Identification and characterization of resistance gene analog (*RGA*) and the leaf rust resistance gene *Lr21* from the wheat cultivar Giza168

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ABSTRACT

Leaf (brown) rust caused by Puccinia triticina is a fungal disease of wheat (Triticum aestivum L.) that causes significant yield losses annually in many wheat-growing regions of the world. Host-plant resistance is the most economically viable and environmentally responsible method for controlling Puccinia triticina, the causal agent of leaf rust in wheat. The identification and utilization of new resistance sources is critical to continue the development of improved cultivars. The objective of this work was to identify defense-related genes against rust in the Egyptian rust resistant cultivar Giza168. Specific primers were designed on the basis of converse motifs of cloned resistance genes of the resistance gene analog (RGA) and leaf rust resistance gene (Lr21) in wheat (Triticum aestivum L.). The designed PCR primers were subsequently used for RT-PCR using RNA isolated from a resistant variety to amplify fragments of 445 bp and 235 bp for RGA and Lr21 genes, respectively. The amplified products were cloned, sequenced and submitted to the GenBank. The nucleotide sequences of the amplified fragments were aligned with their corresponding genes using the BLAST. The expressions of the two genes in the infected and healthy plants were studied using RT-PCR. The RGA expression was induced and detected by RT-PCR, which is up-regulated by fungal infection. The Lr21 expression was detected on both healthy and infected plants, although the expression was higher in infected plants.

***Key words**: RGA gene, Lr21 gene, wheat cv. Giza168.*

INTRODUCTION

Hexaploid wheat (*Triticum aestivum* L.) was originated about 8,000 years ago from the hybridization of tetraploid wheat with diploid *Aegilops tauschii* Coss, containing the D-genome. Thus, the bread wheat D-genome is evolutionary young and shows a low degree of polymorphism in the bread wheat gene pool (Bossolini *et al.*, 2006).

Wheat production is constantly challenged by different diseases and pests; the most prominent ones among them are the rust diseases (leaf, stem, and stripe rust). However, the production of wheat is significantly affected by different diseases (Ling *et al.*, 2003). Leaf rust (causal agent *Puccinia triticina*) is the most widely distributed disease of wheat, causing an average annual yield loss of about 3% worldwide (equivalent to US $2 billions) (Huang *et al.*, 2003). Host resistance is the most effective and economical method of disease control and supplementing conventional breeding with direct gene

transfer by molecular methods promises to enhance the efficiency of plant breeding (Huang *et al.*, 2003).

Over 100 individual rust resistance genes have been identified, the majority of these are of an economic importance and can confer protection against races of stripe (*Puccinia striiformis*), stem (*Puccinia graminis*) and leaf rust (*Puccinia triticina*) pathogens (Spielmeyer *et al.*, 1998). The harmfulness of rust fungi is attributed to its wide distribution and long-distance dissemination, as well as its ability to mutate, forming new races and attacking previously resistant cultivars. Epidemics of this disease can lead to severe losses of grain yield and decreased nutritional quality (Ling *et al.*, 2004).

The majority of the cloned and sequenced resistance genes (R genes) are part of the nucleotide binding site-leucine rich repeat (NBS-LRR) gene family (Rommens and Kishore, 2000). The NBS-LRR gene products are generally composed of three main domains: a) a variable N-terminal domain of approximately 200 amino acids; b) a NBS domain of 300 amino acids, and c) a more variable tandem array of approximately 10 to 40 short LRR (leucine-rich repeat) motifs (Cannon *et al.*, 2002). The NBS domain is believed to participate in signal transduction, while the LRR domain is thought to be involved in ligand binding and pathogen recognition (Young, 2000). P-loop, RNBS-A, kinase 2, RNBS-B, RNBS-C, GLPL, and RNBS-D are also highly conserved motifs generally present in the NBS domain of the R genes (Lee *et al.*, 2003). The use of resistance genes is an effective, economical and ecological method to control epidemics of leaf rust disease.

Many disease resistance (R) genes have been cloned from diverse plant species (Hulbert *et al.*, 2001). Although their protein products share several conserved domains (Dangl and Jones, 2001), R genes are very diverse at the DNA level and colinearity across the grass lineages is often difficult to discern, possibly owing to their fast evolution (Leister *et al.*, 1996). To date, efforts to isolate R genes from wheat on the basis of domain conservation among species or by using model plants with small genomes such as rice as surrogates in positional cloning have been unsuccessful (Kilian *et al.*, 1997). More than 50 leaf rust resistance (*Lr*) genes against the fungal pathogen *Puccinia triticina* have been identified in the wheat gene pool, and a large number of them have been extensively used in breeding. All the 50 *Lr* genes are only known from their phenotype and/or map position except *Lr21*, which was cloned recently (Bossolini *et al.*, 2006).

A new PCR-based strategy using degenerated primers designed for these conserved motifs has resulted in the isolation of numerous resistance gene analogs (*RGAs*) by PCR from plant genomes. This strategy was used successfully to isolate resistance gene analogs from potato (Leister *et al.*, 1996), soybean (Kanazin *et al.*, 1996; Yu *et al.*, 1996), rice (Leister and Katagiri, 2000) and bean (Ferrier-Cana *et al.*, 2003). Nowadays this strategy is being used by different laboratories to isolate and map *RGAs* in several other plant species. Since the gene was found, a series of uses were assigned to this type of marker: a) as a probe to screen BAC or cDNA libraries, in the process of searching for R genes; b) as a marker to be applied in marker assisted selection; and c) to obtain resistance by its overexpression in the plant genome.

MATERIALS AND METHODS

Plant material and rust inoculation

Seven days old seedlings of *Triticum aestivum* L. cv. Giza 168 were inoculated with

diseased specimens obtained from Plant Pathology Institute, ARC. Inocula from the leaf rust (*Puccinia triticina*) were used to inoculate healthy susceptible plants (cultivar Thatcher) and diseased specimens were used to inoculate 7 days old healthy seedlings of the resistant cultivar Giza 168 in the greenhouse. Seedling leaves were rubbed gently between moistened fingers with tap water, sprayed in the inoculation chambers with water, inoculated by shaking and brushing rusted materials over the plants, then sprayed gently again with water in order to induce initial "dew" on the plants. The inoculated plants were kept in moist chamber (18°C) for 24 hr to allow the rust spores to germinate and cause infection. The plants were then moved to their respective benches in the greenhouse and kept for 12-15 days under 22-24°C and relative humidity 80%.

Isolation of total RNA

The leaves of Giza 168 cv at 4 days old after mock- and rust-inoculation were used to isolate RNA. Total RNA was extracted using TriPure Isolation Reagent (Roche, Indianpolis, USA). A volume of 100 mg of each tissue was frozen in liquid nitrogen, transferred to a clean autoclaved mortar, treated with 0.1 % (v/v) diethylpyrocarbonate (DEPC), and ground to a fine powder. The resulting powder was transferred into a polypropylene centrifuge tube at room temperature containing 1 ml TriPure Isolation Reagent. Samples were homogenized using vortex then incubated for 5 min at room temperature to ensure the complete dissociation of nucleoprotein complexes and 0.2 ml chloroform was added and the tube was vigorously shaked for 15 sec. The tube was incubated at room temperature for 10 min and centrifuged at 12,000 *xg* for 15 minutes at 4°C. The colorless upper aqueous phase was transferred to a new polypropylene centrifuge tube. Half ml of isopropanol was added and the tube was inverted several times to mix it thoroughly. Samples were incubated for 10 min at room temperature to allow the RNA precipitate to form, centrifuged at 12,000 *xg* for 10 min at 4°C and the supernatant was discarded. Pellet was washed with 1 ml of 75% ethanol by vortexing and centrifuged at 7500 *xg* for 5 min at 4°C and the supernatant was discarded. The excess ethanol was removed from the RNA pellet by air-drying. The RNA pellet was resuspended in diethylpyrocarbonate (DEPC) treated with RNase-free water, dissolved by passing the solution through a pipette tip several times then incubated for 15 min at 60°C. RNA was either used immediately or kept at -80 until used.

RT-PCR

Isolated RNA was used to detect the presence and expression of genes under study using RT-PCR technique. PCR amplification with *actin*II specific primers was used with the isolated RNA to detect genomic DNA contamination. Reverse transcription using One-Step RT-PCR Kit (Qiagen, Germany) was performed on 2 μg of DNA-free RNA as described by O'Rourke *et al.* (2000). Two sets of primers were designed from the GenBank to amplify fragments of the *Lr21* and *RGA* genes. All primer sequences used in this work are illustrated in Table (1). Total cDNA subjected to PCR was normalized according to the amount of wheat *actin* mRNA. Reaction products were submitted to electrophoresis in a 1.8% agarose gel in 1X TAE buffer as described by Sambrook *et al.* (1989). Ethidium bromide was added to the gel solution at a concentration of 0.1 μg /ml to stain the DNA for visualization after the run using a Transilluminator (λ=375) and photographed under UV light.

Table (1): The nucleotide sequences of the oligonucleotide primers.

Name	Nucleotide sequences 5'→3'	Expected fragment (bp)
Act-F	GATATTCAACCTCTTGTTTG	650
Act-R	ACAATTTCCCGCTCTGCTG	
Lr21-F	GCATCTTGCAGGAGAATGCC	235
Lr21-R	ATATGATCACGCAGCCCCTC	
RGA-F	GGATTTGGGAAGGCCGACGC	445
RGA-R	CCGCGGGATTCGATTCACAT	

Cloning and sequence analysis

The PCR products were cloned in pGEMT Easy plasmid (Promega, Madison, WI) and transformed into *Escherichia coli* DH5α. White colonies were picked and screened for the presence of the cloned fragments by digestion with *Eco*RI. The recombinant clones were sequenced using a Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 DNA sequencer (Applied Biosystems). A homology search was performed using BLASTX against the NCBI protein database (http://www.ncbi.nlm.nih.gov).

Sequences of plant *RGA* genes that showed similarity to the *TaRGA*eg gene were obtained from the NCBI non-redundant and dbEST data sets using BLASTX or BLASTP (ver. 2.0.10) (Altschul *et al.*, 1997). The full amino acid sequences of the proteins were aligned using CLUSTAL W ver. 1.8 (Thompson *et al.*, 1994) and subjected to phylogenetic analysis. Phylogenic trees were constructed by MEGA software (Sudhir *et al.*, 2001) using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with parsimony and heuristic search criteria and 1000 bootstrap replications to assess branching confidence.

RESULTS AND DISCUSSION

Isolation and characterization of a NBS-LRR resistance gene analog from wheat

Total RNA isolated from the fungal resistant wheat cultivar Giza 168 was first tested for its purity from contaminated genomic DNA by PCR analysis using the *actin* specific primers and DNA extract was used as a positive control. Since there are no previous publications for the complete sequence of *RGA* gene isolated from wheat, therefore, *RGA* genes were downloaded from GenBank and aligned together using CLUSTAL W ver. 1.8 (Thompson *et al.*, 1994). Primers were designed according to the conservative region of the genes, and were used to amplify the partial *RGA* gene. In the case of *Lr21*, primers were designed from the wheat (*Triticum aestivum* L.) gene presented in the GenBank (AY139587). The purified RNA was used in the RT-PCR to amplify the partial cDNA of the *RGA* and *Lr21* in addition to PCR to confirm that the amplified fragments are not genomic DNA. The specific *RGA* and *Lr21* primers successfully amplified fragments of 445 and 235 bp, respectively with RT-PCR, but not with PCR indicating that the amplified fragments are cDNA (Fig. 1 A&B). The amplified *RGA* cDNA fragment was purified, cloned into the pGEM®-T Easy Vector, and sequenced. The nucleotide sequences proved that we successfully isolated a cDNA clone with 445 bp. Partial length cDNA of *RGA* gene was submitted to GenBank under the accession number AB304484.

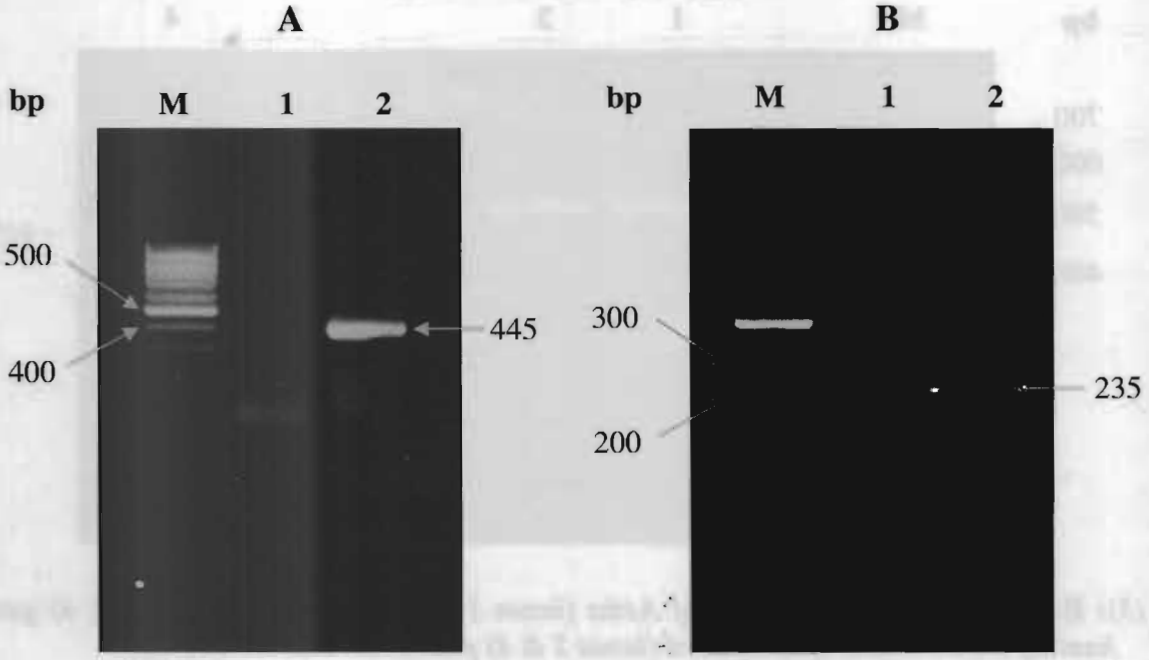

Fig. (1): Amplification (A) RGA gene and (B) Lr21 genes using PCR (lane 1) and RT-PCR (lane 2) analysis, M: marker 100 bp.

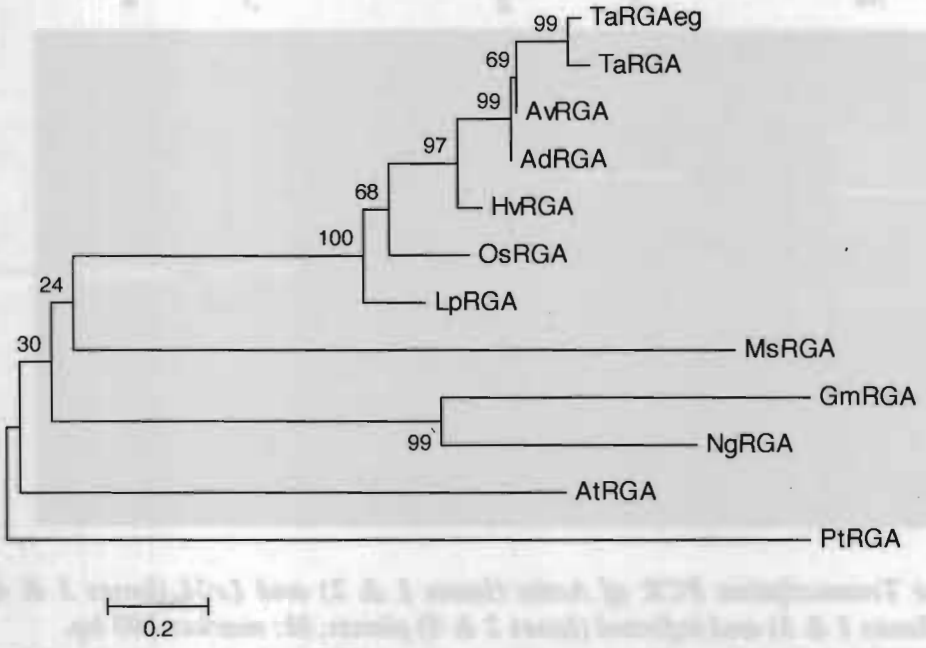

Fig. (2): Phylogenetic analysis of RGAs. A tree generated from the alignment of the amino acid sequence of TaRGAeg with those of plant-encoded isozymes of the RGA protein family was subjected to phylogenetic analysis. Os, Oryza sativa; At, Arabidopsis thaliana; Pt, Pinus taeda; Ta, Triticum aestivum, Av, Aegilops ventricosa, Ad, Avena damascena, Hv, Hordeum vulgare, Gm, Glycine max. Lp, Lolium perenne; Ng, Nicotiana glutinosa; Ms, Medicago sativa.

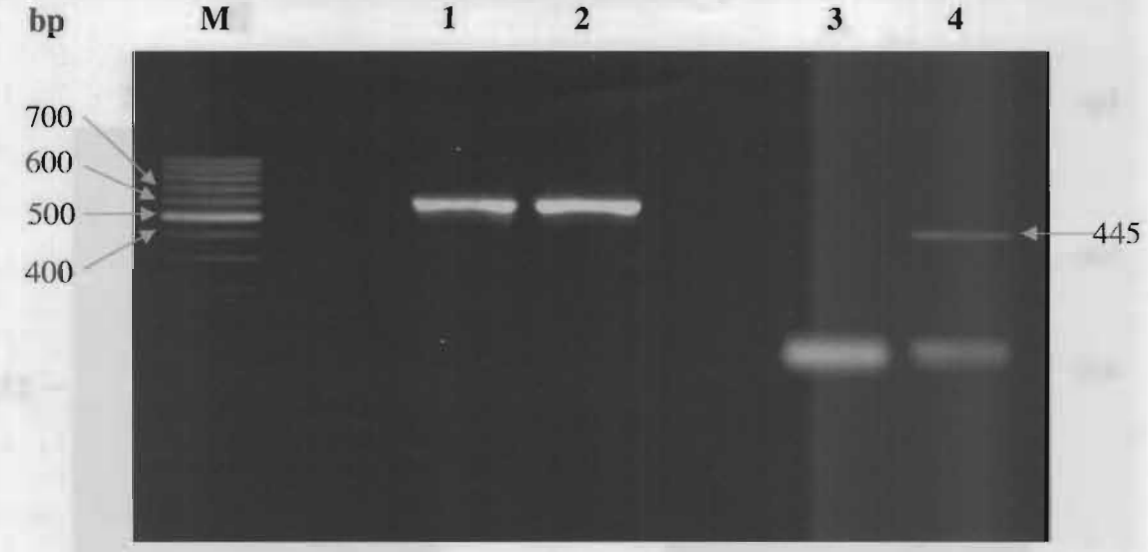

Fig. (3): Reverse Transcription PCR of Actin (lanes 1 & 2) and RGA (lanes 3 & 4) genes for healthy (lanes 1 & 3) and infected (lanes 2 & 4) plants, M: marker 100 bp.

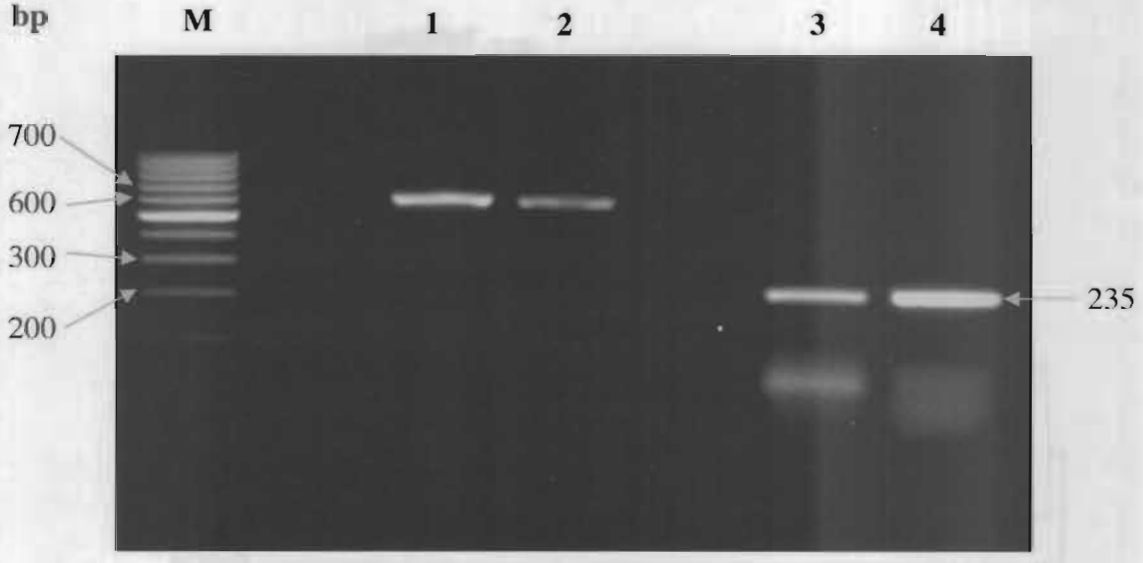

Fig. (4): Reverse Transcription PCR of Actin (lanes 1 & 2) and Lr21 (lanes 3 & 4) genes for healthy (lanes 1 & 3) and infected (lanes 2 & 4) plants, M: marker 100 bp.

The amplified *Lr21* cDNA fragment was purified and directly sequenced using its specific primers. Partial length of *Lr21* cDNA was submitted to GenBank under the accession number AB325481. The BLASTX results confirmed that the isolated gene was *Lr21*. BLASTX results showed 100% similarity to the isolated *Lr21* gene from *Aegilops tauschii*, 63% similarity with NBS-LRR class *RGA* from *Aegilops tauschii* and only 47% similarity with *Lr21* gene isolated from *Oryza sativa*.

To determine the evolutionary relatedness of *TaRGA* to *RGA* proteins isolated from other species, the neighbor-joining method (NJ) was used to generate a gene tree based on amino acid sequence homology of the *RGA* region of the isolated gene to those of *RGA* proteins (Fig. 2). The tree showed that *TaRGAeg* protein forms a distinct clade on phylogenetic tree derived from various *RGA* sequences isolated from monocots. *TaRGAeg* showed the highest degree of similarity with the *RGA* isolated from *Triticum aestivum* (91%), *RGA* from *Aegilops ventricosa* (86%), *RGA* from *Hordeum vulgare* (74%) and *RGA* from *Oryza sativa*, (58%). The obtained *RGA*-eg fragment of the *T. aestivum* cv. Giza 168 showed high similarity with *A. ventricosa* and this may be attributed to the origin of *T. aestivum*, one of its parents is *A. tauschii* which has a genomic similarity with *A. ventricosa*.

Effect of fungus *Puccinia triticina* on the expression of *RGA*-eg and *Lr21*

Reverse-Transcription PCR was used to test the expression patterns of *TaRGA*eg and *TaLr21*eg in healthy and fungus-infected plants (Figs. 3& 4). The Reverse-Transcription PCR was performed using total RNA isolated from both healthy and four-days- old fungus-infected plants. The levels of expression of the isolated genes were calculated relatively to that of the constitutively expressed *Actin* gene. *Lr21* was expressed in both healthy and infected plants (Fig. 3). Interestingly, *TaRGAeg* was expressed only in the infected plants indicating up-regulation expression due to the fungal infection but no RT-PCR product was produced with RNA from healthy plants (Fig. 3). This confirms that the *TaRGA* expression is induced due to the infection of the *Triticum aestivum* plant with the fungus *Puccinia triticina*. In the case of *Lr21*, expression was detected in both infected and healthy plants although the expression was higher with RNA from infected plants as the intensity of the band is almost double of the healthy one. This result may indicate that the gene has higher expression due to fungal infection.

The obtained results indicated that *Lr21*-eg and *RGA*-eg genes are presented in the *Triticum aestivum* cv. Giza168 and their expressions are induced and increased due to fungal infection.

The obtained results are in agreement with Huang *et al.* (2006) who amplified, cloned and sequenced the *WRGA1, WRGA2* and *WRGA14* genes from wheat (*Triticum aestivum* L.). They also found that the wheat *RGA*s contain some conserved motifs such as Kinase-2a, Kinase-3a and HD present in known NBS-LRR resistance genes from other plants and share between 46.0%-9.9% amino acid sequence identity with them. The amino acid sequence identity among three *RGA*s is 80.7%-56.8%. The *WRGA1* expression was induced and detected by Northern blotting, which was positively regulated by salicylic acid. Also, Jiang *et al.* (2005) have applied homology-based gene/gene-analog cloning method in isolation of *RGA*s (resistance gene analogs) in various plant species. A total of seven *RGAs* were obtained and sequenced

from wheat and Thinopyrum genomes. Sequence showed that they have a constitutively expressed single-copy NBS-LRR type and homology analyses showed that the above *RGAs* were highly homologous with known resistance genes or resistance gene analogs and belonged to NBS-LRR type of R genes.

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الملخص العربي

تعريف وتوصيف جين المقاومة المتناظر (*RGA*) وجين مقاومة صدأ الأوراق (*Lr21*) في صنف القمح جيزة ١٦٨

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صدأ الأوراق (البني) مرض فطري للقمح يسبب خسارة كبيرة سنوية في كمية المحصول للعديد من مناطق زراعة القمح في العالم. وتعتبر مقاومة النبات العائل لهذا المرض أفضل طريقة ذات قيمة إقتصادية وغير ضارة للبيئة، للتحكم في الفطر المسبب لصدأ الورقة في القمح. التعريف والإنتفاع بمصادر مقاومة جديدة مفيد لإستكمال تطوير الأصناف المحسنة. الهدف من هذه الدراسة كان تعريف الجينات الدفاعية ضد الصدأ في الصنف المصري المقاوم للصدأ جيزة ١٦٨. تم تصميم بادئات متخصصة في المواضع المحفوظة لجينات المقاومة المعزولة RGA و Lr21 في نبات القمح. إستخدمت تلك البادئات لتفاعل البلمرة المتسلسل العكسي RT-PCR بإستخدام الحمض النووي RNA المعزول من الصنف المقاوم للمرض لتجسيم قطع بطول 445 و 235 زوجاً من القواعد لجينات RGA و Lr21 علي التوالي. تم عزل النواتج المجسمة وتحديد التتابع النوتيدي لها وسجلت في قاعدة البيانات. تم مقارنة التتابعات النوتيدية للمقاطع المجسمة مع الجينات المتوافقة معها في قاعدة البيانات بإستخدام الـ BLAST. وتم دراسة التعبيرات الجينية لتلك الجينات في النباتات المصابة والسليمة بإستخدام RT-PCR. لوحظ إستحثاث للتعبير الجيني للجين *RGA* في النباتات المقاومة نتيجة الإصابة الفطرية. بينما لوحظ حدوث تعبير جيني للجين *Lr21* في النباتات المصابة والسليمة ولكن التعبير الجيني كان أعلى في النباتات المصابة.