Journal of Plant Physiology 193 (2016) 47–56

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Physiology

Exogenous melatonin improves corn (*Zea mays* L.) embryo proteome in seeds subjected to chilling stress

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ARTICLE INFO

Article history:
Received 21 September 2015
Received in revised form 15 January 2016
Accepted 18 January 2016
Available online 20 February 2016

Keywords:
Antioxidant
Chilling stress
Melatonin
Proteome analysis
Seed priming
Zea mays

ABSTRACT

Melatonin (MEL; *N*-acetyl-5-methoxytryptamine) plays an important role in plant stress defense. Various plant species rich in this indoleamine have shown a higher capacity for stress tolerance. Moreover, it has great potential for plant biostimulation, is biodegradable and non-toxic for the environment. All this indicates that our concept of seed enrichment with exogenous MEL is justified. This work concerns the effects of corn (*Zea mays* L.) seed pre-sowing treatments supplemented with MEL. Non-treated seeds (nt), and those hydroprimed with water (H) or with MEL solutions 50 and 500 μM (HMel50, HMel500) were compared. Positive effects of seed priming are particularly apparent during germination under suboptimal conditions. The impact of MEL applied by priming on seed protein profiles during imbibition/germination at low temperature has not been investigated to date. In order to identify changes in the corn seed proteome after applying hydropriming techniques, purified protein extracts of chilling stressed seed embryos (14 days, 5 °C) were separated by two-dimensional electrophoresis. Then proteome maps were graphically and statistically compared and selected protein spots were qualitatively analyzed using mass spectrometry techniques and identified. This study aimed to analyze the priming-induced changes in maize embryo proteome and at identifying priming-associated and MEL-associated proteins in maize seeds subjected to chilling. We attempt to explain how MEL expands plant capacity for stress tolerance.

1. Introduction

Crop production technologies based only on the improvement of cultivation techniques are beginning to face limitations resulting from an inability to use the biological potential inherent in the cultivar. Climate change is already beginning to impair maize and wheat production. Higher average temperature fluctuations in most major grain-producing countries are beginning to work against the progress made by improving seeds and farming practices, according to the study published in *Science* by Lobell et al. (2011). Thus, there is a constant search for new solutions to ensure the most favorable conditions for plant growth and development, for instance by limiting various biotic and abiotic stresses, and ultimately to increase yield. The most preferable solution seems to be search for and then the use of natural bio-stimulators together with seed priming.

The latest results obtained by our group suggest that MEL has a great potential for plant biostimulation (Janas and Posmyk,

Abbreviations: ACN, acetonitrile; AFMK, *N*1-acetyl-*N*2-formyl-5-methoxykynuramine; Btf3, basal transcription factor 3; CHIP, carboxyl terminus of Hsc70-interacting protein; DDT, DL-dithiothreitol; DOXC, deoxycholate sodium salt; DP, declustering potential; eIF5A, eukaryotic translation initiation factor 5A; EMS, enhanced MS scan; EPI, enhanced product ion scan; ER, enhanced resolution scan; GLPs, germin-like proteins; GSH, reduced glutathione; H, hydroprimed seeds; HMel50, HMel500; HSP, heat shock proteins; IDA, information dependent acquisition method; LEA, late embryogeneis abundant; LMW, low molecular weight; MEL, melatonin; MG, methylglyoxal; NAC, nascent polypeptide-associated complex; NDP, nucleoside diphosphate; NDPk, nucleoside diphosphate kinase; NDPk, nucleoside diphosphate kinases; nt, non-treated seeds; NTP, nucleoside triphosphate; PDIa, protein disulfide isomerase; PMSF, phenylmethanesulfonylfluoride; Prx, peroxiredoxin; RING, really interesting new gene; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDR, short-chain dehydrogenases/reductases; SMP, seed maturation proteins; TCA, trichlorocetic acid; TFA, trifluoroacetic acid; TIM, triosephosphate isomerase; TRX, thioredoxin; UPS, proteins degradation system by ubiquitination.

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http://dx.doi.org/10.1016/j.jplph.2016.01.012
0176-1617/

2013). It is a natural, biodegradable and non-toxic, highly conserved molecule present in evolutionarily distant organisms including plants. The pre-sowing seed treatment with MEL protected red cabbage seedlings against toxic Cu ion concentrations (Posmyk et al., 2008) and MEL application to cucumber and corn seeds had a beneficial effect on the seedling growth and crop production of plants that germinated from them, especially those subjected to cold (Posmyk et al., 2009a,b) and water stress (Zhang et al., 2013). MEL plays an important role in plant stress defense (Zhang et al., 2015). Recently, a several papers have tried to explain the molecular mechanism of MEL action in this case (Shi and Chan, 2014; Shi et al., 2015). It was shown that, in *Arabidopsis thaliana* leaves, freezing stress increased endogenous MEL content and it was correlated with the induction of At-ZAT6 expression. Then, the activation of AtZAT6 increased the expression of CBF1-3 directly binding to TACAAT elements of CBF1-3 promoters, and up-regulation of CBF1-3 improved freezing stress resistance in *Arabidopsis* (Shi and Chan, 2014).

Another model of MEL-mediated action in *A. thaliana* leaves assumes that, upon abiotic stresses (cold, salt, and drought) and biotic stress (pathogen infection), an increase in MEL levels is followed by an increase in the transcripts of CBF/DREB1s. This overexpression results in higher transcript levels of multiple stress-responsive genes (COR15A, RD22, and KIN1) and enhanced soluble sugar accumulation (sucrose), thus improving resistance to the above-mentioned stresses (Shi et al., 2015). A complete explanation of all molecular pathways that MEL stimulates and through which it acts in plants will not be easy because MEL appears to be involved into a very dense network of connections in different parts of plants.

Different plant species rich in MEL exhibit a higher capacity for stress tolerance (Park et al., 2013; Bajwa et al., 2014). An increase in melatonin content was also detected in sunflower seeds during sprouting (Cho et al., 2008). Since the germ and reproductive tissues are highly vulnerable to oxidative damage, MEL might be an important component of its antioxidant defense system as a free radical scavenger, especially in dormant and relatively dry tissues of seeds, where enzymes are poorly effective and cannot be up-regulated (Manchester et al., 2000).

Since MEL is soluble in both water and lipids, it may be a hydrophilic and hydrophobic antioxidant. This fact, together with MEL's small size make it particularly able to migrate easily between cell compartments in order to protect them against excessive ROS and RNS. Moreover, recent evidence indicates that the primary MEL metabolites, especially AFMK, also have strong antioxidant abilities. It is documented that the free radical scavenging capacity of MEL extends to its secondary, tertiary and quaternary metabolites (Tan et al., 2007). This process is referred to as the free radical scavenging cascade, which makes MEL, even at low concentrations, highly effective at protecting organisms against oxidative stress. It seems that in an evolutionary sense, the strong antioxidant properties of MEL (Terrón et al., 2001) were its primary role in the defense against unfavorable conditions and in plant stress tolerance, but our results presented in this paper indicate other beneficial effects.

In addition to *in vivo* synthesis, plants can also absorb exogenously provided MEL from the environment and accumulate it at high concentrations (Tan et al., 2007). This, and particularly the evidence that MEL induces resistance to stresses in plants, indicates that our concept of seed enrichment with exogenous MEL is justified.

Despite methodological difficulties, interest in plant proteome research is still growing. Elucidation of changes in protein synthesis and expression under the influence of biotic and abiotic factors can provide a clear understanding of the processes and mechanisms of the plant response to stress. This is a particularly useful and important research tool when the purpose of the study is to find ways to improve the vigor of plants under suboptimal environmental conditions. There is no information concerning the impact of MEL applied by priming on seed protein profiles during germination under low temperature conditions.

This study aimed to analyze the priming-induced changes in the maize embryo proteome and to identify priming-associated and MEL-associated proteins in maize seeds subjected to chilling stress during germination. Comparative proteomic analysis was used to identify differentially expressed proteins in the unprimed seeds (nt) and those hydroprimed (H) and hydroprimed with MEL (HMel50 and HMel500).

2. Materials and methods

2.1. Plant material

Corn seeds (*Zea mays* L. var. Ambrozja) were provided by TORSEED (Torun, Poland). They were stored in the dark, under dry conditions at room temperature, in tightly closed containers before the experiments started.

2.2. Hydroconditioning

Hydroconditioning was performed by the soaking corn seeds at 25 °C in darkness for 3 h, using distilled water or melatonin (50 or 500 μM) water solutions. Seed water content was increased from an initial value of 8.8% (±0.2) to 36.8% (±2.3). The quantity of water needed to increase seed humidity was determined experimentally. Corn seed variants: non-treated (nt-gel A), hydroprimed (H-gel B), hydroprimed with melatonin 50, and 500 μM (HMel50-gel C and HMel500-gel D, respectively). The seeds were re-dried for 3 days at room temperature after hydropriming.

2.3. Seed germination

Germination of all seed variants were carried out in darkness at 5 °C for 14 days. Then, axes were isolated, placed into the liquid nitrogen and finally lyophilized.

2.4. Protein extraction and purification

Protein extraction was carried out using a homogenizer in an ice bath. Lyophilized tissue (200 mg) was treated with 1.5 mL of extraction buffer 50 mM Tris–HCl (pH 7.7) containing: 0.5 mM DOXC; 10 mM DDT; 10 mM EDTA; 1 mM PMSF. The homogenate was centrifuged: 20 000 g/10 min/5 °C. The pellet was discarded. Proteins from the supernatant were precipitated with 10% TCA and 0.07% merkaptoethanol in acetone, in an ice bath for 45 min. The mixture was centrifuged as noted above. The resulting pellet containing precipitated proteins was washed twice with 0.07% mercaptoethanol in acetone. After the second centrifugation: 3000 × g/5 min./5 °C; sediment was vacuum-dried and then diluted in Rabilloud Buffer containing: 8 M Urea; 4% CHAPS and 65 mM DTT. The protein content was determined in 96-microplates using Bradford's (1976) method. The protein solution was stored at −20 °C and then used for 2-D electrophoresis.

2.5. 2-D protein separation and visualization

Quantified proteins (300 mg) were applied to an 11 cm IPG strip (pH 3–10, non-linear gradient ReadyStrip) in a Protean IEF cell (Bio-Rad, Hercules, CA) and in-gel rehydrated for 12 h. The proteins were separated according to their isoelectric point in the IEF-SYS (Scie-Plas Ltd., GB). Isoelectric focusing was performed at 18 °C for 72 h at 0–3000 V gradually changing (first 24 h:0–1500 V; second: 1500–3000; third 3000 V). Prior to the second dimension