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Glyoxylate cycle and metabolism of organic acids in the scutellum of barley seeds during germination

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ABSTRACT

During the developmental processes from dry seeds to seedling establishment, the glyoxylate cycle becomes active in the mobilization of stored oils in the scutellum of barley (*Hordeum vulgare* L.) seeds, as indicated by the activities of isocitrate lyase and malate synthase. The succinate produced is converted to carbohydrates via phosphoenolpyruvate carboxykinase and to amino acids via aminotransferases, while free organic acids may participate in acidifying the endosperm tissue, releasing stored starch into metabolism. The abundant organic acid in the scutellum was citrate, while malate concentration declined during the first three days of germination, and succinate concentration was low both in scutellum and endosperm. Malate was more abundant in endosperm tissue during the first three days of germination; before citrate became predominant, indicating that malate may be the main acid acidifying the endosperm. The operation of the glyoxylate cycle coincided with an increase in the ATP/ADP ratio, a buildup of H_2O_2 and changes in the redox state of ascorbate and glutathione. It is concluded that operation of the glyoxylate cycle in the scutellum of cereals may be important not only for conversion of fatty acids to carbohydrates, but also for the acidification of endosperm and amino acid synthesis.

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1. Introduction

During seed germination, mobilization of stored lipids, carbohydrates and proteins takes place. In cereal seeds, the reserve of carbohydrates (starch) is found in the endosperm, while the reserve of lipids and proteins is found in the scutellum, which represents a single cotyledon of monocotyledonous plants. Starch and proteins are mobilized via the activation of corresponding amylases and proteases, while for starch breakdown an acid environment is important. Acidification of the endosperm is achieved via secretion of organic acids from the scutellum and aleurone layer into endosperm, i.e., the "digestive" process [1–3]. Organic acids are formed primarily during oxidative metabolic processes, such as the TCA cycle and the glyoxylate cycle. The latter becomes active during germination of oil-containing seeds, which includes the seeds of cereal plants, where the process of β-oxidation of fatty acids is localized in the scutellum and aleurone layer [4]. Aleurone layers, according to the estimations of Newman and Briggs [5] contain approximately 2/3 of triacylglycerols of barley grain, with their concentration close to 15% of fresh weight of this tissue; the remaining 1/3 is localized in the scutellum in which lipid concentration can be even higher, up to 40% of fresh weight in maize scutellum [6]. During germination, the lipid content in these tissues decreases in correspondence with the development of β-oxidation and the glyoxylate cycle. Meanwhile, the remobilization of storage reserves towards formation of carbohydrates and amino acids takes place [3,4,7].

Starchy barley seeds [8] are different from Arabidopsis oily seeds [9]. Therefore, even though the glyoxylate cycle plays a crucial role in taking advantage of acetyl-CoA from breakdown of lipids in oily seeds [10,11], it could have different functions in cereal seeds. The function of the glyoxylate cycle is usually attributed to the

Abbreviations: AlaAT, alanine aminotransferase; Asc, ascorbate; DHA, dehydroascorbate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; GSH, reduced glutathione; GSO, putative glyoxysomal succinate oxidase; GSSG, oxidized glutathione; ICL, isocitrate lyase; MS, malate synthase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, PEP carboxykinase; PVPP, polyvinylpolypyrrolidone; SDH, succinate dehydrogenase; TCA cycle, tricarboxylic acid cycle.

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necessity of carbohydrate formation from lipids. This occurs via succinate synthesis from acetyl-CoA by the glyoxylate cycle in glyoxysomes, followed by its conversion to malate and oxaloacetate (OAA) by the TCA cycle in mitochondria, and synthesis of phosphoenolpyruvate (PEP) by PEP carboxykinase (PEPCK) in the cytosol. The latter becomes the substrate for reverse glycolysis (gluconeogenesis), in which carbohydrates are built.

The glyoxylate cycle can be of main importance in the tissues of many plants, especially dicotyledons such as sunflower or castor bean, where carbohydrate reserves in seeds are low or absent. However, in cereal plants the endosperm represents a major reserve of carbohydrates, and therefore the glyoxylate cycle could serve other metabolic purposes than converting lipids to carbohydrate. Using ^{14}C-labelled acetate, which is easily converted to ^{14}C-acetyl-CoA, it was shown that in maize scutellum only a small amount of exogenous acetate is used for carbohydrate synthesis, while the main portion of it is detected in amino acids and organic acids [12]. This led to the conclusion that the function of the glyoxylate cycle in cereals consists mainly in providing substrates for amino acid synthesis and for acidification of the endosperm (reviewed in Ref. [13]).

The function of the glyoxylate cycle to convert acetyl-CoA produced during oxidation of fatty acids of stored lipids to succinate is well established in the oily tissues of germinating seeds of dicotyledonous plants such as sunflower cotyledons and castor bean endosperm [14]. In the seeds of cereal plants the glyoxylate cycle operates in the scutellum, which represents a single cotyledon, or more precisely it has a homology to the third leaf of nymphealean seedlings [15]. The glyoxylate cycle supplies low molecular weight organic acids for the acidification of the endosperm to “digest” the stored starch, which is used as a carbohydrate supply by the developing embryo [3,13]. While the supply of carbohydrates is provided by the endosperm, the role of the glyoxylate cycle in cereals may be expanded to include the supply of organic acids and amino acid synthesis.

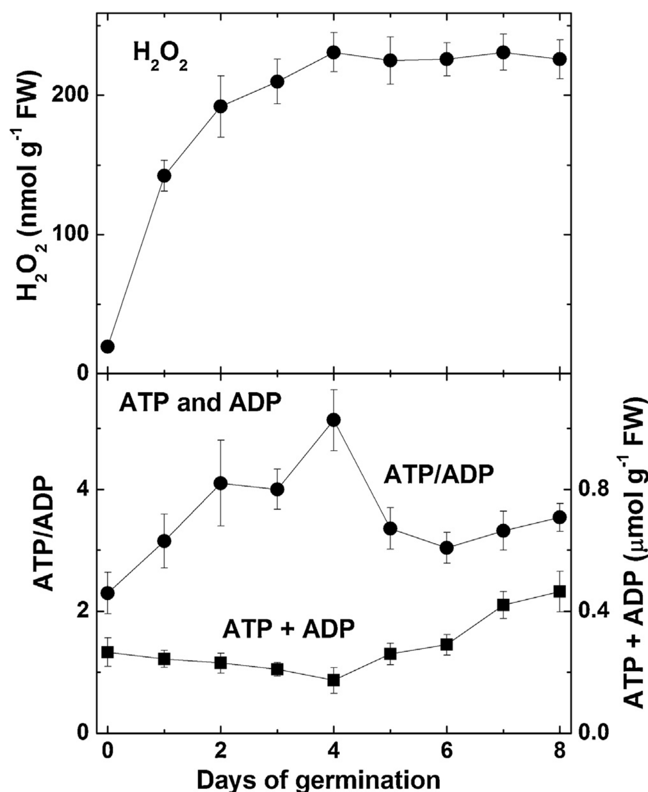

Fig. 1. Changes in H_2O_2 content, ATP + ADP and ATP/ADP ratio in scutellum of barley seeds during germination. H_2O_2 and ATP contents were measured by chemiluminescent methods; ADP was converted to ATP by pyruvate kinase as described in the text. The data are the means of three biological replicates ± SD.

Operation of the glyoxylate cycle in glyoxysomes (which represents a special type of peroxisome) is accompanied by the buildup of hydrogen peroxide during the flavin-dependent oxidation of fatty acids and in other processes. While excess H_2O_2 is normally scavenged by catalase, its generation affects the reduction levels of ascorbate and glutathione in the cell, which also participate in H_2O_2 scavenging in the ascorbate-glutathione cycle [16]. Besides the flavin oxidation-dependent generation of H_2O_2, the formation of NADH and succinate in glyoxysomes, via transport mechanisms that bring reducing equivalents to the mitochondria, may result in the synthesis of ATP.

In this study, we measured the activities of enzymes, redox levels and organic acid content in the scutellum of germinating barley seeds. While our previous study [17] was concentrated on the early germination events (first 48 h post imbibition) associated with breakage of dormancy, our current study deals with heterotrophic metabolism of germinated barley seeds that utilize storage reserves for germination and seedling growth. The results indicate that during glyoxylate cycle operation, organic acids can be used to acidify the endosperm, converted to carbohydrates or transaminated to produce amino acids. This means that the function of the glyoxylate cycle in cereals may be broad and not restricted to the conversion of storage fats to carbohydrates.

2. Material and methods

2.1. Plant material

Barley (*Hordeum vulgare* L., cv. Harrington) seeds were soaked in ddH_2O and germinated in darkness at 25 °C on two layers of filter paper in Petri dishes for 8 days. Endosperm and scutellum tissues were isolated every day and ground into fine powder in liquid nitrogen with a mortar and pestle. Aleurone layer was removed from the endosperm by a sharp blade. To prevent any contamination by endosperm, scutella were cleaned by wet paper towel, rinsed gently by ddH_2O, and dried on filter paper. The tissue powder was stored at −80 °C and used within a week. For enzyme and metabolite measurements the powder was homogenized in the extraction buffer in the ratio of 50 mg tissue fresh weight to 1 mL buffer.

2.2. Measurement of protein concentration

Protein concentration was measured using a standard Bradford protocol and a commercially available reagent (Sigma), according to the supplier's protocol. Protein was extracted from tissue powder by 0.1 M HEPES (pH 7.0) containing 0.5% CHAPS and 0.1% SDS on ice. Bovine serum albumin was used as a standard.

2.3. Measurement of hydrogen peroxide

The concentration of hydrogen peroxide was measured according to the method of Lu et al. [18]. The fine powder of isolated scutella was homogenized in 6% trichloroacetic acid for 30 min at 4 °C, centrifuged at 15,000*g* for 10 min, and then insoluble polyvinylpyrrolidone (PVPP) (50 mg mL^{-1}) was added. The samples were centrifuged at 15,000*g* for an additional 3 min. The preparation of reagents followed the method of Pérez and Rubio [19] and Lu et al. [18]. 10 mL of 6.5 mM luminol and 2 mL of 3 mM $CoCl_2$ in 0.1 M sodium carbonate buffer (pH 10.2) were mixed, diluted to 1 L in the same buffer and stored at 4 °C in the dark overnight before use. Samples, 40 µL, were mixed with 10 µL of the sodium carbonate buffer and the mixture was incubated at 30 °C for 15 min. Catalase (EC 1.11.1.6; Sigma; 500 units) was added and incubated at the

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