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REVIEW PAPER

Root carbon and protein metabolism associated with heat tolerance

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Abstract

Extensive past efforts have been taken toward understanding heat tolerance mechanisms of the aboveground organs. Root systems play critical roles in whole-plant adaptation to heat stress, but are less studied. This review discusses recent research results revealing some critical physiological and metabolic factors underlying root thermotolerance, with a focus on temperate perennial grass species. Comparative analysis of differential root responses to supraoptimal temperatures by a heat-adapted temperate C3 species, *Agrostis scabra*, which can survive high soil temperatures up to 45 °C in geothermal areas in Yellow Stone National Park, and a heat-sensitive cogeneric species, *Agrostis stolonifera*, suggested that efficient carbon and protein metabolism is critical for root thermotolerance. Superior root thermotolerance in a perennial grass was associated with a greater capacity to control respiratory costs through respiratory acclimation, lowering carbon investment in maintenance for protein turnover, and efficiently partitioning carbon into different metabolic pools and alternative respiration pathways. Proteomic analysis demonstrated that root thermotolerance was associated with an increased maintenance of stability and less degradation of proteins, particularly those important for metabolism and energy production. In addition, thermotolerant roots are better able to maintain growth and activity during heat stress by activating stress defence proteins such as those participating in antioxidant defence (i.e. superoxide dismutase, peroxidase, glutathione S-transferase) and chaperoning protection (i.e. heat shock protein).

Key words: Carbohydrate, proteins, respiration, root growth, root mortality.

Introduction

Temperature is one of the major factors affecting plant growth and development. The optimal temperatures for plant growth generally range from 15 to 24 °C for shoots and 10 to 18 °C for roots in temperate or cool-season plant species (DiPaola, 1992; Paulsen, 1994). However, air and soil temperatures often exceed these optimal levels during summer, injuring various physiological and metabolic processes. High temperature is one of the most detrimental abiotic stresses for shoot and root growth of cool-season plant species, and it is expected to become a significant stress in the future as the global temperature is predicted to rise 1–4.5 °C over the next 50 years (Hansen *et al.*, 2006). In recent years, significant progress has been made in understanding the plant adaptation mechanisms to heat stress. However, the majority of current research focuses on the aboveground plant components (reviewed by Wahid *et al.*, 2007) The mechanisms involved with root tolerance to heat stress (thermotolerance) are far less investigated, despite the importance of functional root systems in whole-plant adaptation to high temperatures. The significance of the roots in plant adaptation to high temperature is due to their roles in water and nutrient uptake, as well as their involvement in hormone synthesis affecting shoot growth and development (Nielsen, 1974; McMichael and Burke, 1994, 2002).

Root growth is more sensitive than shoot growth to elevated temperatures because of their lower optimal growth temperature. A decline in root growth and physiological function often precedes shoot growth inhibition at high soil temperatures (Huang and Gao, 2000; Xu and Huang, 2000a,b). Studies which exposed roots and shoots to differential air and soil temperatures demonstrated that increased soil temperature was more detrimental than high air temperature for root and shoot growth and that shoot growth inhibition can be induced by exposing only roots to high temperatures (Kuroyanagi and Paulsen, 1988; Ruter and Ingram, 1990, 1992; Udomprasert *et al.*, 1995; Huang and Xu, 2000; Xu and Huang, 2000a,b, 2001). In a heat-sensitive perennial grass species, *Agrostis stolonifera* var. *palustris* Huds., which is used as turfgrass or forage grass in cool climatic regions, high soil temperature (35 °C) reduced leaf chlorophyll content and photosynthetic efficiency and increased electrolyte leakage of cell membranes in leaves even though shoots were exposed to the optimum temperature (20 °C) (Huang *et al.*, 2001; Xu and Huang, 2001). In these studies, heating roots alone impaired the activities of antioxidant enzymes and enhanced lipid peroxidation in leaves to a greater extent in a heat-sensitive cultivar ('Penncross') than in a heat-tolerant cultivar ('L-93'). Inducing heat injury to plant shoots by exposing the root system to high soil temperature is attributed to the disruption of root functions, including a limitation of water and nutrient uptake and transport to leaves (Graves *et al.*, 1991; Klock *et al.*, 1997; Huang and Xu, 2000) as well as a disruption of cytokinin synthesis in roots (Kuroyanagi and Paulsen, 1988; Udomprasert *et al.*, 1995; Liu *et al.*, 2002). Conversely, inhibition of plant processes by high air temperatures can be alleviated to a great extent if the root system is maintained at a desirable temperature (Kuroyanagi and Paulsen, 1988; Ruter and Ingram, 1992). Reducing soil temperature from 35 °C to 20 °C while maintaining air temperature at 35 °C increased leaf chlorophyll content, photosynthetic rate, total nonstructural carbohydrate content, and growth rates of roots and shoots for *A. stolonifera* up to the same level as the rate at optimal air and soil temperatures (Xu and Huang, 2000a,b, 2001; Xu *et al.*, 2002).

The heat tolerance of roots exhibits interspecies and intraspecific variation, which has been related to the genetic variations in whole-plant heat tolerance. For example, heat-tolerant cultivars of *A. stolonifera* had increased production of new roots and lower root mortality compared to heat-sensitive cultivars under heat stress (Huang and Liu, 2003). Improved whole-plant tolerance of Kentucky bluegrass (*Poa pratensis* L.) to combined heat and drought stress has been associated with greater root viability at greater depth in the soil profile (15–30 cm in depth) that resulted in cooler canopy temperatures and higher leaf transpiration through active root water uptake in the stress-tolerant cultivars (Bonos and Murphy, 1999). Lehman and Engelke (1993) also found that higher root number and increased root length contributed to differences in heat tolerance among cultivars of Kentucky bluegrass. There is no doubt that roots play a critical role in plant tolerance to heat stress and that maintaining viable and active root growth is essential for plant growth and productivity during periods of elevated air and soil temperatures.

Plant adaptation to environmental stresses such as heat stress is dependent upon a variety of responses at the physiological, cellular, and molecular levels (Vierling, 1991; Wahid *et al.*, 2007). A comprehensive understanding of mechanistic root adaptation will prove crucial in devising new strategies for improving plant tolerance to heat stress, especially for warm climatic areas where growth of temperate plant species is often limited. This review addresses mechanisms of plant adaptation to heat stress, with emphasis on carbon and protein metabolism governing root thermotolerance in temperate perennial grass species.

Root growth and viability as affected by high temperatures

Injurious effects of heat on the plant root system are typically characterized by a decrease in root biomass, root number, total length, and metabolic activities (Nielsen, 1974; McMichael and Burke, 2002; Xu and Huang, 2000a,b; Huang and Liu, 2003). Heat stress also accelerates the rate of root mortality and reduces individual life spans. Forbes *et al.* (1997) reported 70% of roots of perennial ryegrass (*Lolium perenesis* L.) survived for over 35 d at 15 °C of air and soil temperature whereas only 16% survived when both air and soil temperatures were elevated to 27 °C. Using a minirhizotron imaging technique, Liu and Huang (2000) examined seasonal patterns of root production and mortality for *A. stolonifera* in natural field conditions. It was found that root mortality increased dramatically and new root production decreased significantly as soil temperatures increased from 20 to 35 °C. Xu and Huang (2000a, 2001) have found that root mortality of *A. stolonifera* was 2–5 times greater at air/soil temperatures 20/35 °C and 35/35 °C, compared to 20/20 °C. Even though growth decline and root mortality with elevated temperatures is widely observed, the mechanisms controlling root growth and survivability under high soil temperatures are far from being completely understood.

The ability of plants to tolerate heat stress varies within and between species. This provides opportunities to elucidate on the underlying mechanisms of plant stress tolerance due to genetic variation (Wahid *et al.*, 2007). One approach to understand these mechanisms is by examining plants already well adapted to extremely stressful environments. For example, studies of desert succulents have identified important mechanisms of drought tolerance (Nobel, 1996). Several temperate (C_3) grass species, including *Agrostis scabra* ('thermal' rough bentgrass) have been identified growing in geothermally heated areas in Yellowstone National Park (Stout and Al-Niemi, 2002; Tercek *et al.*, 2003). *A. scabra* is found in the upper soil surface near geothermal vents where soil temperatures range from 20 to 50 °C, whereas air temperatures ranges from 15 to 27 °C

during May to August (Huang, unpublished data). These geothermal grass species can survive and even thrive in soils permeated by steam at temperatures up to 50 °C, although they may avoid temperatures exceeding 50 °C by flowering early and entering dormancy (Tercek *et al.*, 2003). Most cool-season grass species are sensitive to heat stress (DiPaola, 1992; Paulsen, 1994) and most tissues of higher plants cannot survive temperatures above 45 °C for extended periods (Taiz and Zeiger, 2006). For example, in a congeneric cool-season grass species, *A. stolonifera*, root physiological injury and eventual death occurred when soil temperatures increased to 23 °C, while shoot death was observed as temperatures increased above 30 °C (Fry and Huang, 2004; Pote *et al.*, 2006). Compared to *A. stolonifera*, *A. scabra* was able to maintain greater levels of root viability and root elongation in high-temperature soils (35 °C; Rachmilevitch *et al.*, 2006a,b, 2008; Lyons *et al.*, 2007), as well as more root production (Fig. 1). Roots continued to proliferate at 35 °C soil temperature in *A. scabra*, but creased production in *A. stolonifera* (Fig. 1). *A. scabra* roots also had greater membrane stability compared to roots of *A. stolonifera* under high soil temperatures (Lyons *et al.*, 2007). Therefore, strong evidence exists suggesting that roots of *A. scabra* may possess unique mechanisms for survival in extremely high temperatures that are detrimental to most cool-season grass species, such as *A. stolonifera*. The fundamental questions raised are: how can these roots maintain viability and functionality during long-term heat stress? and which metabolic processes may be involved in root tolerance to heat stress?

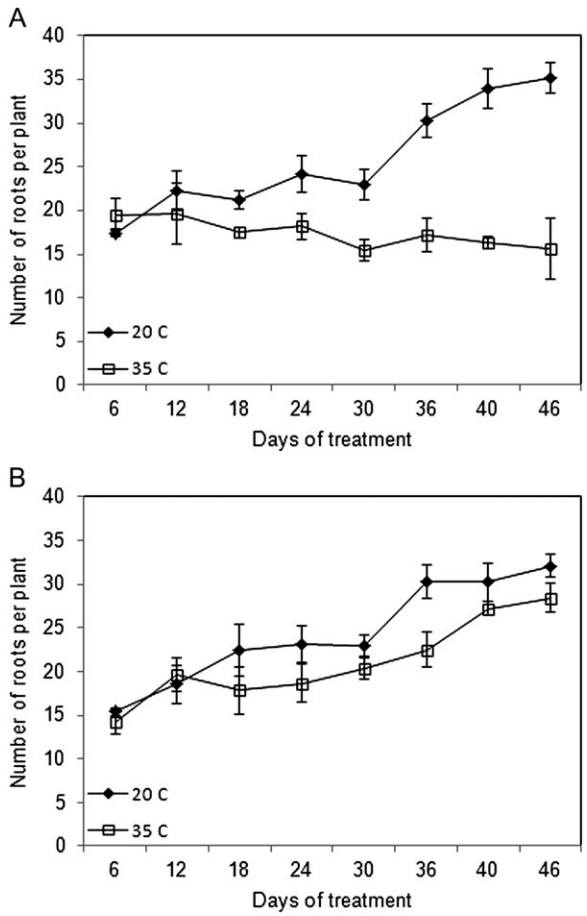

Fig. 1. Root growth, expressed as number of roots per plant, of *A. stolonifera* (cultivar 'Penncross') (A) and *A. scabra* (B) exposed to soil at 20 or 35 °C. Bars are standard errors of the mean ($n = 4$).

Carbon availability and allocation in relation to root thermotolerance

Carbohydrate availability in roots through the regulation of carbon translocation and utilization is a controlling factor for root growth and function because roots are completely dependent upon carbon supply provided by shoots (Lambers *et al.*, 1998). The ability for plants to sustain root growth is regulated by the total amount of carbon fixed during photosynthesis and by the amount allocated to below-ground plant parts (Bloomfield *et al.*, 1996). Interruption of carbohydrate metabolism in the root system has been suggested as a primary factor responsible for growth inhibition and root dysfunction for plants grown at high soil temperatures (Du and Tachibana, 1994a,b). In order to survive extended durations of high soil temperature, roots must possess mechanisms to maintain adequate carbon supply or be especially efficient in carbon utilization when supplies are limited. The effects of heat stress on net carbon fixation during photosynthesis are relatively well understood. However, the factors controlling carbon translocation and allocation to roots under heat stress with elevated air or soil temperatures are not well studied.

Various studies have reported that carbon translocation from shoots to roots is inhibited at high soil temperatures (Ruter and Ingram, 1990; Aloni *et al.*, 1992; Xu and Huang, 2000a,b). For studies which utilized *A. stolonifera* (Xu and Huang, 2000a,b), carbon allocation to roots and total nonstructural carbohydrate content in roots were both significantly reduced by increasing the soil temperature to 35 °C, even though shoots were exposed to the a lower temperature of 20 °C. Contrasting this, carbon availability in roots increased when soil temperature was reduced to 20 °C and air temperature maintained at 35 °C. In addition, under high soil temperatures alone or a combined high air and soil temperatures, nonstructural carbohydrate content in roots of heat-tolerant cultivars was significantly higher than in roots of heat-sensitive cultivars. Root mortality increased and nonstructural carbohydrate content declined for heat sensitive cultivars exposed to a temperature increase above 20 °C during July and August (Xu and Huang, 2000b). Roots of *A. stolonifera* maintained at a lower canopy cutting height had greater mortality than plants with higher canopy height, suggesting that the carbohydrate shortage induced by removal of photosynthesizing leaves may accelerate root mortality (Huang and Liu, 2003). Studies in woody plants also suggest that root mortality is associated with the availability of carbohydrates (Marshall, 1986). Marshall (1986) reported that the amount of starch and sugar reserves is the primary physiological factor controlling fine-root mortality in Douglas

fir [*Pseudotsuga menziesii* (Mirb.) Franco] seedlings. Much evidence suggests that carbohydrate supply may govern root growth and mortality rate. Considering this, a shortage of carbohydrates could substantially contribute to the death of grass roots under high soil temperatures.

Root survival in soils of high temperature depends not only on the total amount of carbon supplied to and accumulated by the roots, but may also be related to the plants ability for efficient carbon partitioning for various metabolic or structural constituents within the roots. Decreased rate of root growth at high temperatures has been correlated to a reduced amount of carbon incorporation into root cellular structures, particularly for protein synthesis and cell-wall maintenance. High soil temperatures impede metabolism and translocation of photosynthates to roots for incorporation into cellular structural components, as demonstrated by decreased amounts of root pectic substances at high soil temperatures (Du and Tachibana, 1994a). Du and Tachibana (1994b) determined the proportion of newly fixed ^{14}C through photosynthesis into sugars, organic acids, and amino acids, showing that a very large proportion of ^{14}C was found in the sugar fraction in roots exposed to 38 °C whereas the soluble ^{14}C was distributed evenly in the three fractions in roots at 25 °C. Using a substrate-based model of plant acclimation to various temperatures, Demar *et al.* (1999) predicted that plant adaptation to increasing temperatures depends mainly on the internal allocation of carbohydrates for protein synthesis. Using ^{14}C-labelling to trace the amount of carbon fixed in photosynthesis that was allocated to protein synthesis in roots, the present study found that the proportion of carbon allocated to proteins in roots (percentage of ^{14}C detected in root protein relative to total ^{14}C in the whole plant) decreased in the two *Agrostis* species under heat stress (37 °C) (Fig. 2). In roots of *A. scabra*, the decline occurred after a longer exposure to high temperatures and the extent of the reduction in the carbon allocation to proteins were significantly less pronounced than in roots of *A. stolonifera* (Fig. 2). These results suggest that roots may be able to adapt to high soil temperatures through the efficient partitioning of carbon to protein synthesis.

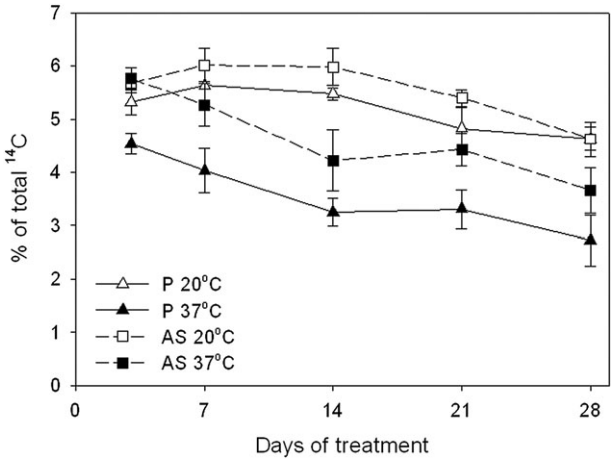

Fig. 2. Proportion of newly synthesized carbon (^{14}C-labelled) allocated to proteins in roots of *A. stolonifera* 'Penncross' (P) and *A. scabra* (AS) exposed to soil at 20 or 37 °C. Bars are standard errors of the mean ($n = 4$).

Respiratory acclimation in relation to heat stress adaptation

Proper control of carbon consumption may play an important role for maintaining adequate root growth and promoting plant survival when carbon supply or allocation to roots is limited under high soil temperatures. Respiration is a major avenue of carbohydrate consumption in a plant. For example, young plants of wheat (*Triticum aestivum*) respired up to 50% of the daily carbon gain by photosynthesis (Morgan and Austin, 1983). Temperature is an important factor affecting the respiratory rate of plants. Sensitivity of respiration to changes in temperature can be expressed as Q_{10}, the proportional change in respiration with every 10 °C increase in air temperature (Lambers, 1985). Root Q_{10} values vary from 1.1 (Higgins and Spomer, 1976) to 2.9 (Tjoelker *et al.*, 1999), which may reflect differences in growth environments, measurement temperatures, and physiological status of the tissues (Loveys *et al.*, 2003). In a previous study which investigated root respiratory responses to elevated soil temperature for *A. scabra* and *A. stolonifera*, Rachmilevitch *et al.* (2006b) reported that roots of *A. scabra*, having a higher growth rate under elevated temperatures, had a lower Q_{10} (1.4) than roots of *A. stolonifera*, themselves having a decreased growth rate and Q_{10} varying with cultivars from 2.1 to 2.2. Carbohydrates used in respiration provide the energy required for maintenance of metabolic activities in all living tissues of plants, but high respirational carbon loss may lead to the depletion of carbohydrate reserves. Therefore, lowering root respiratory rates under elevated soil temperature could better control the total carbon consumption and contribute to long-term plant survival.

Long-term plant exposure to a given temperature range can result in respiratory acclimation, in which respiration rates are less responsive to changing temperatures (Lambers *et al.*, 1998; Atkin *et al.*, 2000; Atkin and Tjoelker, 2003; Kurimoto *et al.*, 2004). Luo *et al.* (2001) reported the acclimation of soil respiration from roots and other organisms to elevated temperatures in tall grass prairie and speculated that the decreased sensitivity of soil respiration in response to warming could be related to less root respiration. Roots with enhanced thermotolerance may be able to control respiratory rates at a lower rate instead of increasing with temperatures or utilize more efficient respiratory pathways. Increasing air and soil temperature by 4 °C (from 16 to 20 °C) caused a 30% increase in root respiration in three perennial grasses, *Bellis perennis*, *Dactylis glomerata*, and *Poa annua*. *B. perennis*, which was acclimated to higher temperatures, maintained a lower rate of respiration compared to the other species under high temperature conditions (Gunn and Farrar, 1999). How do

plants that are adapted or acclimated to high soil temperatures control root respiration rates and otherwise compensate for higher temperatures? Temperature acclimation governed by root respiration has received far less attention than acclimation via processes in above-ground plant parts. Lyons *et al.* (2007) compared the rate responses of total root respiration during short-term (24 h) exposure to low (20 °C) or high soil temperature (37 °C) for *A. scabra* and *A. stolonifera*. When plants previously grown at 20 °C were transferred to 37 °C, root respiration rates increased dramatically for *A. stolonifera*, whereas root respiration rates for *A. scabra* were less responsive to this treatment. In addition under the same temperature regime (37 °C), *A. scabra* maintained lower root respiration rates than *A. stolonifera*. Other researchers have reported that some plant species such as *Poa costiniana* (Loveys *et al.*, 2002) and *D. glomerata* (Gunn and Farrar, 1999) show no temperature acclimation of root respiration, whereas other species such as *Festuca ovina* (Fitter *et al.*, 1998) and *P. annua* (Gunn and Farrar, 1999) exhibit complete acclimation or homeostasis. Plants displaying a large degree of acclimation showed less variation of relative growth rate upon changing temperatures than those with a lesser degree of acclimation. This demonstrates that acclimation via respiratory changes coincides with acclimation via growth rate (Gunn and Farrar, 1999; Kurimoto *et al.*, 2004). Plants expressing root respiration homeostasis have a greater ability to maintain relative growth rate at stressful temperatures by utilizing more efficient respiratory pathways. (Kurimoto *et al.*, 2004). Atkin *et al.* (2000) estimated that the amount of CO_2 released per year by those plants exhibiting respiratory homeostasis is half of that for plants that lack the ability of homeostasis upon increasing temperatures. Therefore, respiratory acclimation is an important factor controlling plant adaptation to long-term exposure to high temperatures.

Respiration consists of two electron transport pathways from ubiquinone to O_2: the ATP-generating cytochrome pathway (CP), which is coupled with oxidative phosphorylation, and the cyanide-insensitive electron transport pathway or alternative pathway (AP), which branches off the CP at the level of ubiquinone (Moore and Siedow, 1991). The AP respiration consumes O_2 and oxidizes NADH without the production of ATP beyond the first phosphorylation site. The CP and AP respiration pathways are known to respond differently to changing temperatures (Vanlerberghe and McIntosh, 1992; Gonzalez-Meler *et al.*, 1999; Fiorani *et al.*, 2005; Rachmilevitch *et al.*, 2007). Increased root respiration with heightened temperatures has been attributed to an increase in the AP component (Du and Tachibana, 1994b). A comparative analysis between *A. scabra* and *A. stolonifera* for respiration partitioning to AP versus CP in response to heat stress found that a greater proportion of total root respiration was attributed to the AP pathway in *A. scabra* compared to *A. stolonifera* (Rachmilevitch *et al.*, 2007). Maintaining a higher proportion of AP in soils of elevated temperature was correlated to higher root viability in *A. scabra* compared to *A. stolonifera*, suggesting that alternative respiration may be beneficial for root adaptation to heat stress.

However, increased AP respiration decreases the efficiency of respiratory ATP production and thus significantly contributes to carbon costs of roots (Lambers, 1982). AP respiration is an energetically wasteful process in terms of carbon consumption and energy production but facilitates continuous electron flow in the mitochondrial membranes when CP respiration is otherwise restricted by environmental stresses (Fiorani *et al.*, 2005). AP respiration has also been associated with the removal of excess electrons in the mitochondrial electron transport chain which would otherwise induce the production of reactive oxygen species causing oxidative damage (Day *et al.*, 1996; Maxwell *et al.*, 1999). With this, increasing alternative respiration may be excessively carbon consuming but also has protective effects for roots to survive high temperatures that may otherwise induce oxidative stress.

Maintenance respiratory costs and metabolic factors associated with root thermotolerance

Respiration involves three major energy-requiring processes: maintenance, growth, and ion transport (Lambers *et al.*, 1998). The term 'maintenance' includes all processes which maintain cellular structures and gradients of ions and metabolites, as well as physiological adaptation processes which maintain cells as active units in a changing environment (Penning De Vries, 1975). Respiratory maintenance costs account for 20–60% of daily assimilates in herbaceous plants (De Visser *et al.*, 1992). Maintenance respiration increases with increasing temperatures (Penning De Vries, 1975; Kase and Catsky, 1984). Increased respiratory carbon consumption with heightened temperatures has been found to derive from increased maintenance respiration rates (Lawrence and Oechel, 1983; Lambers *et al.*, 2002). Plants maintaining low root respiration rates, particularly maintenance costs, have displayed faster growth rates (Poorter *et al.*, 1991; Lambers *et al.*, 1998) and high temperature adaptation and root survival (Bouma *et al.*, 1997; Gunn and Farrar, 1999; Rachmilevitch *et al.*, 2006b). *A. scabra* was able to control root maintenance respiration rates at a significantly lower level under high soil temperatures than *A. stolonifera*, suggesting that thermotolerant roots of *A. scabra* may govern respiratory costs and increase respiratory efficiency by lowering overall maintenance costs (Rachmilevitch *et al.*, 2006b). Down-regulation of maintenance respiration can conserve carbon, which may prolong root survival during periods of high temperatures. Therefore, plants that are adapted to high temperatures may reduce their individual maintenance energy requirement and/or redirect the limited amount of energy towards energy-consuming processes otherwise critical to survival when net energy supplies become limited.

A major proportion of maintenance energy costs is associated with protein turnover, although other processes, such as ion gradient, cytoplasmic streaming, membrane

turnover, and turnover of other cellular constituents, also contribute to energy costs (Lambers *et al.*, 1998). Current evidence suggests that protein turnover is one of the largest maintenance costs in plant tissues (Bouma and De Visser, 1993; Lambers *et al.*, 1998). Protein turnover is a dynamic process involving degradation and synthesis. Approximately 2–5% of all the proteins are replaced daily and values as high as 20% have been reported (Bouma *et al.*, 1994). It has been estimated that approximately 75% of amino acids are recycled from degraded proteins (Davies, 1979) and the remaining 25% are synthesized from basic carbon skeletons. The total cost of protein turnover is estimated at about 3–5% of dry mass per day. Assuming a protein half-life of 5 d, the respiratory costs to sustain normal protein turnover was calculated to be approximately 7% of the total respiratory energy produced in roots of *D. glomerata* (Lambers *et al.*, 1998). Scheurwater *et al.* (2000) studied protein turnover related to maintenance respiration in roots of a fast-growing versus slow-growing grass species and found that roots of both species spent between 22–30% of their daily ATP production on protein turnover, corresponding to 11–15% of the total root ATP production per day. This calculation was based on the assumption that half the total recycling of the ^{14}C-labelled leucine took place in the roots of both species. High respiratory costs have been attributed to increased protein turnover in petals of *Petunia* × *hybrida* when plants were exposed to high temperatures (Hachiya *et al.*, 2007). Metabolic factors which contribute to rapid protein turnover may lead to increased maintenance respiration in roots exposed to high temperatures. Therefore, in terms of net energy and carbon costs, maintaining stable proteins or slowing protein degradation may be important factors promoting root survival during high temperature stress.

Protein metabolism in relation to root thermotolerance

Protein turnover and degradative processes are typically accelerated by environmental stresses as well as during natural tissue senescence (Huffaker, 1990). To assess whether slower protein turnover in roots of *A. scabra* was associated with superior heat tolerance, the content of newly synthesized proteins and rate of protein degradation (labelled with ^{14}C-leucine) were determined during periods of heat stress. Roots of *A. stolonifera* exposed to heat stress at 37 °C exhibited significant decline in content of newly synthesized proteins (Fig. 2) and total protein content (Fig. 3). In contrast, *A. scabra* roots had a less severe decline, significantly higher total protein content, and newly synthesized proteins compared to roots of *A. stolonifera* during prolonged period of heat stress. The decline in the content of total or newly synthesized proteins suggests that protein degradation rates exceed protein synthesis rates during heat stress and that *A. scabra* roots may be better able to sustain protein synthesis and/or possess relatively more thermostable proteins during heat stress. The degradation rate of ^{14}C-labelled protein was significantly lower (30%) while the protein half-life was significantly longer

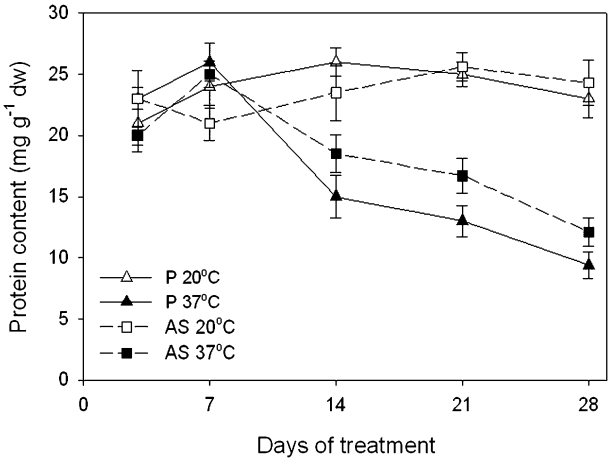

Fig. 3. Total protein content in *A. stolonifera* 'Penncross' (P) and *A. scabra* (AS) roots exposed to soil at 20 or 37 °C. Bars are standard errors of mean ($n = 4$).

(2 d) in *A. scabra* compared to *A. stolonifera* roots after 14 d of heat stress (Table 1), suggesting that the newly synthesized proteins of *A. scabra* roots degraded slower. This could be due to either the presence of more thermostable proteins or lower proteolytic activities. The differential responses of induction and degradation of three selected heat shock proteins (HSPs), which have been found to play critical roles in protecting plants from heat stress (Vierling, 1991), were examined in *A. scabra* and *A. stolonifera*. Western blot analysis of HSP16, HSP60, and HSP70 demonstrated that HSP16 was induced by heat stress, whereas constitutive HSP60 and HSP70 exhibited accelerated degradation at 40 °C. This degradation was slower in *A. scabra* roots compared to roots of *A. stolonifera* (Fig. 4A). The combination of both processes may contribute to the superior root thermotolerance in *A. scabra*.

Stress-induced protein degradation could be accelerated by stimulating the synthesis of proteolytic enzymes (Cooke *et al.*, 1980). Protein degradation is carried out by proteases, which catalyse the hydrolysis of peptide bonds. Several mRNAs responsible for encoding proteins with amino acid sequence identity matching to known proteases, mostly cysteine protease, have been shown to accumulate when plant tissues are exposed to environmental stresses such as high temperature, drought, and salinity (Callis, 1995). Cysteine proteases are among the most extensively studied proteolytic enzymes because they appear to play a central role for a wide range of proteolytic functions of higher plants. Enhanced expression of cysteine protease is involved in the regulation of cell death during tissue senescence (Solomon *et al.*, 1999). When subtraction suppression hybridization analysis was performed to identify cDNA differentially expressed in *A. scabra* roots exposed to both heat stress (35 °C) and optimal temperature conditions, four genes, encoding metallopeptidase, a peptidase family M1 containing protein, a microsomal signal peptidase, and cysteine protease (*AsCP1*), were identified (Tian *et al.*, 2007). The reverse-transcription (RT) PCR analysis

Table 1. Protein degradation constant (k_d), half-life ($t_{0.5}$), and protease activity in roots of *A. scabra* and *A. stolonifera* after 14 d of exposure to soil at 20 or 35 °C

Values followed by the same-case letters are not significantly different ($P > 0.05$) between temperature treatments and between species at a given temperature treatment (lowercase and uppercase, respectively).

Species	Temperature (° C)	Degradation constant, k_d (g protein^{-1} d^{-1})	Half-life, $t_{0.5}$ (d)	Protease activity (μg protein^{-1} h^{-1})
A. stolonifera	20	0.071^{bA}	10^{bA}	7.25^{bA}
	35	0.136^{aA}	4^{aB}	15.58^{aA}
A. scabra	20	0.076^{bA}	9^{aA}	7.96^{bA}
	35	0.098^{aB}	7^{aA}	12.09^{aB}

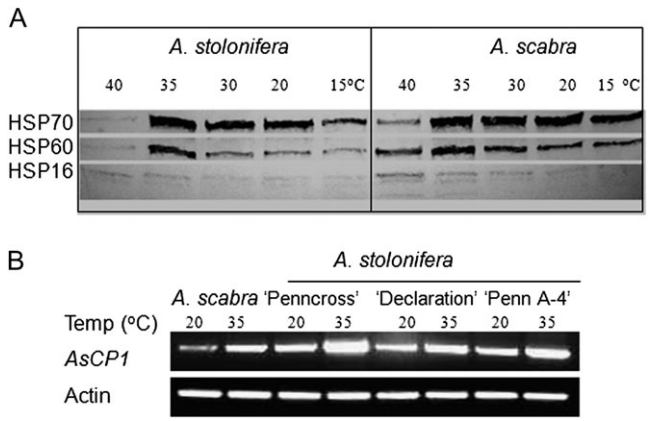

Fig. 4. (A) Western blot analysis of HSP70, HSP60, and HSP16 in roots of *A. stolonifera* and *A. scabra* exposed to soil at 15, 20, 30, 35, and 40 °C for 24 hours. (B) Reverse-transcription PCR of root *AsCP1* encoding cysteine protease in *A. scabra* and three cultivars of *A. stolonifera* roots exposed to soil at 20 or 35 °C for 14 d.

(Fig. 4B) showed that expression levels of *AsCP1* were significantly increased for roots exposed to heat stress, but the gene expression level was lower in roots of *A. scabra* than in *A. stolonifera* cultivars. In addition, roots of *A. scabra* maintained a significantly lower cysteine protease activity under heat stress compared to *A. stolonifera* (Table 1). These results suggest that lower levels of gene expression and decreased cysteine protease activity may contribute to slower proteolytic degradation processes, and therefore improved root thermotolerance. The results from grass species were consistent with other studies in which cysteine protease has been shown to accumulate when plants were exposed to environmental stresses, including high temperature (Callis, 1995). Further characterization of the *AsCP1* may reveal a mechanism of cysteine protease regulating root thermotolerance. However, it should be pointed out that only one gene, encoding cysteine protease (*AsCP1*), was examined in the grass species of this study. Multiple proteases may be involved with protein degradation and root adaptation to heat stress, and therefore the specific ways in which different proteases may be involved in heat tolerance deserves further investigation.

Current knowledge of which specific proteins are involved in root thermotolerance is very limited. Comprehensive profiling of stress-associated proteins is important for understanding the metabolic and molecular factors controlling heat tolerance. Proteomics, the study of global changes in proteins, offers a powerful approach to discover which metabolic pathways are crucial for stress responsiveness and overall tolerance. Two-dimensional polyacrylamide gel electrophoresis in combination with mass spectrometry allows for rapid and reliable protein identification and can provide information about abundance as well as post-translation modification (Van Wijk, 2001). In recent years, these technologies have been successfully applied to the systematic study of proteomic responses for many plant species exposed to a wide range of abiotic stresses, including heat (Ferreira *et al.*, 2006; Lee *et al.*, 2007). However, most research focuses on the proteomic responses in above-ground organs. Proteomic changes associated with root tolerance to heat stress are much less investigated thus far. Proteomic profiling associated with root-based heat tolerance will allow for further molecular dissection of heat-tolerance mechanisms and will generate new strategies for improving plant tolerance to heat stress. This is especially important for warm climatic areas where growth and productivity of temperate plants is often limited during summer months.

Xu and Huang (2009) investigated differential proteomic responses to heat stress in roots of *A. scabra* and *A. stolonifera* by exposing plants to increasing temperature (20 °C, optimal temperature; 30 °C, moderate heat stress; and 40 °C, severe heat stress). The abundance of 70 protein spots changed under heat stress in at least one species and 66 heat-responsive proteins were identified and classified into different functional categories described by Bevan *et al.* (1998). Heat-responsive proteins included 11 proteins involved in primary metabolism, 22 in energy metabolism, six in protein destination and storage, seven in stress defence, and seven in secondary metabolism. Moderate or severe heat stress caused a reduction in the abundance of a total of 47 proteins, including those changes either in one species or in both. The down-regulated proteins included those involved in primary metabolism (cytosolic glutamine synthetase, methionine synthase, serine hydroxymethyltransferase, nucleotide-sugar dehydratase), energy metabolism (aconitate hydratase, fumarase, malate dehydrogenase, sucrose synthase isoforms, pyrophosphate-dependent phosphofructokinase, pyruvate kinase, fructose-1,6-bisphosphate aldolase, GAPDH isoforms, phosphogluconate dehydrogenase, NADH2 dehydrogenase), protein destination and storage (26S protease regulatory subunit 7, phosphogluconate

dehydrogenase, mitochondrial processing peptidase, disulphide-isomerase), stress defence proteins (peroxidase), and secondary metabolism (phenylalanine ammonia-lyase, dDTP-glucose 4-6-dehydratase-like protein, adenosylhomocysteinase, and S-adenosylmethionine synthase). A total of 22 proteins exhibited abundance increases under moderate and severe heat stress in one or both *Agrostis* species. The up-regulated proteins included those involved in primary metabolism (phosphoserine aminotransferase, plastidic ATP sulphurylase), energy (sucrose synthase isoforms, GAPDH isoforms), protein destination and storage (HSP stress inducible protein, peptidyl-prolyl *cis-trans* isomerase), stress defence (glutathione S-transferase, superoxide dismutase), and secondary metabolism (UDP-glucose 6-dehydrogenase). The changes in abundance level of the previously mentioned proteins indicate that roots were able to adjust metabolic processes for metabolism, energy, and stress defence in response to high temperatures. Those heat-responsive proteins controlling metabolism, energy production, and stress protection may contribute to better root adaptability to high temperatures in *A. scabra*.

Root thermotolerance-related proteins were also identified through the comparison of differential proteomic profiles of roots of *A. scabra* and *A. stolonifera* exposed to the same levels of high temperature (30 and 40 °C) (Xu and Huang, 2009). Root thermotolerance-related proteins were mainly classified into three functional categories: (1) stress defence; (2) primary metabolism; and (3) protein repair and stability.

Regarding stress defence, the abundance of glutathione S-transferase (GST), superoxide dismutase (SOD), peroxidase, and HSP stress inducible protein was significantly higher in *A. scabra* compared to *A. stolonifera* under moderate or severe heat stress. In addition, GST was induced by heat stress only in *A. scabra*. RT-PCR analysis confirmed a stronger expression level for a gene encoding a small HSP (*HSP16*) and *HSP70* in roots of *A. scabra* compared to *A. stolonifera* cultivars (Fig. 5). GST, SOD, peroxidase, and HSPs are known for being involved in stress defence via their antioxidant ability and/or as a chaperone (Vierling, 1991; Dixon *et al.*, 2002; Mittler, 2002). A higher abundance of these proteins in *A. scabra* relative to *A. stolonifera* suggests that roots expressing thermotolerance were better able to activate stress defence mechanisms and promote survival during heat stress.

Regarding primary metabolism, several proteins were up-regulated by heat stress in *A. scabara* only. Two proteins involved in primary metabolism, plastidic ATP sulphurylase and phosphoserine aminotransferase, were up-regulated in roots of *A. scabra* only following 10 d of moderate or severe heat stress. This suggests the importance of serine and sulphur metabolism in root thermotolerance, particularly during prolonged periods of stress. *A. scabra* and *A. stolonifera* had differential expression patterns of sucrose synthase in response to heat stress. Sucrose synthase was down-regulated in *A. stolonifera* but up-regulated in *A. scabra* during heat stress. The increased accumulation of sucrose synthase in *A. scabra* could be reflective of a more active sucrose metabolism for roots under heat stress, which may provide energy reserves for root survival during extended periods of high temperatures.

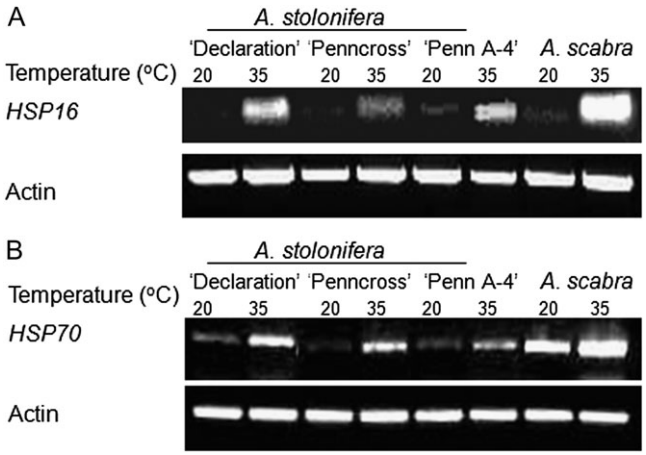

Fig. 5. Reverse-transcription PCR analysis of *HSP16* and *HSP70* expression in *A. scabra* and three cultivars of *A. stolonifera* roots exposed to soil at 20 or 35 °C for 14 d.

Regarding protein repair and stability, protein disulphide isomerase (PDI) was down-regulated in *A. stolonifera* only. The decline in PDI abundance indicates that heat damage in roots may be related to the disruption of protein folding and stability, governed by the degradation of disulphide isomerase. PDI is known to be an essential protein that controls cell viability in yeast by stabilizing the tertiary and quarternary structures of many proteins (Farquhar *et al.*, 1991). It is reasonable to assume that the maintenance of PDI accumulation in roots of *A. scabra* could contribute to its superior root viability during heat stress.

Concluding remarks

Significant progress has been made over the past decade for a better understanding of the mechanisms involved with root thermotolerance. Root responses to high temperatures vary between and within plant species, which provides an opportunity for the identification of mechanisms in stress tolerance. The comparative analysis of a heat-tolerant species, *A. scabra*, which can survive extremely high soil temperatures found in geothermal areas, and a congeneric heat-sensitive species, *A. stolonifera*, revealed that efficient carbon use and protein metabolism are important factors contributing to superior root thermotolerance. Many questions still remain as to how roots may maintain active growth and survive under high temperatures. For example, how do changes in other metabolic processes involving hormones, lipids, and secondary metabolites affect root thermotolerance? And how is root thermotolerance controlled at the molecular level? Comprehensive transcriptomic and metabolomic profilings of root responses to elevated temperatures will enable further dissection of metabolic and molecular factors underlying root thermotolerance and aid in devising new strategies for improving plant tolerance to heat stress.

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