The Effect of Temperature on the Hypersensitive Response (HR) in the *Brassica napus*–*Leptosphaeria maculans* Pathosystem

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**Abstract:** Temperature is considered one of the crucial environmental elements in plant pathological interactions, and previous studies have indicated that there is a relationship between temperature change and host–pathogen interactions. The objective of this research is to investigate the link between temperature and the incompatible interactions of the host and pathogen. In this study, two *Leptosphaeria maculans* isolates (HCRT75 8-1 and HCRT77 7-2) and two *Brassica napus* genotypes (Surpass400 and 01-23-2-1) were selected. The selected *B. napus* genotypes displayed intermediate and resistant phenotypes. The inoculated seedlings were tested under three temperature conditions: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C (day/night: 16 h/8 h). Lesion measurements demonstrated that the necrotic lesions from the 28 °C/22 °C treatment were enlarged compared with the other two temperature treatments (i.e., 16 °C/10 °C and 22 °C/16 °C). The results of expression analysis indicated that the three temperature treatments displayed distinct differences in two marker genes (PATHOGENESIS–RELATED (PR) 1 and 2) for plant defense and one temperature-sensitive gene BONZAI1 (BONI). Additionally, seven dpi at 22 °C/16 °C appeared to be the optimal pre-condition for the induction of PRI and 2. These findings suggest that *B. napus* responds to temperature changes when infected with *L. maculans*.

**Keywords:** *Brassica napus*; *Leptosphaeria maculans*; gene-for-gene interaction; temperature; BONZAI1 (BONI); pathogenesis-related protein (PR)

1. Introduction

Plants develop sets of mechanisms to combat the threat from phytopathogens. Plants secrete a set of metabolites, proteins and gene factors after the triggering of defense responses. According to two studies on the *Arabidopsis thaliana*–*Pseudomonas syringae* pv. *tomato DC3000*, the elicitation of innate immunity induces salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) responsive genes [1,2]; *Brassica napus* was also found to express hormone/ROS-related signals when coping with the fungal pathogen *Leptosphaeria maculans* [3–5].

Plant disease epidemics are affected by various environmental factors, including the temperature, humidity and wind; and these factors can be crucial elements influencing the development of disease in nature. Among these factors, temperature is an important element which influences both hosts and pathogens. Moreover, there is molecular evidence suggesting that these organisms (i.e., hosts and pathogens) have developed various adaptive genetic backgrounds to interact with changes in temperature by evolution; as such, certain genes related to infection/defense can be regulated to change the general physiology of an organism when temperatures reach specific levels.

In nature, there is plenty of evidence indicating that a change in temperature alters disease epidemics by changing the infectivity (from pathogens) and defense (from hosts). For example, the rust pathogen *Puccinia striiformis* is less effective when the temperature increases (starting at 15.4 °C), and the pathogen is unable to infect seedlings of wheat...
when the temperature is over 21 °C. Shayka et al., (2015) [6], by studying potato late blight, suggested that an oscillation of temperature as low as 5 °C was able to increase the infection efficiency, lesion growth development and sporulation. Iglesias et al., (2010) [7], found the maximal spor concentration of Phytophthora infestans within a temperature range of from 16 to 23 °C in Spain, and the optimal temperature for oomycete formation was 21 °C. In the Brassica napus–Leptosphaeria maculans pathosystem, an alteration in the temperature could also change the interaction between the host and fungus. One study based on the Effector–Triggered Immunity (ETI) between Arabidopsis thaliana and Pseudomonas syringae revealed that the hypersensitive response (HR) was suppressed at an elevated temperature of 28–30 °C compared with the ambient temperature (21–24 °C) [8]. Based on the B. napus–L. maculans pathosystem, Huang et al. (2006) [9], suggested that the fungus could grow faster and become more aggressive with an increased incubation temperature, and this happened in the case of both compatible and incompatible interactions. Studies have shown the connection between temperature and the expression of plant defense signaling pathways. A study regarding tobacco resistance against Tobacco Mosaic Virus (TMV) in 1992 [10] suggested that, at an elevated temperature (32 °C), tobacco compromised its salicylic acid (SA)-related resistance with a reduction in the PR1 gene expression. Compared with a lower temperature (22 °C), both free SA and conjugated SA were increased and PR1 expression was induced, which was able to cause necrotic lesions [10]. In Arabidopsis, the gene BONZAI1 (BON1) has been found to modulate the plant defense in a temperature-sensitive manner. The BON1 gene is part of the COPINE gene family, which supports plant growth and development, and negatively regulates plant defense and programmed cell death (PCD) [11–14]. The family of BON genes consists of three homologs: BON1, 2 and 3. BON1 plays a major role while the other two BON genes are more redundant. Moreover, the triple mutant of all three BON genes (bon1bon2bon3) of Arabidopsis also displays difficulty in germination [14]. Studies have suggested that BON1 positively regulates plant growth at lower temperatures, and the mutation of BON genes induces excessive cell death at 22 °C [14–16]. BON1 also represses an R gene named SNC1 (suppressor of NPR1, constitutive 1), and the BON1 mutant bon1–1 induces constitutive resistant responses [17,18]. In the field, the temperature may change in each growing season and the strength of host resistance may be affected because of changes in the climate [19]. By considering all of the information mentioned above, it is possible to conduct a set of experiments to detect the effects of temperature on the defense of B. napus against the blackleg pathogen.

In this study, a set of experiments were conducted to explore the effects of temperature upon HR resistance against L. maculans races on B. napus genotypes at the seedling stage. By analyzing the inoculated B. napus seedlings at three different growing temperatures, distinct patterns of fungal development were observed and molecular evidence suggested that the intrinsic signaling also responded differently at those temperatures.

2. Results

By measuring the lesions on the cotyledons after three temperature treatments, both Surpass400–H75 8-1/H77 7-2 and 01-23-2-1–H75 8-1/H77 7-2 exhibited phenotypes of incompatible interactions; as such, brownish necrotic lesions formed around the sites of inoculation (Figure 1). The L. maculans isolate HCRT75 8-1 (Genotype: avrLm1, AvrLm2, avrLm3, avrLm4, AvrLm1-5, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1 and AvrLepR2) induced incompatible interaction upon Surpass400 (BLMR1/LepR3 and BLMR2/LepR2) by AvrLepR2-BLMR2/LepR2 interaction, and upon 01-23-2-1 (Rlm7) by AvrLm4–7–Rlm7 interaction, respectively. The L. maculans isolate HCRT77 7-2 (Genotype: AvrLm1, avrLm2, avrLm3, AvrLm4, AvrLm1-5, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1 and avrLepR2) induced incompatible interaction upon Surpass400 (BLMR1/LepR3 and BLMR2/LepR2) by AvrLm1–BLMR1/LepR3 and upon 01-23-2-1 (Rlm7) by AvrLm4–7–Rlm7 interaction, respectively [20–22]. Generally, the results indicated that a higher temperature caused larger lesion sizes and, at 28 °C/22 °C, the lesions were the largest compared with the other two temperature treatments (Figure 2). Surpass400–H75 8-1, as the inoculation
combination showing an intermediate phenotype (22 °C/16 °C), displayed an apparent increase in lesion development when responding to an increasing temperature. The other three cases, which were usually identified as resistant interactions (i.e., Surpass400–H77 7-2 and 01-23-2-1-H75 8-1/H77 7-2), displayed relatively mild increases in lesion development as the temperature became higher.

**Figure 1.** Lesion development from six pairs of *Brassica napus* cotyledon–*Leptosphaeria maculans* isolate inoculation: Surpass400–HCRT75 8-1/HCRT77 7-2 and 01-23-2-1–HCRT75 8-1/HCRT77 7-2 at 11 days post-inoculation (dpi) with three temperature treatments: 16 °C/10 °C, 22 °C/16 °C, and 28 °C/22 °C (day/night: 16 h/8 h). Bar = 1cm.

**Figure 2.** The extent of blackleg lesion development at 11 dpi from Surpass400 and 01-23-2-1 inoculated by the isolates HCRT75 8-1 and HCRT77 7-2. The inoculated plants were exposed to three temperature treatments: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C (day/night: 16 h/8 h). The lesion development was calculated by the ratio between the area of the lesion and the area of the cotyledon. Error bars represent standard error of the mean. Different lowercase letters suggest the significant differences among mean values (Fisher’s Least Significant Difference; p < 0.05). The results are based on three replicates in three independent experiments.
Studies have suggested that BON genes are related to the growth/defense balance at a low temperature (22 °C), and BON1 has been found to play a dominant role in repressing the plant defense [11,15]. As can be seen by the expression of BON1 in Figure 3, BON1 was more pronounced at the 22 °C/16 °C condition compared with the other two conditions at 7 dpi. Similar to at 7 dpi, BON1 was still induced at 11 dpi when the plant was treated with the 28 °C/22 °C condition (Figure 4). Moreover, Surpass400–H75 8-1/H77 7-2 maintained a pronounced up-regulation in all three conditions at 11 dpi, while 01-23-2-1–H75 8-1 appeared to have higher levels of BON1 at 16 °C/10 °C than at 22 °C/16 °C. Additionally, the gene also exhibited a high level of induction at 28 °C/22 °C.

Figure 3. Gene expression of temperature-dependent regulator BONZAI1 (BON1), PATHOGENESIS-RELATED (PR)1 and PATHOGENESIS-RELATED (PR)2 (in B. napus) in the regulation of hormonal signals at 7 dpi from Surpass400 and 01-23-2-1 inoculated by the blackleg isolates HCRT75 8-1 and HCRT77 7-2. The inoculated plants were exposed to three temperature treatments: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C (day/night: 16 h/8 h). The levels of the bars are the expression levels obtained from the inoculated cotyledons (inoculated by H75 8-1 and H77 7-2) compared to the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). Error bars represent standard error of the mean. Different lowercase letters suggest the significant differences among mean values (Fisher’s Least Significant Difference; p < 0.05). The results are based on three replicates in three independent experiments.
Figure 4. Gene expression of BONZAI1 (BON1), PATHOGENESIS-RELATED (PRI1 and PRI2) in B. napus in the regulation of hormonal signals at 11 dpi from Surpass400 and 01-23-2-1 inoculated by the blackleg isolates HCRT75 8-1 and HCRT77 7-2. The inoculated plants were exposed to three temperature treatments: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C (day/night: 16 h/8 h). The levels of the bars are the expression levels obtained from the inoculated cotyledons (inoculated by H75 8-1 and H77 7-2) compared to the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). Error bars represent standard error of the mean. Different lowercase letters suggest the significant differences among mean values (Fisher’s Least Significant Difference; p < 0.05). The results are based on three replicates in three independent experiments.

According to previous studies on Arabidopsis, the up-regulation of the PRI gene was observed in the bon1 mutants at the lower temperature (22 °C), suggesting that this gene might have the function of defense suppression in some conditions, such as temperature [23]. Looking at the PRI expression in Figures 3 and 4, the genotype 01-23-2-1 exhibited the strongest induction at 7 dpi when the temperature was 22 °C/16 °C. On the other hand, for Surpass400, the optimal circumstance for the highest induction of PRI appeared to be at 11 dpi, at 28 °C/22 °C. It seemed that PRI expression in 01-23-2-1 synchronized with the expression of BON1, in which the trends of up- and down-regulation between these two genes were similar (both 7 and 11 dpi). On the other hand, PRI and BON1 generally exhibited an antagonistic relationship in Surpass400, which matched with previous studies in Arabidopsis.
Another PR gene tested was PR2. PR2 encodes an enzyme called beta-1, 3 glucanase 2. It is SA-responsive and functions as a regulator of sugar metabolism and fungal cell wall degradation [23–25]. According to Figures 3 and 4, thermal fluctuation did not seem to have apparent effects on PR2 expression. Except for Surpass400–H77 7-2 (at 7 dpi) and 01-23-2-1 – H77 7-2 (11 dpi), PR2 was highly induced at 22 °C/16 °C, suggesting that, unlike PR1, PR2 expression was only induced at 22 °C/16 °C. It seemed that PR2 had no apparent connection with BON1, but it had generally lower expression in the 28 °C/22 °C condition, which indicated that PR2 expression might be affected by other factors besides temperature and/or BON1 regulation.

3. Discussion

Our experiments suggested that the HR lesions were increased at the higher temperature treatment (28 °C/22 °C). The expression profiles of BON1, PR1 and PR2 were distinct among three temperature conditions. The temperature 22 °C/16 °C is generally optimal for PR1/2 to express. The PR1/2 was also induced (higher than water inoculation) when the temperature treatment reached 28 °C/22 °C.

Previous evidence has suggested that a change in temperature is able to alter the general defense in plants. Malamy et al., (1992) [10], observed a reduction in the SA level and PR1 expression in tobacco leaves (inoculated with tobacco mosaic virus (TMV)) when the temperature was increased to 32 °C from 22 °C; on the other hand, a higher temperature (i.e., 32 °C) enabled the virus to replicate and infect the host. Other signaling regulators, such as BON family genes, are involved in temperature-dependent regulation of plant defense.

According to previous studies on Arabidopsis, BONZAI1 (BON1) is expressed at a lower temperature (22 °C) to regulate the general plant defense [14,15]. BON genes appear to regulate SA-related signals, such as PR1, and the expression of BON1 represses SA-related pathogenesis-related proteins (PR1, PR2, and PR5) [12,15]. The results from lesion measurement matched those presented in previous studies of pathogenic development, with an increasing temperature-promoting pathogenic development and suppressing host defense [10,19,26,27]. The lesion size, especially that obtained from the Surpas400–H75 8-1 case, increased significantly at 28 °C/22 °C compared with 16 °C/10 °C and 22 °C/16 °C, whilst the other three cases (Surpass400–H77 7-2 and 01-23-2-1–H77 7-2) also exhibited moderate increases in the lesion size. The enlargement of the lesion size on those genotypes suggested that the effect of the hypersensitive response (HR) on the suppression of fungal growth was mitigated when the temperature was raised. Previous studies have indicated that the temperature has effects on the strength of Effector–Triggered Immunity (ETI). SNC1 (suppressor of NPR1, constitutive 1), as an R gene in Arabidopsis, is repressed by BON1 on its promoter region [18]. SNC1 has been found to play a role in defense against Nicotiana benthamiana, together with the N gene (another R gene specific for N. benthamiana). Moreover, the nuclear accumulation of SNC1 and N genes is reduced when the temperature is elevated [28]. SCN1 is also suppressed by factors other than BON1 at a higher temperature. In Arabidopsis, HOPZ-ETI-DEFICIENT 1 (ZED1) and ZED1-related kinases (ZRKs) suppress SNC1 expression at a temperature of 25 °C, and the mutation of ZED1 was shown to activate defense genes PR1 and PR2 at 25 °C [29].

According to the qPCR results, the genes BON1, PR1 and PR2 were found to react to the thermal changes by observing the transcriptional analyses; however, some changes did not follow the indicated rules set out in previous studies. One remarkable discrepancy is that both BON1 and PR1 displayed a high level of induction at 28 °C/22 °C. Conversely, in previous research, the expression of these genes was shown to be lower than in the other low-temperature conditions.

In the 22 °C/16 °C condition, an antagonistic relationship between BON1 and PR1 appeared to be shown. PR1 displayed a very high expression when the expression of BON1 was not very high, it was more obvious from Surpass400 (except for 11 dpi, Surpass400 H75 8-1), and this finding matched that of the repression of BON1 upon defense genes and
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4. Materials and Methods

4.1. Plant Cultivation and Temperature Treatments

Two Brassica napus genotypes (Surpass400 (BLMR1/LepR3 and BLMR2/RlmS) and 01-23-2-1 (Rlm7)) were grown under three temperature conditions: 16 °C/10 °C (day/night: 16 h/8 h); 22 °C/16 °C (day/night: 16 h/8 h); 28 °C/22 °C (day/night: 16 h/8 h). All flats with seedlings were grown under 22 °C/16 °C (day/night: 16 h/8 h) first, and the seedlings to be tested under the other two conditions were moved into the growth cabinets 24 h before inoculation.

4.2. Pathogen Inoculation

Two L. maculans isolates were selected for inoculation: HCRT75 8-1 (Genotype: avrLm1, AvrLm2, avrLm3, avrLm4, AvrLmJ1, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1 and AvrLepR2) and HCRT77 7-2 (Genotype: AvrLm1, avrLm2, avrLm3, AvrLm4, AvrLmJ1, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1 and avrLepR2).

The cotyledons of B. napus cultivars were inoculated seven days after sowing (cotyledon stage) by puncture inoculation. Each lobe of cotyledons was punctured by a sterile needle twice from each side, to have four inoculation points on each seedling of the canola plant.

4.3. Lesion Measurement

The cotyledons at 11 days post-inoculation (dpi) were scanned and the lesion size was measured by ImageJ (National Institutes of Health, Bethesda, MD, USA).

4.4. Gene Expression Analysis

Frozen cotyledons (7 and 11 dpi) were ground in liquid nitrogen with pestles and mortars. The total RNA was extracted with TRI reagent (Sigma-Aldrich) (St. Louis, MO, USA). According to the manual, the total RNA was purified by DNaseI treatment with a
recombinant DNaseI, RNase-free kit (Roche). Purified RNA was used to synthesize cDNA by employing the GOScript Reverse Transcription System (Promega). The cDNA stock solution was diluted to 100 ng/µL. Quantitative-PCR was performed by loading 1 µL of cDNA (100 ng) into the 10 µL reaction system of the IQ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA). The experiments were based on three biological replicates. The RT-qPCR experiments were run by Touch Real-Time PCR System (BioRad, Hercules, CA, USA).

The qPCR program used for all of the analysed genes (except for COI1 and ACO1) was 95 °C for 3 min, followed by 39 cycles of 95 °C for 15 s and 60 °C for 20 s. This was followed by a melting curve analysis.

All qPCR primers are compiled in Supplementary Table S1. The relative level of gene expression was analysed with the 2-ΔΔCT method described by Livak and Schmittgen, (2001) [32]. Actin was used as a reference gene to normalize the expression of the target genes.

4.5. Statistical Analysis

Unless specified, the analyses of the samples used at least three biological replicates. The statistical analyses were performed using the Tukey ANOVA method with SAS 9.4 software.

5. Conclusions

Taken together, the evidence displayed in this article suggests that infected genotypes growing at a higher temperature cause larger lesion sizes on the resistant genotype by shaping the effects of the hypersensitive response. Expression analysis revealed that, in a higher temperature condition (28 °C/22 °C), both BON1 and PRI were triggered at 7 and 11 dpi, and were presumed to have an antagonistic relationship with each other based on previous studies on Arabidopsis. The results indicated that, at a higher temperature, B. napus seems to display a balance between the plant defense and growth mechanism, at the same time as exhibiting incompatible interactions, from which the expression of defense-repressing factor BON1 and defense gene PRI coincides during a pathogen attack.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10050843/s1, Table S1: Primer list used for quantitative-PCR.

Author Contributions: Conceptualization, C.Y. and W.G.D.F.; methodology, C.Y.; validation, C.Y. and W.G.D.F.; formal analysis, C.Y.; investigation, C.Y.; resources, Z.Z.; data curation, C.Y.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y. and W.G.D.F.; supervision, W.G.D.F.; project administration, W.G.D.F.; funding acquisition, W.G.D.F. All authors have read and agreed to the published version of the manuscript. Z.Z. did data curation for the manuscript.

Funding: This research was funded by NSERC (Discovery) and NSERC-CRD.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Acknowledgments: The authors wish to thank a NSERC Discovery grant and a NSERC-CRD grant awarded to W.G.D. Fernando to carry out this work. We acknowledge the assistance of the Plant Science greenhouse staff in assisting with watering and maintaining our experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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