Plant Science 238 (2015) 148–157

Contents lists available at ScienceDirect

Plant Science

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

Early events induced by the toxin deoxynivalenol lead to programmed cell death in *Nicotiana tabacum* cells

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ARTICLE INFO

Article history:
Received 28 April 2015
Received in revised form 4 June 2015
Accepted 5 June 2015
Available online 12 June 2015

Keywords:
Programmed cell death
Deoxynivalenol
Fusarium
Nicotiana tabacum
Ion channels

ABSTRACT

Deoxynivalenol (DON) is a mycotoxin affecting animals and plants. This toxin synthesized by *Fusarium culmorum* and *Fusarium graminearum* is currently believed to play a decisive role in the fungal phytopathogenesis as a virulence factor. Using cultured cells of *Nicotiana tabacum* BY2, we showed that DON-induced programmed cell death (PCD) could require transcription and translation processes, in contrast to what was observed in animal cells. DON could induce different cross-linked pathways involving (i) reactive oxygen species (ROS) generation linked, at least partly, to a mitochondrial dysfunction and a transcriptional down-regulation of the alternative oxidase (*Aox1*) gene and (ii) regulation of ion channel activities participating in cell shrinkage, to achieve PCD.

1. Introduction

Phytopathogenic *Fusarium culmorum* and *Fusarium graminearum* synthesize various mycotoxins *in planta*, including B-type sesquiterpene epoxide trichothecene, deoxynivalenol (DON). This toxin acts as a virulence factor by increasing the aggressiveness and symptoms of disease [1,2] and is also toxic to animals [3].

In animal cells, DON alters basic metabolic cell processes, including an inhibition of nucleic acid and protein synthesis by a binding to the ribosomal peptidyltransferase [3,4]. DON also induces a variety of events leading to apoptosis, such as nuclear DNA fragmentation, membrane structure and integrity disturbance, generation of reactive oxygen species (ROS) and reduction of mitochondrial transmembrane potential ($\Delta\psi_m$) [3,5,6]. Trichothecenes have effectively been shown to target mitochondria, where they can inhibit mitochondrial protein synthesis and increasing mitochondrial ROS levels [7]. In plant cells, responses to DON include alteration of cell membrane structure [8], inhibition of some enzymatic activities linked to mitochondrial functions [9], overproduction of hydrogen peroxide (H_2O_2) and PCD [10]. However, low concentrations of DON have recently been shown to inhibit the PCD induced by abiotic stress in Arabidopsis cell cultures [11]. The DON level during the infection process seems to depend on the pathogen trophic stage and participates in a sophisticated strategy to circumvent and hijack the plant's defence system [12]. Low concentrations of DON can inhibit host cell PCD during the early biotrophic phase of infection, whereas higher DON concentrations participate in the second necrotrophic phase [11–13]. Desmond et al. [10] provided evidence that DON may not only induce inhibition of protein synthesis activity. The authors demonstrated induction of a suite of defence gene transcripts and proteins in wheat seedlings. Through a priming experiment that enhanced plant defence against *F. graminearum* in wheat, DON production has also been shown to be boosted [14]. Thus, the effects of DON appear to be complex and the mechanism of toxicity remains poorly understood in plant. The intention in the present study was to further elucidate early cellular and molecular mechanisms induced in BY2 cells in response to a high level of DON and examine their

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http://dx.doi.org/10.1016/j.plantsci.2015.06.004
0168-9452/

contribution to plant cell death. We found that, in addition to ROS generation and mitochondrial dysfunction, DON-induced PCD requires regulation of ion channels participating in cell shrinkage as observed for other fungi-derived toxins such as cryptogein or oxalic acid [15,16].

2. Materials and methods

2.1. Plant cell culture conditions

Nicotiana tabacum BY2 cell suspensions were grown in Murashige and Skoog medium (MS medium) at pH 5.8 [17]. They were maintained at 22 ± 2 °C in darkness with continuous shaking at 120 rpm. Cell suspensions were sub-cultured weekly using a 1:40 dilution. All experiments were performed at 22 ± 2 °C using log-phase cells (6–7 days after sub-culture).

2.2. Cell protoplast preparation

BY2-protoplasts were isolated from suspension cultures. Two millilitre of cell suspension was used. After cell self-sedimentation (5 min), the supernatant was removed and replaced by 1 ml of fresh MS medium containing 0.02 g cellulysin, 0.01 g macerase and 0.6 M sorbitol. The digestion was carried out under shaking at 120 rpm −22 °C for 60 min. After incubation, protoplasts were collected by centrifugation at 200 rpm for 3 min and re-suspended in 1 ml of MS medium containing 0.3 M sorbitol to liberate protoplast, then centrifuged again under the same conditions. Finally, the supernatant was removed and the remaining protoplasts were re-suspended in 5 ml of fresh MS medium.

2.3. Evaluation of protoplasts volume

To determine whether DON induced a modification of protoplast volume, BY2 protoplasts exposed to DON were photographed at 15 min intervals for 3 h. The images obtained were then subjected to quantitative evaluation of the area of the protoplasts, which gave an estimation of their whole volume, on the basis of signal intensity analysis using Image J software. The area was expressed as a relative percentage (%) of the original protoplast area (at $t = 0$) in order to normalize the diversity of cell protoplast size (ranging from 20 to 100 μm).

2.4. Cell viability assay

Cell viability was assessed using the fluorescein diacetate (FDA) spectrofluorimetric method [18]. After the appropriate DON-treatment, 1 ml of cell suspension (0.05 g FW) was gently stirred with a magnetic stirrer before FDA was added at a final concentration of 12 μM. The fluorescence increase was monitored over 120 s using an F-2000 spectrofluorimeter (Hitachi, Japan). The slope obtained corresponded to the esterase activity. The results are presented as the percentage of cell death = slope of treated cells/slope of control cells × 100. Control cells were cells added with a water volume equivalent to that of the DON treatment. The time course of death for control cells was evaluated by comparing the slopes obtained with control cells at each considered time with the slope for control cells at $t = 0$. The experiment was repeated at least 3 times for each condition.

Cell and protoplast viability was also checked using the vital dyes Evans blue (EB) or neutral red (NR) after DON treatment for different time periods with or without the appropriate pharmacological effectors. Cells (50 μl) were incubated for 5 min in 1 ml phosphate buffer pH 7 supplemented with EB or NR at a final concentration of 0.005% or 0.01%, respectively. Cells that accumulated EB or did not accumulate NR were considered dead. At least 500 cells were observed and counted under a light microscope for each treatment and the experiments were repeated at least 3 times for each condition.

2.5. Measurement of reactive oxygen species production

Reactive oxygen species (ROS) release in the medium was quantified by measuring the chemiluminescence of luminol reacting with ROS [17]. Briefly, 10 ml of the *N. tabacum* BY2 cell suspension was inoculated with 50 μg ml^{-1} of DON alone or with the appropriate chemical effectors. Before measurements were made, 5 μl luminol (1.1 mM) was added to each 200 μl of cell suspension (0.05 g FW ml^{-1}). Chemiluminescence was monitored every 30 min using a FB12-Berthold luminometer (signal integrating time of 0.2 s). The measurement was repeated at least 3 times for each condition.

2.6. Aequorin luminescence measurements

Cytoplasmic Ca^{2+} variations were recorded from BY2 cell suspensions expressing the apoaequorin gene [19]. For calcium measurement, aequorin was reconstituted by an overnight incubation of the cell suspension in MS medium supplemented with 2.5 μM native coelenterazine. Cell culture aliquots (450 μl in MS; 0.05 g FW ml^{-1}) were carefully transferred to a luminometer glass tube and luminescence was recorded continuously at 0.2 s intervals using an FB12-Berthold luminometer (Berthold Technologies, Bad Wildbad, Germany). Treatments were performed by pipette injections of 50 μl containing the effectors. At the end of each experiment, residual aequorin was discharged by addition of 500 μl of a 1 M $CaCl_2$ solution dissolved in 100% methanol. The resulting luminescence was used to estimate the total amount of aequorin in each experiment. Calibration of the calcium measurement was performed using the equation: $pCa = 0.332588\,(-\log k) + 5.5593$, where k is a rate constant equal to the luminescence counts per second divided by total remaining counts. Data were expressed as μM.

2.7. Electrophysiology

Individual cells were impaled and voltage-clamped in the MS medium (main ions 28 mM NO_3^-, 16 mM K^+) using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) for discontinuous single electrode voltage clamp (dSEVC) experiments as previously described [15,20]. This allowed electrophysiological variations in living cells with their cell wall to be determined in non-stressing conditions. Voltage and current were digitized using a computer fitted with a Digidata 1320A acquisition board (Axon Instruments). The electrometer was driven by pClamp software (pCLAMP8, Axon Instruments).

2.8. Mitochondrial membrane potential measurement

The mitochondrial membrane potential was monitored using JC-1 fluorochrome [21]. Briefly, before treatment, cells were first stained with the mitochondrial membrane potential probe JC-1 by incubating 2 ml of cell suspensions (0.05 g FW ml^{-1}) for 15 min (22 °C in the dark) with 2 μg ml^{-1} JC-1 (3 μM). Treated cells were then analysed using a Hitachi F-2000 spectrofluorimeter. The excitation wavelength used was 500 nm. Fluorescence signals were collected using a band pass filter centred at 530 and 590 nm. Then, fluorescence ratios (high versus low) were evaluated. Valinomycin (a drug known to affect mitochondrial membrane potential) at 1 μM was used as a positive control.