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Full Length Article

Reducing *RbcS* Expression by RNAi Technology Causes Diverse Responses to Nematode in Wheat

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Abstract

The reaction of four wheat *RbcS* RNAi mutants and wild type to the infection of *M. incognita* was investigated in a greenhouse pot experiment using plant growth parameters as well as nematode reproduction. Obtained results revealed that all estimated growth parameters of lines under study were reduced by *M. incognita* infection to various extents. RSS13.2 line showed the highest percentage of reduction of plant total fresh weight, length, and shoot dry weight. It did act as good host for root-knot nematode; therefore, it was classified as susceptible host. In addition, the lowest reduction percentage of fresh and dry weights was observed in RSS8.5 line although it showed high values for nematode development and reproduction parameters; therefore, it seemed to be as a tolerant host. The maximum reduction in chlorophyll content was recorded in RSS7.6 line, whereas, the minimum was obtained from RSS11.5 line. It was evident that none of the five tested wheat lines was immune to *M. incognita* infection. The lowest numbers of second stage juveniles, galls, and egg-masses were observed in soil and on roots of RSS11.5 line although its plant growth was slightly affected; therefore, it is considered as partially resistant line. It could be concluded from the results of this study that, RNAi mutants of the *RbcS* were partially effective in suppressing root-knot nematode development. © 2019 Friends Science Publishers

Key words: Wheat; Ribulose-1; 5-bisphosphate (Rubisco); *Meloidogyne incognita; Host suitability*

Introduction

Wheat, *Triticum aestivum*, is the world's most important source of food. It was reported that almost 80% of all nitrogen in plant is to be found in the form of protein molecules in the chloroplast (Adam *et al*., 2001). Rubisco enzyme is the most abundant massive protein in plant's chloroplast, as it represents about 50% and 30% of the total leaf protein and N contents, respectively (Mae *et al*., 1993). Therefore, it is recognized as a crucial investment and resource for nitrogen storage in plants (Millard, 1988). A slight decrease in the content and activity of this enzyme is a main target in crops to improve nitrogen use efficiency (NUE) and wheat yield as well as environmental safety with less nitrogen input. Furthermore, Rubisco has a slow catalytic rate that mainly located in the chloroplast's stroma and acts essentially to catalyze the two competitive reactions: carboxylation and oxygenation. Therefore, it is required in large amounts for maintaining a sufficient rate of the plant's photosynthesis. This is one limiting factor of the maximum photosynthetic rate (Evans, 1989). The Rubisco enzyme consists of two subunit polypeptides: eight dimeric large subunits (55 kDa) and eight tetrameric small subunits (15 kDa). The large subunits are encoded by the rbcL gene in the chloroplast and include the catalytic site and binding sites of the enzyme. However, the nuclear rbcS gene encodes the small subunits, and they do not carry the enzyme catalytic site. It acts to catalyze the oxygenation reaction on RuBP, as well as to form one molecule of 2-PGA and one molecule of 3-PG during photorespiration (Spreitzer, 1993). It was found that there is an over-investment of N in Rubisco for maintaining the photosynthetic process in wheat (Mitchell *et al*., 2000). Transgenic studies on plants with reduced levels of Rubisco grown in high CO_2 environments showed that a reduction of 15–20% in the levels of the Rubisco protein could definitely cause a slight reduction in the N demand by about 10% without any negative impact on the plant photosynthetic efficiency (Parry *et al*., 2013). It has been identified as an important target for genetic engineering using RNAi technology to the rbcS subunit in order to investigate the limitations imposed by its inhibition on photosynthetic capacity as well as growth performance in an attempt to improve NUE (Rodermel *et al*., 1988; Hudson *et al*., 1992).

Plant-parasitic nematodes are common limiting factor

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of wheat production in fields with high infestation by these microscopic parasites. It is documented that root-invading nematodes reduce the plant efficiency to obtain water and nutrients from soil. Also, they facilitate the formation of disease complexes with other pathogenic fungi (Smiley and Nicol, 2009). There are number of nematode pests associated with wheat causing most of the global crop damage, such as *Meloidogyne* spp. *M. graminicola* is widely spread, and is considered a serious soil borne pathogen reducing the productivity of the rice-wheat system in Southeast Asia (Padgham *et al*., 2004). Previous studies showed that some cultivars of wheat were susceptible to *M. incognita* which can invade, develop, and reproduce on winter grown bread wheat under field conditions (Roberts *et al*., 1981; Opperman *et al*., 1988). *M. incognita* attacks wheat roots, forming small galls in the area around where they begin feeding. *M. incognita* infection causes suppression of plant growth (stunting), chlorosis, and loss of water (wilting) in hot weather (Thomas and Kirkpatrick, 2001). It also can reduce the rate of photosynthesis in certain plants. For example, after two days of infection, it caused reduction in the rate of photosynthesis compared to the non-inoculated tomato plants (Loveys and Bird, 1973). Photosynthesis efficiency estimated by fresh weight, leaf size, and total content of chlorophyll was reduced during early infection stages (Loveys and Bird, 1973). In another study, *M. incognita* infection of henbane (*Hyoscyamus niger*) had reduction impact on several growth parameters including, chlorophyll content, yield, rate of photosynthesis, and nutrient content. Reduction was positively correlated with nematode count (Haseeb *et al*., 1990). It is established that leaf chlorophyll content is associated with the efficiency of photosynthesis which reflects the plant nitrogen content (Evans, 1989). Therefore, chlorophyll content (as a measure of nitrogen content), can be used as an indicator of the damage impact on the infected plants by *M. incognita*. Infection with *M. incognita* can reduce photosynthesis by hindering transport of water and nutrients, especially nitrogen (Carneiro *et al*., 2002). Control of plant nematode infection is a difficult task, yet integrated management protocols that integrate crop rotation with resistant crops (Stapleton *et al*., 2010) contribute in the reduction of nematode populations (Molinari, 2011). Although plant resistance to nematodes differs among different genotypes, elements of plant genetic resistance to particular nematodes have been identified (Karajeh *et al*., 2011; Williamson *et al*., 2013). In wheat, a number of six wheat genotypes showed resistant to *M. javanica* (Curto *et al*., 2012). In a gene transfer study, transfer of resistance gene from *Aegilops ventricosa* to LassiK wheat variety showed resistance to both *M. incognita* (virulent) and *M. javanica* (avirulent) (Williamson *et al*., 2013). Developing and use of resistant varieties for control of nematode infection represents the major achievement of recent research efforts for detection and evaluation of resistance sources, integrating them in commercial crop varieties, and using them in integrated control programs (Ferris, 1992).

Reduction of RbcS gene expression has two benefits; the reduction in the undesired oxygenation reaction during photosynthesis and the increase of the available N through the reduction of Rubisco protein content. Therefore, this study focused on the evaluation of host suitability of four independent transgenic *RbcS* RNAi wheat lines to *M. incognita* infection under the greenhouse conditions.

Materials and Methods

Plant Material and Growth Conditions

Wheat seeds of wild type and *RbcS* RNAi transgenic plants including RSS11.5, RSS8.5, RSS7.6, and RSS13.2 were obtained from Prof. Christine Raines; School of Biological Sciences (University of Essex, UK). These four lines were transformed with a gene construct containing RbcS RNAi. These lines were previously screened and shown to have various reduction levels in Rubisco protein. All transgenic wheat seeds were germinated and grown in 15 cm diameter pots in steam-sterilized mixture of peat moos and sand (1:1, v:v) under controlled glasshouse conditions at 25± 2°C day/18± 2°C night for 3 weeks. A total of 8 replicates per line (two seeds/pot) were grown, and after one month were thinned to one seedling/pot. Leave samples were collected, lyophilized at -58°C, ground to fine powder, and stored at -20°C until used.

Detection of *RbcS* RNAi Construct in Transgenic Wheat Lines

DNA isolation: Leaf tissues from wheat lines were lyophilized at -58°C for 48 h. Tissues were ground to fine powder and used for DNA isolation. DNA was extracted of leaf samples by the CTAB method (Saghai-Maroof *et al*., 1984) with the optimization of the protocol for lyophilized ground tissues. Warm (65ºC) CTAB buffer (1% CITAB, 50 mM Tris pH 8.0, 0.7 M NaCl, 10 mM EDTA, 0.1% BME), 0.5 mL, was added to 5 mg of lyophilized ground leaf powder, mixed, and incubated for 1 h at 65ºC. Equal volume (0.5 mL) of 24:1 Chloroform: Isoamyl was added and mixed. The mixture was centrifuged for 10 min at 12000 rpm at 4ºC. The upper aqueous phase was transferred to new 1.5 mL tube. Isopropanol, 1 volume, was added, mixed, and centrifuged for 10 min at 12000 rpm at 4ºC. DNA pellet was washed with 70% ethanol, air dried for 10 min at room temperature, and dissolved in 50 µL DH_2O. DNA was kept at -20°C until used for PCR.

PCR Detection of *RbcS* RNAi Construct in Transgenic Wheat Lines

The transgenic status of plants carrying the *RbcS RNAi* construct was confirmed by detection of rice RTVP promoter driving the *RNAi* of *RcbS* gene construct using specific primers for *RbcS* RNAi (Forward:

AAGAAGTTCGAGACCCTGTCTTA, Reverse: GTAAATTGCACTCTAGATTTTGCTT). PCR amplification was carried out for 30 cycles that included 50 s at 94°C, 40 s at 57°C and 1 min at 72°C. The final volume of PCR reaction was 25 µL containing 12.5 µL of 2X Master mix (Promega, Wisconsin, USA), 15 ng of DNA template, and 10 pmole of primers (forward and reverse). PCR products were electrophoresed in agarose gel, stained with ethidium bromide, and photographed.

Determination of the Expression Level of *RbcS* Gene in Transgenic T3 Wheat Plants using qPCR

The RNAs were extracted from the second leaves at the seedling stage from T3 plants. *RbcS* RNAi construct was amplified using specific primers (Forward: agttcagcaaggttggcttc, Reverse: cctcgttgagcacctgtgta). The gene expression was normalized against actin reference gene (Forward: gaatccatgagaccacctac, Reverse: aatccagacactgtacttcc). Mixture reactions were prepared as following: 7.5 µL of Sybre green, 2 µL of forward and reverse primers (10 pmol). Total 9 µL of the master mix were added in triplicate into 96 wells plates including 6 µL of cDNAs. Followed by, the amplification cycles of q-PCR as following: 95°C for 3 min, (95°C for 10 sec, 62°C for 30 sec) (45 cycles).

Nematode Source and Inocula

The root-knot nematode was obtained from a pure culture that was generated from a single egg-mass of *M. incognita* which was identified based on its perineal pattern (Taylor and Sasser, 1978). It was propagated and sub-cultured on tomato plants cv Super Strain B to get enough inoculum at Biology Department, Faculty of Science, Taif University. Egg-masses were isolated from tomato infected roots, kept in 0.5% sodium hypochlorite for 5 min (McClure *et al*., 1973). The obtained second-stage juveniles (J2) were kept in a hatching chamber. The number of juveniles collected within 48 h were counted using a Hawksely counting slide and then used directly for plant inoculation.

Plant Growth and Treatments

Four transgenic lines and one wild type line of wheat were screened for their susceptibility to *M. incognita* infection. Plants were grown in plastic pots, eight for each line. After one week of seedling thinning, four plants of the control and mutant lines were infected with 2000 J_2s of *M. incognita*. The other four plants of each line were used as uninfected control for each line. Pots were distributed in complete block design in the greenhouse at 25±2ºC. After infection, 30 days, plants were removed and roots were cleaned from soil. Plant growth parameters including length, shoot and root fresh weight, and shoot dry weight were determined for individual plants. The reduction percentage in growth parameters of infected plants was calculated in relation to the uninfected plants for each line. Infected roots were stained with acid fuchsin in lactic acid (Byrd *et al*., 1993). Number of galls and egg-masses were counted in the stained roots. Both of root gall index (RGI) and egg-mass index (EI) were estimated according to a scale of 0 – 5 (Taylor and Sasser, 1978).

Estimation of Total Chlorophyll Content

Chlorophyll content was estimated using MC-100 Chlorophyll Concentration Meter (Apogee, UT, USA). Measurements were repeated three times for each plant and the average reading was used.

Statistical Analysis

Data were statistically analyzed (ANOVA) (Gomez and Gomez, 1984) and means were compared using Duncan's multiple range tests (Duncan, 1955).

Results

Detection of *RbcS* RNAi Construct in Wheat T3 Plants

To confirm the presence of the *RbcS* RNAi construct in the T3 generation, the first leaf of each independent line was harvested for DNA extractions and PCR screening. As a result, several positive bands were identified at approximately 750 bp, which indicated the presence of the *RbcS* RNAi construct compared to the WT line with no bands (Fig. 1).

Gene Expression Analysis

To analyze the expression of the *RbcS* RNAi construct in transgenic T3 wheat plants, total RNA from the second leaf of 4 plants within the five independent transgenic lines: WT, RSS7.6, RSS8.5, RSS11.5 and RSS13.2, was extracted and the cDNA was synthesized. The *RbcS* cDNA of the different plants within the five independent lines was amplified using qPCR. The results showed that the relative expression levels were decreased in several individual *RbcS* RNAi plants, and that a range of reductions in *RbcS* gene expression levels was evident in the different independent *RbcS* RNAi lines compared to the WT line (Fig. 2).

Response of RbcS RNAi Wheat T3 Lines to *Meloidogyne incognita* Infection

Data in (Table 1) show the influence of *M. incognita* on growth parameters of four transgenic wheat lines and wild plants. Results revealed that *M. incognita* infection reduced the growth of the tested plants at different extents. The highest reduction in plant length as affected by nematode infection was recorded in line RSS13.2 as 35.1%, whereas, RSS11.5 line showed the least percentage of length reduction

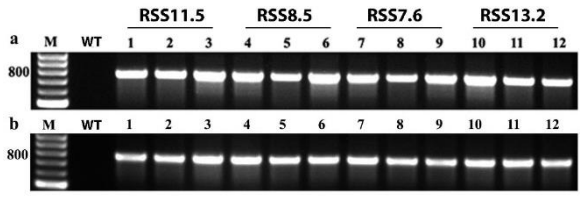

Fig. 1: Detection of RbcS RNAi construct in T3 transgenic wheat plants

The plants containing the *RbcS* RNAi construct represent a band at approximately 750 bp compared to the wild type (WT). M: molecular size marker

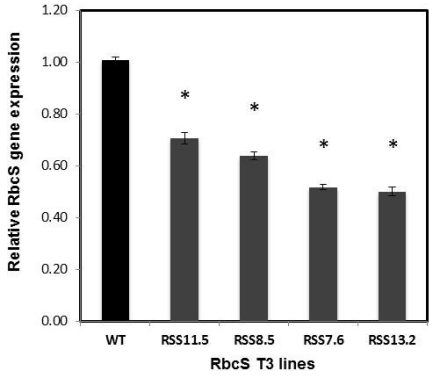

Fig. 2: Expression of RbcS gene in T3 wheat lines expressing RbcS RNAi. Values represent average of four plants from four individual transgenic lines compared to WT line. Stars indicate significant differences from WT ($P<0.05$)

(3.5%). However, remarkable reductions were obtained in lines RSS7.6, wild type, and RSS8.5 with average values 21.8, 18.5 and 15.6%, respectively. Among the five lines tested, RSS8.5 showed the lowest reduction percentage in fresh as well as dry weight with values of 12.1 and 6.5%, respectively followed by RSS11.5 line with values of 17.8 and 8.8%. Wild type showed average reduction percentage of 23.5 and 9.1%, whereas RSS13.2 line gave the highest reduction percentage for the tested parameters (39.8 and 17.2%). A similar trend was noticed in case of chlorophyll content. Significant reduction in leaf chlorophyll content of infected plants compared with the uninfected ones. The maximum reduction in chlorophyll content was recorded in RSS 7.6 line with value of 32.7%, while the minimum reduction was recorded in RSS11.5 line with average value of 19.8%. However, chlorophyll content was moderately reduced in wild type, RSS8.5 and RSS13.2 lines with values of 24.5, 26.8 and 27.3, respectively (Table 2).

Galling and Reproduction of *M. incognita* on T3 RbcS RNAi Lines

Data in (Table 3) revealed that galls and egg-masses were significantly formed on roots of all examined wheat lines infected with *M. incognita*. The highest number of second stage juveniles (324.8), galls (23) and egg-masses (15.8) were observed on roots of RSS8.5 line, followed by RSS13.2 with values of 265.3, 16 and 10, respectively. The least values of nematode number (25), galling (2) and egg-mass number (1) were recorded in RSS11.5 line plants. Root gall indices values ranged from 1.3 to 3, whereas, egg-mass index ranged from 1 to 3.

Discussion

The majority of the plant developmental growth parameters of the wheat lines were considerably adversely correlated with the number of *M. incognita* juveniles in the soil as well as the galls number and egg-masses on the investigated roots. The obtained results correspond with those recorded by Abad *et al*., (2003) who mentioned that *Meloidogyne* spp. induce galling in the infected roots and form giant cells at the stellar region. This leads to a severe damage in the xylem and finally significantly reduce the absorption of water and nutrients from the soil. This effect, limited water and nutrients, represent the first direct effect of nematode infection on plant physiology and metabolism (Lu *et al*., 2014). Our findings are contrary to the conclusion of De Brida *et al*. (2017) that wheat was resistant to both of *M. incognita* and *M. javanica* (Birchfield, 1983). In the current study, none of the five screened wheat lines showed resistance to *M. incognita* infection, yet the pathogen was able to form galls and egg-masses on all of them. However, it relatively failed to reproduce and multiply on the line RSS11.5 and exhibited low reduction in growth parameters. Therefore, RSS11.5 was considered as relatively resistant to *M. incognita* infestation. The lack of significant plant development reduction due to nematode infection indicates that RSS8.5 line is a tolerant host, even when seedlings are severely attacked. RSS13.2 and RSS7.6 lines acted as good hosts for root-knot nematode multiplication and were more severe than that observed for other lines; therefore, they could be classified as susceptible and slightly susceptible, respectively. A reduction in total chlorophyll has been recorded from all tested lines because of nematode infection. The minimum reduction in chlorophyll was observed in RSS11.5 line, whereas, the maximum was obtained from RSS7.6 line. In this respect, our results confirmed the results obtained from other studies (Melakeberhan *et al*., 1986; Swain and Prasad, 1988) carried out on French bean and rice challenged with *M. javanica*. Despite the variance in the *RbcS* gene expression levels among wheat lines, RSS13.2 and RSS7.6 lines had lower expression, showing an evident susceptibility to nematode infection, whereas, this gene was higher expressed in RSS11.5 line, suggesting that resistance in this transgenic line had been stimulated.

Enhanced resistance of *RbcS* RNAi lines could be due to the inhibition of RbcS and consequently reduction in Rubisco protein, which is reflected in the increase of the available N for plant growth, development, storage of seed protein. Wheat plants with RbcS RNAi construct showed different levels of Rubisco, photosynthetic rate, biomass,

Table 1: Plant growth responses of wheat RbcS RNAi lines to infection by *M. incognita* infection under greenhouse conditions

Lines	Nematode Infection	*Plant Growth Response									
		Length (cm)		Plant length (cm)	Red. %	Fresh weight (g)		Whole plant fresh weight (g)	Red. %	Shoot dry weight (g)	Red. %
		Shoot	Root			Shoot	Root				
Wild	U	36.3 a	42.8 a	79.1		35.1 a	40.8 a	75.9 a		12.1 a	
	I	32 b	32.5 b	64.5	18.5	29.3 b	28.8 b	58.1 b	23.5	11 a	9.1
RSS11.5	U	38.5 a	33.8 a	72.3		30.6 a	36.1 a	66.7 a		9.1 a	
	I	37.8 a	32 a	69.8	3.5	26.8 b	26.8 b	53.5 b	17.8	8.3 a	8.8
RSS8.5	U	32.5 a	39.5 a	72		31.2 a	51.7 a	82.8 a		10.8 a	
	I	31 a	29.8 b	60.8	15.6	27.9 a	44.9 b	72.8 b	12.1	10.1 a	6.5
RSS7.6	U	40.3 a	47 a	87.3		29 a	50.5 a	79.5 a		10.7 a	
	I	35.5 b	32.8 b	68.3	21.8	21.4 b	30.9 b	52.3 b	34.2	9.4 a	12.1
RSS13.2	U	42.3 a	49.8 a	92.1		30 a	48.3 a	78.3 a		12.2 a	
	I	30.8 b	29 b	59.8	35.1	24 b	23.2 b	47.1 b	39.8	10.1 b	17.2

*Values are the average of 4 replicates. Values with the letter are not significant at P< 0.05 based on Duncan Multiple Range Test. U: Uninfected, I: Infected

Table 2: Chlorophyll content of wheat RbcS RNAi lines as influenced by *Meloidogyne incognita* infection

Lines	Nematode Infection	*Chlorophyll (mg/g)	Red. %
Wild	U	20.4[a]	
	I	15.4[b]	24.5
RSS11.5	U	20.2[a]	
	I	16.2[b]	19.8
RSS8.5	U	19.0[a]	
	I	13.9[b]	26.8
RSS7.6	U	21.4[a]	
	I	14.4[b]	32.7
RSS13.2	U	23.8[a]	
	I	17.3[b]	27.3

*Values are the average of 4 replicates. Values with the letter are not significant at P< 0.05 based on Duncan Multiple Range Test. U: Uninfected, I: Infected

Table 3: Screening of wheat lines to the infection of *Meloidogyne incognita*

Lines	J_2s in 100 g soil	*Galling and Reproduction response			
		No. of G	RGI	No. of EM	EI
Wild	122 c	5 d	2 b	3 d	1.8 c
RSS11.5	25 d	2 e	1.3 c	1 e	1 d
RSS8.5	324.8 a	23 a	3 a	15.8 a	3 a
RSS7.6	139 c	9 c	2 b	6 c	2 bc
RSS13.2	265.3 b	16 b	3 a	10 b	2.3 b

*Values are the average of 4 replicates. Values with the letter are not significant at P< 0.05 based on Duncan Multiple Range Test. U: Uninfected, I: Infected. G: Galls, EM: Egg mass, RGI: Root Gall Index, EI: Egg-mass Index, 0: No G or EM, 1: 1-2 G or EM, 2: 3-10 G or EM, 3= 11-30 G or EM, 4= 31-100 G or EM, 5= more than 100 G or EM

and grain yield. Plants with partial reduction Rubisco protein up to 25% did not have an adverse effect on photosynthesis, growth or grain yield (Unpublished data).

Therefore, the transgenic wheat lines used in this study with little discrepancies in *RbcS* gene expression and resistant to the root-knot nematode could have the potential as a genetic resource for developing resistant wheat varieties to this nematode. The obtained results are promising since resistant wheat lines could be used in crop rotation or as a succession crop for nematodes integrated management control in infested areas. In conclusion, the present study demonstrated that growing of the resistant (RSS 11.5) and tolerant (RSS 8.5) lines in infested areas with *M. incognita* could contribute to limit root-knot nematode reproduction. Further experimental studies are required to develop more resistant wheat varieties and their implementation in commercial resistant wheat cultivars. In conclusion, growing of the resistant (RSS 11.5) and tolerant (RSS 8.5) transgenic wheat lines, with reduced RbcS, in infested areas with *M. incognita* could contribute to limit root-knot nematode reproduction and should be involved in integrated nematode management programs.

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