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ORIGINAL PAPER

Temperature effects on the reactive oxygen species formation and antioxidant defence in roots of two cucurbit species with contrasting root zone temperature optima

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Abstract Suboptimal root zone temperature (14°C) was imposed on chilling-sensitive cucumber (*Cucumis sativus* L.) and chilling-tolerant figleaf gourd (*Cucurbita ficifolia* Bouché) plants. Exposure of roots to low temperature for up to 10 days caused a strong growth inhibition in cucumber compared with figleaf gourd. Physiological analysis showed that generation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion was significantly induced in cucumber plants as fast as 1 day after low root zone temperature treatment. In addition to the significant induction of antioxidant superoxide dismutase activity, low root zone temperature also increased the mitochondrial electron transport allocated to alternative pathway while decreased cytochrome pathway salicylhydroxamic acid-resistant respiration. However, these defense responses could not compensate for the ROS production, resulting in membrane lipid peroxidation and loss of root cell viability in the low root zone temperature treated cucumber roots. In contrast, 14°C root zone temperature had no significant effects on figleaf gourd plant growth, antioxidant enzymes, ROS levels and alternative respiratory pathway. Hence, difference in ROS metabolism would be associated with the remarkable difference in adaptability of cucumber and figleaf gourd plants in response to suboptimal root zone temperature condition.

Keywords Cucumber · Alternative pathway respiration · Figleaf gourd · Oxidative stress · Root zone temperature

Abbreviations

Alt pathway	Alternative pathway
AOX	Alternative oxidase
APX	Ascorbate peroxidase
CAT	Catalase
CN-resistant R	Cyanide-resistant respiration
Cyt pathway	Cytochrome pathway
GPOD	Guaiacol peroxidase
H_2O_2	Hydrogen peroxide
KCN	Potassium cyanide
MDA	Malonylaldehyde
$O_2^{\cdot -}$	Superoxide anion
ROS	Reactive oxygen species
SHAM	Salicylhydroxamic acid
SHAM-resistant R	Salicylhydroxamic acid-resistant respiration
SOD	Superoxide dismutase

Introduction

In nature, chilling is one of the most important environmental factors limiting the geographical distribution and

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performance of many plant species, including important agricultural and horticultural crops. Exposure to sub-optimal temperature induces significant changes in physiological processes, such as photosynthesis, ion uptake, and mitochondrial respiration (Hendrickson et al. 2004; Munro et al. 2004). The cellular homeostasis may be disrupted during temperature stress, because different pathways that reside in different organelles have a different temperature optimum (Suzuki and Mittler 2006). The most extensively investigated physiological changes to arise from chilling is membrane lipid peroxidation caused by the generation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals as well as the cellular defense and detoxification systems via low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds and tocopherols) and antioxidant enzymes, e.g. superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPOD), and catalase (CAT) (Prasad et al. 1994; Apel and Hirt 2004; Almeselmani et al. 2006). Accordingly, the balance between ROS production and its elimination in response to environmental stimuli is now an important research topic in modern plant biology and agricultural sciences (Ghanati et al. 2005; Gechev et al. 2006).

Owing to the high specific heat capacity of the soil, soils warm up much more slowly than ambient air in some seasons such as winter or early spring, thus, continual root chilling over the short term (several days) is probably more prevalent in the field situation than continual shoot chilling (Huang et al. 2005). Root zone temperature has also been found to be a very important factor for leaves to alter the response and susceptibility to chilling stress (Suzuki et al. 2008). In particular, low soil temperature affects plant growth due to limited absorption of water and essential mineral nutrients even when the aerial environment is adequate (Ahn et al. 1999). However, compared to the intensive available information about the sub-optimal temperatures on the physiological metabolism and functioning in the aerial parts (Hendrickson et al. 2004; Munro et al. 2004; Dwyer et al. 2007), the responses by plant roots to changes in root-zone temperature remains largely unknown (Rachmilevitch et al. 2006).

Cucumber (*Cucumis sativus* L.) is an important crop plant for many parts of the world, in particular, Asian countries. Since most cultivated genotypes are of tropical origin, growing in unheated greenhouse runs the risk of prolonged root chilling during winter and early spring. Efforts have been made to introduce low-temperature resistance into cucumber plants by means such as resistant cultivars, chemical application, and others (HorvaÂth et al. 1983; Bulder et al. 1991). Another promising way to broaden the temperature optimum of cucumber cultivars is grafting. Because figleaf gourd can grow well at a relatively low root zone temperature of around 14°C (Ahn et al. 2000; Lee et al. 2002; Zhang et al. 2007), the grafting between cucumber (as a scion) and figleaf gourd (as a rootstock) species has become a common horticultural practice for successful cucumber production in polythene greenhouse during cold season (Tachibana 1987; Lee 1994). Nevertheless, the physiological basis for the difference in low soil temperature resistance in cucumber and figleaf gourd plants is poorly understood. Most researchers focused on water metabolism, unsaturated fatty acids content, gas exchange, and micro-structure of root tip (Ahn et al. 2000; Lee et al. 2002; Zhang et al. 2007); however, few attempts have been made to reveal the involvement of root respiration in chilling resistance. It is well known that root respiration is the powerhouse to provide energy needed in nutrient absorption and homeostasis maintenance. Analysis of the respiration changes under low root zone temperature would shed new light on the physiological mechanism underlying the different low root zone temperature tolerance of figleaf gourd and cucumber plants.

In the present study, the effects of low root zone temperature were examined on the chilling-sensitive (cucumber) and on the chilling-tolerant (figleaf gourd) species. The involvement of root respiration as well as other cellular antioxidative system in low root zone temperature response was investigated. Our results strongly indicated that in addition to antioxidant enzymes, the mitochondrial respiration especially alternative pathway (Alt pathway) respiration is also involved in defence response against low root zone temperature stress.

Materials and methods

Plant materials

Seeds of cucumber (*Cucumis sativus* L. cv. Jinyan No. 4) and figleaf gourd (*Cucurbita ficifolia* Bouché) were sown in moist vermiculite in trays in a greenhouse. After 7 days, groups of eight seedlings per species were transplanted to a 13-L tank (39 × 27 × 13 cm) filled with half-strength Enshi nutrient solution (Yu and Matsui 1997) that was continuously aerated with an air-pump. Mean daily maximum and minimum air temperatures were 24 and 18°C, respectively. Two weeks later, roots of plants with three to four leaves were treated with solutions set at 14 ± 1 and 24 ± 1°C. The solution temperatures were maintained by cooling or heating pipe systems connected to individual tanks. After 10 days of treatment, roots were sampled and stored at −80°C until physiological analysis.

$O_2^{\cdot -}$ generation rate and H_2O_2 content measurement

$O_2^{\cdot -}$ generation rate was measured as described by Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of O_2. H_2O_2 content were analyzed as described by Zhou et al. (2004) with some modifications. Root samples (0.3 g) were homogenized in 2.5-fold cold acetone and the extract was centrifuged (3,000×*g*) and then 0.2 ml 20% v/v $TiCl_4$ in HCl was added to the supernatant. After shaking, 0.4 ml of one-fifth strength NH_4OH was added. Then the samples were centrifuged (3,000×*g*) and the precipitates washed three times with two volumes of acetone. The precipitates were solubilized in 3 ml 1 M H_2SO_4 and were measured by monitoring the change of titanium-peroxide complex with at 410 nm.

Antioxidant enzyme activities and malonylaldehyde content measurement

Root tissues were homogenized with 0.05 M sodium phosphate cold buffer (pH 7.8) containing 0.2 mM EDTA, and 2% (w/v) polyvinylpyrrolidone, the homogenate was centrifuged at 12,000×*g* for 20 min. 2 mM ascorbic acid was added in the buffer for extracting APX. The supernatant was used for determination of enzyme activities. The whole extraction procedure was carried out at 4°C.

Total SOD activity was measured by the photochemical method of Giannopolitis and Ries (1977) CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu 1987). APX activity was measured following Amako et al. (1994) by monitoring the rate of ascorbate oxidation at 290 nm ($E = 2.8$ mM cm^{-1}). GPOD activity was measured using a modification of the procedure of MacAdam et al. (1992). The malonylaldehyde concentration of the sample was estimated from the supernatant by the method of Zhou et al. (2004) with some modification. 1 ml supernatants were mixed thoroughly with 2 ml of TCA–TBA–HCl (15% w/v TCA and 0.5% w/v TBA in 0.25 M HCl). The absorbance of the sample was determined at 535 and 600 nm in a double beam spectrophotometer against a suitable blank.

Respiration rate of cucumber and figleaf gourd roots

Roots of cucumber or figleaf gourd (0.1 g fresh mass) were transferred to an air-tight cuvette, respiration was measured at 25°C as a decrease of the oxygen concentration using a Clark-type oxygen electrode (Hansatech, King's Lyn) according to Millenaar et al. (2002). The glass cuvette contained 2 ml of air-saturated, well-stirred 20 mM potassium phosphate buffer (pH 6.8). The total respiration rate was measured without any inhibitions. After a constant rate of oxygen uptake was attained, 1 mM potassium cyanide (KCN) and 20 mM salicylhydroxamic acid (SHAM) was added to obtain cyanide-resistant respiration (CN-resistant R) and SHAM-resistant respiration (SHAM-resistant R), respectively.

Measurement of root viability

Viability of root tips was determined by staining the cells with fluorescein diacetate–propidium iodide (FDA–PI) according to Ishikawa and Wagatsuma (1998). In brief, the roots were stained for 10 min (25°C) with a mixture of FDA (12.5 μg ml^{-1})−PI (5 μg ml^{-1}) and 1 min in distilled water, then the root tips were observed under a Zeiss LSM 510 confocal microscope, excitation 488 nm, emission 515 nm.

Statistical methods

There were at least three independent replicates for each determination. Data were subjected to analysis of variance (ANOVA) and the means were compared using Tukey's test at 5% level.

Results

Effects of low root zone temperature on plant growth and ROS production

Low root zone temperature decreased cucumber plant fresh weight by 66%. In contrast, there was no growth inhibition of figleaf gourd plants in response to 14°C root zone temperature (Fig. 1). For cucumber plants, both H_2O_2 level and $O_2^{\cdot -}$ generation rate were increased after low root zone temperature treatment (Fig. 2). After 1, 5, 10 days of treatment, H_2O_2 level in the low temperature-treated roots was 106, 117 and

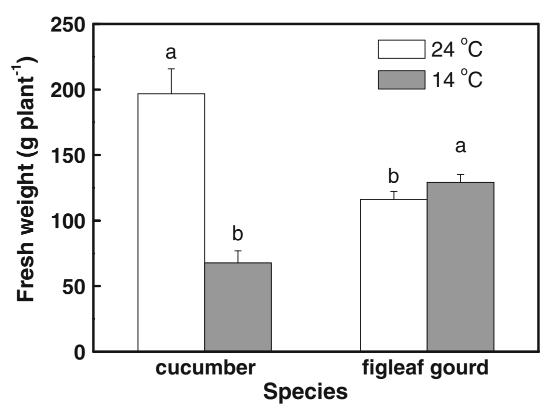

Fig. 1 Effects of 14°C root zone temperature on plant fresh weight of cucumber and figleaf gourd plants after 10 days of treatment. Data are the means of four replicates with standard deviations shown by *vertical bars*. Different *letters* depict significant differences between the treatments ($P < 0.05$)

135%, respectively, of the control plants, while the $O_2^{\cdot-}$ generation rate was increased to 2.3, 3.1 and 4.4-fold of the control plants. In comparison, H_2O_2 level and $O_2^{\cdot-}$ generating rate in figleaf gourd roots did not change significantly during 10 days of low root zone temperature treatment.

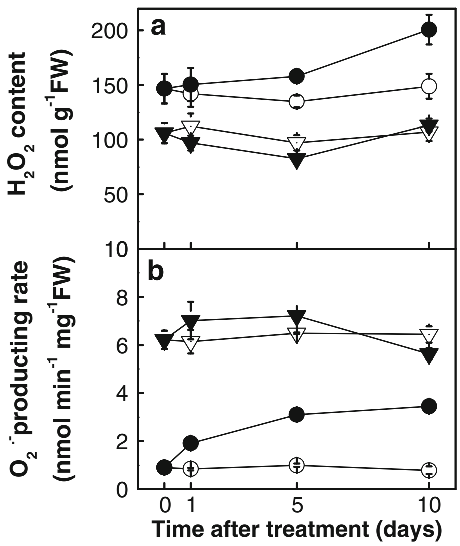

Fig. 2 Changes in hydrogen peroxide (H_2O_2) level (**a**) and superoxide anion ($O_2^{\cdot-}$) generation rate (**b**) in cucumber and figleaf gourd roots as influenced by 14°C root zone temperature. *Open circle* cucumber of 24°C root zone temperature, control; *closed circle* cucumber of 14°C root zone temperature, *inverted open triangle* figleaf gourd of 24°C root zone temperature, *inverted closed triangle* figleaf gourd of 14°C root zone temperature. Data are the means of four replicates with standard deviations shown by *vertical bars*

Effects of low root zone temperature on antioxidant enzyme activities

Low root zone temperature significantly increased SOD activity in cucumber roots from 1 day to the end of the experiments, while did not have any significant effect on other antioxidant enzymes, i.e. APX, GPOD, and CAT activities (Fig. 3). By comparison, 14°C root zone temperature induced a global decrease in APX, GPOD, and CAT activities in figleaf gourd roots, however, only the decrease in CAT activity was distinct, and no evident changes of SOD activity was observed for the 14°C root zone temperature-treated figleaf gourd plants.

Effects of low root-zone temperature on root respiration of cucumber and figleaf gourd plants

The total, SHAM-resistant cytochrome pathway (Cyt pathway), and CN-resistant Alt pathway respiration rates were all higher in figleaf gourd roots than those of cucumber roots (Fig. 4). For figleaf gourd plants, low root zone temperature did not significantly affect total respiration rate and the mitochondrial respiratory electron flux allocated between Cyt and Alt pathways. In contrast, there was no evident effect of low root zone temperature on cucumber total respiration; however, SHAM-resistant R decreased while CN-resistant R increased in stressed cucumber roots. At the end of the experiment, SHAM-resistant R was decreased to 58% and CN-resistant R attained 195%, when compared to the unstressed cucumber plants.

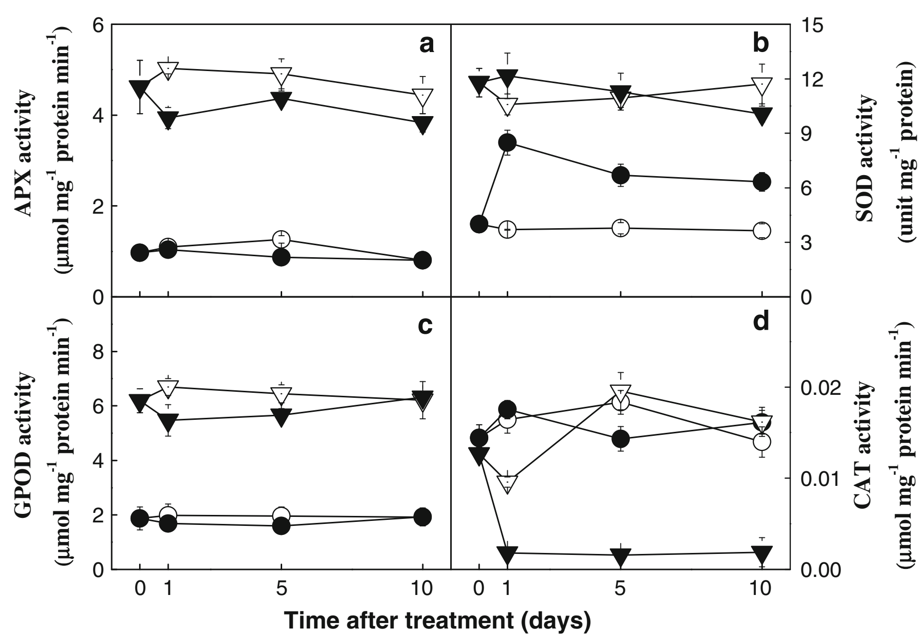

Fig. 3 Changes in the enzyme activities of ascorbate peroxidase (APX, **a**), superoxide dismutase (SOD, **b**), guaiacol peroxidase (GPOD, **c**) and catalase (CAT, **d**) in cucumber and figleaf gourd roots as influenced by 14°C root zone temperature. *Open circle* cucumber of 24°C root zone temperature, control; *closed circle* cucumber of 14°C root zone temperature, *inverted open triangle* figleaf gourd of 24°C root zone temperature, *inverted closed triangle* figleaf gourd of 14°C root zone temperature. Data are the means of four replicates with standard deviations shown by *vertical bars*

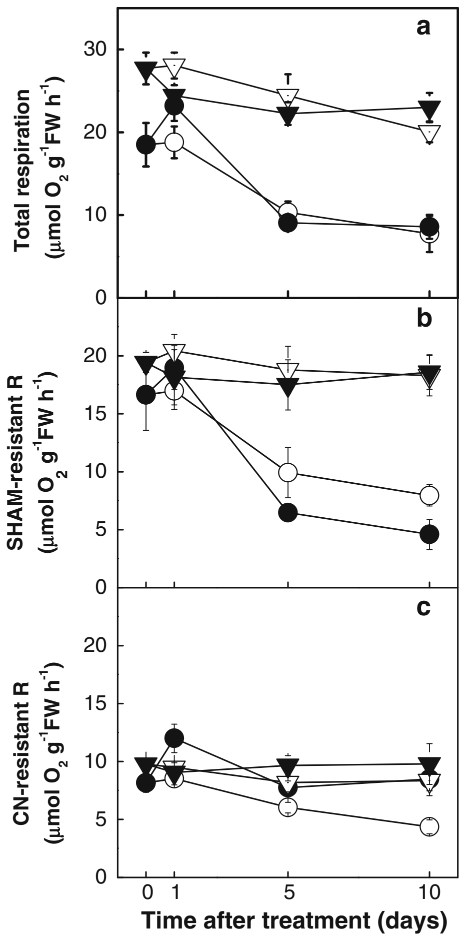

Fig. 4 Changes in respiratory rate of total respiration (**a**), salicylhydroxamic acid-resistant respiration (SHAM-resistent R, **b**), and cyanide-resistant respiration (CN-resistant R, **c**) in cucumber and figleaf gourd roots as influenced by 14°C root zone temperature. *Open circle* cucumber of 24°C root zone temperature, control; *closed circle* cucumber of 14°C root zone temperature, *inverted open triangle* figleaf gourd of 24°C root zone temperature, *inverted closed triangle* figleaf gourd of 14°C root zone temperature. Data are the means of four replicates with standard deviations shown by *vertical bars*

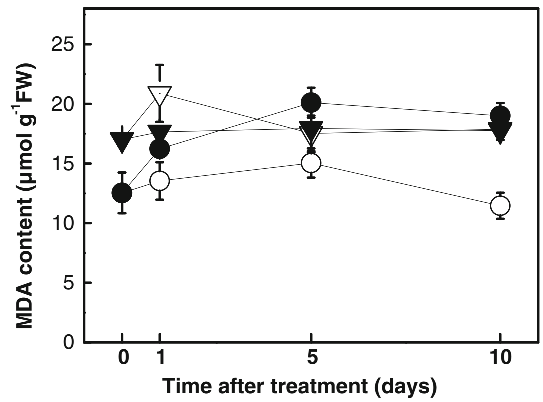

Fig. 5 Changes in malonylaldehyde (MDA) content in cucumber and figleaf gourd roots as influenced by 14°C root zone temperature. *Open circle* cucumber of 24°C root zone temperature, control; *closed circle* cucumber of 14°C root zone temperature, *inverted open triangle* figleaf gourd of 24°C root zone temperature, *inverted closed triangle* figleaf gourd of 14°C root zone temperature. Data are the means of four replicates with standard deviations shown by *vertical bars*

Effects of low root zone temperature on malonylaldehyde content and root cell viability

Similar to the changes in H_2O_2 level and $O_2^{\cdot -}$ generating rate, malonylaldehyde (MDA) content gradually increased in cucumber roots after low root zone temperature treatment, while MDA content of figleaf gourd roots did not show any evident changes (Fig. 5).

Viable cells usually exhibit green fluorescence and dead cells exhibit red fluorescence after staining with dye of FDA–PI. After undergoing 10 days of low root zone temperature treatment, some of the cucumber root-tip cells lost viability, but this phenomenon was not detected in figleaf gourd roots (Fig. 6).

Discussion

In our experimental conditions, cucumber and figleaf gourd were differently affected by 10 days of 14°C root zone temperature treatment. Cucumber growth was inhibited seriously when the root zone temperature was lowered to 14°C, while the growth of figleaf gourd was not affected at low root zone temperature (Fig. 1). This confirms that cucumber is more sensitive than figleaf gourd to low root zone temperature stress and root zone temperature play critical role for different plant species as described by Tachibana (1987). Some researchers even suggest that plant roots are more sensitive to temperature than the overground part of the plant (Ahn et al. 1999), however, the underlying mechanism is not known.

In plants, stress-induced injuries are usually associated with generation of ROS (Almeselmani et al. 2006). Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as chloroplasts, mitochondria, and peroxisomes, while their production is dramatically elevated during stress. In the present study, both H_2O_2 and $O_2^{\cdot -}$ analysis detected increased ROS in low root zone temperature-treated cucumber plants, but not in figleaf gourd plants as compared with their corresponding control (Fig. 2). Enhanced production of ROS under stress conditions will induce both cellular damages and protective responses. To control the cellular homeostasis, plants contain several antioxidant enzymes to eliminate the generated ROS, the scavenging of $O_2^{\cdot -}$ is achieved through an upstream enzyme of SOD. The intracellular level of H_2O_2 is regulated by a wide range of enzymes including CAT, POD, and others (Willekens et al. 1995). In the present

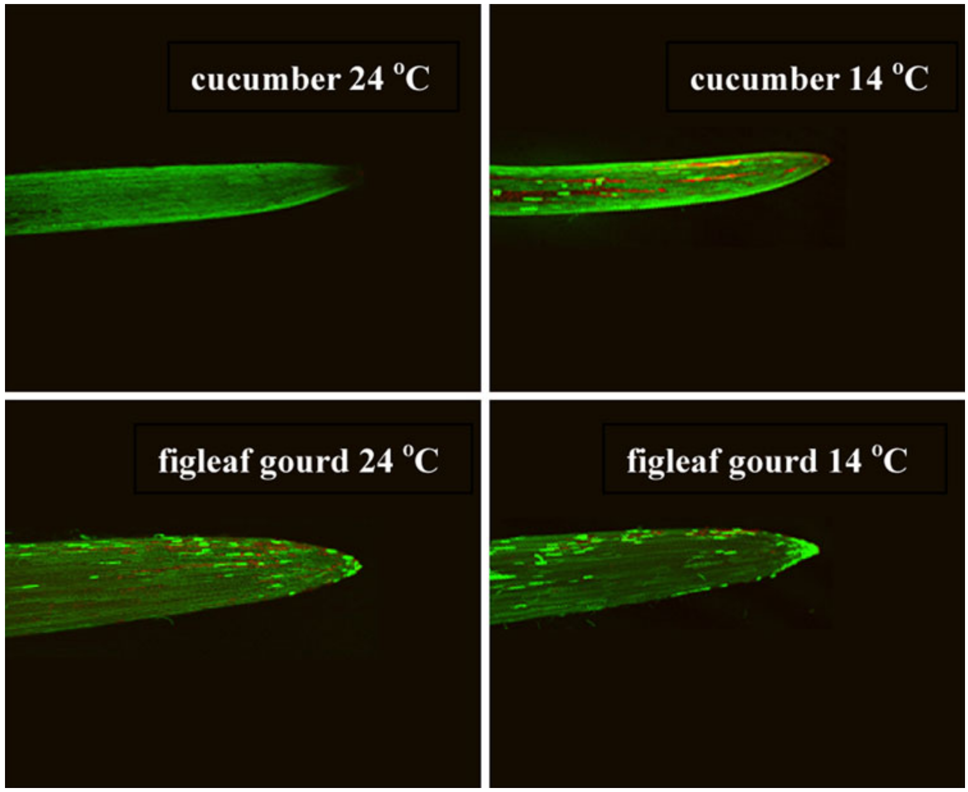

Fig. 6 Confocal microscopic observation of root viability after 10 days of 14°C root zone temperature treatment. *Green fluorescence* and *red fluorescence* exhibit viable and dead cells in root tips, respectively (color figure online)

study, the activities of APX, GPOD, and SOD stayed much higher in figleaf gourd roots than that in cucumber in both stressed and control condition. The antioxidant enzymes were in regulated differently in cucumber and figleaf gourd roots in response to low root zone temperature. For cucumber plants, activities of APX, GPOD, and CAT remained unchanged when exposed to sub-optimal root zone temperature. In contrast, low root zone temperature did induce a great increase in SOD activity for cucumber plants, which are well known to eliminate $\dot{O}_2^-$ production beyond plant homeostasis. For figleaf gourd roots, the opposite change was observed, CAT activity was significantly higher in 24°C root zone temperature than that of 14°C (Fig. 3). Previous studies reported that figleaf gourd can grow well at a relatively low root zone temperature of around 14°C (Ahn et al. 2000; Lee et al. 2002), the great increase of CAT activity might be explained by the fact that 14°C but not 24°C was more optimal for these chilling-adaptive plants. Thus, higher antioxidant enzymes works to antagonize the potential oxidative stress under 24°C root zone temperature.

In addition to antioxidant enzymes, a developing idea is that plant mitochondria respiration pathway also has a role in preventing the overgeneration of cellular ROS (Amirsadeghi et al. 2006). Plant mitochondria have two pathways of electron transport: the Cyt pathway with cytochrome *c* oxidase (COX) as terminal oxidase and the Alt pathway with alternative oxidase (AOX) as terminal oxidase. The Cyt pathway couples electron transport to the generation of a proton motive force for the synthesis of ATP, while the Alt pathway bypasses two proton translocation sites at complexes III and IV, transfers electrons directly from the reduced forms of ubiquinone to O_2 and thus yielding only limited ATP production (Juszczuk and Rychter 2003). In the present study, low root zone temperature changed the electrons allocation between the two respiration pathways in cucumber root mitochondria. The SHAM-resistant R decreased while CN-resistant R increased (Fig. 4); this was in accordance with the results obtained in oxidative stress conditions including chilling, ozone, and pathogen attack (Vidal et al. 2007; Watanabe et al. 2008; Király et al. 2008; Fu et al. 2010). The relatively lower Cyt pathway respiration in stressed cucumber would impair energy status for maintaining cellular metabolism and function. However, the increased Alt pathway in these plants might contribute to restrict the ROS production and alleviate the conflicts between the reduced demand and supplying of respiratory substrates and energy in stressed plant cells. Tobacco (*Nicotiana tabacum*) culture cells of anti-sense for AOX produce more ROS than wild-type cells, while AOX-overexpressing cells produce less (Maxwell et al. 1999). In recent years, mitochondrial retrograde regulation (MRR) has been indicated as one of the important mechanism connecting mitochondrial function and cellular response under environmental stress (Rhoads and Subbaiah 2007). Mitochondria are susceptible to oxidative damage caused by ROS when exposed to chilling stress (Szal et al. 2009), thus, low root zone temperature-induced ROS production in cucumber plants might act as signalling molecules and triggered MRR process which subsequently induced expressions of genes encoding AOX protein and antioxidant enzymes, aimed at regaining ROS level/redox homeostasis.

Although low root temperature stress induced increase in antioxidant enzyme activities and CN-resistant R in cucumber plants, these defense responses could not compensate for the ROS production, resulting in higher ROS accumulation, and thus the membrane lipid peroxidation and loss of root cell viability (Figs. 2, 5, 6). On the contrary, 14°C root zone temperature was suitable for figleaf gourd plant growth, and therefore, the low level of ROS is not enough to induce antioxidant enzyme and Alt pathway respiration. Hence, difference in ROS accumulation might be one of the physiological reasons for the remarkable difference between cucumber and figleaf gourd plant growth in response to suboptimal low root zone temperature condition.

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