Plant Science 250 (2016) 59–68

Contents lists available at ScienceDirect

Plant Science

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

Photosynthetic responses mediate the adaptation of two *Lotus japonicus* ecotypes to low temperature

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ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 2 June 2016
Accepted 2 June 2016
Available online 3 June 2016

Keywords:
Chilling
D2 protein
Photoinhibition
Legumes
PSII
ROS

ABSTRACT

Lotus species are important forage legumes due to their high nutritional value and adaptability to marginal conditions. However, the dry matter production and regrowth rate of cultivable *Lotus* spp. is drastically reduced during colder seasons. In this work, we evaluated the chilling response of *Lotus japonicus* ecotypes MG-1 and MG-20. No significant increases were observed in reactive oxygen species and nitric oxide production or in lipid peroxidation, although a chilling-induced redox imbalance was suggested through NADPH/NADP$^+$ ratio alterations. Antioxidant enzyme catalase, ascorbate peroxidase, and superoxide dismutase activities were also measured. Superoxide dismutase, in particular the chloroplastic isoform, showed different activity for different ecotypes and treatments. Stress-induced photoinhibition also differentially influenced both ecotypes, with MG-1 more affected than MG-20. Data showed that the D2 PSII subunit was more affected than D1 after 1 d of low temperature exposure, although its protein levels recovered over the course of the experiment. Interestingly, D2 recovery was accompanied by improvements in photosynthetic parameters (A*sat* and Fv/Fm) and the NADPH/NADP$^+$ ratio. Our results suggest that the D2 protein is involved in the acclimation response of *L. japonicus* to low temperature. This may provide a deeper insight into the chilling tolerance mechanisms of the *Lotus* genus.

1. Introduction

The *Lotus* genus (Fabaceae) comprises about 120 annual and perennial species distributed worldwide. Several of them are important forage crops because of their high nutritional value and tolerance to different abiotic stresses [1]. Within the genus, *Lotus japonicus* (Regel) K. Larsen is commonly used as a model legume for genetic studies due to its relatively small, diploid genome (n = 6), ability to self-fertilize, and short life cycle [2]. The accessibility of mutant databases [3] and complete genome sequencing [4] make this legume a robust tool for plant research, specifically with respect to plant response to biotic and abiotic stress [5,6]. However, few works have addressed the effect of low temperature stress on species of this genus [7].

Lotus species are found in temperate climates, meaning that they are frequently exposed to low temperatures that affect their growth capacity and crop yield [8]. Low temperatures reduce metabolic reaction kinetics, induce membrane rigidification, increase reactive oxygen species (ROS) production, and promote the formation of secondary RNA structures that affect gene and protein expression [9]. Additionally, chilling, defined as cooling to a temperature below the minimum growth temperature but above the freezing point, affects photosynthesis, resulting in photoinhibition [10]. This phenomenon is ascribed to an impairment of photosystem II (PSII) activity [11] due to an imbalance between photodamage and repair

Abbreviations: PSII, photosystem II; TBA, thiobarbituric acid; MDA, malondialdehyde; DAB, 3,3′-diaminobenzidine; NBT, nitro blue tetrazolium; APx, ascorbate peroxidase; CAT, catalase; SOD, superoxide dismutase; NADK, NAD$^+$kinase; ROS, reactive oxygen species; RNS, reactive nitrogen species.

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http://dx.doi.org/10.1016/j.plantsci.2016.06.003
0168-9452/

rates; in this case, damage most often occurs at the reaction center proteins D1 and D2 [12,13]. D1 in particular has been described as having a key role during photoinhibition under low temperature stress [14]. However, over the last few years, effects at D2 have also been reported for *Lotus* species [15,16], although the relevance of this with respect to chilling-induced photoinhibition remains poorly understood.

Recently, a number of *L. japonicus* ecotypes were collected and characterized according to their morphological traits [17], providing a source of genetic variability within the species. In order to better understand photosynthetic acclimation of *L. japonicus* under low temperature stress, we studied the physiological and biochemical response of two of these ecotypes that show contrasting behavior under this environmental constraint.

2. Materials and methods

2.1. Plant material selection

L. japonicus ecotypes were screened in order to find those with differential germination at low temperature: MG-1, MG-2, MG-4, MG-5, MG-17, MG-20, MG-34, MG-36, MG-52, MG-57, MG-61, MG-62, MG-65, MG-72, MG-75, MG-83, and Gifu B-129.

Seeds were scarified with sulfuric acid (100%) for 3 min, washed 10 times with sterile distilled water, and sown in Petri dishes containing water/agar (0.8%). Then, seeds were incubated in dark growth chambers at either 8 °C (low temperature treatment) or 22 °C (control treatment) for 7 d. The low temperature treatment was set to 8 °C because it was determined to be the minimal temperature allowing *L. japonicus* Gifu seeds to germinate (data not shown). We defined germination as the emergence of at least 1 mm of radicle from the seed coat. Overall, 50 seeds were evaluated per replicate and 3 replicates were performed per ecotype for each treatment. Germination percentages were normalized, at low temperature, using the control germination percentages for each ecotype. Only ecotypes with more than 50% germination at control temperatures were considered in the screening.

Two ecotypes, one with the highest (MG-20) and another with the lowest (MG-1) germination capacities, were selected and used in the following experiments.

2.2. Plant material, growth conditions, and treatments

Seeds of the MG-20 and MG-1 ecotypes were incubated for 7 d in a growth chamber with a 16/8 h day/night cycle at 24 °C/21 °C ± 2 °C and 55/65 ± 5% relative humidity. Light (at an intensity of 250 μmol m^{-2} s^{-1}) was provided by Grolux F 40 W fluorescent lamps. Seedlings were transferred to 110 mL volume pots containing sterilized sand-perlite (2:1), irrigated with half-strength Hoagland's nutrient solution [18], and cultivated under the same temperature, relative humidity, and light conditions already described. Plants with 4–6 fully-developed leaves were used for all experiments, in order to stress both ecotypes at the same developmental stage.

Chilling treatment was performed in a growth chamber (Percival I60-DL) with a 16/8 h day/night cycle at 9 °C/5 °C for 7 d. Illumination was provided by LED lamps, with an intensity of 250 μmol m^2 s^{-1}. The control treatment conditions were identical to the incubation conditions, but were performed in the same growth chamber as the chilling treatment and with the same LED lamps. The experimental design was completely randomized in all cases.

Total fresh weight for each plant and the presence of chlorotic areas were recorded at the end of each treatment for both ecotypes.

2.3. Chlorophyll determination

Chlorophyll was extracted with 4:1 (v/v) acetone/water and quantified according to the method described by Inskeep and Bloom [19]. Measurements were performed in shoots over 7 d for both the low temperature and control treatments, using 4 plants per treatment for each ecotype.

2.4. Lipid peroxidation

Lipid peroxidation was measured through the thiobarbituric acid reactive substances (TBARS) assay using the method proposed by Hodges et al. [20], which corrects for sugars and anthocyanins. The tissue was homogenized in a mortar, after which 40 mg of the homogenate was added to 1 mL of an extraction solution consisting of ethanol/water 4:1 (v/v) including 0.01% butyl-hydroxytoluene. After centrifugation at 10000*g* for 10 min, 0.2 mL of the supernatant was heated at 95 °C for 30 min in a mixture consisting of 0.2 mL of 0.65% TBA in 20% trichloroacetic acid, 0.1 mL of 0.1 N HCl, and 0.1 mL of distilled water. A similar treatment was completed that was identical except for the absence of TBA. Then, 0.4 mL of distilled water was added and the resulting mixture was centrifuged at 10000*g* for 15 min, after which the supernatant was measured at 440, 532, and 600 nm using a Hitachi U-1100 UV–vis spectrophotometer (Hitachi, Japan). Data were expressed as malondialdehyde (MDA) equivalents (nmol g^{-1} FW). Measurements were performed for shoots after 1, 4, and 7 d of low temperature treatment, and after 7 d of control treatment. In each case, 4 plants were tested for each treatment mode in each ecotype.

2.5. Superoxide and H_2O_2 in situ measurements

In situ detection of H_2O_2 was performed according to a procedure by Thordal-Christensen et al. [21]. Detached leaves were vacuum infiltrated under dark conditions with 10 mM potassium phosphate buffer (for a pH of 7.8), 10 mM NaN_3, and 0.1% (w/v) 3,3′-diaminobenzidine (DAB). The infiltrated leaves were then incubated overnight under dark conditions, after which they were clarified with 0.15% (w/v) trichloroacetic acid in 4:1 (v/v) ethanol:chloroform for 48 h before being photographed.

In situ detection of superoxide was performed as described by Jabs et al. [22]. Detached leaves were vacuum infiltrated with 10 mM potassium phosphate buffer (for a pH of 7.8), 10 mM NaN_3, 0.1% (w/v) nitro blue tetrazolium (NBT), and 0.05% (v/v) Tween 20. The detached, infiltrated, and NBT-treated leaves were then kept under daylight conditions for 30 min, after which they were discolored using the same method described above for the detection of H_2O_2.

Measurements were performed on 5 plants for each treatment mode in each ecotype. For each plant, the penultimate developed leaf was tested at 1, 4, and 7 d for the low temperature treatment, and at 7 d for the control.

2.6. Antioxidant enzyme measurements

Ascorbate peroxidase (APx), catalase (CAT), and superoxide dismutase (SOD) were analyzed in total shoots at 1, 4, and 7 d for the low temperature treatment and at 7 d for the control. Measurements were performed on 5 plants for each treatment mode and ecotype.

Leaf samples were ground in a mortar with liquid N_2. From the resulting homogenized powder, 300 mg were transferred to a microtube. Then, 1 mL of buffer extract (a solution with a pH of 7 containing 0.1 M potassium phosphate, 0.1% Triton 100x, 15% glycerol, 0.2% ascorbic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethanesulfonyl fluoride) was added. The