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Wheat blast and phenylalanine ammonia-lyase gene expression in young plants

Abstract − The objective of this work was to quantify the expression of the *PAL* gene, designed from TraesCS1D03G0078900 (Chr1D), in young plants of the BRS Louro wheat cultivar challenged with *Pyricularia oryzae*. The first symptoms of blast were observed at 48 hours after inoculation (HAI). At 120 HAI, severity was 90%. The expression of the *PAL* gene was upregulated in infected plants at 48, 72, 96, and 120 HAI. After 48 HAI, there was a reduction in gene expression, which continued until 120 HAI. The *PAL* gene located on chromosome 1D is upregulated in BRS Louro cultivar; however, it is not efficient in restricting the colonization by *P. oryzae*.

Index terms: *Pyricularia oryzae*, biotic stress, reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Brusone do trigo e expressão do gene fenilalanina amônia-liase em plantas jovens

Resumo − O objetivo deste trabalho foi quantificar a expressão do gene *PAL*, desenhado de TraesCS1D03G0078900 (Chr1D), em plantas jovens da cultivar de trigo BRS Louro desafiadas com *Pyricularia oryzae*. Os primeiros sintomas de brusone foram observados às 48 horas após a inoculação (HAI). Às 120 HAI, a severidade foi de 90%. A expressão do gene *PAL* nas plantas infectadas foi regulada positivamente às 48, 72, 96 e 120 HAI. Após 48 HAI, houve redução da expressão do gene, que continuou até 120 HAI. O gene *PAL* localizado no cromossomo 1D é regulado positivamente na cultivar BRS Louro; no entanto, não é eficiente em restringir a colonização por *P. oryzae*.

Termos para indexação: *Pyricularia oryzae*, estresse biótico, reação em cadeia da polimerase com transcrição reversa quantitativa (RT-qPCR).

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Phenylalanine ammonia-lyase (PAL, EC:4.3.1.5) is the first in the phenylpropaoids metabolism pathway. It produces secondary metabolites such as lignin, phytoalexins, and phenolic compounds, including flavonoids, isoflavonoids, coumarins, and stilbenes. PAL activity in plants is induced in response to tissue injury, the presence of pathogens, hormones, and changes in environmental factors, such as light and temperature (Ferrer et al., 2008; Huang et al., 2010).

In grasses such as barley, the *PAL* gene expression has been found to be higher in resistant cultivars inoculated with *Pyrenophora teres* and *Pyrenophora graminea* than in susceptible cultivars, despite the variation of the expression level depending on the pathogen (Arabi et al., 2020).

OsPAL1 and *OsPAL6* genes were upregulated in the resistant rice cultivar Shennong 9819, when challenged with *Rhizoctonia solani*. According to the authors, the *PAL* genes contribute to the cultivar's resistance response, along with PR genes and the transcription factor *OsWRKY30* (Yang et al., 2022).

In a study on the wide wheat (*Triticum aestivum* L.) genome, Rasool et al. (2021) identified 37 *PAL* genes distributed across chromosomes 1, 2, 6 ABD, 4A, and 5B; and high levels of *PAL* expression levels were verified in the roots of drought-tolerant wheat genotypes; however, a variation in the *PAL* gene expression was observed between genotypes. In a study on the wheat genome for *PAL* gene expression and biotic stress, Zhan et al. (2022) found that different *PAL* genes are upregulated or downregulated in wheat plants challenged with *Fusarium graminearum* or *Puccinia striiformis*; among the 54 genes identified, *TaPAL32* and *TaPAL42* were observed as being involved in stripe rust resistance (Zhan et al., 2022).

Wheat blast, caused by *Pyricularia oryzae*, is one of the most damaging diseases affecting wheat crops. In Brazil, the disease was identified first in Paraná state, in 1985, and it has since been reported in different regions worldwide, most recently in Zambia, Africa (Ceresini et al., 2019; Torres et al., 2022). Climatic changes, including prolonged rainy and warm periods, create an environment more favorable for the development of the pathogen and contribute to the spread of wheat blast in regions previously free from the disease. A disease simulation data projected the spread of wheat blast for the Southern Hemisphere and Tropics by 2040–2070 as potentially resulting in a 13% annual reduction in global wheat production (Pequeno et al., 2024). Few cultivars show a lasting resistance to the disease, which is nonexistent at the young plant stage (Cruz et al., 2010; Torres et al., 2022). There is no correlation between the severity of blast in young and adult plants. Nonetheless, recognizing *PAL* gene and understanding the changes in its expression during infection could be promising for the development of new strategies, to contain the spread of wheat blast.

The aim of this work was to quantify the expression of the *PAL* gene, designed from TraesCS1D03G0078900 (located on Chr1D), in young wheat plants challenged with *Pyricularia oryzae*.

The work was carried out at the Universidade Federal do Pampa, in the municipality of Itaqui, RS, Brazil (29º09'22"S, 56º33'03"W, at 57 m altitude). Seed of the wheat cultivar BRS Louro were surface-disinfected in NaOCl (10%) for 3 min, rinsed thoroughly in sterilized water, and sown in plastic pots (400 mL) filled with substrate composed of biosynthesized *Pinnus* bark, vermiculite, charcoal mill, water and phenolic foam. The plants were fertilized weekly with Hoagland & Arnon (1950) nutrient solution. Plants with three fully expanded leaves at stage 13 (Zadoks et al., 1974) were inoculated with a conidial suspension at 1 x 10^5 conidia mL^{-1} *P. oryzae*. Thirty minutes after inoculation, the plants were transferred to a growth chamber set to 25±2 ºC, at 90±5% relative humidity, and darkness for 24 hours. After that, the plants were transferred to the laboratory and kept under ambient condition (±23 ºC) until the end of the experiment.

For gene expression analyses, the sections showing blast symptoms on the third leaf of each plant were collected at 48, 72, 96, and 120 HAI. Leaves were frozen in liquid nitrogen, and total RNA was extracted using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA), following the manufacturer's recommendations. The concentration of total RNA was measured using a NanoVue Plus spectrophotometer (GE Healthcare Ltd, Little Chalfont, Buckinghamshire, UK), and RNA quality was assessed by examining the integrity of ribosomal RNA bands in 1.5% agarose gel. First-strand cDNA synthesis was performed from 5 µg of total RNA, using the GoScript reverse transcriptase (Promega) and an Oligo $(dT)_{12-18}$ primer, according to the manufacturer's recommendations. The *PAL* (E4.3.1.24) gene sequence was obtained from the literature (Zhang et al., 2011). The forward (5-3) primer sequence was CGTCAAGAGCTGTGTGAAGATGG, and the reverse (5-3) primer sequence was G G T A G T T G G A G C T G C A A G G G T C . These primers were designed based on the accession TraesCS1D03G0078900, transcript TraesCS1D03G0078900.1, located on Chr1D20552688.20561537 reverse. Ubiquitin was used as the internal reference gene (Van Riet et al., 2006). The forward (5-3) primer sequence for ubiquitin was CCTTCACTTGGTTCTCCGTCT, and the reverse (5-3) primer sequence was AACGACCAGGACGACAGACACA. Reactions using RT-qPCR (20 µL) were prepared with the following components: 10 µL of 2x SYBR Green PCR Master Mix (Qiagen, Germantown, MD, USA), 5 µL cDNA

(20 ng μL^{-1}), 2 µL ROX, 0.8 µL *PAL* primers (10 µmol L^{-1}), 1.2 µL of *UBI* primers (10 µ mol L^{-1}), and water, to reach the final volume. The amplification conditions were performed according to the manufacturer's recommendations on a StepOne Real-Time PCR System instrument (Life Technologies, Carlsbad, CA, USA). Single-fragment amplification was verified by dissociation curve analysis. Relative expression levels were calculated using the $2^{\Delta\Delta ct}$ method (Livak & Schmittgen, 2001). The experiment was carried out in a completely randomized design, with four replicates. Each replicate consisted of six plants in a plastic pot. Noninoculated plants were designated as the control. Gene expression data were subjected to the analysis of variance. To study the relationship between HAI (x) and gene expression (y), the exponential nonlinear regression model was used, whose equation is $y = a \times \exp(b \times x)$, where the constants a and b are parameters estimated by the least squares method. The model adjustment and the parameter estimations were done using the R software (R Core Team, 2024). Blast severity was evaluated on the third leaf from the base of the plant, using a diagrammatic scale with severity values from 0 to 100%. The experiment was repeated once.

At 48 HAI, plants exhibited 10% to 15% of the leaf area with chlorotic lesions, which gradually evolved into necrotic lesions, covering approximately 90% of the leaf area by 120 HAI (Figures 1A and 1B). The expression of the *PAL* gene was higher in plants inoculated with *P. oryzae* than the noninoculated control plants. No significant difference in the *PAL* gene expression (ranging from 3.6 to 0.84 fold) was observed across the collection periods of the experiment. However, an exponential reduction of the expression was noted as the HAI increased (Figure 1 C). For each additional HAI, the expression decreased by 0.0291, approximately 3%. At 48 HAI, the first blast symptoms appeared on the leaves, and the *PAL* expression increased (3.6 fold).

According to Cruz et al. (2016) the infection process by *P. oryzae* on wheat is characterized by the germination of conidia around 6 HAI, appressorium differentiation at 12 HAI, and degradation of waxes on the leaves surface, caused by enzymes released by the fungus. And, from 36 HAI to 72 HAI, conidium and appressorium were dehydrated and withered (Cruz et al., 2016). At 48 HAI, the percentage of colony formation of *P. oryzae oryzae* in the leaves of the wheat cultivars Anahuac (susceptible), Ônix, BRS 229, and BR 18 was 71%, 43%, 18%, and 2%, respectively (Chaves et al., 2022). Therefore, the expression at 48 HAI is closely linked to the recognition of the pathogen by the plant and to the plant attempt to activate its defense pathways to prevent or reduce colonization, even in a susceptible genotype.

Rios et al. (2014) verified an increase in PAL enzyme activity at 48 HAI in young plants of the BR 18

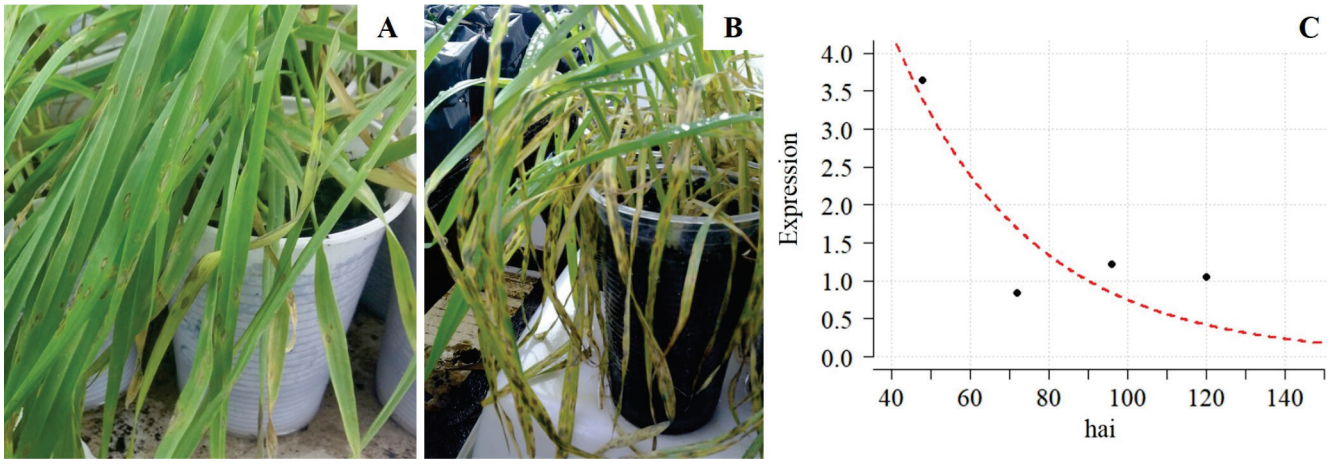

Figure 1. Wheat (*Triticum aestivum*) BRS Louro cultivar inoculated with *Pyricularia oryzae* at the following times: A, 48 hours after inoculation (HAI); B, 120 HAI; C, relative quantification of the *PAL* gene expression on leaves, from 48 to 120 HAI. Photos by Maria Fernanda Antunes da Cruz.

(susceptible) and BRS 229 (moderately resistant) wheat cultivars. This observation indicates that the gene expression is effectively translated into protein, at the beginning of the infectious process in both cultivars, especially in the susceptible one.

Using scanning electron microscopy (SEM), Cruz et al. (2016) observed an extensive colonization of *P. oryzae* on wheat leaf tissues, and the initiation of leaf degradation at 96 HAI, and by 120 HAI, numerous conidiophores and conidia occupied part of the necrotic tissue, along with germinated conidia.

In the present study, the expression levels were downregulated, after 48 HAI, by approximately 2.5 fold until the end of the experiment 120 HAI, suggesting that the reduced expression correlates with a greater extent of pathogen colonization and disease severity in the BRS Louro. This cultivar shows variation for the degree of severity in young plants, when challenged with different *Pyricularia* isolates (Lorenset et al., 2021); however, in the present study, it was highly susceptible.

Recently, Zhan et al. (2022) conducted a multigenome analysis of the *PAL* gene family in wheat, and they verified different expression patterns for various diseases. When subjected to the inoculation of *Fusarium graminearum*, *Puccinia striiformis*, and powdery mildew, ten *PAL* genes were highly induced, which were identified as *TaPAL3, TaPAL10, TaPAL13, TaPAL14, TaPAL17, TaPAL30, TaPAL31, TaPAL32, TaPAL42,* and *TaPAL48*.

The gene used in the present experiment corresponds to the *TaPAL11* from the study conducted by Zhan et al. (2022), whose RNA-seq analysis showed that this gene was not differentially expressed in the following situations: in wheat spikelets at anthesis, inoculated with *F. graminearum* at 3, 6, 12, 24, 36, and 48 HAI; and in 7-day-old leaves inoculated with rust and powdery mildew at 24, 48, and 72 HAI.

Similar results were found in the present study from young plants inoculated with *P. oryzae*. Putative cis-regulatory elements were identified in the promoter region of *TaPAL* genes (Zhan et al., 2022). In *TaPAL11*, regulatory elements were associated with growth and development (33), biotic and abiotic stress (6), and phytohormones (10).

In the present work, no differential expression was observed for *PAL* gene in young plants. However, in an experiment with adult plants, Cruz et al. (2015) observed differential expression of the gene in the flag leaves of the BR 18 wheat cultivar challenged with *Pyricularia* at 24, 48, 72, 96 HAI, in comparison with control plants.

BR 18 Terena is a Brazilian wheat cultivar that has shown quantitative resistance to blast in adult wheat, and it has been widely used in breeding programs since its release in 1986 (Ceresine et al., 2019; Goddard et al., 2020; Torres et al., 2022). When analyzing the transcriptome of 14-day-old wheat seedlings inoculated with adapted and nonadapted *Pyricularia* isolates, Tufan et al. (2009) found differential expression of *PAL* only at 4 HAI, in seedlings challenged with the nonadapted isolates. There was a difference in the expression between the adapted isolates at sampling times, and the highest expression occurred between 18 and 24 HAI. However, there was no difference in the expression of the *PAL* gene at 48 and 72 HAI, in the genotype considered partially resistant to blast (Tufan et al., 2009). Early induction of *PAL* may indicate the deposition of phenolic compounds in the halo structure, beneath the non-virulent isolate only. Our research data corroborates with the observations made by Tufan et al. (2009) at 48 and 72 HAI in the genotype BRS Louro.

According to Rasool et al. (2021) and Zhan et al. (2022), more than 50 *PAL* genes have been identified in wheat. However, it is not yet known which specific genes they are or what expression patterns to expect in their interactions with different pathogens.

The *PAL* gene sequence on 1D chromosome used in the present study was upregulated, comparison with the control plants. Even in susceptible plants, there was an increase in the gene transcription above basal levels up to 120 HAI with *Pyricularia*. The reduction of the gene expression after 48 HAI reflects an increased tissue colonization by the pathogen. In other words, without the activation of the phenylpropanoid pathway, which leads to the production of flavonoids and lignin, the fungal hyphae can spread more easily through the tissue, causing extensive areas of necrosis.

Although *PAL* genes are not the only ones involved in plant defense responses to pathogens, their activation in young wheat plants at the beginning of *Pyricularia* infection appears to be related to a potential resistance response to blast (Tufan et al., 2009). In young plants, wheat genotypes resistant to blast have limited the defense mechanisms, which are related to the inhibition

of conidial germination, as well as to the growth of infective structures, the formation of appressoria, and the production of physical and chemical barriers (Chaves et al., 2022). In this context, some questions still need further investigation, such as follows: Which genes from the *PAL* family are involved in the wheat blast resistance? Would these genes be efficient for selecting resistant cultivars at different stages of plant development? What are the transcription factors involved in *PAL* gene expression in the wheat-*Pyricularia* interaction? How do these transcription factors modulate the expression of the *PAL* gene? Are these transcription factors present in both young and adult plants?

Therefore, the *PAL* gene located on chromosome 1D is upregulated in BRS Louro cultivar; however, it is not efficient in restricting *Pyricularia oryzae* colonization.

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Author contributions

Maria Fernanda Antunes da Cruz: conceptualization, investigation, methodology, data curation, writing – original draft, writing – review & editing; **Franciele Cabral Pinheiro**: investigation, methodology; **Gilberto Rodrigues Liska**: methodology, statistical analyses, writing. conceptualization, investigation, methodology, writing – original draft.

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Data availability statement

The data supporting the findings of this study are available in the article. Should any raw data be needed, they will be provided by the corresponding author upon reasonable request.

Declaration of use of AI technologies

No generative artificial intelligence (AI) was used in this study.

Conflict of interest statement

The authors declare no conflicts of interest.

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