Plant Science 239 (2015) 180–191

Contents lists available at ScienceDirect

Plant Science

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

Metabolite profiling of *Ricinus communis* germination at different temperatures provides new insights into thermo-mediated requirements for successful seedling establishment

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ARTICLE INFO

Article history:
Received 25 June 2015
Accepted 3 August 2015
Available online 4 August 2015

Keywords:
Carbon-nitrogen balance
GABA
Gluconeogenesis
Glyoxylate cycle
Seedling establishment
Stress responsive metabolites

ABSTRACT

Ricinus communis seeds germinate to a high percentage and faster at 35 °C than at lower temperatures, but with compromised seedling establishment. However, seedlings are able to cope with high temperatures at later stages of seedling establishment if germination occurred at lower temperatures. Our objective was to assess the biochemical and molecular requirements of *R. communis* germination for successful seedling establishment at varying temperatures. For that, we performed metabolite profiling (GC-TOF-MS) and measured transcript levels of key genes involved in several energy-generating pathways, such as storage oil mobilization, β-oxidation and gluconeogenesis of seeds germinated at three different temperatures. We identified a thermo-sensitive window during seed germination in which high temperatures compromise seedling development, most likely by down-regulating some energy-generating pathways. Overexpression of malate synthase (*MLS*) and glycerol kinase (*GK*) genes resulted in higher starch levels in *Nicotiana benthamiana* leaves, which highlights the importance of these genes in energy-generating pathways for seedling establishment. Additionally, we showed that GABA, which is a stress-responsive metabolite, accumulated in response to the water content of the seeds during the initial phase of imbibition. Herewith, we provide new insights into the molecular requirements for vigorous seedling growth of *R. communis* under different environmental conditions.

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1. Introduction

Seed germination is a crucial process in the life cycle of higher plants and determines whether seedling establishment will be successful or not. Germination begins with the uptake of water by the seed (imbibition), is followed by reactivation of metabolism, and ends with visible protrusion of the radicle through the surrounding layers [1]. In higher plants, seed germination is controlled by a combination of genetic and environmental factors. Temperature is one of the main environmental factors influencing seed germination and plant growth [2]. In general, a progressive increase in temperature shortens the time required for germination until it reaches an optimum temperature at which the maximum germination percentage is achieved. This optimum temperature may vary between species [3,4]. Following imbibition, cells of the embryo change to a metabolically active state in which several physiological, molecular, and biochemical events occur such as cell elongation, cell cycle activation, transcription, translation, cellular respiration, repair mechanisms, and organelle reassembly [5–9]. These processes are generally supported by the initiation of central metabolism for energy generation and the production of building blocks for cellular structures [8]. Therefore, seed germination also determines whether seedling establishment will be successful or not.

Seed reserves that are mobilized upon germination are utilized as energy resources and new building blocks to support the development of the seedling until it becomes photo-autotrophic [1,8]. Fatty acid β-oxidation and gluconeogenesis are essential for the catabolism of storage lipid reserves in oilseed species, providing

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http://dx.doi.org/10.1016/j.plantsci.2015.08.002
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metabolic energy and carbon skeletons to fuel germination and early post-germination growth [10,11]. Although these biochemical pathways are well characterized, the effect of temperature on these pathways and the consequences for seed germination and seedling establishment are still unclear.

Ricinus communis L. is a member of the Euphorbiaceae and is also known as castor bean. It is widespread throughout tropical, sub-tropical and warm temperate regions [12,13]. The oil extracted from its seeds is mainly used for pharmaceutical and industrial applications due to its unique chemical composition [14]. *R. communis* can be grown in dry and hot environments, where most other crops would not grow, and still display good yield [15]. It has been shown that *R. communis* seedlings have a specific metabolic signature associated with adjusted growth and a likely role in maintaining cellular homeostasis at higher temperatures. A shift in their carbon-nitrogen metabolism is the main biochemical response to high temperatures [16]. However, there is a lack of studies that assess the effect of temperature on *R. communis* seed germination and seedling growth. Therefore, we addressed the question whether different temperatures during seed germination have an effect on important biochemical and molecular mechanisms required for seedling establishment. Our results provide leads for the understanding of the underlying mechanisms that are not only required to support vigorous seedling growth, but also for adaptation to harsh environmental conditions in semi-arid areas worldwide.

2. Materials and methods

2.1. Plant material and germination conditions

R. communis (cv. MPB01) used in this study was developed by the breeding program of the *Empresa Baiana de Desenvolvimento Agrícola S.A* (EBDA-Brazil). This genotype is 1 m high, has a short flowering time and has high seed yield, making it an attractive cash crop alternative for poor family farmers in the semi-arid regions of Brazil. These farmers have limited resources (land, labor, inputs and capital) and therefore face a number of setbacks which hamper their engagement in oil crop production [17].

After seed coat removal, seeds were allowed to imbibe using paper rolls as substrate at three different temperatures (20, 25 and 35 °C) in the dark. Four different stages were defined and sampled for follow-up experiments: dry seeds (Dry), 6-hour-imbibed seeds (6hIS), seeds at radicle protrusion (RP) and seeds with a radicle of 2 cm (R2) (Supplementary Fig. S1). Germination percentage was scored on daily basis for 9 days. For germination under water restricted conditions, seeds were sown in trays with filter paper as substrate and imbibed in water (control) or PEG 8000 solution (−2.0 MPa) at 35 °C for 6 h. Water content of the seeds was measured every hour during the first 6 h of imbibition. Additionally, seeds with water content of 24.4% (fresh weight basis) were sampled for follow-up experiments. For this, seeds were imbibed for 6 h at 35 °C, for 7 h at 25 °C, and for 8 h at 20 °C.

2.2. Primary metabolite profiling

Primary metabolites were analyzed by gas chromatography coupled to a quadrupole time of flight mass spectrometry system (GC-TOF-MS) as TMS derivatives as described previously [16,18]. Approximately 20 mg of freeze-dried seeds were used. Ribitol (1 mg/mL) was used as internal standard.

Fatty acids were extracted in methanol:chloroform (1:1) and injected in an Agilent 7809A gas chromatograph (Agilent Technologies) coupled to a Triple-Axis detector (Agilent 5975C), using a ZB-5 (Phenomenex; 30 m × 0.25 mm) capillary column (0.25 mm film thickness) using helium as carrier gas as described previously [16]. Approximately 10 mg of freeze-dried seeds were used and a mixture of hexadecane and heptadecane (1:1) was used as internal standard. GABA levels were additionally measured by GC-TOF-MS in seeds after they had taken up water to 24.4% of their dry weight. All analyses were performed on three biological replicates of 20 seeds each.

2.3. Soluble carbohydrate analysis

Soluble carbohydrates were determined as described previously [16]. Approximately 20 mg of freeze-dried seeds were used. Melezitose (40 μg/mL) was used as internal standard. Samples were injected into a Dionex HPLC system (Dionex, Sunnyvale, CA) using a CarboPac PA100 4− × 250-mm column (Dionex) preceded by a guard column (CarboPac PA100, 4 × 50 mm), a gradient pump module (model GP40, Dionex) and followed by an ED40-pulsed electrochemical detector (Dionex). Peaks were identified by co-elution of standards. Three biological replicates of 20 seeds each were used for this analysis.

2.4. Starch analysis

The pellets remaining from the previous carbohydrate analysis were used to quantify starch. Starch was determined as glucose produced by enzymatic digestion with α-amylase, as described previously [16]. Approximately 10 mg of dried material was used for the analysis. A standard curve ranging from 1 to 40 μg/mL starch was used to calculate the absolute concentration in the samples. Lactose (10 μg/mL) was used as internal standard. Three biological replicates of 20 seeds each were used for this analysis.

2.5. GC–MS data processing and compound identification

Data processing and compound identification were performed as described previously [16]. Raw data was processed by ChromaTOF software 2.0 (Leco Instruments) followed by alignment of the chromatograms using the MetAlign software. MSClust was used to remove metabolite signal redundancy in aligned mass peak tables and to retrieve mass spectral information of metabolites using mass peak clustering. The mass spectra of the representative masses were used for tentative identification by matching to spectral libraries (National Institute of Standards and Technology [NIST08] and Golm metabolome database [http://gmd.mpimp-golm.mpg.de/]) and by comparison with the retention index calculated by using a series of alkanes. Authentic reference standards were used to confirm the identity of the metabolites. Levels of identification according to Sumner [19] are presented in Supplementary Table S1.

2.6. Multivariate statistical analysis

Normalized data were uploaded at MetaboAnalyst 2.0; a web-based analytical pipeline for high-throughput metabolomics studies as described previously [16,18]. Before data analysis, a data integrity check was performed to ensure that all the necessary information was present. Row-wise normalization was performed to allow general-purpose adjustment for differences among samples. Log transformation and auto-scaling were performed to allow comparison of features. Uni- and multivariate analysis were performed using log transformed and auto-scaled data. ANOVA was performed to assess the overall variation in metabolite levels, followed by post-hoc analyses (Bonferroni correction, FDR < 0.05).

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