Plant Physiology and Biochemistry 77 (2014) 84–89

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Grape marc extract causes early perception events, defence reactions and hypersensitive response in cultured tobacco cells

R. Benouaret, E. Goujon, P. Goupil*

Clermont Université, Université Blaise Pascal, UMR INRA 547 PIAF, 24 avenue des Landais, BP 10448, 63000 Clermont-Ferrand, France

ARTICLE INFO

Article history:
Received 2 January 2014
Accepted 28 January 2014
Available online 6 February 2014

Keywords:
Cell death
Grape marc
Hypersensitive reaction
Plant defence
Protease inhibitors
Tobacco BY-2

ABSTRACT

Grape marc extract (GME) showed elicitor activity on suspension-cultured cells of tobacco. The BY-2 cells reacted to GME (0.25% and 0.125%) with a long-sustained pH rise in their growth medium. Using EGTA or $LaCl_3$, we showed that extracellular alkalinization depended on Ca^{2+} mobilization. The tobacco BY-2 cells challenged with GME promoted cell death and the upregulation of defence-related genes such as *PR3*, *PAL* and *CCoAOMT*. Cell death rate was quantified using an experimental calibrated Evans Blue assay. The GME-induced cell death was dose-dependent and occurred in 24 h. Longer exposure increased the extent of tobacco cell death. To investigate a potential hypersensitive reaction, we tested the effect of various inhibitors of protein synthesis (cycloheximide) and proteases (aprotinin, pepstatin and E-64) on GME-induced cell death. All these chemicals reduced GME-induced cell death rate in 30 min. Overall, our findings indicate that GME elicits early perception events, defence reactions and cell death requiring protein synthesis and proteases.

1. Introduction

In plant cells, pathogen-derived substances elicit a cascade of reactions conferring plant disease resistance. Resistant plants respond rapidly to elicitors at the infection site by the hypersensitive response (HR), forming a localized cell collapse in order to restrict the systemic spread of a virulent pathogen. Hypersensitive cell death, which is distinct from necrosis caused by metabolic toxins or severe trauma, is genetically programmed (programmed cell death, PCD), and requires active host cell metabolism (Van Doorn et al., 2011). In plants, cell death during HR is similar in some features to apoptosis (a specialized form of PCD) in animals (Coll et al., 2011).

During plant–pathogen interactions, the perception of elicitor substances by plant cells, and before the HR reaction, leads to specific physiological perturbations such as ion fluxes across the plasma membrane (Ca^{2+} influx and K^+, Cl^- efflux), pH changes, plasma membrane depolarization, oxidative burst and induction of rapid cell death. The HR is subsequently accompanied by defence gene activation, leading to synthesis of phytoalexins and accumulation of pathogenesis-related (PR) proteins (Garcia-Brugger et al., 2006; Yang et al., 2011). Elicitors include oligosaccharides, glycoproteins, peptides, lipopolysaccharides, and sterols derived from microorganisms or challenged plants (Kasparovsky et al., 2004; Montesano et al., 2003). Highly sophisticated and complex biological processes underlie the interaction between these compounds and the host plants. Early events occurring in the host have largely been deciphered using tobacco cell suspensions (Garcia-Brugger et al., 2006). Modification of membrane properties is not systematically correlated with the activation of downstream defence events. Also, resistance to pathogen invasion is not necessarily associated with cell death (Gilchrist, 1998; Klarzynski et al., 2000). The structural diversity of elicitors can be expected to have a significant influence on plant cell perception, cell death induction and/or defence gene activation.

The prospect of disease control using a plant's own resistance mechanisms has prompted increasing interest in the development of agents that can mimic natural inducers of plant defence systems. These are named plant defence inducers (PDIs), and act at various points in the signalling pathway leading to disease resistance. PDIs can be organic, inorganic, botanical or synthetic (Lyon, 2007; Walters et al., 2005). Although many of these compounds are non-specific and induce resistance in a wide range of crop species against a diverse range of plant pathogens, differences in efficiency are reported (Lyon, 2007). Grape marc extract (GME) is a plant extract that acts as an efficient PDI. On application to tobacco leaves, this wine by-product elicits a variety of defence reactions such as local injury, biochemical changes and systemic molecular response with upregulation of PR proteins (Goupil et al., 2012). As a potent defence elicitor in tobacco, *Arabidopsis* or tomato (Benouaret

* Corresponding author. Tel.: +33 (0)4 73 40 79 40; fax: +33 (0)4 73 40 79 42.
E-mail address: Pascale.GOUPIL@univ-bpclermont.fr (P. Goupil).

0981-9428/$ – see front matter © 2014 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.plaphy.2014.01.021

et al., 2013), GME might thus prove to be a useful alternative tool for environmentally oriented phytoprotection.

The present report investigates the ability of GME to initiate chemoperception and induce defence mechanisms in tobacco cultured cells. We show that GME triggered rapid extracellular alkalinization and activated defence-related genes, ultimately leading to cell death. Using pharmacological inhibition of proteases, we evidenced a hypersensitive status of tobacco BY-2 cells that led to cell death on GME treatment.

2. Materials and methods

2.1. Biological compounds and chemicals

The grape marc extract (GME), a *Vitis vinifera* L. hydroalcoholic extract, was supplied as a red powder by Grap'Sud (Cruviers-Lascours, France) as described by Goupil et al. (2012). Batch #11332 used in our experiments was produced from grapes harvested from several red wine varieties in 2011. According to the company, this grape end-product is 95% dry weight (DW) and contains 66.1% polyphenols including 25.4% anthocyanins. The flavonoid-based compounds are responsible for the red colour of the dry extract, and the acidity (pH 4.2) of the aqueous solution when it is dissolved in water. Control experiments were conducted with ultrapure water acidified with HCl to pH 4.2.

2.2. Plant material

Tobacco cell suspensions of *Nicotiana tabacum* L. cv. Bright Yellow-2 (BY-2), were grown at pH 5.8 in MS medium (Duchefa, the Netherlands) supplemented with sucrose (30 $g.L^{-1}$), thiamine (1 $mg.L^{-1}$), myo-inositol (102 $mg.L^{-1}$) and 2,4-dichlorophenoxyacetic acid (0.2 $mg.L^{-1}$). Cells were maintained in the dark on a rotary shaker (140 rpm, 25 °C) in 250 mL conical flasks. Subcultures were made weekly by dilution at a 1:15 ratio in fresh medium. All the experiments were performed using cells in exponential growth phase at day 5 after subculture. For alkalinization analysis, cells were collected by filtration, washed with 175 mM mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 1 mM KCl and 1 mM sucrose, and resuspended at 0.1 g fresh weight (FW)/mL in the same medium. The initial pH was about 4.8. For transcript accumulation analysis, cells were collected by filtration, and resuspended at 0.3 g FW/L in MS medium. Cells were equilibrated for 2 h in open 6-well microplates (Greiner Bio-one, Germany) with continuous stirring, and then elicited with GME.

2.3. Extracellular alkalinization

The pH variations of the culture medium were recorded by introducing a glass microelectrode (Hanna Instrument, HI1330B, France) into 6 ml of the equilibrated cell suspension culture. The pH measurement, performed every 10 min, started immediately after treatment with elicitors or acidified water. The ΔpH was measured at 10 min intervals, relative to the pH measured at t_0 immediately after adding GME or acidified water (control). Each experiment was repeated three to six times to check for reproducibility. Figures describe the results of typical experiments.

2.4. Real-time RT-PCR

Tobacco BY-2 cell samples (100 mg) were ground in liquid nitrogen, and total RNAs were isolated using CTAB extraction buffer according to Chang et al. (1993). Total RNAs were cleaned up with 1 U DNase I solution (Euromedex, France) containing 40 U RNase inhibitor (Euromedex, France). RNA integrity was verified on a 1% agarose gel by detecting ribosomal RNAs. First-strand cDNA was synthesized from 1 μg total RNA using oligo $d(T)_{15}$ primers and Euroscript Reverse Transcriptase (Eurogentec, France) according to the manufacturer's instructions.

PCR reactions were prepared using the qPCR kit Mastermix for SYBR green (Eurogentec) according to the manufacturer's protocol. The quantitative assessment of mRNA levels was performed using an iCycler iQ5 (Bio-Rad). The cDNA concentration used produced a threshold cycle value (C_T) of between 15 and 30 cycles. Amplification specificity was checked by melting-curve analysis. The EF-3 was used as an internal control (Goupil et al., 2012). Quantification of expression ratios was performed according to the mathematical model developed by Pfaffl (2001). Primers and amplicon sizes are given in Appendix S1.

2.5. Cell death assay

The Evans Blue assay was performed according to Amano et al. (2003). Tobacco cells (500 μL) were withdrawn from the culture medium immediately after GME elicitation (t_0) and then every 24 h for 3 days. The cells were spread onto a cotton swab placed at the bottom of a 10 mL plastic syringe. The cells were washed with 10 mL of distilled water and stained with 500 μL of Evans Blue solution (0.25% w/v in distilled water) for 5 min at room temperature. Excess unbound dye was removed by thorough washing with 50 mL of distilled water. The cotton swab containing cells was soaked with 500 μL SDS solution (0.5%, w/v), and the syringe was immediately heated for 3 min in a boiling water bath. The dye bound to dead cells was eluted with 2.5 mL of distilled water, and remaining drops of dye were squeezed out using the syringe plunger. Absorbance at 600 nm was measured to estimate cell death. Each experiment was repeated three times.

2.6. Pharmacological treatments

We used EGTA (2 mM and 5 mM), $LaCl_3$ (2 mM) as chelator and Ca^{2+} channel blocker respectively, pepstatin (1 μM), E-64 (1.5 μM), aprotinin (0.2 μM) as protease inhibitors, and cycloheximide (50 μM) as protein synthesis inhibitor. Pepstatin was dissolved in ethanol and the other chemicals in water. All the chemicals were purchased from Sigma–Aldrich (France).

2.7. Statistics

All the experiments were performed in biological and technical triplicates. The values were expressed as mean ± standard error of the mean (SE). Letters above bars indicate statistically significant differences between samples, according to one-way ANOVA followed by LSD Significant test ($p \leq 0.05$) using Statistix 9® software.

3. Results

3.1. Effect of GME on proton flux and Ca^{2+} mobilization

GME elicits tobacco BY-2 cells. Suspension cells responded to GME by a rapid, sustained alkalinization of the incubation medium (Fig. 1). Medium alkalinization was detected immediately after addition of GME (0.25%), and the pH shift reached a plateau with a ΔpH of 1.6 units at 80 min. No pH shift was detected with the control cells treated with water. GME added to the cell-free medium did not cause any pH modifications. The intensity of the GME effect was dose-dependent. The pH shift reached 0.9 units with 0.125% GME, and the 0.0625% concentration did not induce any extracellular alkalinization.

The involvement of calcium in GME-induced extracellular alkalinization was investigated by adding EGTA (2 mM or 5 mM) or $LaCl_3$ (2 mM) to the culture medium concomitantly with GME. The