Tech Science Press

Deterioration of Antioxidant Competence in Barley Lesion Mimic Mutant 194

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Abstract: A barley mutant, 194, was observed to exhibit a leaf spot phenotype over the whole course of its growing period. In this study, the phenotype and antioxidant competence were studied in the lesion mimic mutant 194. Plant height was slightly higher in mutant 194 than in the wild type (WT). In addition, leaf spot per plant in mutant 194 was significantly higher than in WT. Antioxidant competence, as indicated by reactive oxygen species (ROS) accumulation, antioxidant enzyme activity, and the expression of antioxidant enzyme-encoding genes was also assessed in mutant 194. Compared to the WT, mutant 194 displayed a relatively higher accumulation of ROS, accompanied by lower activities of some antioxidant enzymes and downregulation of antioxidant enzyme-encoding genes. This demonstrated reduced antioxidant competence in mutant 194. The results suggested that this lower antioxidant competence of mutant 194 could lead to the accumulation of excessive ROS. This excess of ROS could induce programmed cell death and has the potential to promote disease resistance in mutant 194.

Keywords: Lesion mimic mutant; ROS accumulation; antioxidant enzymes; barley

1 Introduction

Lesion mimic mutants (LMMs) display spontaneous programmed cell death (PCD) under normal growth conditions, form disease spots on the leaves. LMMs have been widely used as models in the fundamental research on disease resistance mechanisms and for deciphering cell death signaling pathways [1].

The first LMM was discovered in maize in the 1920s [2]. At least 100 LMMs have been identified over the past four decades, including those in *Arabidopsis* thaliana [3], maize [4], rice [5] and barley [6]. With the completion of the genome annotations for each species, more and more lesion mimic genes have been localized and cloned [7]. To date, 49 lesion mimic genes have been cloned and identified; two of them are from barley, namely the *nec1* gene [8] and the *tigrina-d.12* gene [9], which are homologous genes of *hlm1* (At5g54250) and *flu* (At3g14110) in *Arabidopsis thaliana*, respectively. The FLU protein contains a C-terminal tetratricopeptide repeat (TPR) domain that regulates chlorophyll biosynthesis. Hence a lack of this protein in the LMM causes a type of lesion that occurs in mature leaves, and the seedlings appear albino when the conditions change from dark to light [7,10]. In addition, some disease resistance genes appear to be associated with the lesion mimic phenotype. For example, possessing the recessive mutation of the *mlo* gene improves the broad-spectrum resistance of barley to *Blumeria graminis* f. sp. *hordei*; while under the condition of no pathogen infection, *mlo* plants have a phenotype with cell wall thickening and disease spots [6,11]. The LMM genes were classified as either PCD inhibition or PCD excitation pathway genes according to the phenotype of the LMM and its relationship

with PCD. Mutants in which the PCD pathway is inhibited, are also called “lesion propagation type” as they cannot limit the spread of a spot. The PCD excitation pathway LMMs are also called “lesion initiation type” and can randomly and spontaneously develop spots on leaves or other tissues [12].

The mechanism underlying the lesion mimic phenotype is complex; various factors could cause this kind of phenotype, including enzymes, signaling molecules, and PCD [13]. Abnormal expression of disease resistance genes could also disturb the defense response signaling pathway, leading to cell death and the generation of the lesion mimic phenotype. This is seen with the *OsATL* [14] and *NLS1* [15] genes in rice. In some cases, LMMs express cytological and biochemical markers that have been associated with disease-resistant responses, and exhibit local and systemic resistance to a variety of pathogens that usually cause disease [16]. In addition, the accumulation of reactive oxygen species (ROS) and the activities of ROS-related enzymes, such as superoxide dismutase (SOD) and catalase (CAT), play important roles in the formation of the lesion mimic phenotype [17,18]. For example, the light-dependent LMM, *lm3*, shows adult-plant resistance to powdery mildew in common wheat because of the accumulation of ROS [19]. Furthermore, the LMM, *lmm6*, was more resistant than the wild type (WT) to rice blast fungus *Magnaporthe grisea*, and had a higher accumulation of ROS and a lower SOD enzyme activity [20].

This study focused on a novel, stable, inherited LMM of barley (*Hordeum vulgare*), which is both an economically important cereal and a model member of the Triticeae tribe. We compared the phenotype and antioxidant competence of the LMM and the WT. The results clearly showed that the ROS content was upregulated in the LMM, which will provide a basis for further study and application of LMMs.

2 Materials and Methods

2.1 Plant Materials

A barley LMM, 194, was generated via mutagen breeding in our laboratory, using the mutagen EMS (ethyl methane sulfonate) applied to ‘Tamalpais’.

2.2 Statistical of Lesion Mimic in Leaf

The area of lesion mimic was measured using the ImageJ program (ImageJ 1.44p, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

2.3 Determination of Superoxide Radical (O_2^-) and Hydrogen Peroxide (H_2O_2)

The O_2^- assay in leaves was performed using the method, as described by Hui et al. [21]. The H_2O_2 content was determined according to Sairam and Srivastava [22].

2.4 Determinations of Antioxidative Enzyme Activities

The activities catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) were detected based on previously described methods: catalase (CAT) [23], superoxide dismutase (SOD) [24], guaiacol peroxidase (POD) [25], ascorbate peroxidase (APX) [26], All samples were conducted with a Shimadzu (UV-2550) spectrophotometer (Shimadzu, Japan).

2.5 Quantitative Reverse Transcription PCR Analysis

Total RNA was extracted from the leaves with TransZol (TransGen Biotech, China), and treated with DNase I (RNase-free, Promega). qRT-PCR was carried out in a 25 μL reaction volume containing 2 × TransStart Top Green q-PCR SuperMix (TRANS, China). Quantitative analysis was performed using the Bio Rad CFX Manager system. This method normalizes the expression of a specific gene versus a control reference with the formula $2^{-\triangle\triangle CT}$. *Actin* was evaluated as the control genes [27]. Information on the genes analyzed is listed in Tab. 1.

Table 1: Primers used in the current study

Target Genes		Primer sequence (5'-3')	Accession code	Application
HvActin	Forward	TCGCAACTTAGAAGCACTTCCG	AK362208	qRT-PCR
	Reverse	AAGTACAGTGTCTGGATTGGAGGG		qRT-PCR
Cu/Zn SOD	Forward	CCCCTCACCAAGTCAGTCAT	AK252295	qRT-PCR
	Reverse	ATTGCAAGTCGGTGTCCTTC		qRT-PCR
HvCAT1	Forward	TGGACGGATGGTACTGAACA	AF021938	qRT-PCR
	Reverse	GTGCCTTTGGGTATCAGCAT		qRT-PCR
HvAPX1	Forward	CGCCCTCTTGTGGAGAAATA	AS006358	qRT-PCR
	Reverse	CGCGCATAGTAGCAGCAGTA		qRT-PCR

2.6 Statistical Analysis

All experiments and determinations were conducted at least in triplicate. The IBM SPSS statistics program was used to perform the statistical analyses. All pairwise comparisons were analyzed using Duncan's test. Differences between the mean values were compared using Duncan's multiple range tests at 0.05 probability levels.

3 Results

3.1 Comparison Between the Phenotype of WT and Mutant 194

We compared the phenotype of the WT and mutant 194 (Fig. 1(A)). The plant height of mutant 194 was significantly higher (approximately 1.09 times) than WT (Fig. 1(B)), but there was no obvious difference in the tiller number (Fig. 1(C)). Furthermore, the leaf spot per plant of mutant 194 was significantly higher (7.75 times more) than that of WT (Fig. 1(D)).

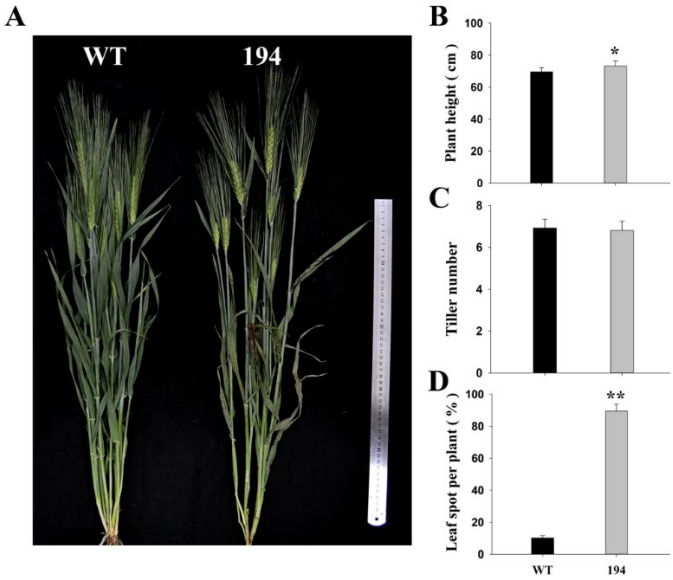

Figure 1: The phenotypic differences between WT and LMM 194 at 10 days after flowering. The parameters include (A) the phenotype; (B) the plant height; (C) the tiller number; and (D) the leaf spot per plant. Values are the means calculated from 30 replicates. Error bars indicate standard deviations, *, $p < 0.05$; **, $p < 0.01$

The spot area per leaf was also observed and quantified between wild-type and mutant 194 plants (Fig. 2(A)). The spot area per leaf in mutant 194 plants was significantly larger than in the WT (Fig. 2(B)), which was consistent with the leaf spot per plant comparison (Fig. 1(D)).

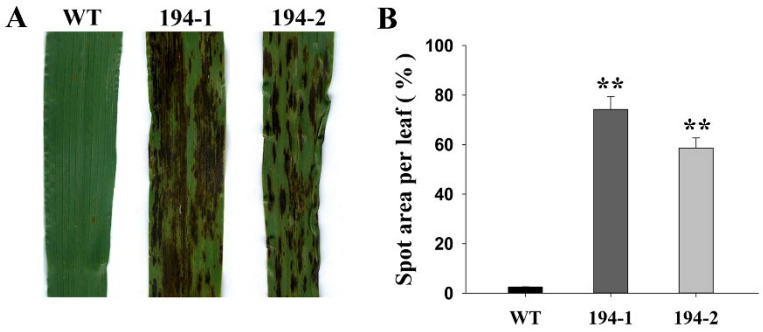

Figure 2: The variation in area of spots per leaf between WT and LMM 194 at 10 days after flowering. The parameters include (A) the spot area phenotype and (B) the spot area per leaf in wild-type and LMM 194 plants. Values are means calculated from 30 replicates. Error bars indicate standard deviations, *, $p < 0.05$; **, $p < 0.01$

3.2 Antioxidant Competence in WT and Mutant 194

The accumulation of ROS was detected in wild-type and mutant 194 plants. As shown in Fig. 3(A), mutant 194 showed relatively higher H_2O_2 accumulation than the WT. The change in O_2^- production rate was consistent with the H_2O_2 accumulation; the mean of the mutant 194 O_2^- production rates was significantly higher (31.2%) than the WT production rate (Fig. 3(B)).

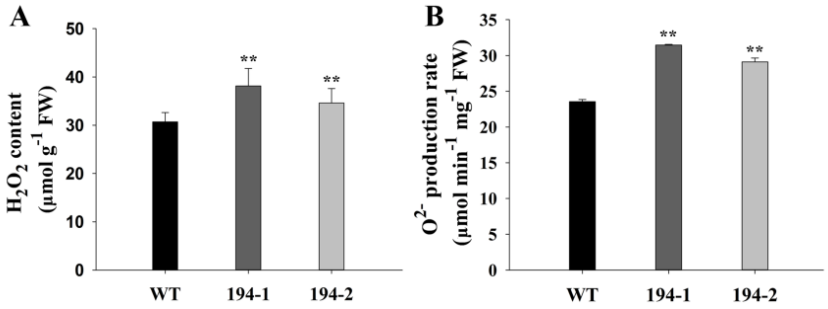

Figure 3: Changes in the accumulation of ROS between WT and LMM 194 at 10 days after flowering. The parameters include (A) the H_2O_2 content and (B) the O_2^- production rate. Values are means calculated from three replicates. Error bars indicate standard deviations, *, $p < 0.05$; **, $p < 0.01$

The activities of antioxidant enzymes were then measured. The change in the trends of CAT and peroxidase (POD) activity was consistent with that of SOD activity. The activities of SOD (Fig. 4(A)), CAT (Fig. 4(B)), and POD (Fig. 4(D)) in the mutant 194 plants were lower than in wild-type plants (approximately 0.89, 0.88, and 0.92 times lower, respectively). There was no significant difference in the activity of ascorbate peroxidase (APX) between wild-type and mutant 194 plants (Fig. 4(C)).

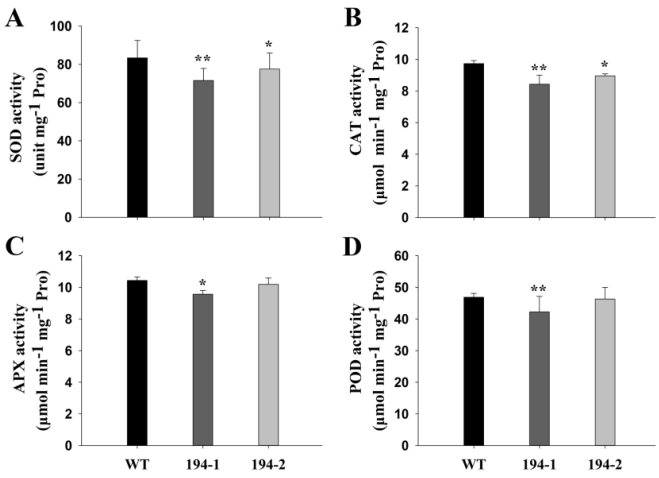

Figure 4: Antioxidant enzyme activities in WT and LMM 194 at 10 days after flowering. The parameters include (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) ascorbate peroxidase (APX), and (D) peroxidase (POD). Values are means calculated from three replicates. Error bars indicate standard deviations, *, $p < 0.05$; **, $p < 0.01$

To validate the changes observed in enzymatic activity, the expressions of parts of the antioxidant enzyme-encoding genes were analyzed. *Cu/Zn-SOD* encodes a chloroplastic copper/zinc superoxide dismutase, and *CAT* encodes a catalase. The expression of *Cu/Zn-SOD* in mutant 194 plants was significantly lower than in the WT (Fig. 5(A)). The trends of *HvCAT2* and *HvAPX* expression were consistent with the changes in the *Cu/Zn-SOD* expression; they were significantly lower in mutant 194 plants than in the WT (Figs. 5(B) and 5(C)).

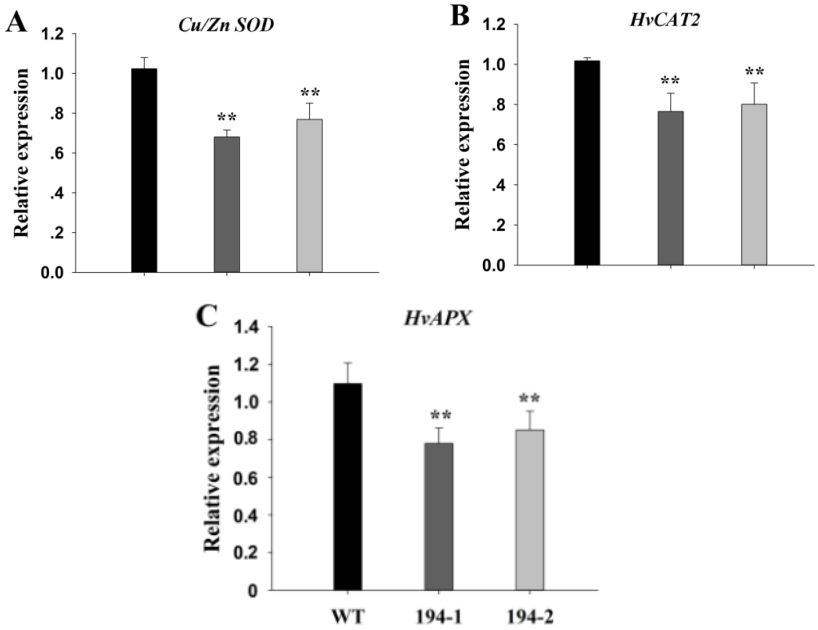

Figure 5: Relative expression of genes related to antioxidant enzymes in WT and LMM 194 at 10 days after flowering. The parameters include the expression of (A) *Cu/Zn-SOD*, (B) *HvCAT2*, and (C) *HvAPX*. Values are means calculated from six replicates. Error bars indicate standard deviations, *, $p < 0.05$; **, $p < 0.01$

4 Discussion

Barley (*H. vulgare*; 2n = 2x = 14) is the fourth largest cereal crop in the world and has many advantageous properties such as stress tolerance and high yield [28]. What's more, the genome of barley is approximately 5 Gb in size, relatively simple, and is highly morphologically and genetically diverse. Therefore, barley is considered to be an ideal model plant within the Triticeae. Here, we constructed an LMM of barley using ethyl methanesulfontae (EMS). Researching the barley mutant will improve our knowledge on the lesion mimic phenotype within the Triticeae tribe.

The disease spot phenotypes of LMMs are diverse, varying in parameters such as shape, color, and size. LMMs have been classified into two types: an "initiation type" and a "propagation type" [29]. For example, the *Arabidopsis* mutants *acd5*, *acd6* [30], and *lsd2* [16] are "initiation type", and *svn1* [31] and *acd11* [32] are "propagation type". In the present study, the spots appeared on the leaf of LMM 194 at the three-leaf stage, then the spots spread to the whole plant as the plant matured. Hence, we categorized mutant 194 as "propagation type".

In wheat, the yield components and plant height of the LMMs are similar with those of the mother line [33], indicating that the presence of the lesions mimic phenotype in the mutant did not have a substantial effect on its agronomic performance. However, lower values were obtained for agronomic traits such as stature, tiller number, and panicle number, and a lower yield potential was detected in the rice *bl2* LMM compared with the WT [34]. Furthermore, the tiller number and seed-setting rate of the wheat LMM *I30* were found not to be significantly different from the WT, but the thousand-seed weight and yield were reduced [35]. In the current study, the plant height of mutant 194 was significantly higher than the WT (Fig. 1(B)), but there was no obvious difference in the tiller number. Apparently, different lesion mimic genes have multiple functions in agronomic performance, which is probably because each gene participates in a different regulation pathway.

The accumulation of ROS is one of the earliest events in plants under pathogen attack [36], and it can induce PCD [37]. A ROS burst has been proven to be associated with various defense responses, such as the salicylic acid [38] and Ca^{2+} signaling [39]. According to previous studies, the content of ROS in LMMs was much higher than in wild-type *Arabidopsis* [40], rice [20], wheat [33], and barley [41]. In the current study, the ROS content in the barley LMM 194 was significantly higher than in the WT (Fig. 3). It is likely that mutant 194 has potential disease resistant properties. ROS can oxidize DNA, cytomembranes, and proteins, which can lead to cell death in the plant [42]. Foyer and Noctor [43] demonstrated that antioxidant enzymes could scavenge the ROS and maintain better plant growth under various stresses. Furthermore, the lower antioxidant competence of mutant 194 manifested as decreased activities of SOD, CAT, and POD enzymes (Fig. 4). These results were consistent with the expression of antioxidant enzyme genes (Fig. 5). These results suggest that the increased ROS content is mainly due to the decrease in antioxidant competence.

5 Conclusions

Based on the results of the present study, we hereby propose a potential mechanism of disease resistance in the barley LMM 194. The lower antioxidant competence of LMMs could cause accumulation of excess ROS. This excess ROS could then induce PCD and has the potential to confer disease resistance in mutant 194.

Acknowledgments: We thank Professor Daolin Fu (University of Idaho) and Jiajie Wu (Shandong Agricultural University) for providing EMS mutants of lesion mimic. This work was supported by the Science and Technology Plan Projects of Zaozhuang (2019NS01), Doctoral Research Initiation Funds of Zaozhuang University (2018BS043), Provincial Science and Technology Plan for Colleges in Shandong Province (No. J17KA151), and National Innovation and Entrepreneurship Training Program for College Students (No. 201710904074).

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