Identification of *TIR-NBS-LRR* gene candidates linked to *Or5* locus for resistance to broomrape (*Orobanche cumana* Wallr.) in sunflower

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Osman E. Radwan*,*; Talaat A. Ahmed** and Steven J. Knapp*****

*Soybean Research Center, 1101 W. Peabody dr., University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801, USA

**Biological and Environmental Sciences Department, College of Arts and Sciences, Qatar University, Doha 2713, Qatar

***Center for Applied Genetic Technologies, University of Georgia, Athens, Georgia 30602-6810 USA

Corresponding author: Osman E. Radwan (oradwan@illinois.edu)

ABSTRACT

Plants use different types of disease resistance genes to detect the presence of pathogens and induce defense responses. NBS-LRR genes confer resistance to pathogens including oomycetes fungi, bacteria, nematodes and virus. However, NBS-LRR genes have not yet been implicated in resistance to any parasitic plant. Here, we identified gene candidates of TIR-NBS-LRR subfamily that linked to Or5 locus, which confer resistance to races A, B, C, D and E of Orobanche cumana. These RGCs are mapped in the upper part of the linkage group 3 of sunflower genetic map. The identified RGCs will be useful in marker-assisted selection programs and positional cloning of Or5 locus. Reverse transcription PCR (RT-PCR) detected the transcription accumulation of RGC192, while it could not detect the transcription accumulation of other RGCs suggesting that either the level of transcription of these genes is very low or their expressions is induced only under parasitic infection. The sunflower-Orobanche analyses proposed here could be the first case and open the way to further understanding how sunflower and other plants resist parasitism by Orobanche.

***Keywords**: Broomrape, Helianthus annuus, resistance genes, RT-PCR and gene expression.*

INTRODUCTION

Disease resistance is commonly mediated by resistance (*R*) genes in plants (Staskawicz *et al.* 1995). Many of the *R* genes discovered in plants encode proteins consisting of nucleotide binding site (*NBS*) domain and leucine-rich repeats (LRRs) (Hammond-Kosack and Jones 1997; Ellis *et al.* 2000). NBS-LRR gene families are abundant in plant genomes and encode proteins necessary for triggering defense or 'guard' systems (Dangl and Jones 2001; Meyers *et al.* 1999; Meyers *et al.* 2003; Holt *et al.* 2003; Dangl and Jones 2006). Since the initial discovery of the role played by *NBS-LRR* proteins in triggering disease resistance in plants, comparative genomic approaches have been widely used to isolate *NBS-LRR* genes conferring resistance to a broad spectrum of bacterial, fungal, nematode, and viral pathogens in plants (Bent 1996; Kanazin *et al.* 1996; Leister *et al.* 1998; Hammond-Kosack and Jones 1997; Dangl and Jones 2001; Meyers *et al.* 2003; Hulbert *et al.* 2001). Of the more than 40 *R* genes cloned from plants, 75% are members of *NBS-LRR*

gene families (Hulbert *et al.* 2001). In sunflower, several *NBS-LRR* gene families have been described (Gedil *et al.* 2001; Bouzidi *et al.* 2002; Radwan *et al.* 2003; Radwan *et al.* 2004; Radwan *et al.*, 2008). These resources supply a wealth of candidates for *R* genes, including downy mildew and rust resistance genes (Radwan *et al.* 2007), and are creating an increasingly more complete picture of the large and complex network of *R* genes found in the sunflower genome.

Orobanche cumana Wallr. (broomrape) is a weedy parasitic plant and serious pest of cultivated sunflower (*Helianthus annuus* L.) in Eastern Europe, the Balkans, and the Mediterranean (Parker and Riches, 1993). Seed yield losses from broomrape infestations in susceptible sunflower genotypes can reach 50% (Dominguez *et al.* 1996). The *Orobanche* spp. biological cycle comprises well-defined steps separated both spatially and temporally, that are potential targets for host defense strategies. Upon germination, stimulated by host root-exuded chemical signals, broomrape seed develops a small seedling that attaches to the host root and differentiates in the attachment organ (appressorium). After host tissue penetration and connection to the vascular system through the haustorium, the parasite becomes a major sink for plant photosynthesis, gradually forming a tubercle from which a shoot arises to emerge from the soil to flower and produce seeds (Parker and Riches, 1993; Westwood, 2000). Because *O. cumana* has a broad host range and produces an extraordinarily large number of small, long-lived, facilely dispersed seeds, control through crop management has been difficult (Ish-Shalom-Gordon *et al.*, 1993; Parker and Riches, 1993; Ruso *et al.*, 1996; Sukno *et al.*, 1999; Roman *et al.*, 2001). The primary line of defense against broomrape, other than quarantine, has been genetic resistance (Sackston, 1992; Ruso *et al.*, 1996; Sukno *et al.*, 1999; Lu *et al.*, 2000).

Despite several attempts to identify markers linked to *Orobanche* resistance genes, the closest reported thus far is 5.6 cM downstream of *Or5*, a gene for resistance to races A to E (Lu *et al.*, 2000). Tang *et al.* (2003) placed *Or5* locus in a telomeric region of linkage group 3 with the closest SSR marker mapping 6.2 cM proximal to *Or5* locus. *Orobanche* as other plant pathogens overcomes the host resistance by producing the new races. This is the case of the new race F of *O. cumana*, which has been recently reported in south Spain (Jan *et al.*, 2002), and quantitative trait loci (QTLs) associated with this trait have been identified (Pérez-Vich *et al.*, 2004). Thus far, about 8 races (A to H) of *Orobanche* have been identified. The aims of this work were to isolate of new families of *NBS-LRR* resistance gene candidates (RGCs), identify of NBS-LRR markers linked to *Or5* locus and understand the resistance to this obligate parasite at a molecular level.

MATERIALS AND METHODS

Plant materials

Two hundred and sixty-two F5 recombinant inbred lines (RILs) were developed by single seed descent from a cross between two inbred lines, PHC is a cytoplasmic-genic male sterile (CMS) maintainer susceptible to *Orobanche* race E; PHD is CMS fertility restorer resistant to *Orobanche* race E (Tang *et al.* 2003).

Sunflower-*Orobanche* infestation and phenotyping

The infestation of the two parents and F5 seedlings of RILs was carried out as described by Tang *et al.* (2003). The broomrape seeds (race E) were homogenously mixed with mixture of sand and peat at the rate of 250 mg/kg. Five seeds of each entry were planted in

plastic pots (6x10x10 cm) filled with the infested soil mixture. The plants were grown under a 14 hour photoperiod with 25 °C day and 18 °C night temperatures and constant ≈ 60% humidity. Two-month-old plants were carefully removed from the pots to phenotype for presence or absence of emerged or underground broomrape stalks. RILs with 100% uninfected plants were scored as resistant (R), while RILs with 100% infected plants were scored as susceptible (S) and RILs with a mixture of infected and uninfected plants were scored as heterozygotes (H).

DNA manipulations and genetic mapping

DNA was extracted from bulked fresh leaf samples using the procedure described by Web and Knapp (1990). Bulk DNA samples from parental genotypes and the susceptible and resistant bulks (Michelmore *et al.* 1991) were screened for polymorphic loci using 200 primer pairs. These primers were developed by mining the sunflower EST database and sequencing genomic DNA fragments amplified from *H. annuus*, *H. tuberosus*, and other wild sunflower species using degenerate primers complementary to conserved amino acid sequence motifs in the NBS domain (Radwan *et al.* 2008). Primers that showed polymorphisms between resistant (resistant parent and resistant bulk) and susceptible (susceptible parent and susceptible bulk) were used to screen all the progenies. The primer sequences used to amplify mapped RGCs are presented in Table (1). The PCR (20 µl total) was carried out using 30 ng of DNA template, 0.65 U Taq polymerase (Qiagen, USA), 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.16 uM of each primer. A 'touchdown' PCR protocol was used. The initial denaturation of 94 °C for 3 min was followed by 1 cycle of 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 60 seconds. In each subsequent cycle, the annealing temperature was decreased by 1 °C till reached 58 °C. The amplification continued for 35 cycles at 94 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 60 seconds. The final extension was carried out at 72 °C for 15 minutes. After verification of PCR product using 1.5% agarose gel, the single strand conformational polymorphism (SSCP) was checked for PCR amplicons as described by Slabaugh *et al.* (1997). Linkage analysis was made with the software Mapmaker 3.0 (Lander *et al.* 1987). Markers were ordered with LOD value threshold of 3.0 and a maximum recombination fraction of 34.

Expression analysis by RT-PCR

Total RNA was extracted from roots, hypocotyls, cotyledons and leaves of 15 day-old PHD (resistant) using the Trizol reagent (Invitrogen), then treated with DNase I (Promega, USA) to remove genomic DNA contaminations. Two µg of DNase treated RNA were reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA). A "minus" reverse transcriptase PCR reaction was used to test each mRNA sample for genomic DNA contaminations, where no reverse transcriptase enzyme was added during the cDNA synthesis reaction. The transcriptional expression of each gene was analyzed using semi-quantitative PCR. *Ef-1α* (For 5' AGGCGAGGTATGATGAAATTGTCA; Rev 5' GTCTCTTGGGCTCATTGATTTGGT 3') was used as an internal control (Radwan et al. 2005a; Radwan *et al.* 2005b). cDNA was diluted 1/10, and then 2 µl was used in 20 µl of the PCR mix containing 0.65 U (0.13 µl) of Taq DNA polymerase (Qiagen, USA), 1 X Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 µM of each dNTP and 0.16 uM of each primer. PCR was carried out under the following conditions: an initial denaturation at 94 °C for 3 min followed by 40 cycles of 20 s at 94 °C, 30 s at

58 °C and 60 s at 72 °C. PCR amplification products were separated on TBE-Agarose gels (2%). Gels were stained with ethidium bromide (0.5 mg ml^{-1}). All PCR products were cloned and sequenced once to ensure the specificity of amplifications.

RESULTS

Screening for associations between NBS-LRR Loci and *Or5*

By using a bulked segregant analysis method (Michelmore *et al.* 1991), the resistant parent (PHD), resistant bulk, susceptible parent (PHC) and susceptible bulk were screened to detect polymorphic markers. Out of two hundred primer pairs were designed and used for screening (Radwan *et al.* 2008), only four polymorphic markers have been detected between resistant plants (resistant parent and resistant bulk) and susceptible plants (susceptible parent and susceptible bulk). These markers were used for screening all the RIL population of PHC x PHD cross (Fig.1).

SSCP markers for the newly identified NBS-LRR loci (RGC172, RGC181, RGC206 and RGC192) mapped to the upper segment of LG3 and were linked to Or_5, a telomeric *Orobanche* resistance locus previously mapped by Tang *et al.* (2003) in the PHC x PHD recombinant inbred line (RIL) population (n = 262). Or_5 is one of several alleles or tightly linked *Orobanche* resistance loci (Or_1-Or_5) found in the upper end of LG 3. None of the candidate NBS-LRR loci (RGC172, RGC181, RGC206 and RGC192) completely co-segregated with Or_5 (Fig. 2). Finally, three of four NBS-LRR loci linked to Or_5 (RGC172, RGC181 and RGC192) were identified from *H. tuberosus* ESTs while the fourth one was identified from common wild *H. annuus*. *H. tuberosus* has been a rich source of *Orobanche R* genes and was the source of Or_1, the first *Orobanche R* gene identified in sunflower (Vranceanu *et a*l. 1980; Parker and Riches 1993). The closest marker (2.6 cM) to *Or5* locus is RGC181, while RGC172 is located at 7.5 cM from *Or5* locus. On the other hand, RGC206 and RGC192 mapped 33 cM proximal to *Or5* locus.

Table (1): Accession numbers, sequence type and primer sequences that used for amplification of RGCs linked to Or5 locus.

RGC	Accession N.	Sequence type	5'---------------------------------------3'
172	EL452113	TIR-NBS-LRR	For: TACCATGTGTCTCCCTCTGATGT
			Rev: GATGTCCTTGATGTTCCCAATAA
181	EL455612	NBS-LRR	For: TGGCATTAGATATGAGGTTTGGA
			Rev: TTGGACAAGAAGTCAAGTCAAGG
206	DY908907	TIR-NBS-LRR	For: CGGTCTTCTATGATGTGGAACCT
			Rev: GGTTTCCTGACATTTCCCTTACA
192	EL468211	TIR-NBS-LRR	For: ACGATCAAAGTTCTGGGTTCATT
			Rev: AACGACGAACGATATTCTTACCC

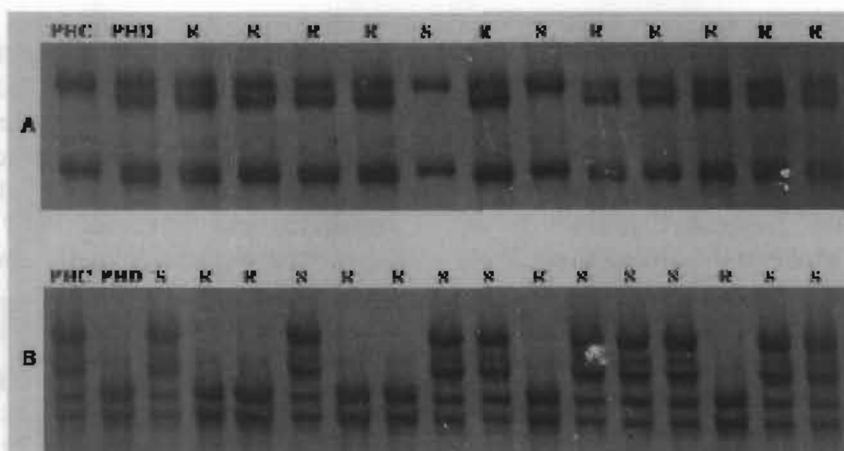

Fig. (1): SSCP gel of RGC181 (A) and RGC172 (B). The two first lanes represent the parents PHC (susceptible) and PHD (resistant), whereas the rest represent different individuals of PHC x PHD RIL population. R=resistant and S=susceptible.

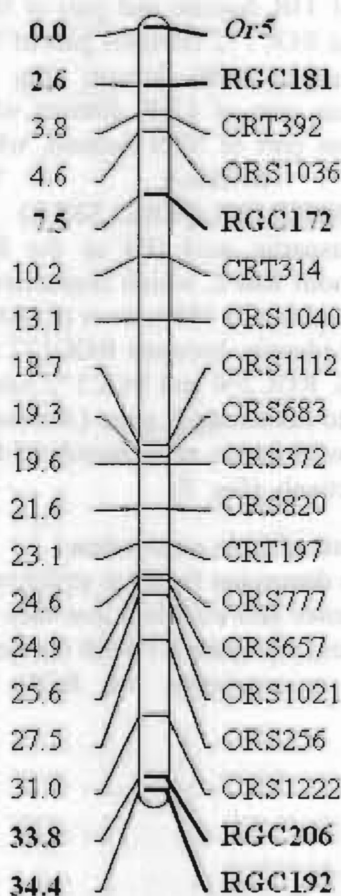

Fig.(2): Genetic map of linkage group 3 of PHC x PHD showing the relative positions of Orobanche resistance locus (Or5), NBS-LRR RGCs (181, 172, 206 and 192) and other previous markers that have been mapped to Or5 locus (Tang et al., 2003). Genetic distances were calculated using the Kosambi map function and are shown in centimorgans (cM).

Sequence analysis of RGCs linked to *Or5* locus

Blast_X results reveal that these RGCs share high homology with other known *R* genes as nematode *R* gene of potato (Accession N. AAP44393), TMV resistance protein N of *Arabidopsis thaliana* (Accession N. BAB11635) and N protein of tobacco (Accession N. BAD12594). All of these plant *R* genes are belonging to TIR-NBS-LRR subfamily that gives strong evidence about sequence type of current RGCs as a TIR-NBS-LRR subfamily. The analysis of amino acid sequences of these RGCs reveals that these RGCs are members of TIR-NBS-LRR subfamily of *R* genes. RGC206 contains the complete part of TIR domain and part of NBS domain, whereas RGC172 contains part of TIR domain and part of NBS domain (Fig. 3). RGC181 contains part of LRR domain while RGC192 contains part of NBS domain, which included RNBS-A TIR (FLENIRExSKKHGLEHLQKKLLSKLL) motif and an aspartic acid (D) as the final amino acid in motif Kin-2, which characterizes TIR-NBS-LRR subfamily (Meyers *et al.* 2003). The amino acid identity between RGC172 and RGC206 is 32%. RGC206 and RGC172 have a high similarity to nematode *R* gene (Accession N. AAP44393) with amino acid identity of 42% and 34%, respectively (Fig. 3).

RT-PCR analysis of gene expression

In order to determine the gene structure of the candidate genes and establish that they are transcribed genes, we used RT-PCR to detect the transcript accumulation of RGCs in different types of resistant parent tissues. RT-PCR detected the transcript accumulation of only one member of this cluster of resistance gene (Fig. 4). The transcript accumulation of RGC192 was detected in roots, hypocotyls, cotyledons and leaves of PHD reflecting the constitutive expression of this gene (Fig. 4).

DISCUSSION

The *Orobanche R* gene pyramid

Many of the *Orobanche R* genes described in sunflower confer resistance to previously described races, thus creating a pyramidal distribution of host-pathogen *R* gene interactions. Vranceanu *et al.* (1980, 1986) identified five races (A to E) of *Orobanche* using differentials carrying dominant genes (Or_1, Or_2, Or_3, Or_4, and Or_5) for resistance to races A, A+B, A+B+C, A+B+C+D, and A+B+C+D+E, respectively. The pyramidal pattern of resistance was found in subsequent analyses of host differentials throughout Europe (Bulbul *et al.* 1991; Saaverdra *et al.* 1994; Shindrova 1994). Race F ultimately defeated the previously described *R* genes and led to discovery of Or_6, a gene for resistance to race F (Fernandez-Martinez *et al.* 2000). Or_6 apparently confers resistance to each of the previously described races (A-E), in addition to race F. The 'pyramid' created by host differentials displaying resistance to previously and newly identified races could be an artifact of the inbred lines selected as host differentials. Moreover, the pyramidal pattern undoubtedly oversimplifies the biological complexity of *Orobanche* races and sunflower *R* genes.

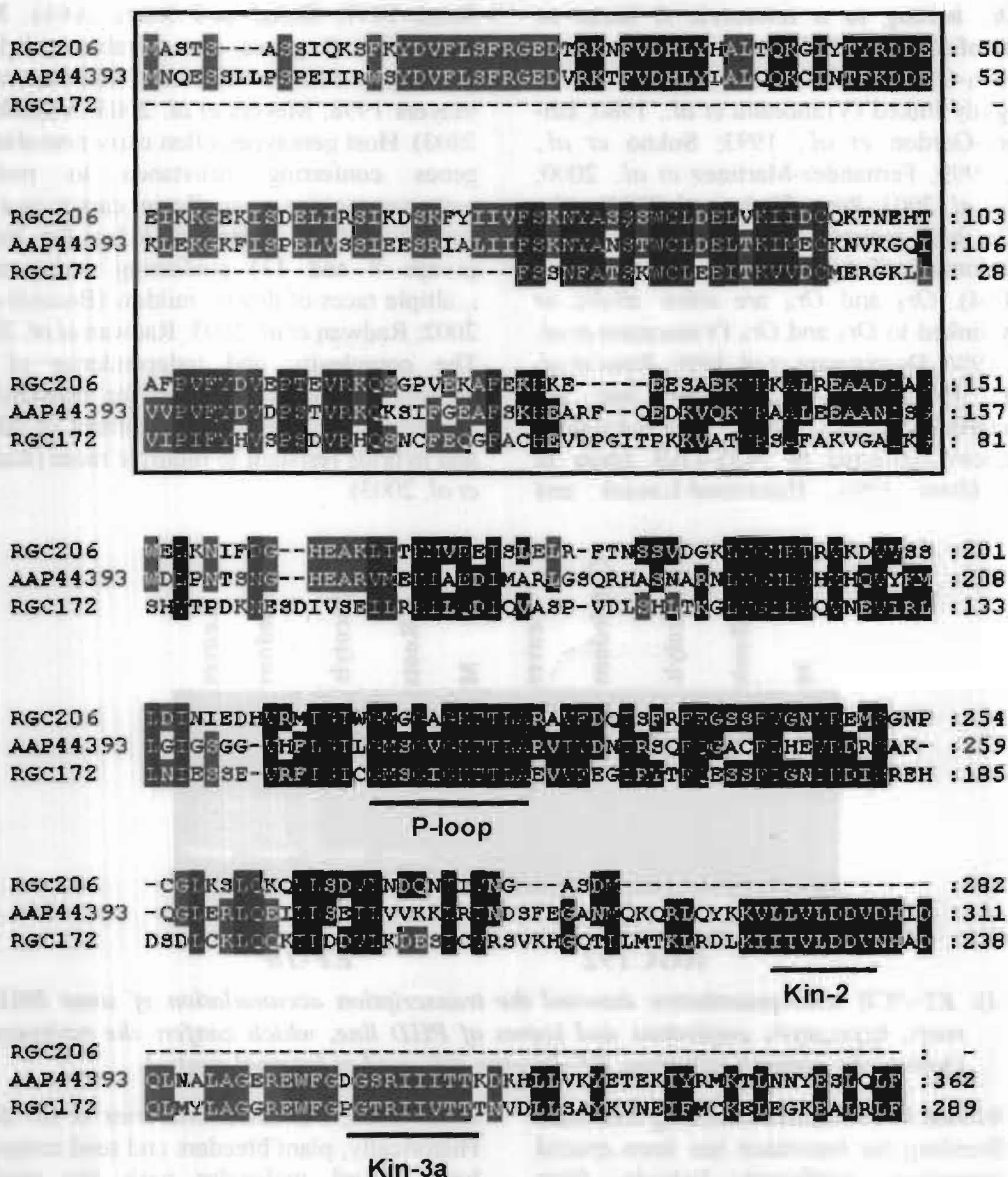

Fig. (3): Partial alignment of deduced amino-acid sequences of two TIR-NBS-LRR RGCs (172 and 181) and nematode resistance gene (Accession N. AAP44393). The computer program CLUSTAL_X was used in alignment analysis. Alignments were shaded using GENEDOC software. P-loop, Kin-2 and Kin-3a motifs are underlined, whereas TIR domain is boxed.

Or_1-Or_6 belong to a telomeric *R* locus or cluster of *R* loci on linkage group 3

Or_1-Or_6 are either allelic or non-allelic but tightly linked (Vranceanu *et al.*, 1980, Ish-Shalom-Gordon *et al.*, 1993; Sukno *et al.*, 1998, 1999; Fernandez-Martinez *et al.*, 2000; Tang *et al.* 2003; Pérez-Vich *et al.* 2004). Or_5 and Or_6 both mapped to linkage group 3 and were telomeric (Tang *et al.* 2003; Pérez-Vich *et al.* 2004). Or_1 and Or_4 are either allelic or tightly linked to Or_5 and Or_6 (Vranceanu *et al.* 1980, 1986; Dominguez *et al.* 1996; Ruso *et al.* 1996). The Or_1- Or_6 cluster has the characteristics of other gene-for-gene resistances conferred by NBS-LRR genes in plants (Bent 1996; Hammond-Kosack and Jones 1997; Dangl and Jones 2001). Many NBS-LRR *R* genes are members of large, genetically complex clusters (Michelmore and Meyers 1998; Meyers *et al.* 2003; Michelmore 2003). Host genotypes often carry non-allelic *R* genes conferring resistance to multiple pathogen races, e.g., sunflower carries two very large NBS-LRR clusters of *R* loci (on linkage groups 8 and 13) conferring resistance to multiple races of downy mildew (Bouzidi *et al.* 2002; Radwan *et al.* 2003; Radwan *et al.* 2004). The complexity and independence of two clusters facilitates selection for resistance to multiple races and the development of inbreds and hybrids resistant to multiple races (Radwan *et al.* 2003).

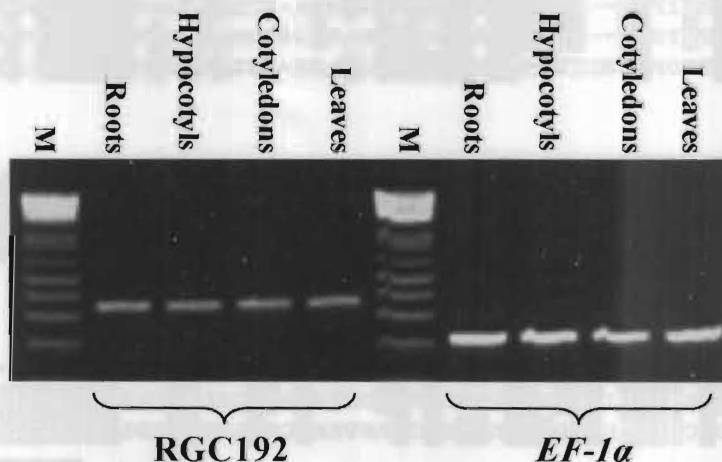

Fig. (4): RT-PCR semi-quantitative detected the transcription accumulation of some RGCs in roots, hypocotyls, cotyledons and leaves of PHD line, which confers the resistance to Orobanche cumana pathogen. EF-1α gene was used as internal control.

The *Orobanche* resistance breeding treadmill

Breeding for resistance has been crucial for protecting sunflower hybrids from *Orobanche* parasitism, it is a challenging task because new races of the pathogen continually emerge and ultimately defeat known *R* genes and, in the process, have created a classic disease resistance breeding treadmill (Sackston 1992; Ruso *et al.* 1996; Sukno *et al.* 1999; Lu *et al.* 2000; Fernandez-Martinez *et al.* 2000). Historically, plant breeders and seed companies have lacked molecular tools for applying marker-assisted selection (MAS) to breeding for resistance to *Orobanche* (Tang *et al.* 2003; Pérez-Vich *et al.* 2004). Out of necessity, traditional breeding methods have been used to identify and transfer *Orobanche R* genes into the parents of commercial hybrids. Because of

the time required for inbred line development by traditional breeding methods, new races of the pathogen often emerge before genes for resistance to previous races have been deployed (Fernandez-Martinez *et al.* 2000). Moreover, the search for new and novel sources of resistance has completely relied on phenotypic screening subsequent to the discovery of new races of the pathogen, thus further delaying the discovery and deployment of new *R* genes. While phenotypic screening is a crucial element of breeding for resistance to *Orobanche*, the process of identifying novel sources of resistance can be accelerated and greatly enhanced by cloning *Orobanche R* genes and developing DNA markers diagnostic for specific loci and alleles, as has been done for the downy mildew NBS-LRR *R* gene clusters in sunflower (Bouzidi *et al.* 2002; Radwan *et al.* 2003; Radwan *et al.* 2004; Radwan *et al.* 2007).

Candidate *Or*$_5$ genes are transcribed at low levels

The function of *R* gene products as receptors interacting with pathogen elicitors in a setting of defence signaling has been suggested by both direct and indirect evidence (Jia *et al.*, 2000; Nimchuk *et al.*, 2001). While *avirulence* genes probably play a role in the fitness or pathogenicity of the pathogen (Vivian and Gibbon, 1997), *R* gene products may have a function in plant development and therefore be expressed in healthy, unchallenged plants, ready to detect any attack (Hammond-Kosack and Jones, 1997). Here, RT-PCR detected the transcript accumulation of RGC192, which constitutively expressed at low levels in healthy roots, hypocotyls, cotyledons and leaves of the resistant plant, whereas RT-PCR could not detect the transcript accumulation of other RGCs. One possibility to interpret this result is that the level of transcription of these genes is very low and was not detected by traditional RT-PCR methods. Another possibility is that these RGCs belong to *R*-genes which are induced by infection such as *Xa1*, a bacterial resistance gene in rice (Yoshimura *et al.*, 1998), the *pib* rice blast resistance gene (Wang *et al.*, 2001), Ha-NTIR11g, a downy mildew resistance gene candidate in sunflower (Radwan *et al.*, 2005a).

In conclusion, NBS-LRR genes found in a cluster on linkage group 3 could play a central role in recognizing *O. cumana* and triggering host defense responses in sunflower. The known *R* genes on linkage group 3 (*Or*$_1$-*Or*$_5$) have the hallmarks of NBS-LRR *R* genes found in plants (Bent 1996; Kanazin *et al.* 1996; Leister *et al.* 1996; Leister *et al.* 1998; Hammond-Kosack and Jones 1997; Dangl and Jones 2001; Meyers *et al.* 2003; Hulbert *et al.* 2001; Dangl and Jones 2006). NBS-LRR loci in the cluster on linkage group 3 are predicted to encode *R* proteins conferring hypersensitive race-specific resistance to infection by *O. cumana*.

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الملخص العربي

تعريف جينات مقاومة TIR-NBS-LRR مرتبطة مع موقع Or5 المقاوم للهالوك (*Orobanche cumana* Wallr.) في عباد الشمس

عثمان عليو رضوان*,* ، طلعت عبد الفتاح أحمد** و ستيفن كناب*****
*مركز بحوث فول الصويا ، جامعة إلينوى ، الولايات المتحدة الامريكية
**قسم العلوم البيولوجية و البيئية ، جامعة قطر ، قطر
***مركز تكنولوجيا الوراثة التطبيقية ، جامعة جورجيا ، الولايات المتحدة الامريكية

تستخدم النباتات أنواع مختلفة من جينات المقاومة للأمراض لتتعرف على وجود الكائنات الممرضة و تستحث النظم الدفاعية في النبات. إن جينات المقاومة NBS-LRR تعمل على مقاومة العديد من المسببات المرضية و التى تشمل الفطريات البيضية ، الفطريات ، البكتريا ، النيماتودا و الفيروسات. جدير بالذكر، جينات NBS-LRR لم يسبق ان تم اكتشاف دورها فى النباتات الطفيلية. هنا فى هذا البحث تم تعريف مجموعة من جينات TIR-NBS-LRR ارتبطت مع موقع *Or5* التي لها دور في مقاومة سلالات الهالوك A, B, C, D, E (*Orobanche cumana*) . تم تحديد مواقع تلك الجينات RGCs على الجزء العلوى من المجموعة الارتباطية الثالثة على الخريطة الوراثية لعباد الشمس. الجينات التى تم تعريفها RGCs سوف تفيد في برامج الانتخاب باستخدام المثبرات الوراثية و عزل جين *Or5*. تكنيك تفاعل البلمرة المتسلسل العكسي RT-PCR أظهر تراكم نواتج جين RGC192 بينما لم يظهر تراكم نواتج لأي جينات أخرى من مجموعة RGCs و يمكن تفسير ذلك اما أن نواتج تلك الجينات منخفض جدا أو أن نواتجهم و تعبيراتهم تستحث فقط تحت تأثير عدوى الطفيل.إن النتائج المتحصل عليها هنا فى هذا البحث الخاصة بالهالوك هي الأولى من نوعها و التي قد تفتح الطريق لفهم كيفية مقاومة عباد الشمس و النباتات الأخرى لتطفل الهالوك.