 2005) and would be a consequence rather than an initiator of PCD (Kinnaly, 2007). Recently, CsA was shown to largely decrease cannabinoid-induced plant cell death displaying necrotic characteristics by inhibiting PTP formation (Morimoto, 2007). Cyclosporin A only slightly reverses the effect of oxalate-

induced mitochondrial depolarization and cell death. Thus, we can not exclude that a small part of the cell population undergoes a necrotic cell death pathway in response to oxalate where as the majority of the cells undergo a PCD pathway possibly involving a CsA independent $\Delta\Psi_m$ decrease. Further studies are needed to elucidate this point but such different behaviors were reported on saffron cultured plant cells in response to fusaric acid (Samadi & Shahsavan Behboodi, 2006). Nevertheless, our data clearly show that oxalate, a toxin from necrotrophic fungi, can induce plant cell PCD in an ET and anion channel activity dependent way (Figure 5).

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Figure legends

Figure 1: Effect of oxalate on A. thaliana suspension cell viability.

Light micrographs of oxalate-treated *A. thaliana* cells. Cells were treated with 6 mM oxalate (OA) for 24 hours and stained with neutral red **(A)** or Evans blue **(B)** before observation. Effect of increasing concentrations of oxalate on cell viability estimated by Evans blue staining **(C)** and the FDA assay **(D)** after a 24 hours treatment. **(E)** Effect of increasing concentrations of acetic acid (pH 5.8) on cell viability estimated FDA assay after a 24 hours treatment. **(F)** Time course of cell death estimated with FDA during a 24 hours treatment with 6 mM oxalate. **(G)** Effect of pretreatment with actinomycin D (AD, 20 µg/ml) or cycloheximide (Chx, 20 µg/ml) on 6 mM oxalate-induced cell death detected by Evans blue staining. The data correspond to means of at least 4 independent replicates and error bars correspond to standard errors. **(H)** Fragmentation of nuclear DNA detected by gel electrophoresis after treatment by 6 mM oxalate with or without actinomycin D (20 µg/ml) or cycloheximide (20 µg/ml). Representative results from three independent experiments are shown. DNA molecular weight markers (bp) are given on the left.

Figure 2: Oxalate-induced depolarization and anion current increase of *A. thaliana* cells.

(A) Typical depolarization of PM observed in response to 6 mM oxalate (OA). **(B)** Mean values of depolarization recorded with increasing concentrations of oxalate with or without anion channel blockers (200 µM 9-anthracen carboxylic acid (9-AC) or 200 µM glibenclamide (gli)). **(C)** Anion currents measured under control conditions and after the addition of 6 mM oxalate. Protocols were as illustrated, holding potential (V_h) was V_m. **(D)** Corresponding current-voltage relationships at 1.8 seconds. **(E)** Mean steady state values of anion current recorded at -200 mV and 1.8 seconds after oxalate addition with or without

200 µM 9-AC in the medium. Current variations are given as a percentage with respect to the control level. Data correspond to mean values ± s.d. of at least six independent experiments. **(F,G)** Effect of pretreatment with anion channel blockers (9-AC, gli or niflumic acid (NA), 200 µM each) on cell death induced by 6 mM oxalate after 24 hours for 9-AC and gli (**F**) and after 6 hours for NA (**G**), due to the toxicity of this anion channel blocker. The data correspond to means of at least 4 independent replicates and error bars correspond to standard errors.

Figure 3: Effect of oxalate on mitochondrial membrane potential ($\Delta\Psi_m$) of *A. thaliana* cells

(A) Mean values of JC-1 fluorescence ratio (high $\Delta\Psi_m$ versus low $\Delta\Psi_m$) measured with increasing concentrations of oxalate after 15 minutes, 1 hour and 2 hours. Valinomycin at 1 µM was used as a positive control. **(B)** Effect of 50 µM cyclosporin A (CsA) on the decrease of JC1 fluorescence ratio induced by 6 mM oxalate after 2 hours. **(C)** Effect of pretreatment with 50 µM CsA on cell death induced by 6 mM oxalate after 24 hours. **(D)** Effect of 200 µM 9-AC or gli on JC-1 fluorescence ratio induced by 6 mM oxalate after 2 hours. Data are representative of at least 4 independent experiments and error bars correspond to standard errors. * Significantly different from the control, P<0,05.

Figure 4: Involvement of ethylene in oxalate induced cell death of *A. thaliana* cells

(A) Effect of pretreatment with 200 µM aminooxyacétique acid (AOA), inhibitor of ACC synthase, or by alpha-aminoisobutyric acid (AIB), inhibitor of ACC oxidase on cell death induced by 6 mM oxalate after 24 hours. **(B)** Oxalate-induced synthesis of ethylene after a 2 hours treatment and its inhibition by anion channel blockers: 9-AC, gli or NA (200

μM each). Data are representative of at least 4 independent experiments and error bars correspond to standard errors.

Figure 5: Hypothetical scheme for oxalate induced signaling in *A. thaliana* cells

Oxalate perception is followed by activation of anion channels. The resulting anion efflux contributes to the depolarization of the plasma membrane. The large efflux of anions leads to K^+ and water effluxes from *A. thaliana* cells, inducing cell shrinkage and participating to cell death process. Anion efflux is also required for ET synthesis. Once synthesized, ET could promote nuclear DNA cleavage and organized PCD. Additionally oxalate might inhibit H_20_2 generation (data not shown but also previously observed on soybean cells (Cessna *et al*., 2000) and depolarization of the mitochondria through PTP formation leading to ATP depletion, thus explaining the alkalization of the external medium (data not shown), leading to cell death.

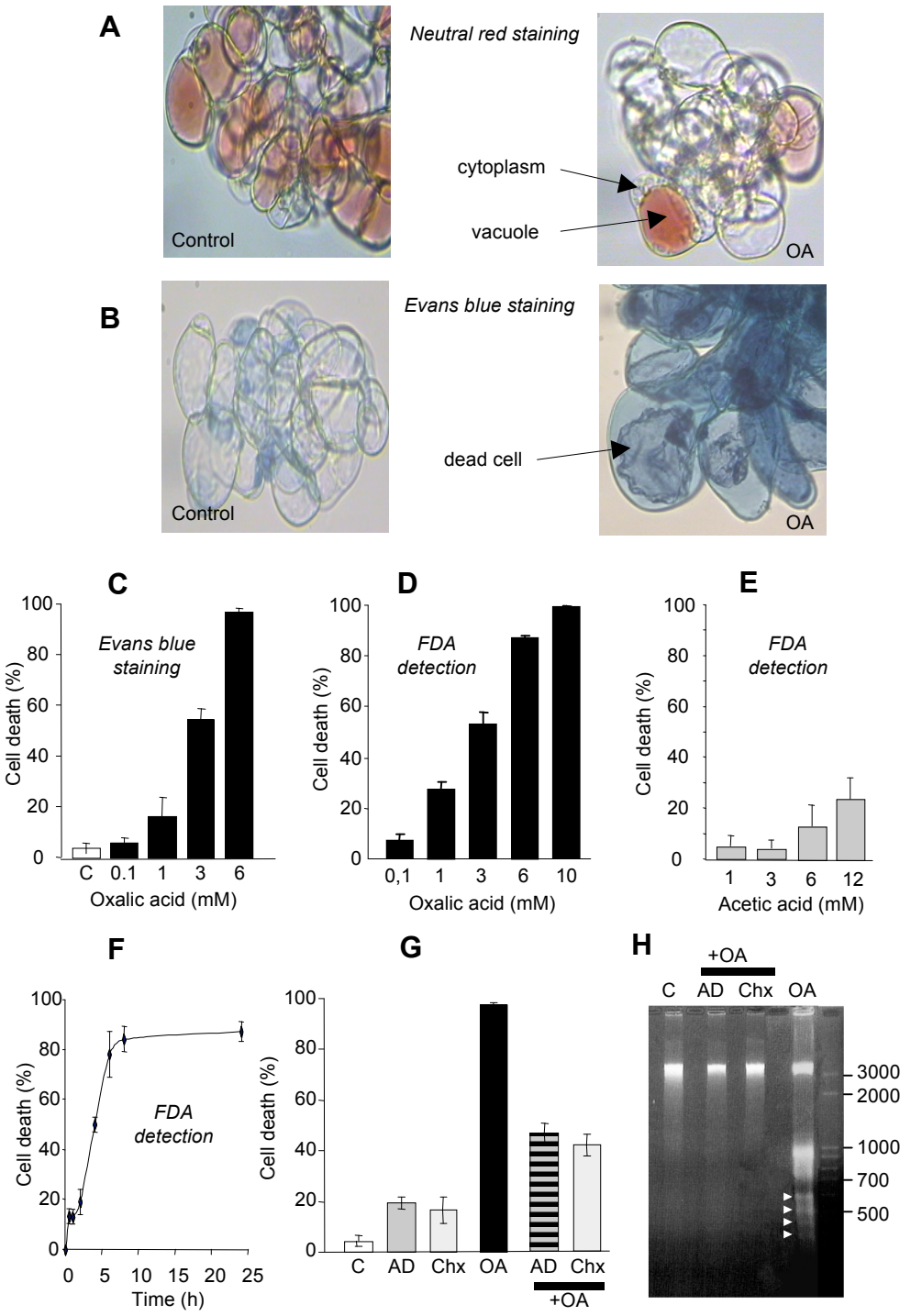
A
Neutral red staining
Control
cytoplasm
vacuole
OA
B
Evans blue staining
Control
dead cell
OA
C
Evans blue staining
Cell death (%)
Oxalic acid (mM)
C 0.1 1 3 6
D
FDA detection
Cell death (%)
Oxalic acid (mM)
0,1 1 3 6 10
E
FDA detection
Cell death (%)
Acetic acid (mM)
1 3 6 12
F
FDA detection
Cell death (%)
Time (h)
G
Cell death (%)
C AD Chx OA AD Chx
+OA
H
+OA
C AD Chx OA
3000
2000
1000
700
500

Figure 1

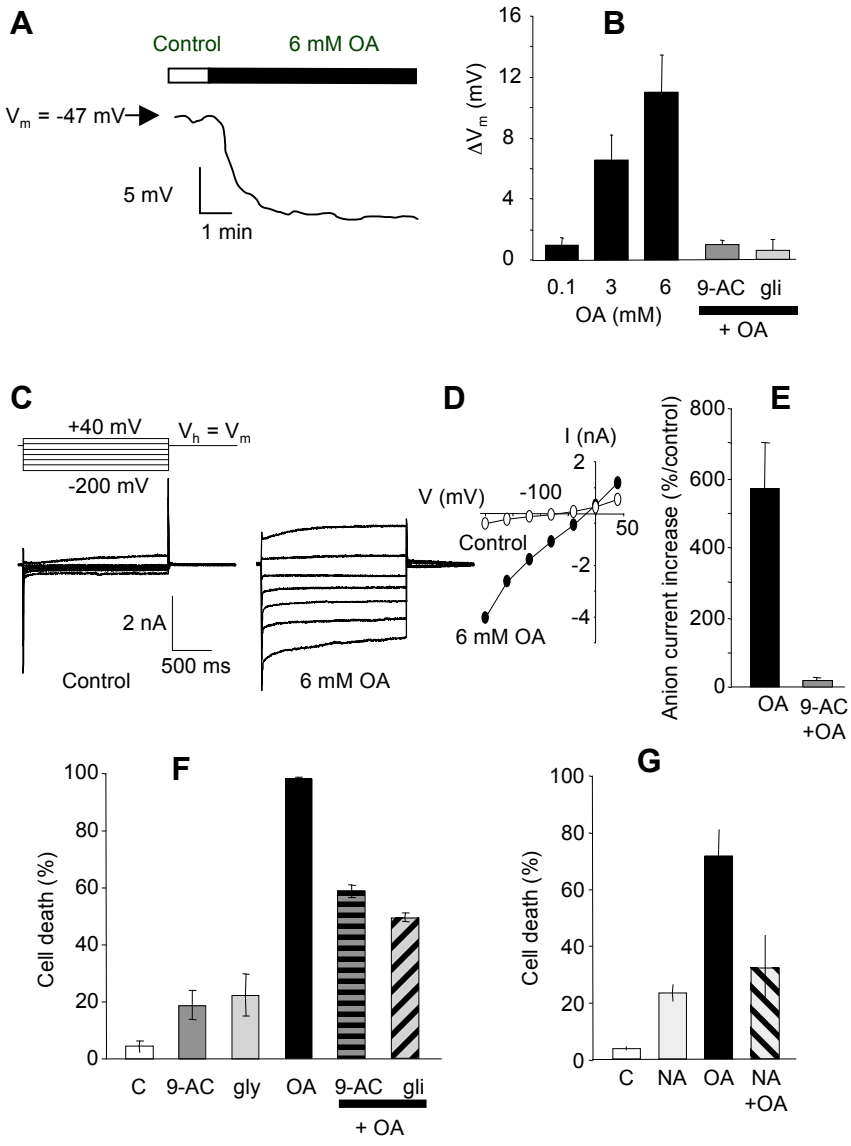
A
Control
6 mM OA
V_m = -47 mV
5 mV
1 min
B
ΔV_m (mV)
16
12
8
4
0
0.1
3
6
9-AC
gli
OA (mM)
+ OA
C
+40 mV
$V_h = V_m$
-200 mV
2 nA
500 ms
Control
6 mM OA
D
I (nA)
2
V (mV)
-100
50
Control
-2
-4
6 mM OA
E
Anion current increase (%/control)
800
600
400
200
0
OA
9-AC
+OA
F
Cell death (%)
100
80
60
40
20
0
C
9-AC
gly
OA
9-AC
gli
+ OA
G
Cell death (%)
100
80
60
40
20
0
C
NA
OA
NA
+OA

Figure 2

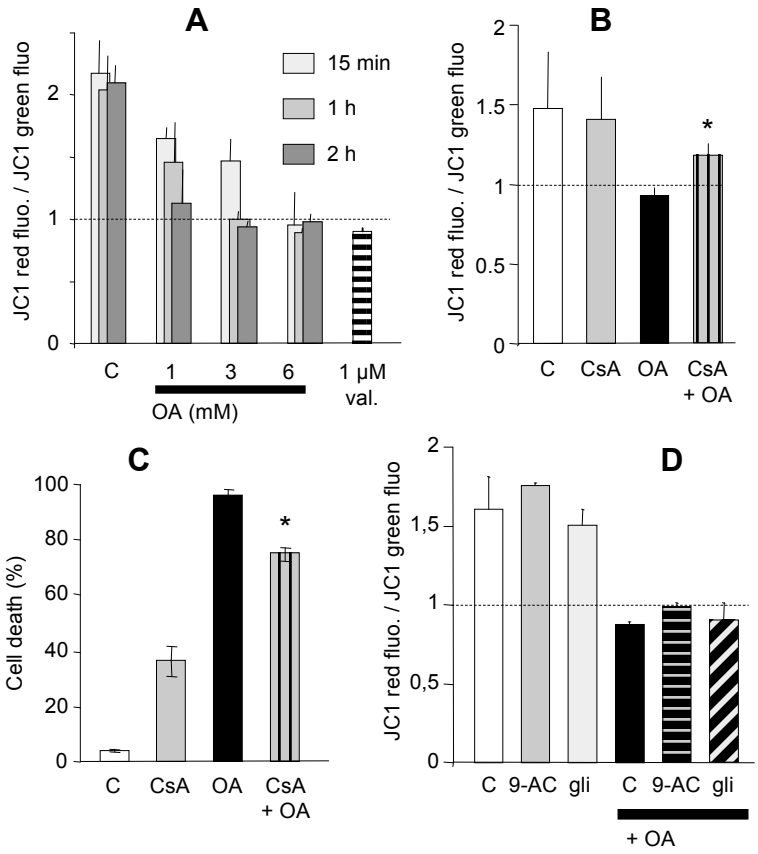
A
15 min
1 h
2 h
JC1 red fluo. / JC1 green fluo
0
1
2
C
1
3
6
1 µM
val.
OA (mM)
B
JC1 red fluo. / JC1 green fluo
0
0.5
1
1.5
2
C
CsA
OA
CsA
+ OA
*
C
Cell death (%)
0
20
40
60
80
100
C
CsA
OA
CsA
+ OA
*
D
JC1 red fluo. / JC1 green fluo
0
0,5
1
1,5
2
C
9-AC
gli
C
9-AC
gli
+ OA

Figure 3

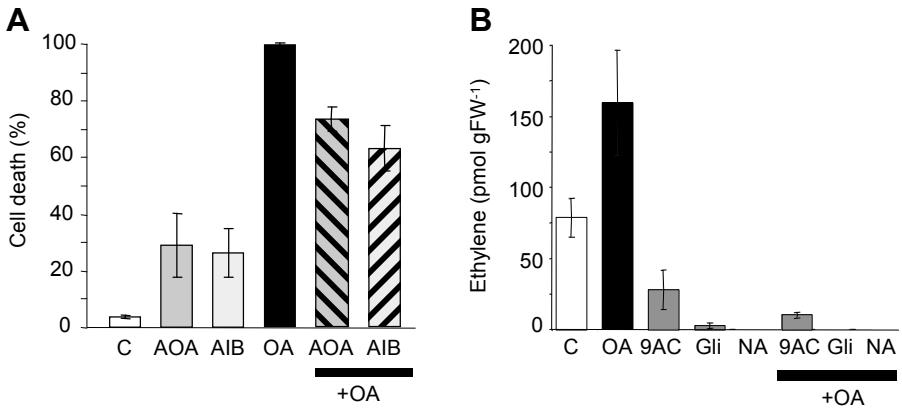
A
Cell death (%)
100
80
60
40
20
0
C
AOA
AIB
OA
AOA
AIB
+OA
B
Ethylene (pmol gFW-1)
200
150
100
50
0
C
OA
9AC
Gli
NA
9AC
Gli
NA
+OA

Figure 4

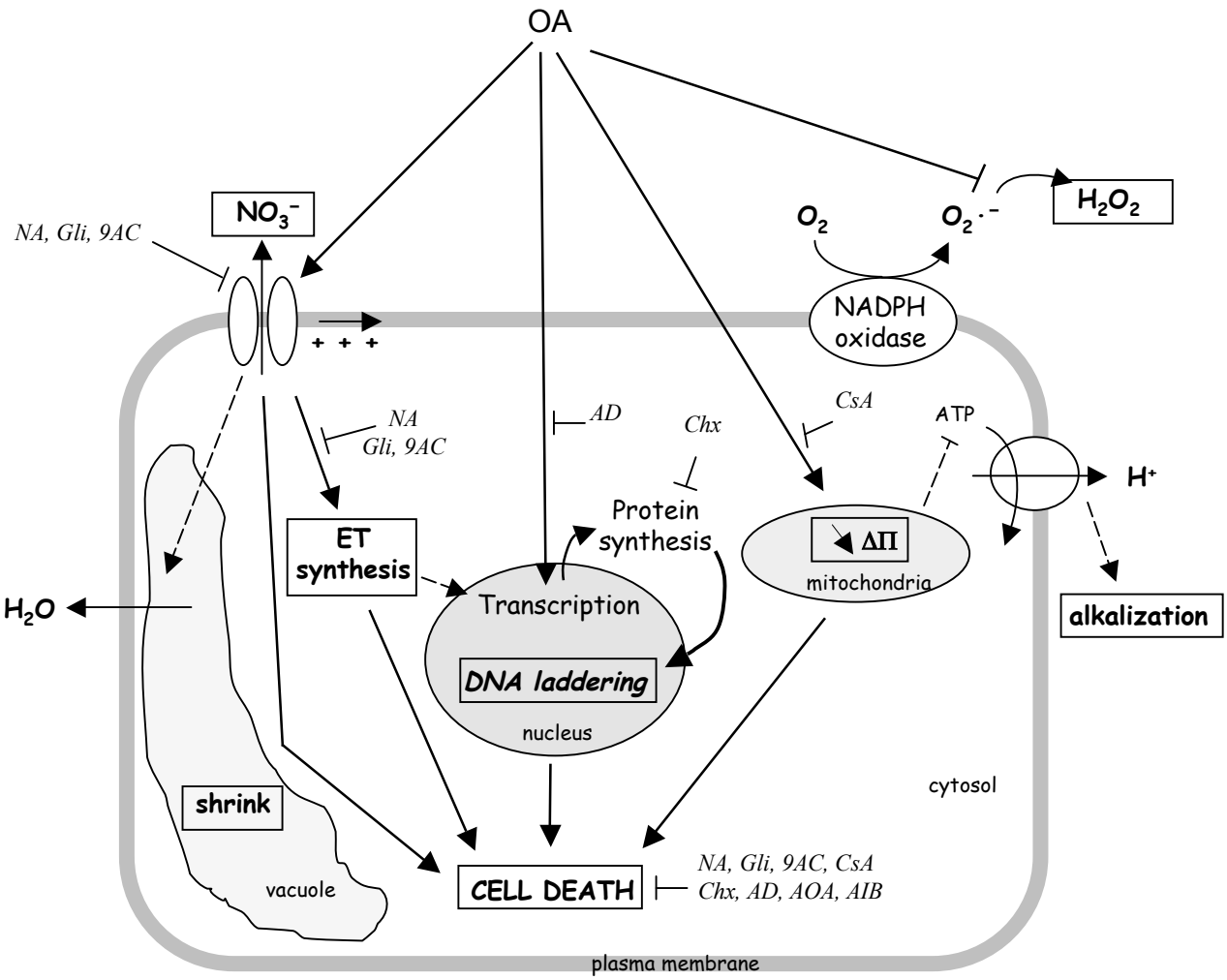
OA
NO_3^-
NA, Gli, 9AC
O_2
$O_2^{\cdot -}$
H_2O_2
NADPH oxidase
+ + +
NA
Gli, 9AC
AD
Chx
CsA
ATP
H^+
ET synthesis
Protein synthesis
ΔΠ
mitochondria
H_2O
Transcription
DNA laddering
nucleus
alkalization
cytosol
shrink
vacuole
CELL DEATH
NA, Gli, 9AC, CsA
Chx, AD, AOA, AIB
plasma membrane

Figure 5