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Theme 4. Biodiversity, conservation and genetic improvement of range and forage species
Sub-theme 4.1. Plant genetic resources and crop improvement

Study on protein related to heat tolerance of *Medicago sativa* L. 'Deqin'

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Introduction

High temperature over the growth threshold of plants can cause their metabolic and developmental changes, and consequently bring about their slow growth (Lobell *et al*., 2008). Alfalfa (*Medicago sativa* L.) is a high-quality legume suitable for growing in semi-arid areas. Its poor thermo tolerance restricts its introduction and popularization in southern China and transitional zone. Fall dormancy is an important indicator for planting planning of alfalfa (Barnes *et al*., 1977. Existing research shows that alfalfa varieties with a higher fall dormancy rate (FDR) have a stronger heat resistance (Lu, 1998). *M. sativa* L. 'Deqin' (hereinafter referred to as 'Deqin') is a wild type alfalfa with a low FDR (1.2) which can grow and proliferate normally in dry-hot valley regions in Deqin County, Yunnan, China. It shows a strong heat resistance. This phenomenon is contradictory to the inference that alfalfa varieties with a higher FDR have a stronger heat resistance. We intend to study physiological and biochemical changes and the change of mRNA expression quantity of differential protein and coding differential protein of 'Deqin' under high-temperature stress and analyze heat resistant physiology and molecular biological mechanism of 'Deqin'. This result will enrich the study theory of alfalfa resources in China and provide theoretical basis for the introduction and popularization of alfalfa with the indicator of fall dormancy rate.

Materials and Methods

Materials: 'Deqin' (FDR=1.2), 'Sardi10' (FDR=9), and 'Algonguin' (FDR=2).
Method: Plant materials were placed in a manual climatic box (PQX-1000B, Ningbo, China) with photoperiod 14/10h (day/night), relative humidity 65% and illumination intensity 400 μmol m^{-2} s^{-1}. 25℃/20℃ (day/night) was set as control (*i.e*. high-temperature stress 0d). They were processed with the method of continuous long-term high-temperature stress (35℃/30℃, day /night) for 35 days. Completely ringent leaves and leaves in the same part were analyzed at days 7, 14, 21, 28 and 35 after treatment. Photochemical efficiency (MAXI Imaging-PAM, Walz, Effeltrich, Germany), malondialdehyde content, relative conductivity, leaf relative water content, content of cytoplasm and cytomembrane proteins, expression profile, endogenous hormone content, antioxidant enzyme activity and isoenzyme profile were measured; differential proteomics analysis (iTRAQ), (Zieske, 2006) and qRT-PCR verification were conducted.

Results and Discussion

In seedling stage, 'Deqin' leaves could maintain the completeness of cytomembrane, have a strong moisture holding capacity, produce a lot of proteins with rich varieties and show a strong heat resistance. After high-temperature stress, the increase extent of relative conductivity and malonaldehyde content of 'Deqin' was higher than that of 'Sardi 10' and significantly lower than that of 'Algonguin'. The decrease extent of its photochemical efficiency, relative water content, cytoplasm and cytomembrane proteins was between those of two controls. A lot of small molecular weight proteins were produced in its leaves in the final stage of stress (day 28). After high-temperature stress, the increase extent of relative conductivity and malonaldehyde content of 'Algongiun' was the highest and its photochemical efficiency, relative water content and cytommbrane protein content decrease to the lowest. Its cytoplasm protein content was slightly higher than that of 'Deqin' (P>0.05). Therefore, 'Sardi 10' was heat resistant, 'Algonguin' was heat sensitive and the thermotolerance of 'Deqin' was intermediate.

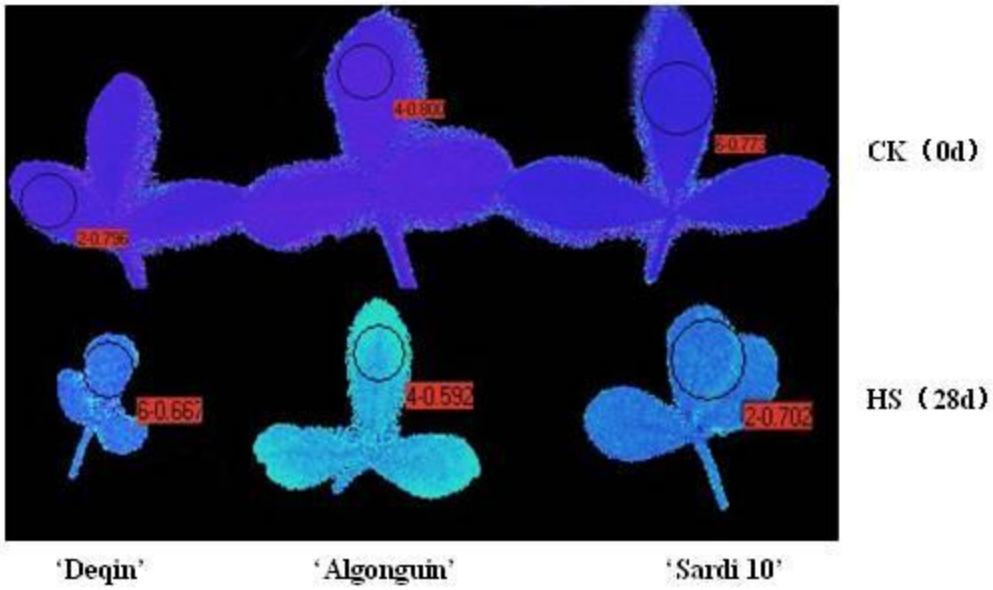

Fig 1 Variation in photochemical efficiency (measured as Fv/Fm) in 'Deqin' under hest stress

'Deqin' responded to high-temperature stress relatively early and its high-level ABA could hold for a certain time. It had high content of IAA and CTK and strong antioxidant enzyme activity, which could clear ROS in cell body in time. Delaying senescence of leaves might be one of the reasons for its strong heat resistance. ABA content of 'Deqin' increased slightly at day 7 after high-temperature stress. During the whole period of high-temperature stress, the cumulative increasing amount of its IAA content was the highest and that of its GA_3 content was lower than that of 'Algonguin'. Heat sensitive 'Algonguin' had the lowest ABA content, relatively lower CTK content and relatively higher GA_3 content during the whole period of high-temperature stress. With the extension of high-temperature stress time, 'Deqin' and 'Algonguin' had similar SOD activity change trend, i.e. decreasing trend. However, SOD activity of 'Deqin' was higher than that of 'Algonguin'. During the stress, CAT activity of 'Algonguin' showed a continuous decreasing trend and the other two varieties had inflexion point of increase. POD activity of 'Algonguin' had the highest degree of reduction and its GR activity showed a decreasing trend during the whole period of treatment. After day 21 of high-temperature stress, APX activity of 'Algonguin' showed a continuous decreasing trend, but 'Deqin' and 'Sardi 10' showed a continuous rising trend. In addition, SOD2, POD4, POD7 and APX3 isoenzymes related to plant heat resistant in 'Deqin' leaves appeared continuously or did not disappear totally during the whole period of high-temperature stress and its SOD, CAT, POD, APX and GR antioxidant enzyme activities were stronger than those of 'Algonguin'. Compared to 'Algonguin', 'Deqin' can clear excessive accumulation of reactive oxygen in cell body caused by high-temperature stress effectively in time, reduce peroxidation of membrane lipid, maintain the semipermeability of cytomembrane and physiological function of membrane and show a strong heat resistance.

A lot of heat resistance-related proteins were produced in leaves of alfalfa varieties. The increase times of seven proteins of 'Deqin' – ClpB, HSP70, DnaK, DnaJ, HSP18.2, HSP17.6 and Cu/Zn SOD was higher than that of 'Algonguin'; the increase times of four enzymes – SOD, APX, GSH-PX and POD was maximum; PS II electron donor side was most stable. 1,727 high-temperature induced alfalfa differential expression proteins were identified with iTRAQ technology. With the extension of high-temperature stress time, PS II electron donor side and acceptor side of 'Algonguin' were obviously damaged by high temperature, ATP synthetaseα- and β-subunits had the poorest stability, and energy-requiring biochemical reactions such as photosynthetic phosphorylation were restricted. Compared to 'Sardi 10', PS II electron donor side of 'Deqin' was most stable (Zhang *et al*., 2011), but its Cyt b6f complex V subunit had the poorest stability. The increase times of seven high-temperature induced proteins of 'Deqin' (ClpB, HSP70, DnaK, DnaJ, HSP18.2, HSP17.6 and Cu/Zn SOD) was higher than that of 'Algonguin' and the increase times of its four antioxidant enzyme-related proteins (SOD, APX, GSH-PX and POD) was higher than that of 'Algonguin' and 'Sardi 10', indicating that heat resistance of 'Deqin' is related to the increase of stress proteins under its high-temperature stress, which maintains the stability of its photosynthetic apparatus and guarantees smooth energy and material metabolism, and the high activity of antioxidant enzyme.

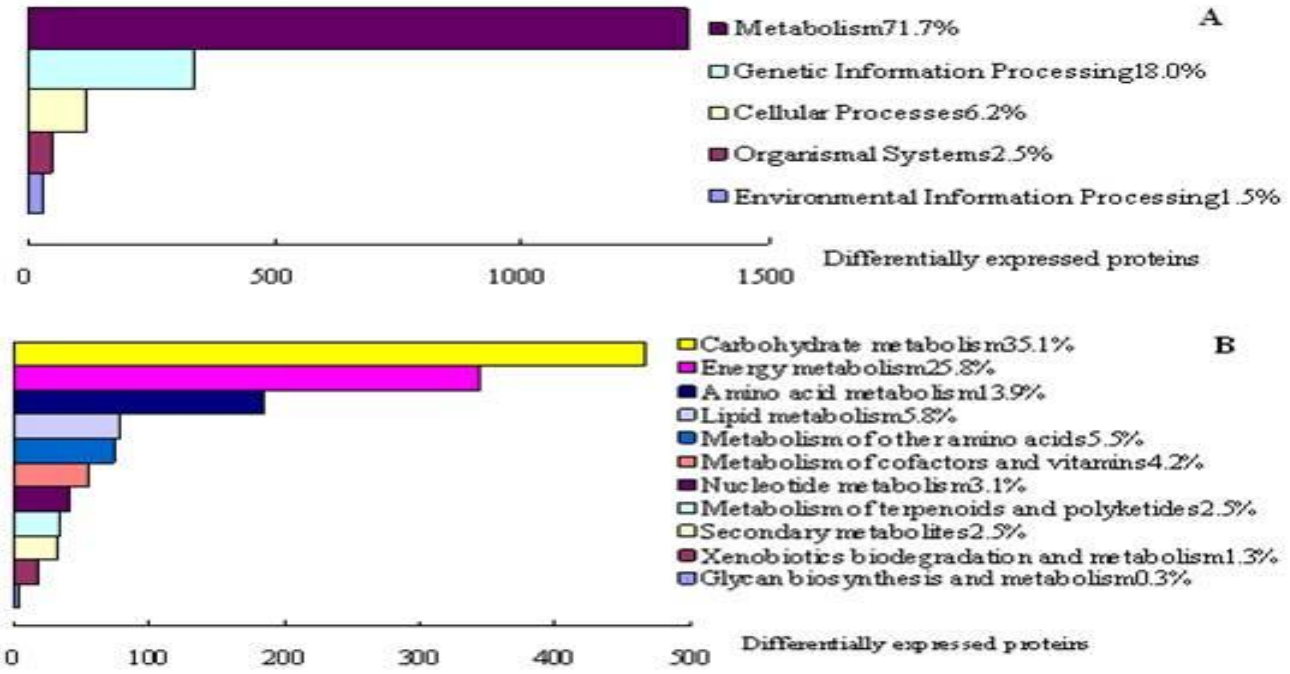

Fig 2 The functional distributions of differentlly expressed proteins in alfalfa under heat stress
A: All differently expressed proteins functional distributions; B: Differently expressed proteins function distribution related to metabolisms

The results of qRT-PCR analysis indicated the validity of iTRAQ analysis. By qRT-PCR, we confirmed expression changes of 23 heat related proteins including antioxidase (Cyt Cu/Zn-SOD, Chl Cu/Zn-SOD, Fe-SOD, Mn-SOD, CAT, PODa, PODd and PODc) and enzymes in the AsA-GSH cycle (Cyt APX, GR, DHAR and Cyt MDHAR). Among these proteins, Four antioxidase (Cu/Zn-SOD, Chl Cu/Zn-SOD, Cyt Fe-SOD and Cyt APX) were up-regulatedly expressed after heat stress (P<0.05). The mRNA of six heat induced molecular chaperones related to thermo tolerance (*Hsp70*, *Hsp18.2*, *Le-Hsp17.6*, *DnaJ*, *DnaK* and *NEFs*) were also up-regulated. Among them, the over expressions of *Hsp 18.2* conform to its change at protein level.

Conclusion

This paper studies physiological changes and the change of mRNA expression quantity of differential protein and coding differential protein of 'Deqin' under high-temperature stress with 'Sardi 10' and 'Algonguin' as control. The result shows that 'Deqin' responds to high-temperature stress relatively early and its high-level ABA can hold for a certain time. It has high content of IAA and CTK and strong antioxidant enzyme activity, which can clear ROS in cell body in time. Delaying senescence of leaves might be one of the reasons for its strong heat resistance. 1,727 high-temperature induced alfalfa differential expression proteins have been identified with iTRAQ technology. The heat resistance of 'Deqin' is related to the increase of stress proteins under its high-temperature stress, which makes its photosynthetic apparatus stable and non-degradable and guarantees smooth energy and material metabolism, and the high activity of antioxidant enzyme. qRT-PCR analysis result proves the effectiveness of iTRAQ analysis.

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