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RESEARCH ARTICLE

Transcript analysis of telomerase enzyme gene in sunflower infected by sclerotinia stem rot disease

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ABSTRACT: Sclerotinia stem rot disease caused by *Sclerotinia sclerotiorum* is one of the most important diseases of sunflower. Telomeres are nucleoprotein structures at the ends of chromosomes that are essential for maintaining the integrity of the genome. The aim of this study was to determine the alteration of telomerase enzyme gene (*tert*) expression under fungal infection stress. The expression of *tert* gene in both susceptible (SDR19) and resistant (LC1064-C) genotypes of sunflower was evaluated by qRT-PCR after infection with A37 isolate of *S. sclerotiorum*. The results showed significant and drastic decreased levels of *tert* expression in both susceptible and resistant genotypes of sunflower, immediately after fungal infection. This depletion followed by mild variation when infection continued for more hours, which was more constant in resistant line, compared with the susceptible one. In conclusion, the expression of *tert* gene in sunflower is downregulated in response to Sclerotinia rot disease.

KEYWORDS: Fungal infection, Plants, Telomerase gene expression, qRT-PCR

INTRODUCTION

Sunflower (*Helianthus annuus* L.), a plant with the origin of North America, is one of the most important oil seed crops in the world. One of the unique features of this plant is its short growth period and adaptation to various weather conditions. Therefore, it is suitable for cultivation in dry and low rainfall areas (1,2). Recombinant sunflower plants are highly economical and nutritive, so that 12% of the world's largest dietary oils are derived from this plant (3). In addition to produce nutritious oil, this valuable ingredient is also used as one of the main materials of polymers, biofuels and lubricants in the industry (4). In recent years, private and public sectors have focused more and more on the accurate genetic information of sunflowers and their collection as to improve the productivity of this strategic product. Today, the estimated value of sunflowers breeding budget in the world is 20 billion dollars a year (5). However, this plant is still exposed to the invasive pathogens such as *Sclerotinia sclerotiorum* (Lib.). This pathogen is a necrotrophic fungus that has high affinity to infect a wide range of different species of sunflower (6). Aside from sunflower, this pathogen can also infect 400 species of 75 different plant families, led to eliminate the important products such as soybeans, canola and all kinds of legumes (7). Being adopted in the wide range of hosts and conserved proliferation in tough environmental conditions, this fungus considers as one of the most important causes of plant diseases (6). Infected plants show dry rots, include white crown in stems, one of the most destructive infections aroused by *S. sclerotiorum* (8). Over the past years, several studies have been made to clarify the resistant mechanisms against the pathogenic

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fungus *S. sclerotiorum*, but the exact and possible implication of mechanisms in growth arrest of infected area in plant have not been reported (9). Telomerase is a ribonucleoprotein with reverse transcriptase activity, using internal RNA subunit as template. This enzyme expresses through cell cycle, as to add the tandem telomeric sequence motif at the end of DNA (10,11). Telomeres usually contain short and repetitive G-rich nucleoprotein structures at the ends of linear eukaryotic chromosomes, include 5'(TTAGGG)3' in mammals, and 5'(TTTAGGG)3' in plants which was first characterized in *Arabidopsis thaliana* (12). As a matter of fact, telomeres represent functional elements; meanwhile its appropriate elongation is necessary to maintain integrity and vital stability of the genome. The telomerase compensates telomere shortening using its catalytic protein subunit TERT, and an RNA subunit TER (13). Thereby telomerase leads the primary mechanism of replenishing the lost terminal sequences in eukaryotic DNA telomeres, essential for self-renewing of the cells. Improper telomerase expression provokes domino-like effects, finally led to abnormal proliferation. Taken together, sufficient telomerase expression is inevitable to trigger normal proliferation of regenerative tissues and restrain stem cell-related diseases (14). In plants, this enzyme is present in embryonic and primary developmental stages, associated with reproduction, as well as it is detectable in dividing meristem cells, which have the highest enzyme activity in the early roots of the three-day and flowering plants (15, 16). It was shown that transcription of the *tert* gene acts directly as the main factor determining telomerase activity in plant tissues (15).

Unlike animal cells, alteration of telomerase activity through plant diseases has not apparently been detected. Inspired by this fact, we attempt to study the variation of *tert* gene expression in Sclerotinia rot in sunflower for the first time.

MATERIALS AND METHODS

Plant material and inoculation

Two genotypes of sunflower, SDR19 and LC1064-C lines with different sensitivity to *S. sclerotiorum* isolate A37 were selected based on the results of previous studies (17,18) and prepared from the French Agronomic Research Institute. Seeds of both genotypes were planted in 30 cm clay flowerpots of 20 × 60 cm containing sterilized soil and farm manure mixture. The plants were grown in controlled conditions at 25 ± 1 °C, 65% relative humidity and 12h dark-light photoperiod for six weeks until the 6th-8th leaf stage (19). For inoculation purpose, the fungus isolate cultured in potato dextrose agar (PDA) medium. After growing the isolate, discs of mycelium were cut in 3 mm from the growing colony and subjected over the exposed base of healthy stems. Afterwards, the base of plant stems was covered gently with parafilm and cotton pad for 72h to maintain moisture. Experimental groups were categorized to control, 3, 6, 12, 24 and 48h after infection with fungal isolates. Prepared samples transferred to liquid nitrogen as to storage. In order to control the inoculation efficiency along with the observation of necrotic trends, several inoculated SDR19 and LC1064-C genotypes were grown for a few days (17). The experiment was performed as a completely randomized block design to arrange the pots with 3 replicates per treatment.

qRT-PCR analysis

RNA extraction kit RNX-plus™ (Sinaclon Co., Iran) was used according to the manufacturer's protocol. At first, leaf samples in amounts of 50-100 mg were homogenized and purified. Then, 600 µl of RNX-plus buffer was added to the tube containing sample powder and vortex for 10-15 s. the tube was placed at room temperature for 5 min. Then 200 µl of chloroform was added to the solution and placed on the ice for 15 min. The solution was centrifuged at 13000 rpm for 15 min. After removing the supernatant phase, isopropanol was added and the mixed sample was placed at -20 ° C for 30 min. Final pellet was dissolved in nuclease-free water. One-percent agarose gel electrophoresis and spectrophotometer were used to verify the quality and quantity of the extracted RNA respectively.

Complementary DNA (cDNA) synthesis Kit (Fermentas LIFE SCIENCE # K1621) was used according to the manufacturer's instructions to perform reverse transcription reaction, using six micrograms of total RNA with the oligo-dT primers.

Quantitative reverse transcription-PCR (qRT-PCR) was performed in duplicate using 6.25 µl of Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) (Thermo Fisher Scientific, Germany), 5 pM of each primers and 50ng of cDNA in a total volume of 12.5µl. The primer pairs of *tert* gene (F: ttgcctcgcatgtatatggttg, R: tctgcttcttccctgatcgag) and *actin* (F: gcagggatgagcacaagtg, R: cccaccactgagcacacaatgt) were designed by Oligo 7

software. Relative gene expression was analyzed by comparative Ct method, $2^{-\Delta\Delta C}$ (20). Target gene was normalized by the reference gene, *actin* and calibrated for each sample against the control. qRT-PCR data were expressed as mean±SD, and the differences of the mean values were statistically analyzed by SPSS software (Version 20) using one-way ANOVA followed by Tukey's HSD test. P-values less than 0.05 were considered statistically significant.

RESULTS

Based on previous phenotypic studies, the LC1064-C genotype was resistant to A37 isolate, but SDR19 was highly susceptible (17).

To evaluate the efficiency of our experimental groups versus control one in induction of telomerase gene, TERT expression, we used qRT-PCR. Significant reduction ($P \leq 0.05$) was observed in the expression of *tert* gene immediately after beginning the infection with fungi through different hours of treatment in both susceptible and resistant genotypes (Fig. 1a and b). However, the reduction rate was significantly more in resistant genotype compared to susceptible one 3h after inoculation. This reduction was efficient enough to prove the direct effect of fungal infection on telomerase gene expression. Although this repletion showed mild pattern later, but no remarkable difference observed in the levels of *tert* expression between susceptible and resistant genotypes (Fig. 1c), which indicates the same effects of resistant and susceptible cultivars on the expression of *tert* gene.

DISCUSSION

Biological stresses such as various fungal, microbial and viral diseases are one of the most important factors in reducing crop production in the world. Sclerotinia rot is one of the most important fungal dependent disease in sunflower with high prevalence worldwide, which has made it as a main problem in the cultivation of oilseeds (21, 22). One of the most economical methods for controlling such disease is utilization of resistant genotypes. It is necessary to understand molecular mechanisms and gene expression patterns affecting the growth and development of the plants as to improve plant resistance to Sclerotinia rot. Recent studies have demonstrated the probable molecular mechanisms of plant resistance to this pathogen. However, there are no molecular assessments on how the growth of plant is affected in the presence of this and the other necrotrophic pathogens. For example, *pdf1.2* gene, regulated by jasmonic acid, is known as a resistant factor to necrotrophic agents (23, 24).

Different expression levels of phenylalanine ammonia-lyase are involved in the sensitivity of plant varieties to disease (25). Another study has reported the increased expression of *pal-2* gene through rapidly inducing cell death (26). Upregulation of WRKY15 and WRKY33 transcription factors as well as growth hormones are correlated respectively with increased resistance of weeds

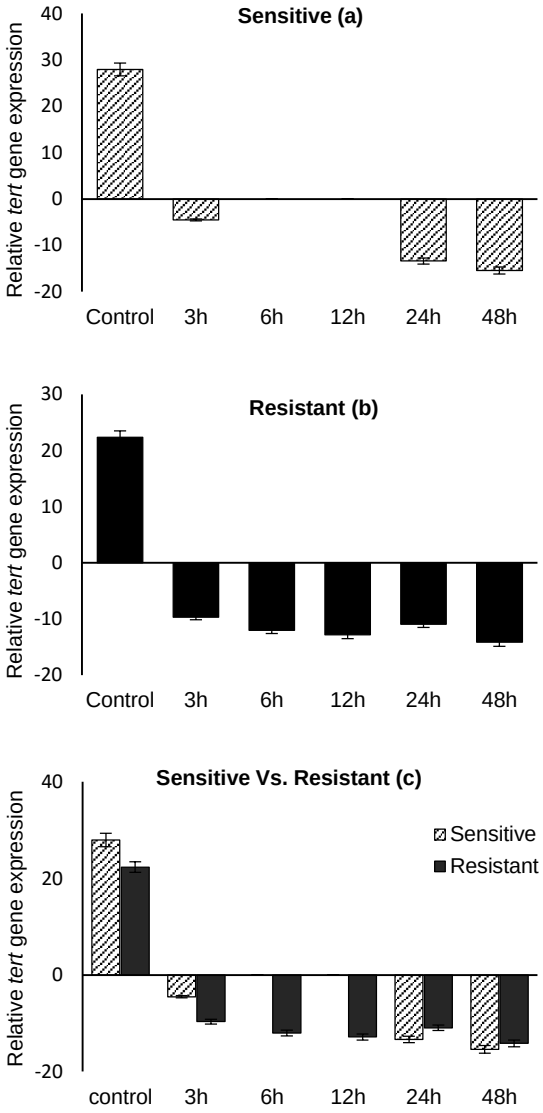

Figure 1. Relative expression of *tert* gene on 3, 6, 12, 24 and 48 hours after treatment with *S. sclerotiorum*. Significant downregulation of *tert* gene in sensitive (a) and resistant (b) genotypes ($P \leq 0.05$) at the first time of treatment. No significant difference be observed when comparing sensitive and resistant genotypes (c).

and Olive-oily seeds versus Sclerotinia rot (27). Additionally, downregulation of endo-β-1 and 4-xylanase genes can decrease the rate of growth and development of plants through such disease (28). It has also been confirmed that there are relations between increased expression of *Pvpgip* gene in the bean and *Pvpr3*, *Pvpr2*, and *Pvpr1* genes in *phaseolus vulgaris* when facing the Sclerotinia rot (29). However, a similar hallmark of molecular investigations about how the growth of plants affected by *S. sclerotiorum*, had not been described prior to this report.

Given the importance of investigation on the involved molecular factors that are effective on plant growth under different biological stresses, as well as having insufficient information about it, our research aimed to study a new gene expression pattern in response to this pathogen.

Overall, it has been shown that Sclerotinia rot is a necrotrophic disease, causes cell death, and as a result, infected tissues are not able to reproduce. According to studies on animal cells, the expression of telomerase enzyme decreases when cell death, but increases when cell reproduction.

Inspired by animal studies, one interesting finding is that plant tissues also show lower levels of telomerase enzyme activity when infected with necrotrophic disease include Sclerotinia rot, due to loss of reproductive capacity and finally death of cells. The results presented here demonstrate for the first time that *tert* expression is disturbed in plant when facing disease. According to our results, it is clear that both of resistant and susceptible genotypes of sunflower show decreased rate of *tert* expression through disease. In the sensitive genotype, immediate and significant decrease of *tert* expression is seen through the first hours after beginning disease. This depletion, then followed by gradual increased rate of this gene expression at 6 and 12 hours after contamination, which indicate a kind of struggling of the plant to be survive. However, no significant depletion of *tert* expression dominates again at 24 and 46 hours after contamination, which resume the necrotic processes, finally led the plant to death. *tert* expression in the resistant genotype was lower than sensitive one in compare.

Overall, these results confirm the direct effects of *S. sclerotiorum* on the expression of *tert* gene, maybe correlated with cell death.

CONCLUSION

It can be concluded that the rate of *tert* gene expression is significantly reduced in sunflower when respect to the sclerotinia fungus. Additionally, cell death in infected plant is induced from the first hours of contamination in both sensitive and resistant genotypes. Interestingly, both of genotypes show low difference with each other in the depletion rate of *tert* expression during the contamination. It can be propounding that resistance genes have little correlation with the process of *tert* expression, an issue which evokes further investigation in future. Furthermore, understanding the relationship between *tert* and necrotic genes in necrotic diseases such as Sclerotinia rot requires further consideration.

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CONFLICT OF INTEREST

The authors indicate no potential conflict of interest.

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آنالیز رونوشت آنزیم تلومراز تحت تنش قارچ اسکلروتینیا در گیاه آفتابگردان

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چکیده

بیماری پوسیدگی ساقه اسکلروتینیا ناشی از قارچ *Sclerotinia sclerotiorum* یکی از مهم‌ترین بیماری‌های آفتابگردان است. تلومرها ساختارهای نوکلئوپروتئینی موجود در انتهای کروموزوم‌ها هستند که برای حفاظت از تمامیت ژنوم ضروری می‌باشند. هدف از این مطالعه تعیین تغییر میزان بیان ژن آنزیم تلومراز (*tert*) تحت استرس عفونت قارچی بود. در این تحقیق بیان ژن *tert* در هر دو ژنوتیپ حساس (SDR19) و مقاوم (LC1064-C) آفتابگردان پس از آلودگی با جدایه A37 قارچ *S. sclerotiorum* توسط تکنیک qRT-PCR بررسی شد. نتایج حاصل، کاهش معنی‌دار و شدیدی را در میزان بیان ژن *tert*، در هر دو ژنوتیپ حساس و مقاوم آفتابگردان بلافاصله پس از عفونت قارچی انشان داد. با افزایش مدت زمان آلودگی، این کاهش بیان تغییرات کمی را نشان داد که این تغییرات در لاین مقاوم نسبت به لاین حساس بیشتر بود. در نتیجه گیری کلی می‌توان گفت که ژن *tert* یکی دیگر از ژن‌های القا شونده با بیماری پوسیدگی اسکلروتینیا در آفتابگردان می‌باشد که الگوی کاهش بیان را نشان می‌دهد.

کلمات کلیدی: عفونت قارچی، آفتابگردان، بیان ژن تلومراز، qRT-PCR