Isolation and characterization of glutathione s-transferase and glutathione reductase in Kabarla strawberry variety

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Abstract
Reactive oxygen species (ROS) increase in plants due to abiotic and biotic stresses. ROS interferes with intracellular substances and cell membrane to damaged plants. Plants have enzymatic and non-enzymatic antioxidant systems to protect themselves against ROS. Glutathione reductase (GR) and glutathione s-transferase (GST) are two of the antioxidant enzymes. The sequence information of these enzymes, which are extremely important for coping with stress, has not been investigated in many plants including strawberries. In this study, GR and GST antioxidant enzyme genes were isolated and characterized from Kabarla strawberry variety. For this purpose, degenerated primers were designed and partial cDNAs were amplified by PCR, and then 3' and 5' RACE analysis were performed to obtain their full length nucleotide sequences.

According to the sequence results, the GR and GST genes were composed of 2091 and 1007 nucleotides respectively and were found to have open reading frames of 557 and 287 amino acids. In addition, the isolated GR gene has shown high similarity to the genes isolated from Pisum sativum, Medicago truncatula and Cicer arietinum; the GST gene demonstrated high sequence similarity to the genes obtained from Arabidopsis thaliana, Zea mays and Prosopis juliflora.

Keywords: Kabarla, antioxidant enzyme, glutathione s-transferase, glutathione reductase.

Introduction

The species of the genus Fragaria in the Rosaceae Family of Rosales is called strawberry. As strawberry, whose homeland is north and south America, has adapted to different ecological conditions, it can grow naturally in some places. Strawberry species are divided into 3 categories as short days, neutral days and long days according to photoperiodism. Strawberry mostly loves sandy-loam, active lime content and low salinity, slightly acidic soils[1].

Kabarla, which is obtained by crossing of Early Sweet and Selva cultivars, is a short-day variety. Short-day strawberries begin to bloom when the day length is less than 12 hours. While short-day strawberries begin to bear fruit in June in the regions where the winters are cold, they bear fruit from December in the regions where winters are mild. Kabarla is a fertile strawberry variety and its fruits are medium size and bright. In many studies, it is determined that the yield of the Kabarla strawberry variety is higher than the other varieties[15]. In many studies, it is found that the strawberry is a fruit rich in reduced glutathione (GSH), B3 (nicotinic acid), B6 (pyridoxine hydrochloric), B9 (folic acid), C (ascorbic acid) vitamins and antioxidans[6].

As a result of making oxygen respiration by the organism, oxygen in the cells is involved in the destruction of many nutrients together with enzymes as a result of various biochemical activities and as a result of this destruction, a variety of end-products are generated. Free radicals are found in these end-products. Free radicals are found in organisms both for beneficial metabolic purposes and as a result of leakage from the intermediate steps of chemical destruction reactions. Mitochondria, cell membrane, cytochrome P450 and active leukocytes are the endogenous radical sources found in the organisms[7-9].

Great majorities of abiotic stresses increase the amount of harmful oxygen species (ROS), which are superoxide, hydrogen peroxide and hydroxyl. Plants fight with ROSs through enzymatic and non-enzymatic systems available in their structures[10]. Glutathione reductase (GR) and glutathione s-transferase (GST) enzymes are also found in many living organisms including prokaryotes and eukaryotes. GR is an enzyme that has FAD in its structure and is bound to NADPH and its catalytic cycle consists of two phases. The first semi-reactive phase converts NADPH into $NADP^+$, and the other active phase converts GSSG into GSH. Most of the glutathione reductases are present in chloroplasts.

GSH generated by GR ensures the stable operation of the enzymes in the chloroplast. GR is also included in the ascorbate-glutathione cycle surrounding H_2O_2. When low amounts of GSH are present in the environment, it is indicated that fructose diphosphatase enzyme does not work properly in chloroplast[10-12].

Glutathione is found in many organisms including plants, and has three amino acids in its structure. One of them is sulfur-containing cysteine and others are glutamine and glycine. Glutathione is a multifunctional protein that regulates gene expression, cell signalization, cell cycle, plant growth and cell death in plants[13-15].

Glutathione transferases are also called glutathione s-transferase. Glutathione s-transferase (GST) is present in many organisms like GR. GST is encoded by a large gene family and has multiple functions. Plant GSTs detoxify the types of oxidative reactions (ROS) resulting from various stresses. There are tau, lambda, phi, theta, zeta, beta and delta class GTSs in the plants. Beta, delta, tau and phi are also found in bacteria and insects[13,16,17]. GSTs also convert the maleylacetoacetate to fumarylacetoacetate in the cytoplasm during tyrosine catabolism. Another role in the cytoplasm is to bind flavonoids[13]. Plant GSTs encoded by a large gene family play an important role in eliminating the harms of xenobiotics and oxidative stress metabolism[17].

The aim of this study was to isolate and characterize the genes encoding the glutathione s-transferase (GST) and glutathione reductase (GR) enzymes in the Kabarla strawberry variety.

Material and Methods

Materials: Plant materials were obtained from the Isparta University of Applied Sciences, Faculty of Agricultural Sciences and Technologies, Department of Horticulture. Strawberry fruits were used for the isolation of the genes.

Methods

cDNA synthesis: The strawberry fruit (1 gram) was ground to powder using liquid nitrogen. Total RNA was isolated with the guanidine isothiocyanate method[18]. Possible DNA pollution was eliminated by Dnase kit (ambion). The obtained Dnase-free RNA samples were transformed into cDNA using the Advantage RT-PCR kit (clontech) protocol.

Synthesis of degenerate primers: Degenerate primers were designed based on the unmodified portions of the GST and GR gene sequences previously obtained from other plants. These were synthesized by the Iontech company. Designed primers are presented in the table 1.

PCR analysis: Obtained PCR products were run at 90 V for 2.5 hours on a 1% agarose gel prepared with 1X TBE buffer (89 mM tris, 89 mM boric acid and 2mM EDTA, pH: 8). The products were viewed under UV light. Displayed DNA bands were cut with the help of a scalpel under the UV light and these were weighed by precision scales. Bands were purified using the GeneJet PCR purification kit (Thermo) protocol.

GST PCR analysis: PCR samples were prepared with 8 µL cDNA, 3.5 µL degenerate forward primer, 3.5 µL degenerate reverse primer, 5 µL 10X PCR buffer, 1.2 µL dNTP mix (10mM), 1 µL Taq polymerase and 27.8 µL pure water. The total mixture was 50 µL. All of the reagents used for PCR reactions were obtained from GeneDirex.

Amplification reactions were performed in 40 cycles, each consisting of a denaturation step at 94 °C for 1 min, annealing step at primer-dependent temperatures of 51-44 °C for 1 min, and extension step at 72 °C for 1 min followed by a final extension at 72°C for 10 min. The initial denaturation step was performed for 5 min at 94 °C.

GR PCR analysis: PCR samples were prepared with 5 µL cDNA, 3 µL degenerate forward primer, 3 µL degenerate reverse primer, 5 µL 10X PCR buffer, 1 µL dNTP mix (10mM), 1.2 µL Taq polymerase and 31.8 µL pure water. The total mixture was 50 µL. All of the reagents used for the PCR reactions were obtained from GeneDirex.

Amplification reactions were performed in 40 cycles, each consisting of a denaturation step at 95 °C for 15 sec., annealing step at primer-dependent temperatures of 60-40°C for 40 sec, and extension step at 68 °C for 40 sec followed by a final extension at 60 °C for 10 min. The initial denaturation step was performed for 10 min at 94 °C.

RACE analysis: A race analysis was performed to isolate the full-length gene sequences by using the protocol of the FirstChoice RLM-RACE kit (invitrogen).

Southern blot analysis: Genomic DNA from the fruit was extracted by CTAB protocol[19]. The quality and concentration of isolated DNA were checked using a NanoDrop 2000/c spectrophotometer (Thermo) and 1% agarose gel electrophoresis. Southern blot analysis was performed following the DIG high prime DNA labeling and detection starter kit (Roche). The results were viewed with gel imaging system (Kodak GL 1500).

Results and Discussion

Glutathione s-transferase in plants is known to be involved in the synthesis of secondary metabolites, hormone homeostasis, prevention of pathogens and detoxification of damage caused by harmful compounds. According to the researches, 1107 GST proteins were analyzed from 20 different plant species[20].

Table 1
The designed primers for the isolated genes

GENE	SEQUNCE	TM
Gulutathion reductase (Forward)	GGAGCATCTTATGGAGGTGAAC	60
Glutathion reductase (Reverse)	CAGTTTTTTCTTGTCGCCCAG	58
Gulutathion s-transferase (Forward)	CTTNNCTTKCYCTYAAYCC	55
Glutathion s-transferase (Reverse)	CATMATCWGTKSTCWTRCC	53

However, no GST genes have been isolated from strawberry fruit previously and therefore in this study we have isolated the GST gene from kabarla strawberry fruit.

Dong et al[21] isolated 59 and 49 GTS genes from *Gossypium raimondii* and *Gossypium arboreum* respectively. They reported that the molecular weight of the 59 genes isolated from *G. Raimondii* ranged from 22.25 kDa to 47.69 kDa, while the molecular weights of the 49 genes isolated from *G. Arboreum* varied between 15.40 kDa and 47.65 kDa. In another study, it has been stated that the GST gene isolated from mango consisted of 690 base pairs which had an ORF encoding a protein consisting of 229 amino acids and a molecular weight of 25.5 kDa[22]. The GST gene isolated from the Kabarla was found to contain 1007 base pairs according to the sequence analysis. It was found that the open reading frame (ORF) of this gene encoded 287 amino acids and the gene had an approximate molecular weight of 32.242 kDa (Table 2).

There are ten known classes of GST genes in plants, but the most commons are phi and tau classes[23]. 59 GST genes were detected in *Gossypium raimondii* that Tau contained the largest number of GrGST genes (38) followed by Phi (7). Similarly, 29 of the 49 GST genes were isolated from *Gossypium arboreum* belonging to the tau class, while 6 of them were found to belong to the phi class[21]. In another study, 90 GST genes isolated from tomato were divided into ten classes, of which Tau class contained the most of the genes (57) followed by the phi class (6)[24]. According to the conserved domain analysis of the GST genes isolated from strawberry, the domains were found to belong to the tau and phi classes (Figure 1).

Cells have numerous antioxidant factors that fight against oxidative stress scattered between cytosol, mitochondria and chloroplasts. Glutathione reductase, which catalyzes the reduction of glutathione, is also one of these factors[25,26]. In the Arabidopsis thaliana, 152 genes were detected to control reactive oxygen species and two of them were found to be GR enzyme encoding genes. In the previous study, it was found that the GR (At3g54660) gene had a nucleotide length of 1698 bp encoding 565 amino acids[26-28].

In cowpea, the GR gene was reported to be 1734 bp in length encoding 500 amino acids and it had a molecular weight of 53.6 kDa[29]. In this study, it was found that the GR gene consisted of 2091 base pairs and the open reading frame (ORF) of the gene encoded 557 amino acids. The gene had a molecular weight of about 63.203 kDa.

Upon comparing GR genes in rice and Arabidopsis, the conserved pyridine nucleotide disulfide oxidoreductases class I domain was detected[30]. In this study according to the conserved domain analysis, GR was found to have three reading frames, that contain pyridine nucleotide-disulfide oxidoreductase and dimerization domains (Figure 2).

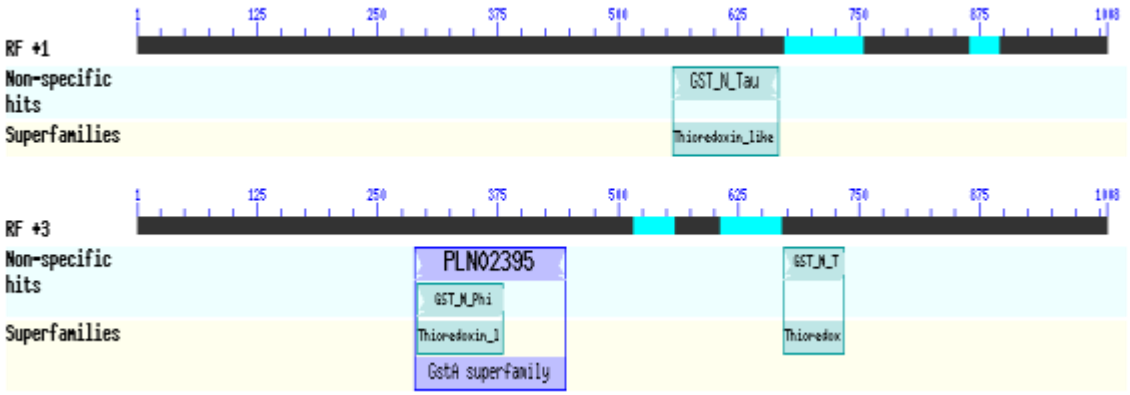

Figure 1: Conserved domain analysis of the GST gene

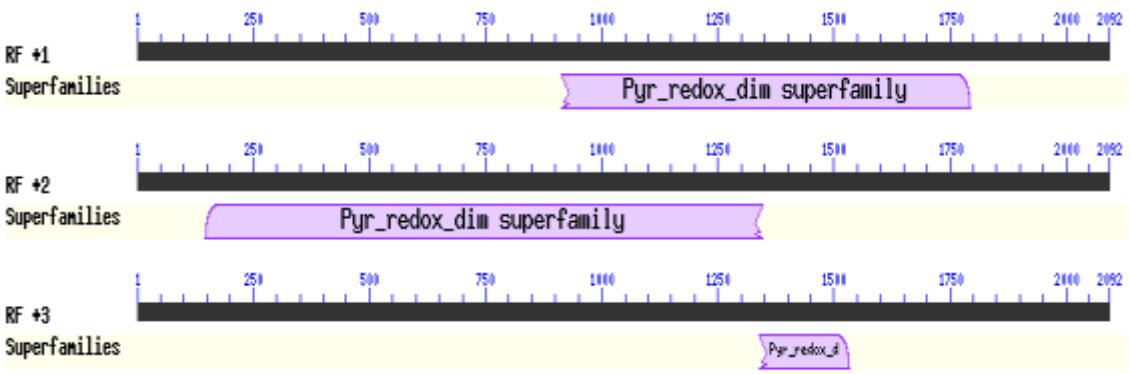

Figure 2: Conserved domain analysis of the GR gene

The GR gene obtained from the Kabarla was compared with the GR genes isolated from about 100 different plants in the gene bank database (NCBI). As a result, there was a 40-59% similarity ratio between GR proteins. 11 different protein sequences, which are the most similar sequences to the GR protein sequence, were used to construct the phylogenetic tree. It has been paid attention that the proteins used in the comparison belonged to different plant species. Strawberry GR protein showed the highest similarity to the protein sequence of the *Pisum sativum* GR enzyme. Full length amino acid sequences were similar to those of *Oryza sativa*, *Panicum hallii*, *Arachis ipaensis* and *Medicago truncatula*. *Eleusine coracana*, *Trifolium repens*, *Brassica oleracea* and *Setaria italica* had less similarity.

Approximately 100 different GST genes were compared to form the phylogenetic tree of the GST gene in the gene bank (NCBI) and similarity rate was determined as 30-75% (Figure 3). In the sequence alignment, 9 different protein sequences that most closely resembled to the GST protein sequence were used. The type that has the highest similarity with the Kabarla GST protein sequence has been *Sorghum bicolor*. Morover, *Aegilops tauschii*, *Nicotiana tabacum* and *Solanum tuberasum* were the species that showed similarity. The less similar species were identified as *Brassica rapa*, *Arabidopsis thaliana* and *Zea mays*. The lowest similarity was observed with the GST protein of *Triticum aestivum* species (Figure 4).

As a result of the southern blot anlysis, it was found that both GST gene and GR gene were available as single copies in strawberry (Figure 5 and 6).

Although GST and GR genes have been isolated and characterized in some plants, these genes have not been investigated in many plants. Studies have shown that these two genes are important for plants to cope with abiotic and biotic stresses. This study shows that the GST and GR genes isolated from the Kabarla variety of strawberry can be used in the isolation and characterization of these genes in other species. it will also contribute significantly to the development of new strawberry varieties with high tolerance to different stresses.

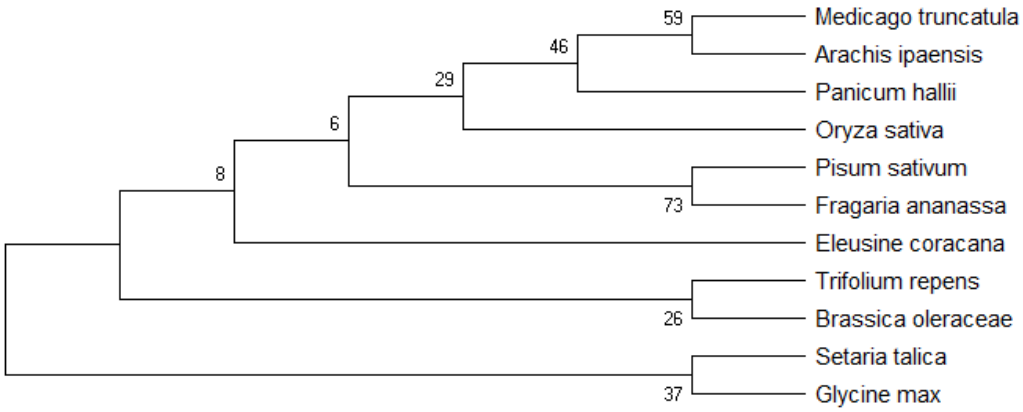

Figure 3: Phylogenetic tree of the GR genes

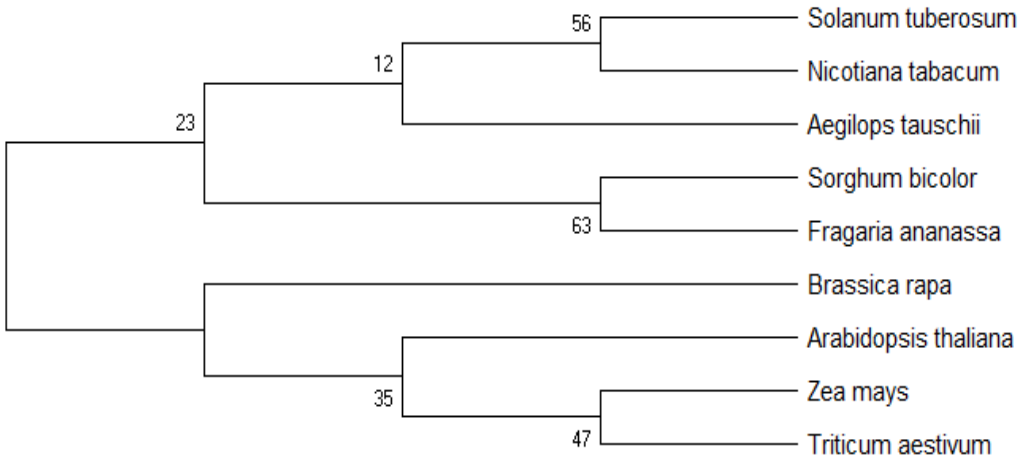

Figure 4: Phylogenetic tree of the GST genes

Table 2
Properties of GR and GST genes isolated from Kabarla fruit

Gene	Nucleotide (baz)	Amino acid	5' Non-coding	3' Non-coding	Moleculer weight (kDa)	pI value
GR	2091	557	81	336	63.203	9.26
GST	1007	287	57	87	32.242	11.28

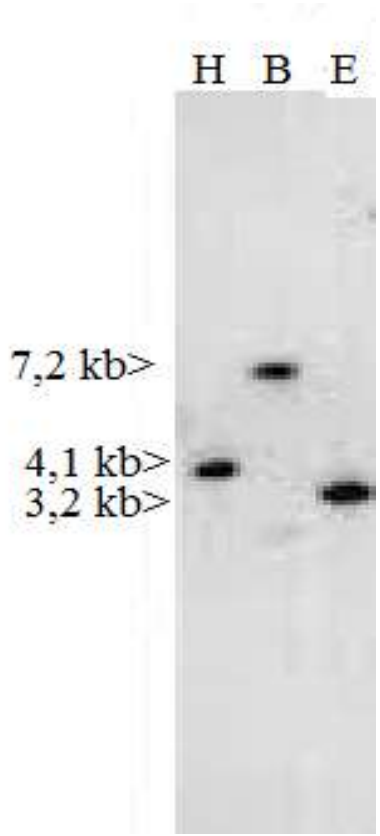

Figure 5: Southern blot analysis of the GR gene.

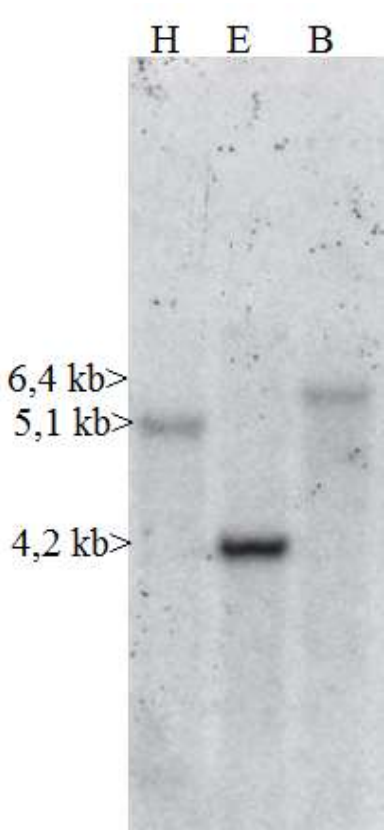

Figure 6: Southern blot analysis of the GST gene.

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