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Detection of Resistance Related Gene to Net Blotch Disease in Some Barley Genotypes

A.I.S. Al-Masry*; K.I. Zaki*; M.F. Salem; Kh.A. El-Halfawy** and S.A. Afiah*****

* *Plant Protect. Dept., Desert Res. Centre, El-Matarya, Cairo, Egypt.*

** *Genetic engineering & Biotechnol. Inst., Menofiya Univ., Sadat City, Egypt.*

*** *Plant Genetic Resources Dept., Desert Res. Centre, El-Matarya, Cairo, Egypt.*

Distinct morphological differences were demonstrated within single-spores of five *Drechslera teres* isolates, obtained from Siwa, Maryout and Ras Sudr Agricultural Experimental Stations of Desert Research Centre (DRC). The morphological parameters data classified *D. teres* isolates to different five isolates which showed great differences among them. Where, *D. teres* isolates spore length ranged from 6.41 µ and 9.49 µ for the isolate No. 3 and No. 2, respectively. In addition, similarity in the number of septa was found, where the spore septa length ranged from 0.79 µ to 5.9 µ for isolates No. 3 and No. 4, respectively. Moreover, there was a slight difference in the length of the germ tube.

Under greenhouse conditions, the five *D. teres* isolates showed different levels of virulence, where isolates No. 3 and No. 4 were the most virulent, while isolate No. 2 was the least virulent. On the other hand, barley genotypes varied in their reaction to *D. teres* isolates, where, genotypes L26, G126, and G131 were classified as moderately resistant while genotypes L3, H6, H7 and H10 were classified as resistant.

The PCR product using degenerate primer of *MLO1* indicated the appearance of one fragment sizes about 500 bp. This was detected in four resistant barley genotypes (L3, H6, H7 and H10), but was absent in the moderately resistant genotypes (L26, G126 and G131). Therefore, this study may add a molecular based value to the application of *MLO1* in plant diseases molecular diagnostics for detection of net blotch resistance in different barley genotypes. This may indicate that such barley genotypes could be cultivated in different geographic locations according to their response to biotic stress by different *D. teres* isolates.

Keywords: Barley genotypes, *Drechslera teres*, gene *MLO1*, morphological parameters, PCR and virulence.

Barley (*Hordeum vulgare* L.) is one of the principal cereal crops in the world and is cultivated in all temperate areas (Von Bothmer *et al.*, 1995). Barley net blotch caused by the Ascomycetes fungus *Pyrenophora teres* f.sp. *teres* Smedeg. [Anamorph: *Drechslera teres* (Sacc.) *Shoem.* f.sp. *teres* Smedge.], is one of the most widely distributed foliar diseases of barley (Steffenson and Webster, 1992). The pathogen causes lesions that initially appear as spots and short yellow streaks on leaves, and the lesions can expand into longer longitudinal and transverse necrotic streaks on susceptible genotypes. Typical yield losses due to net blotch nearing

100% in some highly susceptible barley cultivars but losses of 10% to 40 % are more common with this disease (Mathre, 1982). *Pyrenophora teres* is a haploid fungus, thus it is impossible to characterize dominance. Therefore, these genes could code for virulence gene products used by the pathogen to exploit the host or they could code for avirulence gene products that are recognized by the host to signal a resistance response. Either way these genes are important in the host pathogen interaction and further characterization of these genes is needed (Zhibing *et al.*, 2007).

The identification of resistant germplasm in wild species of agronomic crops and the subsequent introgression into commercial cultivars has been the major focus of many plant breeders for the better part of the 20th century (Adawy *et al.*, 2008 and Saker *et al.*, 2005). Furthermore, most plant breeders chose to employ single dominant and semi-dominant genes in their breeding programs because of the ease and efficacy in which they could be introduced into agronomical acceptable cultivars (Baker *et al.*, 1997). However, plant disease resistance genes (R genes) encode proteins that detect pathogens. R genes have been used in resistance breeding programs for decades, with varying degrees of success. Recent molecular research on R proteins and downstream signal transduction networks has provided exciting insights, which will enhance the use of R genes for disease control (Morel and Dangl, 1997). Definition of conserved structural motifs in R proteins has facilitated the cloning of useful R genes, including several that are functional in multiple crop species and/or provide resistance to a relatively wide range of pathogens. Numerous signal transduction components in the defence network have been defined, and several are being exploited as switches by which resistance can be activated against diverse pathogens (McDowell and Woffenden, 2003).

R gene-mediated resistance has several attractive features for disease control. When induced in a timely manner, the concerted responses can efficiently halt pathogen growth with minimal collateral damage to the plant. No input is required from the farmer and there are no adverse environmental effects. Unfortunately, R genes are often quickly defeated by co-evolving pathogens (Pink, 2002). Many R genes have a narrow range of resistance, often to only one or a few strains of a single pathogen species (although some R genes do provide a wider spectrum, as noted above). One crucial aspect of this strategy lies in selecting the right promoter to drive the *Avr* gene. An ideal promoter would respond rapidly to a wide variety of pathogens and thereby provide broad-spectrum resistance. The promoter must be inactive under disease free conditions to ensure that the plant does not sustain collateral damage from spurious defence responses triggered by leaky expression of the *Avr* transgenic. Datasets from micro-array experiments will aid in the identification of useful promoters. In a different approach, synthetic promoters were engineered by combining *cis* regulatory elements that had been previously associated with defence (Rushton, 2002). Some synthetic configurations were strongly induced by pathogens, while remaining quiescent under disease free conditions. These promoters might prove to be important tools for engineering R/*Avr* resistance as well as for other strategies, such as localized expression of antimicrobial proteins (Pan *et al.*, 2000 and Van der Biezen, 2001).

Monogenic resistance mediated by recessive (*mlo*) alleles of the *Mlo* locus is different. Apart from being recessive, it differs from race-specific incompatibility to single pathogen strains in that (1) it confers a broad spectrum resistance to almost all known isolates of the fungal pathogen, (2) *Mlo* resistance alleles have been obtained by mutagen treatment of any tested susceptible wild-type (*Mlo*) variety, and (3) the resistance is apparently durable in the field despite extensive cultivation in Europe. Finally, under pathogen-free or even axenic conditions, *Mlo* plants exhibit a spontaneous leaf cell death phenotype, preceded by the appearance of characteristic cell wall appositions (Wolter *et al.*, 1993). The main objectives of the present study were to realize the relation between virulence of five *Drechslera teres* isolates and disease resistance of seven barley genotypes, under greenhouse conditions, as well as detection of *MLO1* gene in certain barley genotypes with specific reference to net blotch resistance.

Materials and Methods

1- Plant Materials:

Seven barley genotypes, five newly bred lines and two Egyptian varieties (Table 1), previously selected as divergent genetic material under edaphic and climate conditions of Maryout (North western coast of Egypt), Ras Sudr Agric. Experiment. Station, Siwa Oasis, and Experimental Field of Menofiya Univ. at Sadat City (Al-Masry, 2006). During 2007/2008 growing season, these genotypes were tested under DRC greenhouse conditions for reaction to infection with net blotch disease.

Table 1. Pedigree and classification of barley varieties/lines under investigation

Name	Caryopsis type	Pedigree and/or selection history
Line 3	Hulled	Giza126/(ICB 82-1451-8AP-OAP-9AB-0TR) $F_8$3Sel,Mar.
Line 26	Hulled	Giza126/(Arar//2762/BC-2L-2Y-ICB83-0687-7AP-0AP-1AP) $F_8$26Sel,Mar.
Giza126	Hulled	Baladi Bahteem/SD 729-Por12762-BC
H6	Hulles	Giza126/(ICNB F8 - 654 Sel, 5AP) F_8 H6 Sel, Mar.
H7	Hulles	Giza126/(ICNB F8 - 654 Sel, 5AP) F_8 H7 Sel, Mar.
H10	Hulles	Giza126/(ICNB F8 - 654 Sel, 5AP) F_8 H10 Sel, Mar.
Giza131	Hulles	CM67-B/CENTENO//CAM-B/3/ROW906. 73/4/GLORIA-EAR/COME-B/5/FALCON-BAR/6/LINO

2- Isolation of the pathogen:

Samples of naturally infected barley seeds, with net botch symptoms, were collected from grain yield in season 2006/2007. Also, infected leaves were collected during season 2007/2008 from different barley genotypes grown in three locations; Siwa Oasis, Maryout and Ras Sudr Agric. Experimental Stations of DRC.

a-Isolation from infected seeds:

Seeds of barley genotypes (L3, L26, G126, H6, H7, H10 and G131) were obtained from plants grown in season 2006/2007 at Siwa Oasis, Maryout Agricultural Experimental Station, Ras Sudr Agric. Experiment. Station of DRC. The fungus was isolated from the infected seeds by sanitizing the seeds by immersion in 2% aqueous sodium hypochlorite solution for 2 min, washing them 3 times in sterile water and incubating them on wet filter paper in a tray for 10 days at 24°C with a 12 h photoperiod. Conidia of *D. teres* were collected from each seed and inoculated onto plates of PDA which were also incubated for 7 days at 24°C with a 12 h photoperiod. After confirmation of the vegetative structure of the fungus, conidia were transferred to PDA slants and incubated as mentioned before. All isolates were stored as conidia and hyphae at 4°C.

b- Isolation from infected leaves:

Samples of naturally infected barley leaves showing net blotch symptoms with necrotic lesions were collected from three different locations (Siwa Oasis, Maryout, and Ras Sudr) during season 2007-2008. Samples were saved in paper bags, marked for each growing location and transferred to the laboratory for isolation of the causal organism. Small pieces (5 mm) were cut from each sample and sterilized with sodium hypochlorite 1% for 1 min and dried between folds of sterilized filter papers and placed on potato dextrose agar plats (PDA) supplemented with streptomycin-sulphate (100 µg/ml). Petri dishes were incubated at 24°C for 48-72 hours.

Single spores or hyphal tips were taken from the developed purified colonies and transferred onto PDA medium slants. After 4 days incubation, they were kept at 4°C until use in further experiments.

3-Identification of Drechslera teres isolates:

Identification of the *D. teres* isolates was carried out in the Plant Pathology Unit, Plant Protection Department, DRC, and Plant Pathology Laboratory in the Genetic Engineering and Biotechnology Research Institute (GEBRI), Menofiya University.

4-Morphological characterization of D. teres isolates:

Five representative isolates of *D. teres* (Two isolates from both Siwa and Maryout as well as one isolate from Ras Sudr Agric. Experiment. Stations of DRC) were selected and classified according to the following mycological parameters: spores length and width, number of septa/spore, length of septa and germ tube number & length by staining spores with cotton blue and examined microscopically. The mycological parameters were obtained from 100 observations for each isolate. These isolates represented different morphological variants (Holliday, 2001).

5-Inoculum production:

Fungal cultures of the different *D. teres* isolates grown on PDA slants were transferred to PDA medium in Petri dishes and incubated at 24°C for 7 days. The spore suspensions were prepared by adding aliquots of 20 ml sterilized water to mix with a surface of the *D. teres* fresh culture (7 days) using a brush to facilitate the separation of the spores to suspend in the added water, and the concentration of prepared spore suspension was adjusted to approximately 5000 conidia/ml using haemocytometer slide according to the method of Jonasson *et al.* (1997).

6- Reaction of barley genotypes to D. teres under greenhouse conditions:

Ten grains of different barley genotypes were sown in each of 25 cm diameter pots (2 kg sterilized soil) with six replicates. Separate a grill greenhouse cages were used for each isolate. Three weeks after planting, barley leaves were sprayed with 30 ml of spore suspension (5000 conidia/ml) of each fungal isolate using a hand atomizer. Control plants were sprayed with sterile water. The differentiated leaf spots were evaluated visually for the fungal isolates action on barley leaves one week after inoculation according to Brown (1983).

The rating scale of disease index (severity of infection) was estimated as percentage from 0 to 100 where: $\leq$ 30 resistant (R), $\geq$ 30-50 moderately resistant (MR), $\geq$ 50-70 moderately susceptible (MS) and $\geq$ 70 highly susceptible (S) as described by Tekauz (1985).

$$\text{Disease index} = \frac{(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d) + \ldots\ldots}{a + b + c + d + \ldots\ldots}$$

Whereas: a, b, c, d .... are the numbers of plants which fall in the score of infection categories 0, 1, 2, 3 ....., respectively, (Khan and Boyd, 1982).

7- Molecular detection of resistance related gene:

a) Primer design:

Specific degenerate primers were constructed according to their accession number and sequences *(Mlo-F* 5\` GTG CAT CTG CGT GTG CGT A 3\`; *R* 5\` CAG AAA CTT GTC TCA TCC CTG 3\`) to recover partial-length of interesting genes, and synthesized at Metabion, Germany. Primer sequences were checked for accuracy using the oligonucleotide software Oligo 4.1 (National Biosciences Inc., Plymouth, MN, USA).

B) Polymerase chain reaction:

Total genomic DNAs were isolated from barley (*Hordeum vulgare* L.) seedlings using the Axyprep multisource genomic DNA miniprep kit cat. no. AP-MN-MS-GDNA-50, Axygen, USA, and used as templates in the PCR.

PCR was accomplished by adding 5 µl 10X Taq DNA polymerase buffer, 1 µl dNTPs (10 mm each) (Bioron, Germany), 2.5 units Taq DNA polymerase (Bioron, Germany), 10 pmol of each primer (forward and reverse), 2 µl DNA and RNase free water up to 50 µl. All PCR reactions were performed in a thermal cycler (GeneAmp PCR System 2400) (Perkin Elmer), by pre-heating at 94°C for 5 min followed by 30 cycles of 1 min at 94°C, 1 min at annealing temperature showed in table (3) and 2 min for extension at 72°C. Finally, the amplified DNA was incubated at 72°C for 7 min to accomplish a final extension. 20 µl of PCR reaction were subjected to electrophoresis in 1.5 % agarose gel containing ethidium bromide (0.01%), subjected to 100 volts for 1hr and then photographed using UVP gel documentation system, UVP corporation-UK (Korzun, 2002).

c) Gel electrophoresis:

A volume of 20µl of PCR-products was resolved in 1.5% ultra pure (GIBCOBRL) agarose gel electrophoresis with 1x TAE running buffer. The run was performed at 80 V for 100 min and the gel was stained with ethidium bromide.

8-Statistical analysis:

The data collected for all barley genotypes tested from the pot experiments were subjected to the ordinary analysis of variance of split plot design on ten plants mean basis in each of the three replicates as described by Gomez and Gomez (1984). Means of treatments, genotypes, were compared using the new least significant difference New L.S.D. test shown by Waller and Duncan (1969) at the 5% level of probability.

Results

1- Morphological characterization of D. teres isolates:

The morphological parameters data classified *Drechslera teres* isolates to different five isolates which showed great differences among them. Where, *D. teres* isolates spore length ranged from 6.41 µ to 9.49 µ for the isolate No. 3 and No. 2, respectively (Table 2). In addition, similarity in the number of septa was found. The spore septa length, ranged from 0.79 µ to 5.9 µ for isolates No.3 and No.4, respectively. Moreover, there was a slight difference in the length of the germ tube.

Table 2. Morphological parameters obtained from 100 spore observations of each *D. teres* isolate

D. teres isolate	Spore		No. of Septa	Length of septa (µ)			Germ tube (µ)	
	Length(µ)	Width(µ)		I*	II	III	Length	No
1	7.5	2.76	3	2.68	3.6	1.66	3.6	1
2	9.49	2.81	3	2.78	3.9	2.04	3.9	1
3	6.41	2.82	2	2.82	4.6	0.79	4.55	1
4	7.01	2.57	3	2.55	5.9	1.26	5.97	2
5	7.65	2.49	3	2.44	3.8	1.47	3.8	1

* I, II and III: range of spore septa.

Reaction of barley genotypes to D. teres under greenhouse conditions:

Under greenhouse conditions, the five *D. teres* isolates showed different levels of virulence. Isolates No. 3 and No.4 were the most virulent caused 30.5 and 32.1% disease severity (D.S.), respectively. While isolate No.2 was the least virulent one on tested barley genotypes when recorded 25.0% D.S. (Table 3).

On the other hand, barley genotypes varied in their reaction to *D. teres* isolates, barley genotypes L26, G126, and G131 were classified as moderately resistant and showed the highest average of D.S., 32.9, 42.67 and 41.67%, respectively. While genotypes L3, H6, H7 and H10 were classified as resistant genotypes as D.S. recorded 18.33, 19.67, 21.33 and 21.67%, respectively, (Table 3).

Table 3. Mean performance for disease severity of barley genotypes artificially infected with five isolates of *D. teres* under greenhouse conditions

Barley Genotype	*D. teres* isolate					Control*	Mean	Reaction**
	1	2	3	4	5			
L3	16.7	18.3	20.0	21.7	15.0	1.7	18.3	R
L26	30.0	31.3	33.3	33.3	36.7	3.3	32.9	MR
G126	35.0	45.0	43.3	46.7	43.3	6.7	42.7	MR
H6	21.7	11.7	25.0	16.7	23.3	3.3	19.7	R
H7	23.3	13.3	23.3	26.7	20.0	6.7	21.3	R
H10	23.3	18.3	23.3	23.3	20.0	3.3	21.7	R
G131	31.7	36.7	45.0	56.7	38.3	10.0	41.7	MR
Mean	26.0	25.0	30.5	32.1	28.1	5.0	27.8	-
New L.S.D. at 5% for:						Isolates (I) =	0.33	
						Genotypes(G) =	0.45	
						I×G =	0. 87	

* Control values are not involved in disease severity of the isolates mean in each barley genotype.

** Reaction was estimated as percentage from 0 to 100 where: ≤ 30 resistant (R), 30.0-50 moderately resistant (MR) described by Tekauz (1985).

Detection MLO1 gene in seven barley genotypes with specific reference to net blotch resistance:

Data obtained in this study demonstrated the presence of new approach for detection of *MLO1* gene in the genomic DNA based on the analysis of seven barley genotypes (L3, L26, G126, H6, H7, H10 and G131) with specific reference to net blotch resistance (Fig. 1).

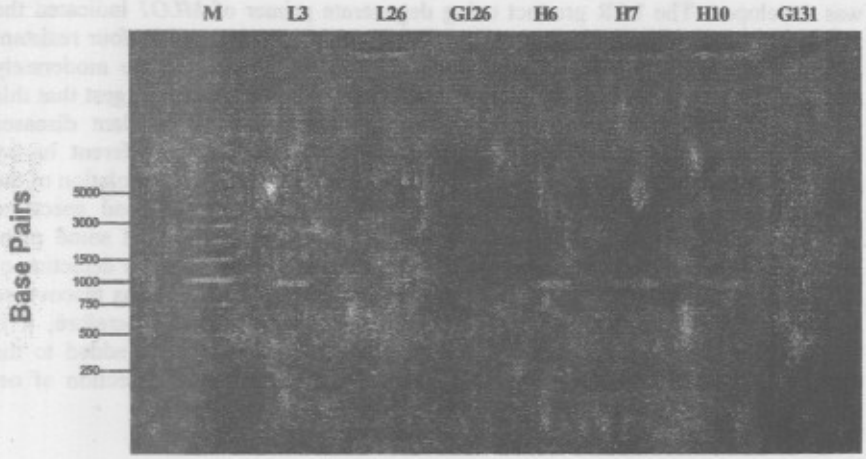

Fig. 1. PCR product of *MLO1* gene of *D. teres* on the seven barley genotypes tested (L3, L26, G126, H6, H7, H10 and G131).

Plant foliar diseases related gene *MLO1* has been selected from gene bank data base (National Centre for Biotechnology Information "NCBI") and primers -which used in the present study, were designed as degenerate primers.

The PCR product, using degenerate primer of *MLO1*, indicated the appearance of one fragment sizes about 500 bp. that detected in four resistant barley genotypes (L3, H6, H7 and H10), while it was absent in the three moderately resistant genotypes (L26, G126 and G131).

Discussion

This study exhibited that morphological parameters data can be used to differentiate among the five *Drechslera teres* isolates from diverse locations in Egypt. Isolates No. 3 and No. 4 were the most virulent. These results gave attention about using the morphological parameters to differentiate among different isolates. Most previous work done on this aspects used virulence phenotypes for assessing genetic variation in fungal pathogens; however, virulence markers are often limited in number and subject to host selection (Steffenson and Webster, 1992).

Under greenhouse conditions, the seven barley genotypes showed significant differences in their response to the five *D. teres* isolates. Barley genotypes, *i.e.* G131, G126, and L26, were classified as moderately resistant genotypes, while L3, H6, H7 and H10 were the most resistant genotypes, which showed the lowest percentage of disease severity and could be cultivated in the different geographic locations, according to their response to biotic stress by different isolates. These results are in harmony with those obtained by Afiah and Zaki (2001), Zaki and Afiah (2002) and Al-Masry (2006).

Recent findings and new approach for detection of *MLO1* gene in the genomic DNA based analysis of seven barley with specific reference to net blotch resistance was developed. The PCR product using degenerate primer of *MLO1* indicated the appearance of one fragment sizes about 500 bp. that was detected in four resistant barley genotypes (L3, H6, H7 and H10), while it was absent in the moderately resistant genotypes (L26, G126 and G131). Hence, the researchers suggest that this is an addition molecular based add value to the application of plant diseases molecular diagnostics for detection of net blotch resistance in different barley genotypes. However, Buschges *et al.* (1997) described the molecular isolation of the *Mlo* gene as a first step toward a molecular interpretation of broad spectrum resistance mediated by recessive host gene as a control element of some plant pathogen resistance. It is worthy to mention that this is the first time for detection of *MLO1* for net blotch resistance, in Egypt. Since, Jorgensen (1992) has discovered and characterized of *Mlo* powdery mildew resistance in barley. Therefore, it is a great value to suggest that a molecular based add value must be added to the application of *MLO1* in plant diseases molecular diagnostics for detection of net blotch resistance in different barley genotypes in Egypt.

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تحديد جين مرتبط بمقاومة مرض التبقع الشبكي في بعض التراكيب الوراثية من الشعير

أحمد إسماعيل المصرى*، خالد إسماعيل زكى*، محمد فتحى سالم**، خليل عبد الحميد الحلفاوى**، سامى عبد العزيز عافية***

* قسم وقاية النبات، مركز بحوث الصحراء، القاهرة – مصر.
** معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية، جامعة المنوفية، مدينة السادات- مصر.
*** قسم الأصول الوراثية النباتية ، مركز بحوث الصحراء، القاهرة- مصر.

تم إجراء تعريف وتوصيف لخمسة من العزلات الفطرية المعزولة من نباتات شعير مصابة بمرض التبقع الشبكى مأخوذة من ٣ مناطق هى سيوه ، رأس سدر بجنوب سيناء ومربوط حيث عرفت جميعا على انها *Drechslera teres* . أثبتت نتائج الفحص وجود اختلافات في الصفات المورفولوجية للجراثيم من خلال تسجيل قراءات تشمل " طول الجرثومة، عرض الجرثومة، عدد الحواجز العرضية/جرثومة ، طول الحواجز العرضية، طول أنبوبة الإنبات وعدد أنابيب الإنبات وذلك من خلال الفحص الميكروسكوبي لعدد ١٠٠ جرثومة لكل عزله من العزلات الفطرية ، وأبدت النتائج اختلافات بين العزلات من حيث طول الجراثيم الذي تراوح بين ٦,٤١ ، ٩,٤٩ ميكرون للعزلتين ٣ و ٢ على التوالي، بينما لا توجد اختلافات بين العزلات من حيث عرض الجرثومة وكذلك عدد أنابيب الإنبات، وبدراسة طول أنابيب الإنبات كانت العزلتان ٤ و ٣ هي أكثر العزلات فى طول أنبوبة الانبات حيث سجلت ٥,٩٧ و ٤,٥٥ ميكرون على التوالي.

أوضحت النتائج وجود تباين في سلوك التراكيب الوراثية المختبرة من الشعير للعدوى الصناعية بخمس عزلات مختلفة من الفطر *D. teres*، وكانت التراكيب الوراثية (L26, G126, G131) هى الأكثر حساسية للمرض وصنفت متوسطة المقاومة (MR) بينما كانت التراكيب الوراثية H6, H7, L3, H10 الأكثر مقاومة للمرض (R). وكانت العزلتان الفطريتان رقم ٣ و ٤ هي أكثر العزلات إمراضية مقارنة ببقية التراكيب الوراثية من الشعير، بينما كانت العزلة رقم ٢ هى اقل العزلات إمراضية.

تم تشخيص وتحديد بادئات جزيئية متخصصة لمقاومة مرض التبقع الشبكي من خلال اختيار الموقع الوراثي *MLO1* والذي له علاقة بمقاومة بعض الأمراض النباتية (البياض الدقيقى) ، وذلك من خلال المتاح بقاعدة البيانات الخاصة بـ "National Centre for Biotechnology Information" (NCBI). والمتاح على شبكة المعلومات الدولية Internet. وللكشف عن هذا الموقع في التراكيب الوراثية للشعير تحت الدراسة ، تم تصميم البادئات المتخصصة لهذا الجين، وبعمل التفريد الكهربي للحامض النووي الـ DNA باستخدام تلك البادئات تم رصد وجود هذا الجين في السلالات الأربعة المرباة حديثاً من الشعير وهى "L3, H6, H7, H10" بينما لم يظهر في أكثر التراكيب الوراثية حساسية للمرض "L26 , G126 وG131" وعليه يمكن التوصية باعتبار هذا الموقع الوراثي *MLO1* كاحد معايير الكشف عن المقاومة لمرض التبقع الشبكي في التراكيب الوراثية المختلفة من الشعير.

وعلى ذلك يمكن التوصية بزراعة السلالات الأربعة المرباة حديثا من الشعير (L3, H6, H7, H10) في المناطق التى تنتشر بها العزلات الفطرية المختبرة "سيوه – مربوط – رأس سدر" وذلك بعد إجراء الدراسات الحقلية اللازمة.