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Asparagine slows down the breakdown of storage lipid and degradation of autophagic bodies in sugar-starved embryo axes of germinating lupin seeds

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ABSTRACT

The research was conducted on embryo axes of yellow lupin (*Lupinus luteus* L.), white lupin (*Lupinus albus* L.) and Andean lupin (*Lupinus mutabilis* Sweet), which were isolated from imbibed seeds and cultured for 96 h in vitro under different conditions of carbon and nitrogen nutrition. Isolated embryo axes were fed with 60 mM sucrose or were sugar-starved. The effect of 35 mM asparagine (a central amino acid in the metabolism of germinating lupin seeds) and 35 mM nitrate (used as an inorganic kind of nitrogen) on growth, storage lipid breakdown and autophagy was investigated. The sugar-starved isolated embryo axes contained more total lipid than axes fed with sucrose, and the content of this storage compound was even higher in sugar-starved isolated embryo axes fed with asparagine. Ultrastructural observations showed that asparagine significantly slowed down decomposition of autophagic bodies, and this allowed detailed analysis of their content. We found peroxisomes inside autophagic bodies in cells of sugar-starved Andean lupin embryo axes fed with asparagine, which led us to conclude that peroxisomes may be degraded during autophagy in sugar-starved isolated lupin embryo axes. One reason for the slower degradation of autophagic bodies was the markedly lower lipolytic activity in axes fed with asparagine.

1. Introduction

The main storage compound in lupin seeds is protein, basically globulins. Yellow lupin seeds contain about 45% protein, white lupin seeds up to 38% protein, and Andean lupin seeds up to 50% protein (Borek et al., 2012a). Consequently, the metabolism of germinating lupin seeds is based on proteins and amino acids. The main respiratory substrates in such tissues are amino acids, which, after hydrolysis of storage protein, undergo deamination and supply the tricarboxylic (TCA) cycle (Lehmann and Ratajczak, 2008; Avila-Ospina et al., 2014). Lupin seeds also contain storage lipids (triacylglycerols), but at a much lower level. Seeds of yellow lupin contain only a small amount of storage lipid (about 6% of seed dry matter). Seeds of white lupin are slightly richer in storage lipid (7–14%), and Andean lupin seeds may contain up to 20% storage lipid (Borek et al., 2012b). Mature, dry lupin seeds contain no starch. However, this storage carbohydrate is transiently accumulated in developing lupin seeds, is absent in mature seeds, and appears again during seed imbibition and germination. Generally, the carbohydrate content in lupin seeds is about 36%, and the basic carbohydrates are oligosaccharides and fiber (Borek et al., 2013a). Lupins can grow on soil poor in mineral nitrogen because they thrive in symbiosis with symbiotic soil bacteria (mostly *Bradyrhizobium*) fixing N_2. However, the symbiosis starts at the seedling stage (about 2–3 weeks after germination), and up to this stage the source of nitrogen is storage protein accumulated in seeds. During lupin seed germination, the storage protein is intensively mobilized, and a portion of the amino acids after deamination is efficiently used as a respiratory substrate. Deamination processes generate toxic ammonium, which is stored as asparagine (Lehmann and Ratajczak, 2008; Avila-Ospina et al., 2014). Ammonium assimilation into asparagine is catalyzed by ammonia-dependent asparagine synthetase or glutamine-dependent asparagine synthetase (Gaufichon

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et al., 2010). In tissues of germinating lupin seeds, the level of asparagine may reach 30% of dry matter (Lehmann and Ratajczak, 2008). Asparagine is accumulated only transiently at this stage because it is used for recovery of the amino acid pool in the seedling when photosynthesis is active. Asparagine and recovered amino acids allow growth and development of the seedling up to the stage when symbiosis with N_2-fixing bacteria is initiated. Asparagine also regulates carbohydrate levels in organs of germinating lupin seeds. It significantly enhances starch accumulation and simultaneously reduces the soluble sugar level, especially in embryo axes. Thus, asparagine intensifies sugar deficiency or sugar starvation in organs of germinating lupin seeds (Borek et al., 2013a). Asparagine is also important in lupins because this is the main transport form of nitrogen from source to sink tissues in these plants (Lea et al., 2007; Lehmann and Ratajczak, 2008; Sulieman and Tran, 2013). Asparagine is one of the most abundant amino acids loaded by the mother plant into developing seeds in legumes (Borek et al., 2015). This amino acid regulates storage protein and lipid accumulation in developing lupin seeds, increasing protein content and simultaneously decreasing lipid content (Borek et al., 2009).

Storage lipid in germinating seeds is used mainly as a respiratory substrate, and it is converted to sugars. As sucrose, it is transported from storage organs to the growing embryo and the developing seedling. During storage lipid breakdown, free fatty acids are liberated by lipases from triglycerides accumulated in oil bodies, and they undergo β-oxidation inside peroxisomes (formerly called glyoxysomes; Pracharoenwattana and Smith, 2008). The next step of fatty acid catabolism is the glyoxylate cycle, which partially takes place in peroxisomes. Operating in the glyoxylate cycle, isocitrate lyase, citrate synthase and malate synthase are located inside the peroxisome, but aconitase and malate dehydrogenase are located in the cytosol. Succinate synthesized through the glyoxylate cycle is transported into the mitochondrion and then, as malate, is used for the synthesis of sugars during gluconeogenesis (Pracharoenwattana and Smith, 2008; Borek et al., 2015). Peroxisomes are especially important in storage lipid breakdown during seed germination and seedling establishment, because, despite harboring the β-oxidation of fatty acids and part of the glyoxylate cycle, they also contain the triacylglycerol lipase SDP1. This lipase is associated with the surface of the peroxisomes, and it is translocated to the oil body surface during seedling establishment (Thazar-Poulot et al., 2015). SDP1 has also been identified as the major triacylglycerol lipase involved in lipid reserve mobilization in seedlings of *Arabidopsis thaliana* (Eastmond, 2006; Thazar-Poulot et al., 2015). The pathway of storage lipid conversion to sugars exists in germinating lupin seeds, and storage lipid is converted to sugars (Borek and Ratajczak, 2010; Borek et al., 2013b, 2015). However, during the germination of lupin seeds, storage lipid is also used for amino acid synthesis. Lipid-derived carbon skeletons are drawn off from the pathway and are directed to amino acid synthesis. Asparagine, glutamine and glutamic acid are mainly synthesized from the storage lipid (Borek et al., 2003; Borek and Ratajczak, 2010). Four alternative routes of carbon flow from lipid to amino acids are described in germinating lupin seeds, and in each of them the lipid-derived carbon skeletons are directed to amino acid synthesis at the stage of the glyoxylate cycle (Borek and Ratajczak, 2010).

Research on the regulation of storage lipid breakdown in germinating lupin seeds showed that mobilization of this storage compound is controlled by the sugar level in tissues. Sugar level may regulate gene expression and activity of several enzymes involved in lipid metabolism in germinating seeds, including lipase, catalase, acyl-CoA oxidase, isocitrate lyase, peroxisomal and mitochondrial aconitase, cytosolic and mitochondrial isocitrate dehydrogenase and phosphoenolpyruvate carboxykinase (Borek et al., 2006; Borek and Nuc, 2011; Borek et al., 2013b). Sugar deficiency in tissues significantly enhances storage lipid breakdown in 96-h-old seedlings as well as in excised and cultured in vitro cotyledons. However, a completely opposite relationship was observed in 96-h-old isolated embryo axes cultured in vitro. The breakdown of storage lipid in isolated sugar-starved embryo axes is less intense, and the content of total lipid is significantly higher than in axes fed with 60 mM sucrose. The total lipid content (in dry matter) in sugar-starved isolated embryo axes of yellow lupin cultured for 96 h in vitro was 43% higher than in axes fed with sucrose, in axes of white lupin it was 44% higher, and in axes of Andean lupin it was 70% higher (Borek et al., 2012b). This result is completely inconsistent with literature data showing significant intensification of the breakdown of storage compounds under sugar starvation conditions (Morkunas et al., 2012). It should be emphasized that this atypical result concerns only the breakdown of storage lipid, because the content of protein, soluble sugars and starch was significantly lower in sugar-starved isolated lupin embryo axes than in organs fed with sucrose (Borek and Ratajczak, 2002; Borek et al., 2006, 2012a, 2013a).

During plant growth and development, many superfluous or damaged cell compounds (e.g. cell organelles or protein complexes) undergo degradation inside the vacuoles during autophagy. The cargo inside a vacuole is called the autophagic body, and it is degraded very quickly by the action of numerous vacuolar lytic enzymes, including proteases, lipases, nucleases, and phosphatases (Avin-Wittenberg et al., 2012; Li and Vierstra, 2012; Liu and Bassham, 2012; Yoshimoto, 2012; Reggiori and Klionsky, 2013; Avila-Ospina et al., 2014; Klionsky et al., 2016). Autophagy is not intense under normal conditions, and it is a housekeeping process then, but it is significantly enhanced under some biotic and abiotic stresses. Autophagy is especially enhanced under sugar or nitrogen starvation and allows the survival of cells by providing respiratory substrates. Many cell components are massively and non-selectively degraded under such circumstances (Avin-Wittenberg et al., 2012; Liu and Bassham, 2012; Reggiori and Klionsky, 2013; Lv et al., 2014; Klionsky et al., 2016). However, cell components also may be selectively degraded during autophagy (Avin-Wittenberg et al., 2012; Floyd et al., 2012; Liu and Bassham, 2012; Reggiori and Klionsky, 2013; Klionsky et al., 2016).

A good system to study autophagy is isolated lupin embryo axes, which can be easily cultured in vitro under sugar- or nitrogen-starvation conditions. In cells of sugar-starved isolated lupin embryo axes cultured in vitro for 96 h, advanced autophagy is observed. Symptoms of autophagy are a huge increase in cell vacuolization (Borek et al., 2006, 2011, 2012a) and a significant decrease in phosphatidylcholine content (Borek et al., 2012b). Based on such results concerning autophagy and clearly higher lipid levels in sugar-starved isolated lupin embryo axes, we formulated the hypothesis that autophagy causes disturbances in action of enzymes involved in storage lipid breakdown. More precisely, the peroxisomes (harboring β-oxidation of fatty acid, part of the glyoxylate cycle, and triacylglycerol lipase SDP1) could be degraded during autophagy under conditions of sugar starvation, which causes the higher lipid content in the sugar-starved isolated embryo axes. In cells of axes fed with sucrose, autophagy does not occur; they grow intensely and lipid reserves are used in larger quantities (Borek et al., 2011, 2012b, 2015). In the research described in this paper, we attempted to verify this hypothesis. We tried to assess the effect of asparagine and nitrate on storage lipid breakdown in germinating lupin seeds, and on autophagy in sugar-starved lupin embryo axes. We used asparagine because it is a central amino acid in metabolism of protein-storing lupin seeds (Lea et al., 2007; Lehmann and Ratajczak, 2008). Nitrate was also applied because this inorganic kind of nitrogen enhances accumulation of storage lipid during development of lupin seeds (Borek et al., 2009). Experiments were conducted on embryo axes grown