Plant Physiology and Biochemistry 70 (2013) 195–203

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Antioxidant and photosystem II responses contribute to explain the drought–heat contrasting tolerance of two forage legumes

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A R T I C L E I N F O

Article history:
Received 18 January 2013
Accepted 16 May 2013
Available online 4 June 2013

Keywords:
Heat stress
Water stress
Photoinhibition
PSII proteins
Proline
Lotus corniculatus
Trifolium pratense

A B S T R A C T

Identification of metabolic targets of environmental stress factors is critical to improve the stress tolerance of plants. Studying the biochemical and physiological responses of plants with different capacities to deal with stress is a valid approach to reach this objective. *Lotus corniculatus* (lotus) and *Trifolium pratense* (clover) are legumes with contrasting summer stress tolerances. In stress conditions, which are defined as drought, heat or a combination of both, we found that differential biochemical responses of leaves explain these behaviours. Lotus and clover showed differences in water loss control, proline accumulation and antioxidant enzymatic capacity. Drought and/or heat stress induced a large accumulation of proline in the tolerant species (lotus), whereas heat stress did not cause proline accumulation in the sensitive species (clover). In lotus, Mn-SOD and Fe-SOD were induced by drought, but in clover, the SOD-isoform profile was not affected by stress. Moreover, lotus has more SOD-isoforms and a higher total SOD activity than clover. The functionality and electrophoretic profile of photosystem II (PSII) proteins under stress also exhibited differences between the two species. In lotus, PSII activity was drastically affected by combined stress and, interestingly, was correlated with D2 protein degradation. Possible implications of this event as an adaption mechanism in tolerant species are discussed. We conclude that the stress-tolerant capability of lotus is related to its ability to respond to oxidative damage and adaption of the photosynthetic machinery. This reveals that these two aspects should be included in the evaluation of the tolerance of species to stress conditions.

1. Introduction

1.1. Biochemical responses to drought

Lotus corniculatus (lotus) and *Trifolium pratense* (clover) are legumes used in agriculture as a forage source. These species are both perennial herbaceous plants used in temperate grassland and can be nodulated by rhizobia. Nevertheless, lotus is better suited to soils with water restriction and has a superior tolerance to drought [1].

In the field, mainly during the summer, these plants are commonly exposed to environmental stresses such as drought and high temperatures, which in fact are considered to be the most important environmental factors limiting plant growth and development [2–5].

Soil water deficits resulting in dehydration and osmotic stress may seriously affect plant growth [6]. In response to a water deficit, plant cells accumulate low-molecular-mass compounds termed compatible solutes, mainly proline, glycine betaine, sugars and polyols, in the cytoplasm to accommodate the ionic balance in the vacuoles [7]. Among these solutes, proline has been associated with different functions, such as being a free radical scavenger, a cell redox balancer, a cytosolic pH buffer and a stabilizer for subcellular structures, especially during osmotic and salt stresses [8–10].

The accumulation of proline is known to be a good indicator of drought in *L. corniculatus* [11]. However, some plants that tend to accumulate proline in drought conditions replace it with sucrose as

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Abbreviations: APX, ascorbate peroxidase; BSA, bovine seroalbumin; CAT, catalase; C, control treatment; D, drought treatment; D + H, drought and heat treatment; GR, glutathione reductase; H, heat treatment; HI, hydric index; H_2O_2, hydrogen peroxide; hydroxyl radicals, lotus; *Lotus corniculatus*, Pro; proline, P5C; pyrroline 5 carboxylate, PSII; photosystem II, ROS; reactive oxygen species, superoxide radical; SOD, superoxide dismutase; clover, *Trifolium pratense*.

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http://dx.doi.org/10.1016/j.plaphy.2013.05.028

the major osmoprotectant when subjected to a combination of drought and heat stress [12]. It is also known that proline accumulation under heat stress decreases the thermotolerance of the plant, probably because of an enhancement in the production of reactive oxygen species (ROS) via the Pro/P5C cycle [13]. Additionally, drought affects the rate of photosynthesis due to an increase in photoinhibition, a process that can be enhanced when two or more types of abiotic stress coexist [14].

Under stress conditions, the possibility of overexcitation of photosystem II (PSII) increases. This can cause a decline in the photosynthetic rate as the process of photoinhibition increases due to the necessity to dissipate, through nonradiative processes, the excess of absorbed energy [14,15].

Because the capacity of photoprotection is limited, certain conditions can lead to damage and loss of active PSII reaction centres. Under severely high temperatures, stress commonly associated with a water deficit during drought, the photosynthetic apparatus has been long considered to be the primary site of damage. On the contrary, photosystem I has been shown to be more resistant to heat than PSII [16–18]. Once photoinhibition is established, the PSII reaction centre is simultaneously repaired via removal, synthesis and replacement of degraded D1 protein [19,20]. The observed photoinhibitory damage is the net result of a balance between photodamage and the repair process [21–23].

Several studies have reported a good correlation between changes in chlorophyll fluorescence parameters in response to environmental stresses, such as heat, chilling, freezing, and salinity [24–27]. Others authors have linked the decrease in the maximum quantum yield of PSII (F_V/F_M) to the physical dissociation of the PSII reaction centres that lead the photoinhibition, and they performed this technique to discriminate tolerant cultivars [28].

It is well known that the effect of a combination of different stresses on plants can be quite different from those generated when plants are subjected to individual types of stress [29], and is motive of studio.

1.2. Water–heat stress and ROS metabolism

Another effect of water and heat stress is an overproduction of ROS, such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$), which occurs mainly in organelles such as the chloroplast, mitochondria and peroxisomes [30,31]. These species are responsible for lipid and protein oxidation, the primary consequences of ROS-mediated oxidative stress [32]. This stress can cause damage to the PSII, mainly when it occurs at the chloroplast level, leading to disassembly of the different structures through proteolysis.

In particular, the D1 protein is damaged because of intrinsic ROS generation by the PSII machinery [33], which can also impair the activity of the Calvin cycle and disrupt electron transport [34]. This situation generates metabolic limitations and, together with the stomatal restriction generated during drought conditions, leads to a decay in carbon assimilation [35–37].

The enzymatic antioxidant systems include superoxide dismutase (SOD; EC 1.15.1.1), which provides the first line of defence against ROS by dismutating $O_2^{\bullet-}$ to H_2O_2, and catalase (CAT; EC 1.11.1.6), which regulates H_2O_2 levels [30]. These enzymes, in combination with enzymes of the ascorbate–glutathione cycle, such as ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2), protect plant cells by reducing the generation of toxic oxygen forms. A number of studies have shown that the antioxidant defence systems are induced and up-regulated to a greater extent in water stress-tolerant cultivars compared to sensitive species such as apple [38], tomato [39] and alfalfa [40] when subjected to water stress, and the induction of these systems is associated with acclimation to water deficit conditions. The generation of oxidative stress induced by abiotic stress and mediated by ROS accumulation is widely documented [30]; however, additive or synergistic effects of two or more environmental stresses on ROS-related photoinhibition of PSII are poorly understood.

According to Boyer (1982) [6], water shortages limit crop production in semi-arid regions more than any other factor. A better understanding of the mechanisms that enable plants to adapt to water deficits and maintain growth and productivity during drought periods will ultimately help in the selection of drought-tolerant varieties.

Studies indicate that even though drought and heat stress can occur together in most regions (particularly the semi-arid tropics), the physiological or biochemical mechanisms operating to induce escape or tolerance to each of these stresses may be different. Furthermore, the characteristics of the traits associated with drought and heat stress might be different. Therefore, the selection–production of genotypes for tolerance to combined drought and heat stress must be performed under stress conditions that include both of these stresses [41].

Lotus and clover have different tolerance to drought and drought–heat combination, stressing environmental conditions frequently found during the summer. We sought to explain the differences in tolerance through the evaluation of the antioxidant and PSII responses of leaves under stress conditions to contribute to the understanding of differential field drought tolerance in two forage legumes.

2. Results

2.1. Differential response to dehydration induced by drought

To confirm the differential behaviour observed between lotus and clover when they are subjected to drought, we first followed

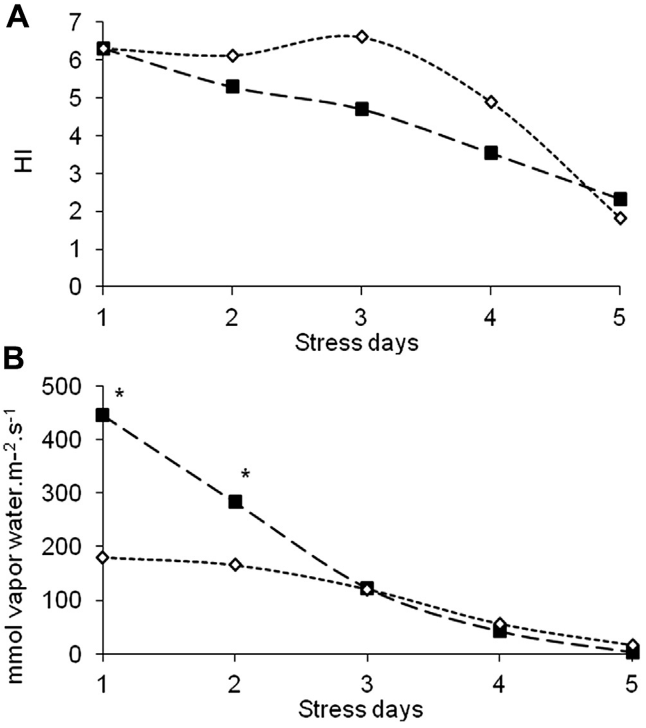

Fig. 1. Hydric index and stomatal conductance. A. Hydric index (HI) in the 5 days of treatments. B. Stomatal conductance during the 5 days of treatments. Lotus is indicated by white diamonds (◇) and clover by black squares (■). Asterisks indicate that differences in the clover values compared to the lotus values were statistically significant at $p \leq 0.05$, Tukey's test.