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CELL DEATH AND ANTI-CELL DEATH IN TWO WHEAT CULTIVARS AND THE IMPLICATION OF THEIR INVOLVEMENT IN DISEASE RESPONSE

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Abstract: Cell death occurs under various developmental and stress conditions. Its involvement in plant response to pathogen attacks has been well studied in model plant *Arabidopsis thaliana.* In our present work, Fumonisin B1, a toxin from *Fusarium verticillioides*, a major fungal pathogen of cereals, was used as a biotic stressor to trigger responses in two wheat cultivars. Fumonisin B1 induced cell death in both Fusarium head blight (FHB) resistant and FHB susceptible cultivars (Frontana and Roblin, respectively). The treatment also triggered DNA smearing in both. However, the expression of two DNA repairing genes was enhanced in Frontana but not in Roblin. Our results have suggested potential regulatory differences in the response to FB1 toxin in FHB resistant and FHB susceptible cultivars.

Keywords: cell death, DNA repair, Fumonisin B1, *Fusarium* head blight, wheat.

Introduction

Programmed cell death (PCD) is recognized as an essential physiological and genetic process during plant development and in response to biotic and abiotic stresses [BEERS & MCDOWELL, 2001; DANEVA & al. 2016]. Localized cell death occurs in both susceptible and immune plants during pathogen attack. In immune plants, a host resistance (R) protein recognizes a pathogen effector leading to hypersensitive response (HR), which is a form of localized PCD [BURKE & al. 2020; POZO & al. 2004]. Many host responses precede the HR, including proteolysis, changes in ion fluxes, production of reactive oxygen species (ROS), and activation of mitogen-activated protein kinase (MAPK) cascades. In susceptible plants, much less is known about the molecular events leading to cell death. Studies suggest that host-controlled PCD plays a role in cell death occurred in different plant tissues [COLL & al. 2011; GREENBERG & YAO, 2004]. For biotrophic pathogens, early activation of host PCD would likely limit pathogen spread, whereas necrotrophic pathogens benefit from host cell death and kill the host by injecting toxins or activating host PCD [COLL & al. 2011; POZO & al. 2004].

Specific DNA fragmentation into oligonucleosomal units or DNA laddering occurs during PCD in both animal and plant cells [DANON & al. 2000]. Necrosis and a DNA smear on agarose gels are normally caused by concurrent nuclease and protease activities [DANON & al. 2000; WYLLIE & al. 1980]. DNA laddering phenomenon has been studied for a long time in different plant developmental processes and under environmental stresses such as cold [KOUKALOVÀ & al. 1997], UV radiation [DANON & GALLOIS, 1998], and heat [FAN & XING, 2004; SWIDZINSKI & al. 2002]. Harsh environmental stresses often cause necrosis that is an accidental cell death, accompanied with DNA smear, rupture of nuclear, organelle and

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plasma membranes [DANON & al. 2000]. Therefore, DNA ladders are currently used to distinguish apoptosis from necrosis at the molecular level [DANON & al. 2000].

Fusarium verticillioides produces Fumonisin B1 (FB1) [ASAI & al. 2000] and it was used as a biotic stressor in our present study. In *Arabidopsis*, FB1 treatment initiated nuclear DNA fragmentation preceding the loss of membrane integrity, which resembled apoptosis typically associated with PCD in animal cells [ASAI & al. 2000; PLETT & al. 2009]. FB1 induced cell death is dependent on active transcription and translation, as well as reversible protein phosphorylation [ASAI & al. 2000]. In *Arabidopsis* protoplasts FB1-elicited PCD was shown to required SA, jasmonic acid (JA) and ethylene (ET)-dependent signaling pathways as well as one or more unknown factors activated by FB1 [ASAI & al. 2000]. There was also a correlation between MAPK activity and cell death during plant-pathogen interactions [POZO & al. 2004; YANG & al. 2001; ZHANG & LIU, 2001]. Our previous study has indicated that *Arabidopsis* five ethylene receptors have different roles in FB1-induced cell death [PLETT & al. 2009]. Here, we have shown that FB1 induced cell death in both FHB resistant and FHB susceptible wheat cultivars (Frontana and Roblin, respectively). Our results have also suggested potential regulatory differences in the response to FB1 toxin including the expression of anti-cell death genes.

Materials and methods

Plant materials and growth conditions

Wheat (*Triticum aestivum*) seeds were sterilized in a solution of 70% ethanol for 2 min, then transferred to a bleach solution of 25 mL of bleach, 25 mL of distilled water and 10 µL of Triton X-100. The seeds were then rinsed 10 times in distilled water. After drying 5-6 seeds were potted in autoclaved Pro-mix BX soil fertilized with 7-9 granular of slow release NPK fertilizer (14:14:14). The seeds were then placed in a growth chamber (Enconnair Technologies Inc., Winnipeg, MB, Canada) set for 16 hrs at 22°C in the light and 8 hrs at 18°C in the dark. Plants were watered every second day.

FB1 treatment

Frontana and Roblin leaves were collected and treated with FB1 for different periods of time. Three to four leaves harvested from the 3-week-old plants were cut to ~2 cm segments and incubated in 5 µM FB1 at same growth conditions after infiltration. Leaf segments were stretched upward on the filter paper in Petri dish so that they were just covered by the solution. Control leaf segments were treated in the same way with an equal volume of distilled water. Samples after 24 hrs, 48 hrs and 72 hrs incubation were collected. The leaf segments without any treatment were taken as the sample at 0 hr for both FB1 and water treatment. Leaf segments were collected in Falcon tubes, and directly used for staining. For DNA or RNA extraction, leaf materials were collected in Falcon tubes and snap frozen in liquid nitrogen. The materials were then stored at -80 °C till use.

Trypan blue staining

Cell death was detected using an Axioplan 2 microscope (Carl Zeiss, Germany). Methods described by TANG & al. (1999) and STONE & al. (2000) were used with slight modifications. Leaf tissues were immersed in 10 mL of ethanol-lactophenol (2 volumes of ethanol and 1 volume of phenol-glycerol-lactic acid-water 1:1:1:1) that contained 0.05% trypan blue. The leaves were placed in 15 mL Falcon tubes and covered with ethanol-lactophenol-

trypan blue. The samples were incubated at 95 °C for 4 min and then kept at room temperature for 20 min. The staining solution was removed and 1.5 mL chloral hydrate destaining solution (2.5 g/mL of nano pure water) was added to each tube. The leaves were cleared for 2 days by replacing the destaining solution twice. After destaining, leaves were suspended in 50% glycerol and examined under microscope with white light.

Aniline blue staining

Wheat leaves were immersed and vacuum-infiltrated in 10 mL of ethanol-lactophenol (2:1 v:v) and then incubated at 60 °C for 30 min. Leaves were rinsed in 50% ethanol and stained overnight with aniline blue (0.01% aniline blue powder in 150 mM K_2HPO_4, pH 9.5). Leaves were equilibrated in 50% glycerol and aniline blue staining was visualized using a UV epifluorescence (Axioplan 2 microscope, Carl Zeiss, Germany) with a DAPI filter.

Isolation of nuclear DNA and DNA laddering analysis

Total DNA was isolated using a modified method of FAN & XING (2004). Briefly, wheat leaves were ground to a fine powder in liquid nitrogen and added to the extraction buffer (200 mM Tris, pH 7.5, 25 mM EDTA, and 0.5% sodium dodecylsulphate). The supernatant was extracted with phenol and chloroform before precipitation with isopropanol. The DNA solution was incubated at 37 °C for 1 hr in the presence of RNase and 10 µg DNA was separated by electrophoresis in a 2% agarose gel. The gel was then stained with ethidium bromide for visualization of DNA.

Expression analysis of DNA repair genes

RNA was extracted from wheat leaves. GAPDH gene (GenBank accession number EU022331.1) was used as an internal standard [LLOYD & al. 2007]. For RT-PCR, the primers were 5'-GTGAGGCTGGTGCTGATTACG-3' (forward) and 5'-TGGTGCAGCTAG CATTTGAGAC-3' (reverse). The following conditions were used in RT-PCR for wheat GAPDH gene: 94 °C for 1 min; 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 seconds for 28 cycles; and then 10 min at 72 ° C. The size of the amplified fragment was 198 bp.

The primers for RAD50 (GenBank accession number EU159424.1) were 5'-CAGGGACACATTGACTGGTG-3' (forward) and 5'-TTTCCTCGGCAAAATGTACC-3' (reverse). The following conditions were used for RT-PCR: 94 °C for 1 min; 94 °C for 1 min, 67 °C for 1 min, 72 °C for 30 seconds for 28 cycles, and then 72 °C for 10 min. The size of the amplified fragment was 176 bp. The primers for RAD51 (GenBank accession number EU915557.1) were 5'-CAGAAGGCACATTCAGACCA-3' (forward) and 5'-GCAAACCTTG TCTCCACCAT-3' (reverse). The RT-PCR setting was 94 °C for 1 min; 94 °C for 1 min, 71 °C for 1 min, 72 °C for 30 second for 28 cycles, and then 72 °C for 10 min. The size of the amplified fragment was 166 bp.

Results

FB1-induced cell death in Frontana and Roblin leaves

Cell death levels were determined after leaves were detached from three-week-old Frontana and Roblin and treated with water or 5 µM FB1 in long day conditions for up to 72 hrs. Trypan blue is commonly used to selectively stain dead tissues or cells blue, and under white light the dead cells appeared to be much darker compared to living cells. These blue dead cells scattered on leaves as clusters without defined margins and the cell death did not seem to

occur in the whole leaf. Microscopic images were taken by focusing on one of several dead cell groups. Water treatment was used as a control for all plants and no dead cells were detected (Figure 1A). Upon FB1 treatment, cell death was first observed at 24 hrs in both Frontana and Roblin, and more dead cells were detected when leaves were incubated longer (Figure 1A).

The accumulation of callose deposition around the site of infection is usually part of the complex cell wall-strengthening process that halts pathogen invasion [WANG & al. 2021]. To determine whether the cell death detected by trypan blue assay is associated with callose deposition, we determined the presence of callose with aniline blue in Frontana and Roblin leaves 0 hr, 24 hrs, 48 hrs, and 72 hrs after FB1 treatment. In water control, cell death was induced at 72 hrs, and the area of dead cells is larger on Roblin leaves than on Frontana leaves (Figure 1B). The earliest cell death induced by FB1 was detected at 24 hrs in both Frontana and Roblin, and more cell death appeared at 72 hrs (Figure 1B). Overall, upon FB1 treatment, cell death occurred earlier in Frontana leaves than in Roblin leaves as detected by trypan blue and aniline blue, but there was more cell death in Roblin leaves than in Frontana leaves at 72 hrs.

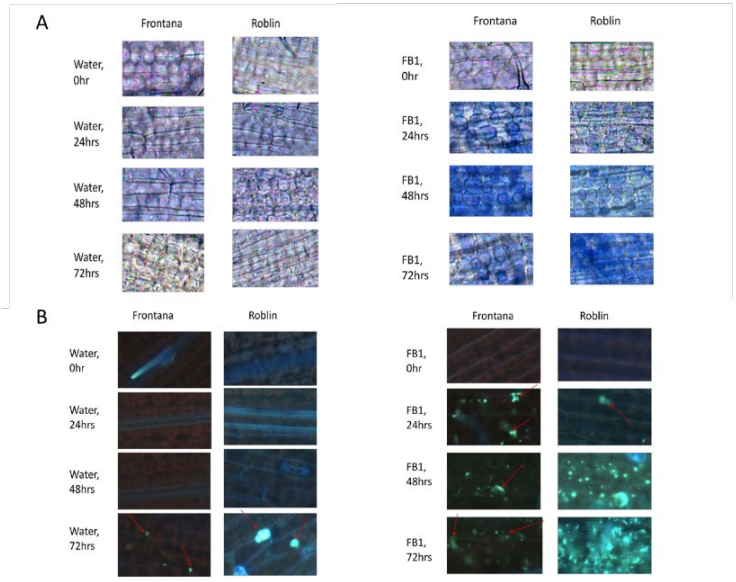

Figure 1. Microscopic images of Frontana and Roblin leaves. Leaves detached from three-week-old plants were treated with water or 5 μM FB1. Half of each type of leaves was then stained with trypan blue. Microscopic images were taken with white light by Axioplan 2 microscope. The other half portion of leaves was stained with aniline blue. Microscopic images were taken using a microscope equipped with a UV light source and DAPI filter (scan range: 320-520 nm). Arrows indicate the sites of fluorescent signals from aniline blue strain. A. Trypan blue detection of cell death. B. Aniline blue detection of cell death. Three experiments were carried out with similar results.

FB1 induced DNA smearing in Frontana and Roblin leaves

DNA ladder phenomenon is one of the most common characteristics of PCD in animals [NING & al. 2002]. We monitored the integrity of DNA by electrophoresis. When Frontana and Roblin leaves were treated with FB1 for different periods of time, a significant DNA smearing was observed (Figure 2).

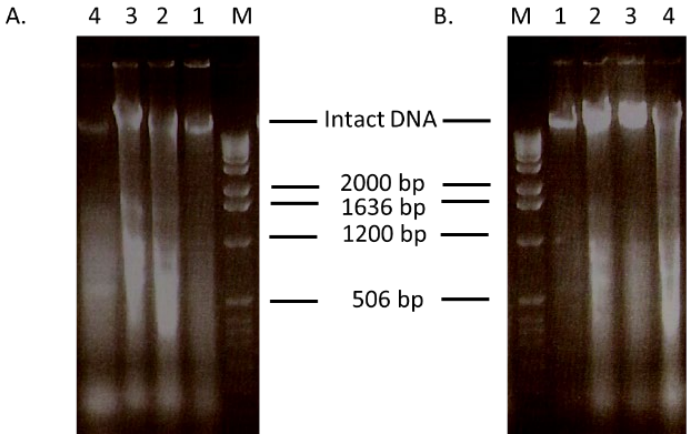

Figure 2. Wheat leaf DNA smearing after FB1 treatments. Ten µg DNA per lane was loaded onto a 2% agarose gel followed by electrophoresis. **A**. Frontana. Lanes 1-4, Frontana leaves after FB1 treatment for 0, 24, 48 and 72 hrs, respectively. **B**. Roblin. Lane 1-4, Roblin leaves after FB1 treatment for 0, 24, 48 and 72 hrs, respectively. M, DNA markers. Three experiments were carried out with similar results.

Expression of DNA repair genes in Frontana and Roblin leaves treated with FB1

DNA repair genes including Radiation sensitive 50 (RAD50) and Radiation sensitive 51 (RAD51) have been identified in yeast, animals and plants, and they are involved in various processes such as DNA damage repair, DNA replication, meiosis, and telomere maintenance [CZORNAK & al. 2008; LAMARCH & al. 2010; LLOYD & al. 2007]. In order to examine whether RAD50 and RAD51 are involved in DNA damage repair after a pathogen attack, DNA integrity of three-week old Frontana and Roblin leaves treated with water or 5 µM FB1 for 0, 24, and 48 hrs was examined. There was no significant change in the expression levels of either RAD50 or RAD51 in Roblin. However, there was an increase in the transcript levels of RAD50 and RAD51 in Frontana at the 24 hrs time interval after the FB1 treatment (Figure 3).

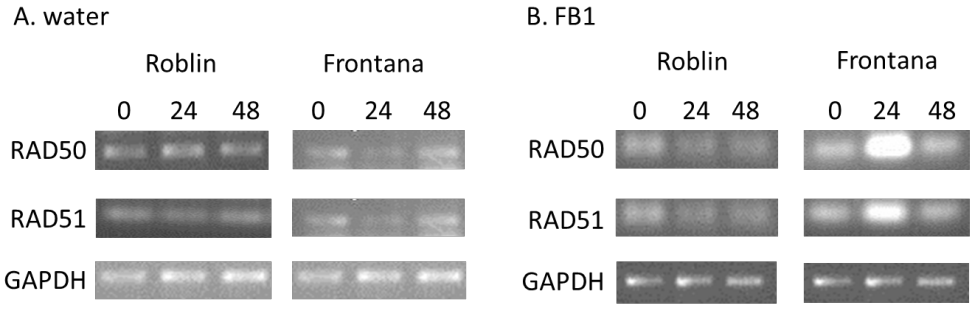

Figure 3. The expression of DNA repair genes. RNA was extracted from wheat leaves treated with water or FB1 for 0, 24 and 48 hrs. **A**. RAD50 and RAD51 gene expression in wheat leaves treated with water (control). **B**. RAD50 and RAD51 gene expression in wheat leaves treated with FB1. GAPDH gene was used as an internal standard. Three experiments were carried out with similar results.

Discussion

Plants mount numerous defense responses to survive pathogen attack. One of these is programmed cell death, which is characterized by the rapid death of plant cells at the site of pathogen infection [LAM, 2004]. Fumonisin B1, a PCD-eliciting fungal toxin, is a sphinganine analogue that has been shown to trigger dosage-dependent cell death in *Arabidopsis* that shares many features with effector-induced cell death [STONE & al. 2000; ASAI & al. 2000]. In this work, we have analyzed FB1-induced cell death in FHB resistant and FHB susceptible wheat cultivars, Frontana and Roblin, respectively. In both cultivars, cell death occurred when leaves were treated with FB1. Cell death occurred earlier in Frontana leaves than in Roblin leaves, suggesting that the particular host-pathogen interaction determines the rapidity of activation of defense responses. This can be explained by the fact that certain levels of disease symptoms in susceptible plants appear over the course of days, whereas defense responses are induced within hours in 'gene-for-gene' immunity [POZO & al. 2004]. Timely activation of cell death is necessary in some host-pathogen interactions for pathogen containment, whereas in other situations cell death is either not essential or not sufficient for disease resistance [LAM & al. 2001]. Our observations have shown more cell death occurred in Roblin leaves than in Frontana leaves after 2 to 3 days treatment with FB1, so it is possible that early activation of host cell death in Frontana would likely limit pathogen proliferation. It should also be noted that upon FB1 treatment the levels of cell death detected by aniline blue assay and by trypan blue assay correlated well, suggesting that the cell death indicated in trypan blue assay is associated with callose deposition, which is triggered by ROS. Therefore, our results suggested that cell death in wheat leaves upon FB1 treatment is associated with concurrent accumulation of ROS.

FB1 induces apoptosis-like PCD in both plants and animals [ASAI & al. 2000]. In *Arabidopsis*, FB1 treatment initiated nuclear DNA fragmentation preceding the loss of membrane integrity, which resembles apoptosis typically associated with PCD in animal cells [STONE & al. 2000]. DNA smear is observed in necrosis, which is the result of severe detrimental changes in the environment of affected cells and is not an active gene-dependent form of cell death [DANON & al. 2000]. Normally, concurrent nuclease and protease activity causes necrosis and a DNA smear on agarose gels [WYLLIE & al. 1980]. FB1 treatments triggered DNA smearing instead of laddering in our current work (Figure 2). It is possible that at certain stages nucleases and proteases are involved in FB1-induced necrosis in wheat leaves. It is also possible that wheat leaves mainly undergo necrosis instead of apoptosis (or both) with FB1 treatment.

Genes that have been identified in wheat that may have a role in DNA repair include RAD50 and RAD51 [LLOYD & al. 2007]. RAD50 was shown to contribute to DNA damage repair, DNA replication, meiosis, and telomere maintenance [CZORNAK & al. 2008; LAMARCHE & al. 2010]. RAD51 was shown to be involved in DNA strand exchange and meiosis [KHOO & al. 2008; SHINOHARA & al. 1992]. It seems possible that DNA repair mechanism may help reduce the damage in Frontana during FB1 treatment. Meanwhile, the mechanisms underlining the observed DNA smearing should be further studied for a better understanding of FB1-induced cell death.

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