Research Article

CHANGES IN H_2O_2 AND PEROXIDASE ACTIVITIES IN STRAWBERRY PLANTS UNDER HEAT STRESS

Sergül ERGİN, Müge KESİCİ, Hatice GÜLEN*

ABSTRACT

Effects of heat stress were investigated in two strawberry cultivars, Redlands Hope (R. Hope) and Cal-Giant 3 (CG3), which are heat-tolerant and heat-sensitive, respectively. Collected leaves from the grown plants were exposed to high temperature increased gradually to 35, 40, 43, 46, 49, 52, 55 and 60 ° C to impose a "gradual heat stress" (GHS). Additional leaves collected from the plants were also exposed directly to each temperature, to impose a "shock heat stress" (SHS). Electrolyte leakage, hydrogen peroxide (H_2O_2) and peroxidase (PRX) activity were evaluated fallowing each temperature. The electrolyte leakage and H_2O_2 levels were found higher in CG3 than R. Hope in both GHS and SHS treatments. A basic PRX band was observed in all treatments except 60ºC on native PAGE with different intensities. The intensities of the band were generally higher in CG3 than R. Hope. In conclusion less H_2O_2 accumulation and cellular damage in heat tolerant cv. R. Hope in spite of less PRX activity can be correlated with the effects of other defense systems.

Key Words: Strawberry, *Fragaria x ananassa*, high temperature, electrolyte leakage, H_2O_2, peroxidase

SICAKLIK STRESİ ALTINDAKİ ÇİLEK BİTKİLERİNDE H_2O_2 VE PEROKSİDAZ AKTİVİTESİNDEKİ DEĞİŞİMLER

ÖZET

Yüksek sıcaklık stresinin etkileri, yüksek sıcaklığa tolerant ve hassas olan Redlands Hope (R. Hope) ve Cal-Giant 3 (CG3) çilek çeşitlerinde araştırılmıştır. Bitkilerden alınan yaprak örnekleri kademeli olarak 35, 40, 43, 46, 49, 52, 55 ve 60°C sıcaklıklara maruz bırakılıp "kademeli yüksek sıcaklık stresi" oluşturulmuştur. Ayrıca, bitkilerden alınan yaprak örnekleri "şok yüksek sıcaklık stresi" oluşturmak amacıyla doğrudan bu sıcaklıklara maruz bırakılmışlardır. Her bir sıcaklık uygulamasında iyon sızıntısı, hidrojen peroksit (H_2O_2) ve peroksidaz (PRX) aktivitesi incelenmiştir. Kademeli ve şok yüksek sıcaklık uygulamalarının her ikisinde de iyon sızıntısı ve H_2O_2 miktarının CG3 çeşidinde R.Hope'a gore daha yüksek olduğu belirlenmiştir. Native-PAGE'de 60ºC haricindeki bütün sıcaklıklarda bazik bir peroksidaz bandı tespit edilmiştir. Belirlenen bandın yoğunluğunun CG3'te R.Hope'a gore daha yüksek olduğu görülmüştür. Sonuçta, yüksek sıcaklığa tolerant olan R.Hope'da PRX aktivitesinin düşük olmasına rağmen, H_2O_2 birikiminin ve hücresel zararlanmanın da düşük olması, diğer savunma sistemleri ile ilişkilendirilmiştir.

Anahtar Kelimeler: Çilek, *Fragaria x ananassa*, yüksek sıcaklık, iyon sızıntısı , H_2O_2, peroksidaz.

Uludağ University, Faculty of Agriculture, Horticulture Department, Görükle, Bursa

* Corresponding author: hsgulen@uludag.edu.tr

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INTRODUCTION

Heat stress is one of the major abiotic stress limiting the growth and development (Havaux, 1993). Transitory or constantly high temperatures cause an array of morpho-anatomical, physiological and biochemical changes in plants and may lead to a drastic reduction in economic yield (Wahid et al., 2007). Heat stress conditions generally can cause to generate reactive oxygen species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Liu and Huang, 2000, Kocsy et al., 2004). These ROS inactivate enzymes and damage important cellular components like proteins, and loss of membrane integrity (Arora et al., 2002; Howarth, 2005).

In plant cells, one such protective mechanism is an antioxidant system, thus, plants have developed a complex antioxidant system to mitigate and repair damage initiated by ROS (Foyer et al., 1994). Antioxidative enzymes such as superoxyde dismutase (SOD), catalase (CAT), peroxidase (PRX), ascorbate peroxidase (APX) and glutathione reductase (GR) are the most important components in the scavenging system of ROS (Liu and Huang, 2000; Gulen and Eris, 2004; Xu et al., 2006). Peroxidases are a family of isozymes found in all plants; they are heme-containing glycoproteins and are usually classified as acidic, neutral, or basic, according to their isoelectric points (Yoshida et al., 2003). Peroxidases have many physiological functions in plants such as removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin cell walls, auxin catabolism, defensive responses to wounding, defense against pathogen or insect attack and some respiratory processes (Gaspar et al., 1982). The H_2O_2 in chloroplasts is scavenged by a PRX which trigger the conversion of H_2O_2 to water and oxygen (Nakano and Asada, 1981; Jeffrey, 2002). More specifically, PRX enzyme has been related to the appearance of physiological injuries caused in plants by thermal stress, and its activity was enhanced by high temperature stress (Chaitanya et al., 2002; Mazorra et al., 2002; Gulen and Eris, 2004). During acclimation to heat stress many of the changes that appear are reversible, yet if the stress is too great, irreversible changes occur and these can lead to cellular death. In this respect electrolyte leakage is an effective means of measuring cell membrane thermostability and has been used as an indicator of direct heat injury (Lester, 1985; Saelim, 2000; Gulen and Eris, 2003, 2004; Kesici, 2009).

Strawberry has an economic importance as a nutritious berry fruit and it is cultivated almost all over the World. Strawberry plants expose to high temperature during its growing period, since it is cultivated all-round the year both under protected and on open field conditions. Although some cultivation techniques have been commonly used to reduce the heat damage in strawberry fields, these are not effective enough to protect the plants. Using the heat-tolerant cultivars is the most effective way of avoiding heat damage. In this respect determination of heat-tolerance of commonly grown strawberry cultivars and understanding of heat-tolerance mechanism in strawberry are the effective way to develop new cultivars. Currently, knowledge of heat-tolerance in strawberry plants is limited in a few studies indicating some physiological and molecular effects of heat stress in a certain strawberry cultivar (Wang and Zheng, 2001; Gulen and Eris, 2003, 2004; Ledesma et al., 2004, 2008; Wang and Lin, 2006). Recently Kesici (2009) and Kesici et al. (2012) reported heat stress tolerance of commonly used strawberry cultivars. Nevertheless, little is known about how shock heat stress and gradually heat stress effects on PRX activity and H_2O_2 accumulation in strawberry. In this study, changes of H_2O_2 content and activities of peroxidases were analyzed under gradual and shock heat stress treatments in two strawberry cultivars known as heat-tolerant and heat-sensitive. The changes related to heat stress were investigated for a better understanding of the general genotypic differences and for providing a basis for further studies.

MATERIALS and METHODS

Plant material and heat stress treatments

Cold stored (frigo) strawberry [*Fragaria x ananassa* cvs. Redlands Hope (R. Hope) and Cal-Giant 3 (CG3)] seedlings were planted in 14x12 cm pots using perlite, torf and garden soil (1:1:1) mixture. R. Hope and CG3 are known to be heat-tolerant and heat-sensitive strawberry cultivars, respectively (Kesici, 2009; Kesici et al., 2012). Plants were grown for eight weeks (plants had 6-7 leaves) in a greenhouse with day/night temperature of 30–15°C, average relative humidity of %65. Plants were watered on need basis to avoid any water stress by Actagro (7-7-7) (Actagro LLC, Biola, CA, USA) nutrient solution.

Controlled heat test were applied to leaf samples based on the method of Arora et al. (1998) with some modifications. Fully

expanded leaves were collected from the plants, placed into parex tubes with cap, and placed into temperature controlled water bath. Temperature was stepwise increased (1°C/10 mins) to 35, 40, 43, 46, 49, 52, 55 and 60 ° C to impose a "gradual heat stress" (GHS) and leaves were exposed to each temperature step for 2h. In addition to leaves already into water bath, new leaves collected from the plants were also placed into the water bath at each temperature step to impose a "shock heat stress" (SHS). Each sample was removed from the water bath at the end of their exposure time to high temperature, frozen in liquid nitrogen immediately, and stored at -80°C for H_2O_2 and PRX analysis.

Relative electrolyte leakage

Membrane thermostability was measured using the procedure of Arora et al. (1998), with the modifications of Gulen and Eris (2004) for strawberry. Leaf discs 2 cm in diameter were cut from each of three plants (replicates) per treatment (unstressed- control and each temperature of GHS and SHS treatments). Discs were lightly rinsed in distilled water, gently blotted with paper, and placed in test tubes (one disc per tube). Then, 20 mL of distilled water was added to each test tube. Samples were then vacuum infiltrated to allow uniform diffusion of electrolytes and shaken on a gyratory shaker at 250 rpm for overnight. After incubation, electrical conductivity of each solution was measured using a conductivity meter (WTW TetraCon 325 model, InoLab Cond Level 1, Weilheim, Germany). After measuring initial electrolyte leakage (C_1), samples were heat-killed (autoclaved at 121°C, 124 kPa for 15 min) and final electrolyte leakage (C_2) was measured at room temperature. Ion leakage was calculated using the equation: % electrolyte leakage=C_1/C_2 x 100.

H_2O_2 concentration

The H_2O_2 levels in heat-stressed and unstressed (control) leaf tissues were determined according to method of Ngo and Lenhoff (1980). Frozen leaf samples (1g) were ground with a mortar and pestle in 4 ml 0.2 N $HClO_4$ and the solution was centrifuged at 10 000 g at 4ºC for 20 min. The supernatant was neutralized to pH 7.5 with 4 N KOH and 1 N HCl and the solution was centrifuged at 1000 g at 4ºC for 1 min to remove insoluble potassium perchlorate. A 400 µl aliquot of the supernatant was applied to column of anion exchange resin (AG 1-X8; Bio-Rad). The column was washed with 1600 µl of distilled water and the eluate was used for the H_2O_2 assay (Okuda et al., 1991). The reaction mixture contained 1 ml of the eluate, 400 µl of 12.5 mM 3-dimethylaminobenzoic acid in 0.375 M sodium phosphate buffer (pH 6.5), 80 µl 1.3 mM 3-methyl-2-benzothiazolinone hydrazone and 20µl (0.25 U) horseradish peroxidase in a total volume of 1.5 ml. The absorbance was recorded using a Beckman UV-DU 520 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) at 590 nm and content of H_2O_2 was determined using standard curve plotted with known content of H_2O_2.

PRX extraction and native polyacrylamide gel electrophoresis (PAGE)

The PRX was extracted from leaf tissues using the extraction methods described by Gulen et al. (2002). Ground leaf tissues (0.1 g) were homogenized at 4ºC in 0.6 ml extraction buffer [0.1 M potassium phosphate pH 7.5, 30 mM boric acid, 50 mM L-ascorbic acid, 17 mM sodium metabisulfite, 16 mM dithiocarbamic acid, 1 mM EDTA and 4% (w/v) PVP-40 and final pH was readjusted to 7.5 with NaOH]. Homogenates were centrifuged at 15 000 rpm for 20 min and supernatant was used for electrophoresis. Discontinuous PAGE was performed with a PROTEAN III vertical electrophoresis unit (Bio-Rad, Hercules, Calif.) for acidic and basic PRX, respectively, according to Davis (1964) and Reisfeld et al. (1962). In PAGE, 5% stacking gels and 10% separating gels were prepared for both systems. For each sample 20 µl of crude extract was loaded to the gel. Electrophoresis was performed at 20 mA for 30 min, followed by 40 mA for 3h. Gels were stained for PRX using the method of Wendel and Weeden (1989). The relative distance (Rf value) of the bands on the gel was calculated as described by Manganaris and Alston (1992) using Rf=1.0, distance to the fastest band and Rf=0.0, the starting point.

Statistics

The experiment was arranged in a randomized block design with three replications. Statistical analyses were performed by SPSS 13.0 for Windows program.

RESULTS and DISCUSSION

The electrolyte leakage from cell membrane, which is indication of cell membrane injury, in leaf tissues of the two strawberry cultivars exposed to GHS and SHS

was shown in Figure 1. In general, heat stress increased electrolyte leakage in both treatments. The electrolyte leakage sharply increased at the temperatures above 40°C in GHS, whereas similar trend was determined at the temperatures above 46°C in SHS. Even though both cultivars were shown very similar trend, CG3 had more electrolyte leakage than R.Hope in both GHS and SHS. In other word CG3 were more injured than R. Hope in cellular. Cell membrane stability has been widely used to express stress tolerance, and higher membrane stability could be correlated with abiotic stress tolerance (Premachandra et al., 1992). Considering heat stress tolerance or heat acclimation, Gulen and Eris (2004) was reported that long term (48 h.) GHS increased heat stress tolerance in leaf tissues of strawberry cv. Camarosa. In addition Chen et al. (1982) stated that continuous high temperatures are necessary for heat acclimation. The electrolyte leakage (as indicated by relative electrical conductivity) following high-temperature exposure was dramatically increased in both GHS and SHS treatments in current study. In addition, GHS leaf tissues did not exhibit heat acclimation in comparison to SHS treatment. It may because of the short term exposure time. Consequently, 2 h. exposure to high temperatures may not be long enough to develop heat-acclimation.

The changes in H_2O_2 concentration of cultivars under heat stress were shown in Figure 2. In general, the heat treatments caused significant increase in H_2O_2 depends on the cultivars and the temperature. In the GHS treatments; the level of H_2O_2 increased after 46ºC in CG3. In contrast, there was no significant difference in H_2O_2 content of R.Hope in all GHS treatments. In the SHS treatments no significant change was observed in the H_2O_2 concentration of both cultivars. On the other hand H_2O_2 accumulation was higher in CG3 than R. Hope in both GHS and SHS treatments. Under heat stress, an increment in the production of H_2O_2 as one of the ROS reported in various plant species in parallel to our results. Rivero et al. (2003) reported that leaves of tomato plants undergoing heat stress with over-produce H_2O_2 at 35ºC. Similarly, Yin et al. (2008) demonstrated that, after 10h treatments H_2O_2 concentration increased by 11%, 14% and 65% at 37ºC, 42ºC and 47ºC, respectively, in comparison with the control in lily. ROS accumulation and cellular damage also closely related to genotype. Data indicated a significant difference between CG3 and R. Hope cultivars in this study. The H_2O_2 contents of CG3 were higher than R.Hope in both GHS and SHS. Therefore the data clearly indicated higher accumulation of H_2O_2 in heat sensitive cv. CG3. Similarly, under heat stress, perennial rye grass (heat sensitive) leaves showed higher H_2O_2 accumulation than tall fescue (moderate) leaves (Xu et al., 2006). On the other hand H_2O_2 levels of CG3 were increased particularly above 46ºC in GHS treatment, while H_2O_2 contents of cultivars were not changed significantly in SHS treatment. This result indicated that ROS accumulation under heat stress depends on the duration of high temperature stress, in other word if the duration of high temperatures takes for a long time it may cause more ROS production in plants.

Native PAGE of PRX was performed to obtain acidic and basic isozyme profiles in GHS and SHS treated leaf tissues. Native PAGE analysis of the samples was repeated at least three times with similar results and data from a single, representative analysis are presented herein. Although no any acidic PRX band was observed in both cultivars under GHS and SHS treatments, a single sharp basic PRX band was obtained (Fig.3 and Fig. 4). In general PRX activities were the highest level in certain temperatures, whereas high temperatures (particularly above 50ºC) inhibited the PRX activity. Only one band with Rf = 0.55 was observed in all treatments except 60ºC treatment. In addition, the band intensity increased until 43ºC in both cultivars under GHS. Indeed PRX band intensities of CG3 were higher than R.Hope (Fig.3B). Similar results were obtained from SHS treatment. Only one band with Rf = 0.55 was observed in all of the treatments except 60ºC treatment (Fig.4A). Band intensity of an Rf = 0.55 isoperoxidase of CG3 showed some fluctuations (up and down), while band intensities of R. Hope showed stepwise decrease followed by constant increase until 40ºC. However, the band intensities of CG3 were generally higher than R.Hope (Fig. 4B). Tolerance to high temperature stress in crop plants has been reported to be associated with an increase in antioxidant enzymes activity (Zhau et al., 1995; Sairam et al., 2000). Regarding PRXs, they are a large group of isoenzymes with an extreme range of isoelectric points, serving a multitude of functions (Huystee, 1987). Each group is thought to have a different function in the cell. Function of basic isoenzymes has been suggested that they might provide H_2O_2 for other PRXs (Walter, 1992). Previous studies show that PRX activities increase during

exposure to heat stress in strawberry and lily (Gulen and Eris, 2004, Yin et al., 2008). Gulen and Eris (2004) reported that one basic isoperoxidase band (Rf=0.22) was correlated with lignification and recovery of cell membrane damage under long term (48 h) heat stress in leaf tissues of strawberry cv. Camarosa. In current study, data from native PAGE of two cultivars indicated one basic isoperoxidases (Rf=55) with different intensities in both GHS and SHS. Different Rf of the PRX bands obtained from previous and current studies are closely related to duration and level of high temperature treatments. Increasing of PRX activity at certain temperatures could explain that it scavenges H_2O_2. Increment in H_2O_2 concentration at higher temperatures, such as 49, 52, 55 and 60ºC, can be due to decrease of activity of PRX. On the other hand less PRX activity in R. Hope than CG3 directly related to heat tolerance of the cultivars and the data from electrolyte leakage and H_2O_2 levels.

In conclusion, since heat stress induced electrolyte leakage and the level of H_2O_2 at cellular level were higher in CG3, the higher activity of PRX was not effective to develop heat tolerance this cultivar. In other words, increase of PRX activity could not stop the deleterious effects of high temperature, but may reduce stress severity in CG3. Indeed CG3 could not show heat acclimation even in GHS treatment in comparison to R. Hope. So this mechanism makes R. Hope more heat tolerant than CG3.

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FIGURES

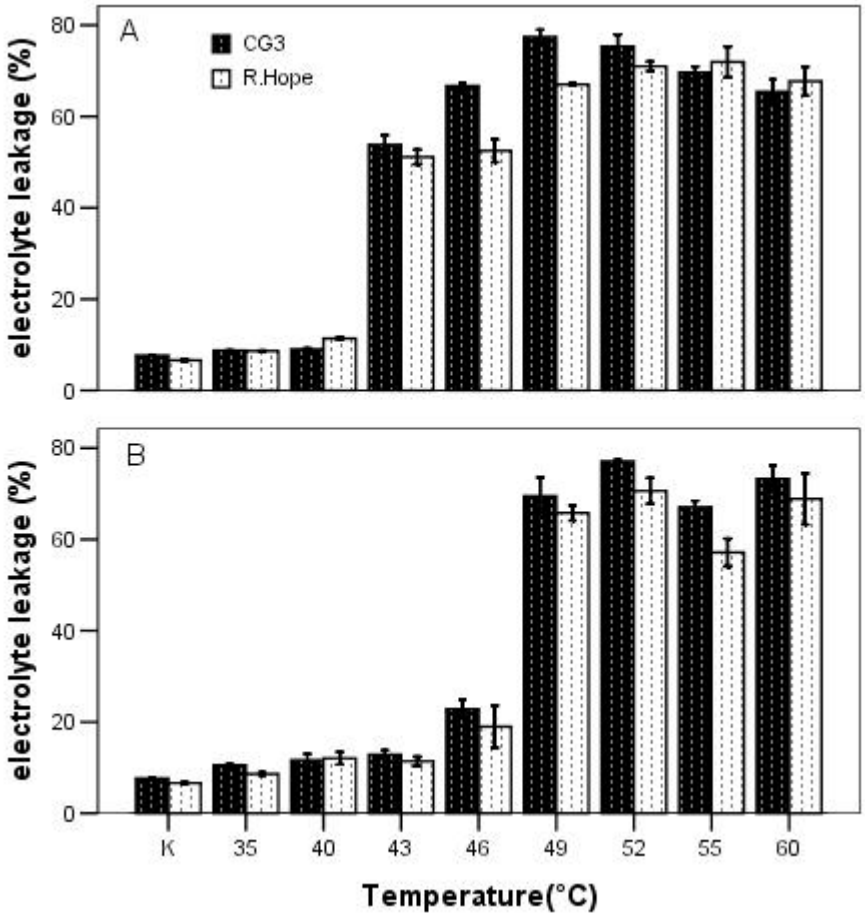

Figure 1. Effect of high temperature on electrolyte leakage in leaves of strawberry plants. Values are means from three replications and vertical bars indicate ± S.E. A: Gradual heat stress (GHS). B: Shock heat stress (SHS).

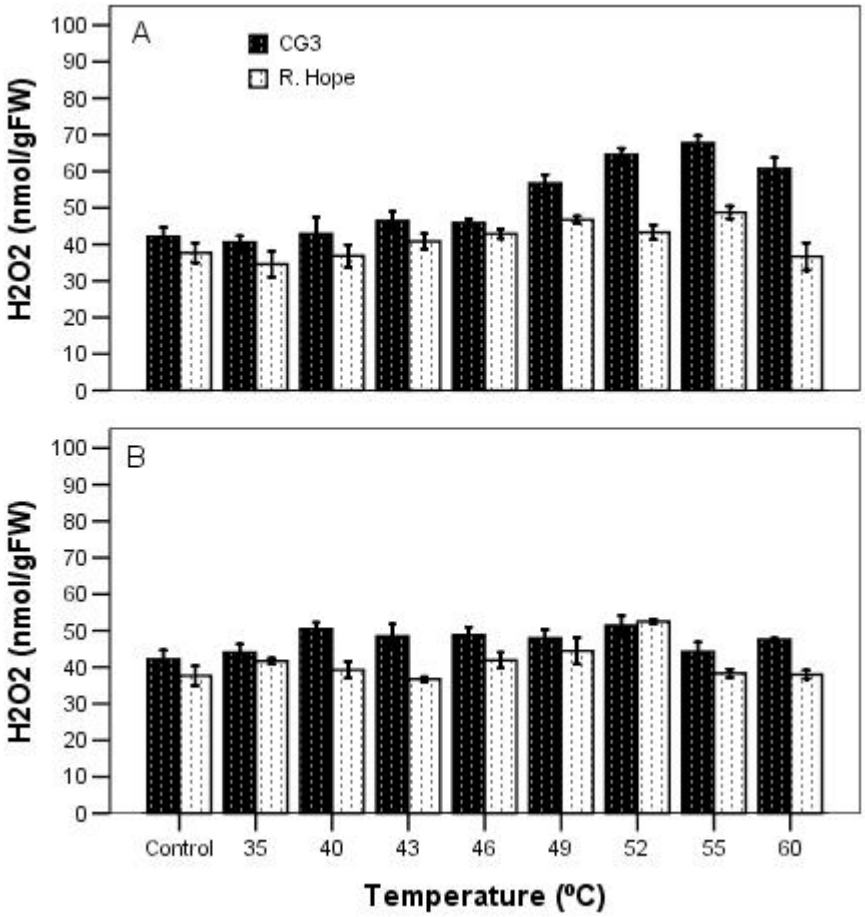

Figure 2. Effect of high temperature on H_2O_2 levels in leaves of strawberry plants. Values are means from three replications and vertical bars indicate ± S.E. A: Gradual heat stress (GHS). B: Shock heat stress (SHS).

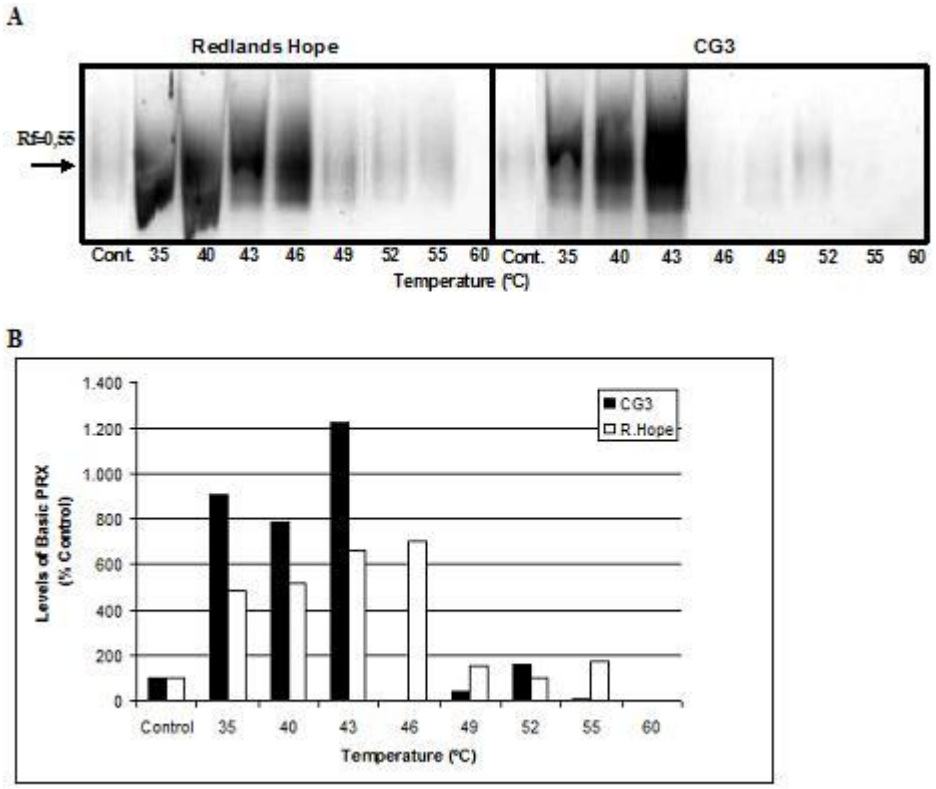

Figure 3. Effect of gradual heat stress (GHS) on basic PRX profiles of CG3 and R. Hope. A: Basic peroxidase activity (arrow on the left indicates the Rf=0.55 isoperoxidase); B: Band intensities of the basic peroxidase activity defined as Rf=0.55. Equal volumes of the crude extracts, 20µl, were loaded in each lane.

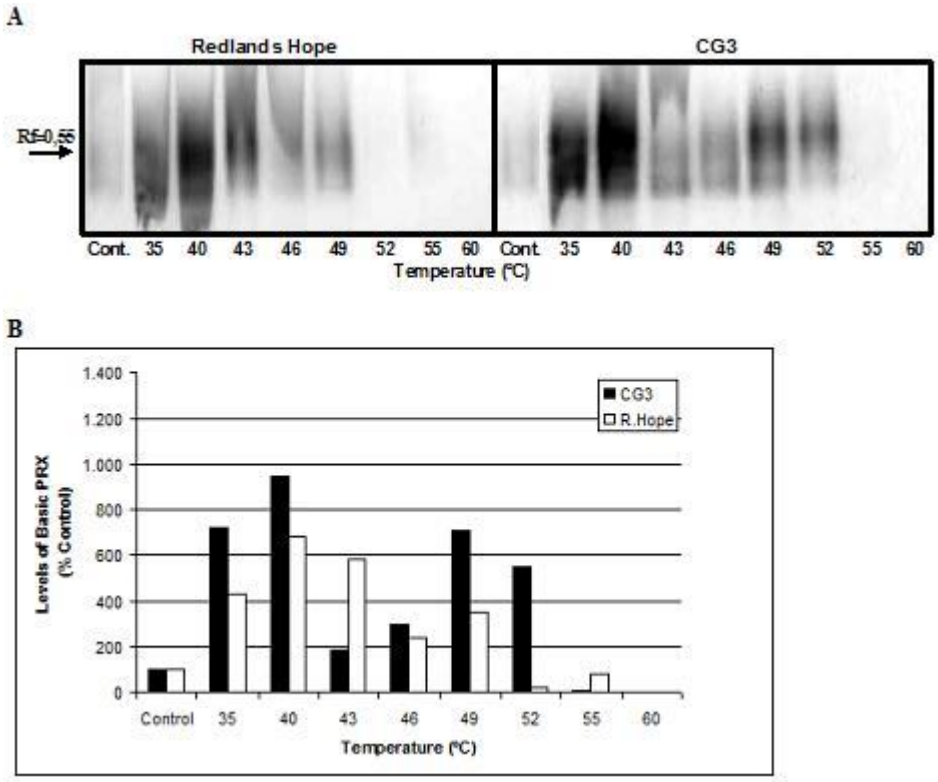

Figure 4. Effect of shock heat stress (SHS) on basic PRX profiles of CG3 and R. Hope. A: Basic peroxidase activity (arrow on the left indicates the Rf=0.55 isoperoxidase); B: Band intensities of the basic peroxidase activity defined as Rf=0.55. Equal volumes of the crude extracts, 20µl, were loaded in each lane.