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Cellular changes during *Medicago truncatula* hypocotyl growth depend on temperature and genotype

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ABSTRACT

Hypocotyl growth is a key characteristic for plant emergence, influenced by environmental conditions, particularly temperature, and varying among genotypes. Cellular changes in *Medicago truncatula* hypocotyl were characterized to study the impact of the environment on heterotrophic growth and analyze differences between genotypes. The number and length of epidermal cells, ploidy levels, and sugar contents were measured in hypocotyls grown in the dark at 20 °C and 10 °C using two genotypes with contrasting maximum hypocotyl length. Hypocotyl elongation in the dark was due to cell elongation and not to an increase in cell number. A marked increase in cell ploidy level was observed just after germination and until mid elongation of the hypocotyl under all treatments. Larger ploidy levels were also observed in the genotype with the shorter hypocotyl and in cold conditions, but they were associated with larger cells. The increase in ploidy level and in cell volume was concomitant with a marked increase in glucose and fructose contents in the hypocotyl. Finally, differences in hypocotyl length were mainly due to different number of epidermal cells in the seed embryo, shown as a key characteristic of genotypic differences, whereas temperature during hypocotyl growth affected cell volume.

1. Introduction

Temperatures during early growth stages are a major limitation for crop area distribution and yield. Germination (defined as protrusion of the radicle) may not occur in some seeds, or seedling growth may be disturbed, which, especially in cold conditions, results in shorter abnormal seedlings with thick glassy shoots that fail to reach the soil surface (e.g., Ref. [1–3]). The heterotrophic growth of the hypocotyl or epicotyl has been widely studied mainly as a standard model system in physiological studies on cell elongation (e.g., Ref. [4–7]), but less frequently in relation with environmental stresses that may occur during this developmental stage.

Plant response to cold conditions, even non-freezing temperatures, is under complex physiological and genetic control, and also likely depends on the stage at which the stress occurs. Effects of cold stress have been widely studied at molecular level in *Arabidopsis thaliana*, but often using young plants grown in the light. This enabled the identification of genes involved in the cold signaling cascade and cold response, such as those encoding CBF transcription factors or ICE, a regulator of these genes (see Thomashow [8,9] for example; [10]). The growth of the hypocotyl has also been widely studied, but mainly to analyze the interplay between light signaling and hormones in the regulation of growth and developmental responses. These studies enabled the identification of genes involved in the phytohormone (brassinosteroid, auxin, ethylene, gibberellins), cryptochrome and phytochrome signaling pathways [11–13]. Differences were found in the hormone interaction networks between seedlings grown in the dark and those grown in the light. The aim of the present study was to improve our knowledge of the cellular and physiological events that lead to changes during seedling growth under cold conditions specifically in the dark, and to differences in growth among genotypes. Light does not penetrate the soil to a depth of more than 1–2 mm [14,15] meaning that after sowing, seedlings grow in dark conditions in the soil for a crucial period until seedlings emerge at the soil surface and start photosynthesis. From the onset of germination, seed reserves are converted into soluble metabolites that are transported to the embryo axis for further metabolism and seedling growth. This is a crucial difference between dark-grown

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seedlings and young photosynthesizing plants, as in the dark, a limited supply of carbon is available for growth from the depletion of seed reserves. Among seed storage compounds, raffinose family oligosaccharides are found in larger quantities in legume seeds than in other species, ranging from 60 to 125 mg g^{-1} of dry seed [16,17], and about 100 mg g^{-1} in *Medicago truncatula* [18]. Sugars may play several physiological roles during germination and heterotrophic growth. They provide energy; as osmoticum help maintain large vacuoles [19,20] and prevent damage to cells under cold stress or water deficiency. They also serve as building blocks for cell wall synthesis [21]. Their amounts and/or the balance between their different forms are possibly signals for regulation of cell division and elongation [22–27]. However, even though soluble sugars may play many different roles, these have not been widely studied during seedling heterotrophic growth, especially in legume seeds.

The size of an organ relies on cell division and/or cell elongation. In some species, it has been shown that hypocotyl growth in the dark is due to elongation of epidermal and cortical cells [4,19,20,28,29]. Like in leaves [30], Kutschera and Niklas [31] elaborated the epidermal-growth-control theory of stem elongation. Hypocotyl growth depends on internal turgor pressure in its individual cells, which causes the extension of the epidermis cell walls. In *A. thaliana*, hypocotyl basal epidermal cells reached up to 1 mm in length, representing a 100-fold increase in length compared to embryo cells in dry seeds before imbibition [4]. Different mechanisms influence cell size, including changes in nuclear DNA content, in the size of the vacuole, and in cell wall plasticity. High rates of endoreduplication (increasing nuclear DNA content without cellular division; [32]) have been observed during germination [33–36] and also in hypocotyls during seedling heterotrophic growth [4]. The level of genome ploidy has been shown to influence cell size and/or growth parameters among genotypes in different plant parts [37–39].

The aim of the present study was to provide more physiological basis necessary for in-depth molecular and genetic analyses of seedling growth under abiotic stresses. We analyzed cell changes (ploidy level, the number and size of cells) during heterotrophic growth in *M. truncatula* hypocotyls. Because of the limited supply of carbon in dark-grown seedlings and of the major role of soluble sugars in cell elongation/division processes, changes in soluble sugar contents were also measured in the different seedling parts. As genetic diversity has been observed in final hypocotyl length under cold conditions [40,41], two contrasting genotypes were compared. This allowed highlighting cellular events modified by cold independently of the genotype, and also differences between the two genotypes. The results improve our understanding of seedling growth and should renew interest in the stages of formation of the embryo.

2. Materials and methods

2.1. Plant material and growth conditions

Two genotypes of *M. truncatula* were used, Jemalong A17 the reference genotype for genomic studies, and F83005.5, an ecotype from a mountainous area (southern French Alps). The latter genotype has shorter hypocotyls than Jemalong A17 under different temperatures [40,41]. Seeds were all produced in 2006 in greenhouses located at the *M. truncatula* Biological Resource Center (Montpellier, France, http://www.montpellier.inra.fr/BRC-MTR). After scarification, five seeds were sown at a depth of 1.5 cm in plastic pots (7.5 cm × 10 cm) filled with 500 g of white sand (150–200 μm) and watered with 100 mL of a nutrient solution adapted for young seedling growth [42]. The pots were wrapped in aluminum foil to maintain dark conditions and limit evaporation, and placed in a dark growth chamber at 20 °C or 10 °C as

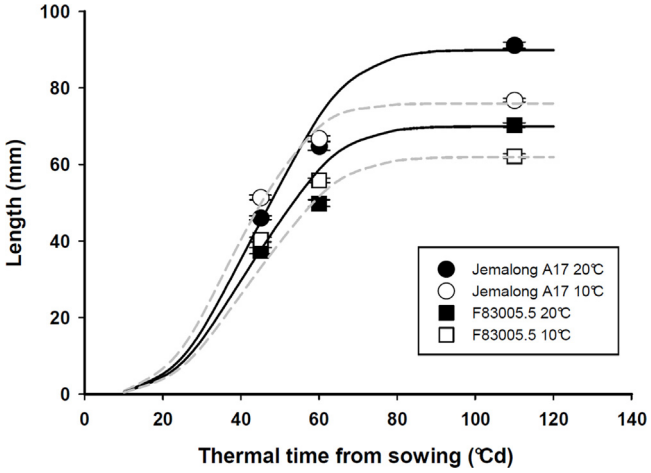

Fig. 1. Length of the hypocotyl during growth in the dark at 20 °C and 10 °C in the two *M. truncatula* genotypes (Jemalong A17 and F83005.5). Vertical bars are standard errors. Lines are fittings to a Weibull function.

cold conditions. Previous results had shown that a temperature of 10 °C reduced hypocotyl elongation, and that below 10 °C, growth was very slow and much impaired, as the threshold temperature value for elongation process in *M. truncatula* is 5–7 °C, depending on the genotype [40]. Based on these previous results, sampling times were chosen after calculation of thermal times (sum of daily mean temperature minus the base temperature threshold value, from sowing to the sampling date) to enable seedlings to be harvested at the same stage of growth after sowing at the two different temperatures [43]. Seedlings were collected three times during hypocotyl growth under each growth temperature regime: twice during the rapid elongation period at 45 and 60 degree-days (°Cd) after sowing, and once when elongation had reached its maximum plateau value at 110 °Cd (Fig. 1). The number, length, and surface area of the cells, ploidy level, and sugar contents in the hypocotyl were measured at each sampling date. Sugar contents were also measured in cotyledons and radicles. In addition, ploidy levels and sugar contents were measured in the embryo axes in dry seeds and at 10 °Cd after sowing (80% germinated seeds).

2.2. Ploidy levels

Flow cytometric analyses were performed on dry seeds and at 80% of germination (10 °Cd) on the whole embryo axis on three replicates comprising 10 seeds. At the three later sampling dates, three replicates of 10 hypocotyls were analyzed. Samples were finely chopped and immersed in 400 μL of extraction buffer (CyStain UV PreciseP, Partec) for 5 min, after which 1.6 mL of DNA staining buffer (CyStain UV PreciseP, Partec) was added. This preparation was filtered on a 30 μm nylon membrane, and analyzed with a flow cytometer (Partec PAII, Görlitz, Deutschland) at 460 nm. At least 5000 nuclei were analyzed for each measurement. Pea leaf samples were added to each sample as an internal diploid (2*C*) reference. DNA contents (peaks in *C* values from 2*C* to 16*C*) were determined according to this reference. Peaks were automatically analyzed to obtain the number of nuclei under each peak. The mean *C*-value of a sample was calculated as proposed in Lemontey et al. [44] according to the equation:

$$\text{Mean } C\text{-value} = \sum_{i=1}^{n} \frac{C_i \times N_i}{N_{\text{sample}}}$$

where n is the number of peaks of DNA content of the sample; C_i is the C value of the nuclei in peak n_i; N_i is the number of nuclei