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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.

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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

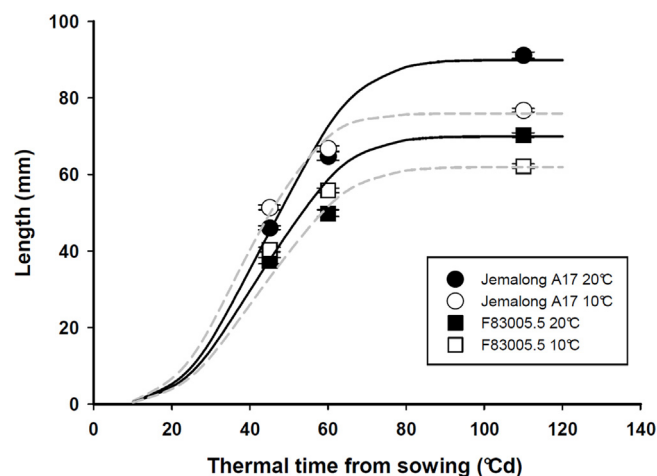


Fig. 1. Length of the hypocotyl during growth in the dark at 20 °C and 10 °C in the two *M. truncatula* genotypes (Jemalong A17 and F83005.5). Vertical bars are standard errors. Lines are fittings to a Weibull function.

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 (2C) reference. DNA contents (peaks in C values from 2C to 16C) 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:

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

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

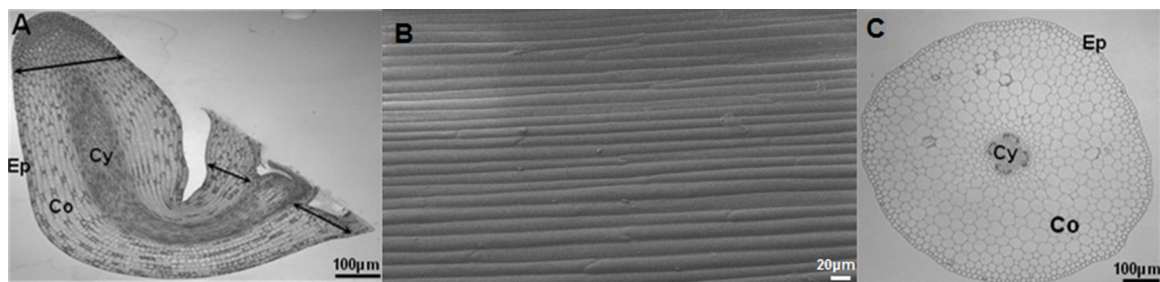


Fig. 2. (A) Longitudinal section of *Medicago truncatula* embryo axis from seeds (imbibed for 12 h). The limits of the hypocotyls are indicated by black arrows. Ep, epidermis; Co, cortex; Cy, central cylinder; (B) SEM imaging of fully elongated cells of hypocotyl epidermis; (C) radial section of hypocotyl. Illustrations show genotype F83005.5 grown at 20 °C.

in peak n_i ; N_{sample} is the number of nuclei in all the peaks of the sample.

2.3. Number and length of hypocotyl epidermal cells in longitudinal sections

Inclusions were made to determine the number of hypocotyl cells in the embryo using three seeds of each genotype imbibed for 12 h at 4 °C (3 replicates for each seed). Samples were fixed in 4% glutaraldehyde in phosphate buffer (0.1 M; pH 7.2) for 2 h at 4 °C under vacuum and then dehydrated in a graded ethanol series and embedded in Technovit 7100 resin (Heraeus K ulzer® embedding kits) according to Kroes et al. [45]. Samples were stored at 40 °C for at least a week. Longitudinal slices (3- μm thick) of the embryo axis were cut with a Leica RM2165 microtome. The slices were mounted on glass slides, stained with toluidine blue O, and, after dehydration, mounted in synthetic resin and examined under a DM1000 Leica microscope equipped with a Qimaging Micropublisher 3.3 RTV camera. The hypocotyl was distinguished from the radicle in the embryo axis by considering the end of the last file of root cap cells (Fig. 2A), as proposed by Esau [46]. Three different tissues were observed in the hypocotyl: the epidermis, comprising a single layer of cells, the cortex, and the central cylinder in which vessels progressively differentiated during hypocotyl growth in the dark. Hypocotyl epidermal cells were counted on both sides of the embryo using ImageJ software [47].

The length of the epidermal cells of fully elongated hypocotyls was measured by scanning electron microscopy (SEM, Zeiss EVO LS10; illustrated in Fig. 2B). Seedlings were harvested at 110 °Cd in the two genotypes at both temperatures. They were placed in nutrient solution to avoid osmotic stress during cutting. Slices (1 cm long, 3 replicates) were cut from the bottom, middle and top sections of the hypocotyls and immediately transferred in a 1 cm diameter plate, covered with graphite, frozen in pasty nitrogen (-220 °C), and placed in the SEM preparation chamber (-130 °C). External residual water remaining on the sample was sublimated for 10 min at -87 °C. Sample metallization was performed with a gold-Palladium anode (2 min). The samples were analyzed under high pressure conditions (10^{-4} Pa) with a voltage of 10 keV, a probe current of 12 pA, and a working distance of 17 mm. Images of the same 1 cm slice of hypocotyl were assembled with Photoshop CS5 (Adobe System) and the length of the epidermal cells measured with ImageJ software.

As the final length of the hypocotyls was too long to allow direct measurements of the total number of epidermal cells with SEM, the cell length measurements were used to estimate the number of epidermal cells in a file of cells from the bottom to the top of the hypocotyl at the end of its growth. First, the number of cells in each 1 cm slice (bottom, middle, top) was determined

using the measured cell lengths. The proportion of each part in the total length of the hypocotyl was estimated to be 25%, 50% and 25% respectively, and these proportions were used to determine the length of each part of the hypocotyl. Then, the number of cells in each of the three parts was calculated using the estimated number of cells in a 1 cm long slice, and the three numbers were summed. A second estimation of the number of cells was also performed, using the total length of the hypocotyl divided by the average cell length calculated from the cell length in each part. These two estimations were compared to the number of hypocotyl cells determined on slices of embryo axis.

2.4. Cell surface area of hypocotyl cells measured in radial cross sections

Radial cross sections of the three 1 cm long slices cut from the bottom, middle and upper end of the hypocotyls were observed (Fig. 2C). Samples were prepared and cut in the same way as for observations of the embryo axis described above. The surface areas of the epidermis and cortex cells were measured with ImageJ software.

2.5. Soluble sugar contents

Soluble sugar contents were measured in the cotyledons and in the embryo axis of dry seeds and at 80% germination (10 °Cd). They were then measured in three seedling parts, the cotyledon, hypocotyl and radicle at 45, 60 and 110 °Cd. At each sampling date, three replicates comprising 30 seedlings were separated into different parts and immediately frozen in liquid nitrogen prior to lyophilization. Dried samples were weighed, and ground. One mL 80% methanol containing 40 μL melezitose as internal sugar standard was added to 10–15 mg of powdered sample. After heating at 76 °C for 15 min, the liquid was evaporated under vacuum in a Speedvac AES1010 (Savant Instruments). The pellet was then dissolved in 1 mL distilled water and centrifuged for 3 min at $14,000 \times g$. The resulting supernatant was removed and dissolved in demineralized water prior to analysis. Sugars were analyzed by HPLC-PAD on a CarboPac PA1 column (Dionex Corp., Sunnyvale, CA, USA).

2.6. Statistical analyses

Analyses of variance were performed with SAS 8.1 software (SAS Institute 2000) using the GLM procedure to detect the effects of time, temperature and genotype and their interactions. All the mean comparisons were made using the Student–Newman–Keuls multiple comparison test. P -values <0.01 were considered to be statistically significant.

Table 1

Means (\pm S.E.) of mean C-values for the embryo axis in dry (0 °Cd) and germinating (10 °Cd) seeds, and for hypocotyls of the two *M. truncatula* genotypes (Jemalong A17 and F83005.5) grown in the dark at 20 °C and 10 °C.

Genotype	Temperature	Embryo axis		Hypocotyl		
		0 °Cd	10 °Cd	45 °Cd	60 °Cd	110 °Cd
Jemalong A17	20 °C	3.16 \pm 0.01 ^{aAα}	3.47 \pm 0.04 ^{aAα}	5.76 \pm 0.11 ^{bAα}	5.63 \pm 0.06 ^{bAα}	5.44 \pm 0.12 ^{bAα}
	10 °C	3.16 \pm 0.01 ^{aAα}	3.33 \pm 0.09 ^{aAα}	5.84 \pm 0.02 ^{bAα}	5.80 \pm 0.08 ^{bAα}	5.56 \pm 0.12 ^{bAα}
F83005.5	20 °C	3.19 \pm 0.01 ^{aAα}	3.51 \pm 0.04 ^{bAα}	6.02 \pm 0.10 ^{cAα}	6.53 \pm 0.04 ^{dAβ}	5.94 \pm 0.06 ^{cAβ}
	10 °C	3.19 \pm 0.01 ^{aAα}	3.65 \pm 0.05 ^{bAα}	7.08 \pm 0.14 ^{cBα}	6.97 \pm 0.08 ^{cBβ}	6.32 \pm 0.14 ^{dAβ}

F-values of overall results of analysis of variance followed by level of significance (** $P < 0.01$, *** $P < 0.001$): thermal time effect, 326.04***; genotype effect, 59.05***; temperature effect, 11.20**; and genotype \times temperature effect, 7.23**.

Different lower case letters indicate a thermal time effect for each genotype at each temperature.

Different capital letters indicate differences between temperatures for each genotype at each thermal time.

Greek letters indicate differences between genotypes at each thermal time for each temperature.

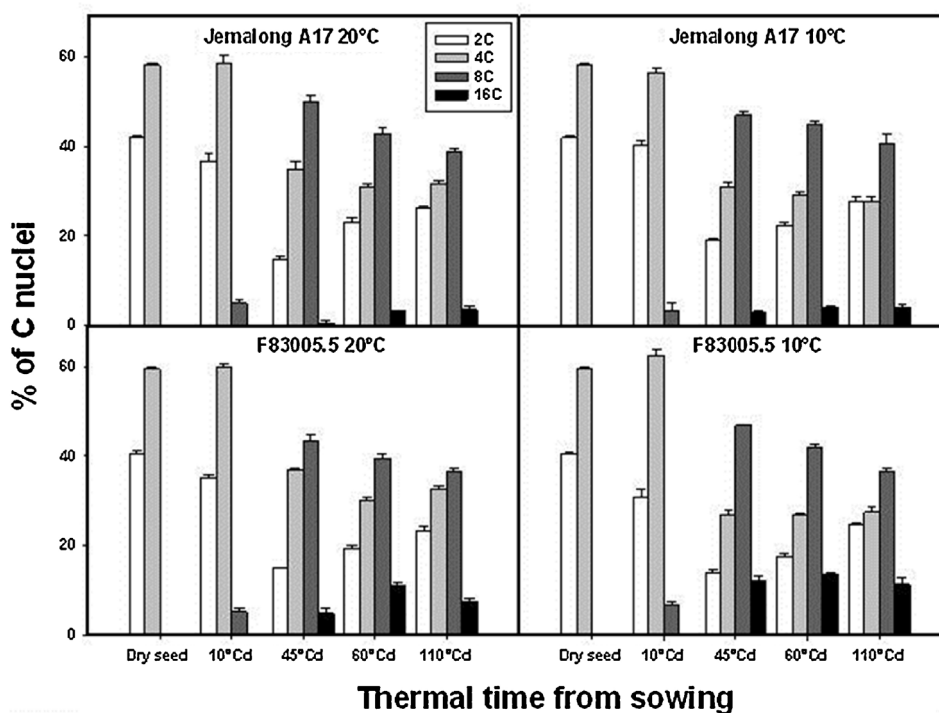


Fig. 3. Changes with time in the proportion of C-value nuclei in the embryo axis during germination (two first points) and then in the hypocotyls of the two *M. truncatula* genotypes (Jemalong A17 and F83005.5) grown in the dark at 20 °C and 10 °C. Vertical bars are standard errors.

3. Results

3.1. Hypocotyl growth in response to temperature

Hypocotyl elongation is shown in Fig. 1. After 60 °Cd, a plateau value was progressively reached and, when sampled at 110 °Cd, the seedlings had reached their maximum length. At this stage, Jemalong A17 hypocotyls grown at 20 °C were 91.2 \pm 0.8 mm long and F83005.5 hypocotyls 70.0 \pm 0.6 mm. The hypocotyls of Jemalong A17 were longer at both temperatures than those of F83005.5, but they shortened more in response to low temperature (16% vs. 12%).

3.2. Hypocotyl ploidy level increased dramatically after the onset of germination and depended on both temperature and genotype

The mean C-value in the embryo axis of dry seeds did not differ in the two genotypes (about 3.2; Table 1), dry seeds were characterized by the same 2C contents and also by high 4C nuclei

contents (nearly 60%, Fig. 3). During germination, mean C-values increased slightly in all treatments (only significantly in F83005.5), with the appearance of a small proportion of 8C in the axes (Fig. 3). After germination, a sharp significant increase in mean C-values was observed in the hypocotyls in all treatments, with the maximum reached around 45–60 °Cd after sowing (Table 1). This was due to a marked increase in the proportion of 8C nuclei and the appearance of 16C nuclei (Fig. 3). After 45 °Cd, mean C-values remained almost stable with a slight decrease at 110 °Cd. Significant differences between genotypes and temperatures, and a significant genotype \times temperature interaction appeared from 45 °Cd onwards (Table 1). F83005.5 had higher mean C-values than Jemalong A17 linked with its higher proportions of 16C nuclei (Table 1 and Fig. 3). Higher mean C-values and proportions of 16C nuclei were also obtained at low temperatures (Table 1 and Fig. 3). Finally, whereas the increase in ploidy level was concomitant with hypocotyl elongation whatever the treatment, the higher ploidy values were obtained for the shorter hypocotyls at the end of growth.

Table 2
Means (\pm S.E.) for (A) cell numbers and (B) cell lengths in the hypocotyl epidermis and for (C) cell surface areas in hypocotyl epidermis and cortex during growth in the dark at 20 °C and 10 °C (110 °Cd corresponded to fully expanded hypocotyl) of the two *M. truncatula* genotypes (Jemalong A17 and F83005.5).

Genotype	Temperature	Seed	110 °Cd Estimation 1	110 °Cd Estimation 2			
A. Number of epidermal cells							
Jemalong A17	20 °C	192 \pm 2 ^{aA}	197 \pm 19 ^{aA}	150 \pm 16 ^{aB}			
	10 °C		229 ^a	151 ^a			
F83005.5	20 °C	158 \pm 6 ^{bA}	162 \pm 14 ^{bA}	111 \pm 5 ^{bB}			
	10 °C		131 \pm 9 ^{bA}	91 \pm 9 ^{bB}			
F-values of the results of analysis of variance followed by level of significance (** P < 0.01, *** P < 0.001): 20 °C, genotype effect 47.87*** and estimation method effect 34.50***; 10 °C, genotype effect 59.37*** and estimation method effect 31.12**; global, genotype effect 83.35***, estimation method effect 75.45***, temperature effect 0.56 NS.							
Different lowercase letters indicate differences between genotypes for each count.							
Different capital letters indicate differences between counts for each genotype.							
Genotype	Temperature	110 °Cd					
		Bottom	Middle	Top			
B. Length of epidermal cells (μm)							
Jemalong A17	20 °C	273 \pm 19 ^{aC}	485 \pm 24 ^{aB}	1050 \pm 77 ^{aA}			
	10 °C	174 \pm 18 ^{aC}	416 \pm 26 ^{aB}	960 \pm 26 ^{aA}			
F83005.5	20 °C	208 \pm 27 ^{aC}	517 \pm 24 ^{aB}	1106 \pm 29 ^{aA}			
	10 °C	195 \pm 107 ^{aC}	742 \pm 88 ^{aB}	1037 \pm 76 ^{aA}			
F-values of the results of analysis of variance followed by level of significance (** P < 0.01, *** P < 0.001, NS, not significant): at 20 °C, genotype effect 0.06 NS and effect of the part of the hypocotyl 243.96***; at 10 °C, genotype effect 2.48 NS and hypocotyl part effect 29.4***; total: genotype effect 0.06 NS, temperature effect 1.20 NS and hypocotyl part 246.29***							
Different lowercase letters indicate differences between genotypes for each part of the hypocotyl at each temperature.							
Different capital letters indicate differences between parts of the hypocotyl for each genotype at each temperature.							
Genotype	Temperature	Bottom			Middle		
		45 °Cd	60 °Cd	110 °Cd	45 °Cd	60 °Cd	110 °Cd
C. Surface areas of radial cells (μm²)							
Epidermis							
Jemalong A17	20 °C	37.5 \pm 0.5	37.8 \pm 0.3	30.3 \pm 1.6	45.1 \pm 5.1	34.7 \pm 4.3	32.2 \pm 0.2
	10 °C	43.6 \pm 2.7	52.6 \pm 3.8	42.6 \pm 5.8	33.6 \pm 3.7	36.5 \pm 3.6	35.8 \pm 1.6
F83005.5	20 °C	37.8 \pm 2.5	37.9 \pm 3.1	40.1 \pm 2.9	42.1 \pm 7.8	30.1 \pm 0.1	44.6 \pm 2.8
	10 °C	56.8 \pm 3.5	68.8 \pm 2.7	51.2 \pm 7.1	50.9 \pm 4.9	50.0 \pm 1.7	43.1 \pm 10.7
F-values of results of analysis of variance followed by level of significance (** P < 0.01, *** P < 0.001, NS, not significant): thermal time effect, 1.21 NS, temperature effect, 20.41***, genotype effect, 12.74***, hypocotyl part effect, 5.19 NS and genotype \times temperature effect, 5.72 NS.							
Cortex							
Jemalong A17	20 °C	373.5 \pm 72.1	333.7 \pm 25.3	266.9 \pm 16.3	243.8 \pm 13.1	298.8 \pm 59.9	242.3 \pm 0.1
	10 °C	347.2 \pm 28.9	342.1 \pm 17.9	302.5 \pm 28.9	345.0 \pm 29.6	284.6 \pm 43.9	201.2 \pm 26.6
F83005.5	20 °C	324.9 \pm 4.5	301.6 \pm 33.2	317.5 \pm 44.2	282.2 \pm 45.7	220.4 \pm 20.0	168.7 \pm 18.6
	10 °C	427.6 \pm 13.2	409.0 \pm 26.3	359.5 \pm 36.9	381.1 \pm 14.3	326.2 \pm 5.0	273.9 \pm 1.2
F-values of results of analysis of variance followed by level of significance (** P < 0.01, *** P < 0.001, NS, not significant): thermal time effect, 12.61***, temperature effect, 18.21***, genotype effect, 2.07 NS, hypocotyl part effect, 32.65*** and genotype \times temperature effect, 11.55**.							

3.3. Histological analyses revealed a huge increase in hypocotyl cell length during heterotrophic growth, but no change in the number of cells, which depends on the genotype

In seeds, the initial number of epidermal cells in a cell file, determined on longitudinal sections of the embryo axis (Fig. 2A), were significantly higher in Jemalong A17 than in F83005.5 (Table 2A). The number of cells estimated at 110 °Cd at the end of hypocotyl elongation, using the mean lengths of epidermal cells measured in the different parts of the hypocotyl, did not significantly differ from the initial counts made on hypocotyls in the embryo axis, whatever the genotype or the temperature. The estimated number of epidermal cells at 110 °Cd was also higher for Jemalong A17 than for F83005.5. The second estimation of the number of epidermal cells, based on the calculated average epidermal cell lengths for Jemalong A17 and F83005.5, was less precise and led to significantly lower values, but to the same differences between genotypes.

As regards cell lengths, the epidermal cells observed with SEM at the end of hypocotyl elongation were very long at both temperatures, with a gradient from the bottom to the top part of the

hypocotyl where they reached about 1 mm in length (Table 2B), but there was no significant difference between the two genotypes. Although the effect of temperature was not generally significant, cells were shorter in Jemalong A17 at the lower temperature (515 vs. 603 μ m on average, significant at P < 0.05) in accordance with the larger difference in the length of the hypocotyl between temperatures in Jemalong A17 than in F83005.5. In F83005.5, the differences in cell length between the two temperatures were smaller.

The surface areas of epidermal cells measured on radial cross sections (Fig. 2C and Table 2C), were small, about 50 μ m² on average. They were generally significantly larger at 10 °C than at 20 °C, and also significantly larger in F83005.5 than in Jemalong A17. In the cortex, the cell surface area was large (on average 300 μ m²). It decreased significantly during elongation, corresponding to a change in the shape of the hypocotyl, which became thinner during elongation. The surface areas of the cortex cell were also significantly larger at 10 °C than at 20 °C, and a genotype \times temperature interaction was found: F83005.5 had larger cells than Jemalong A17 at 10 °C.

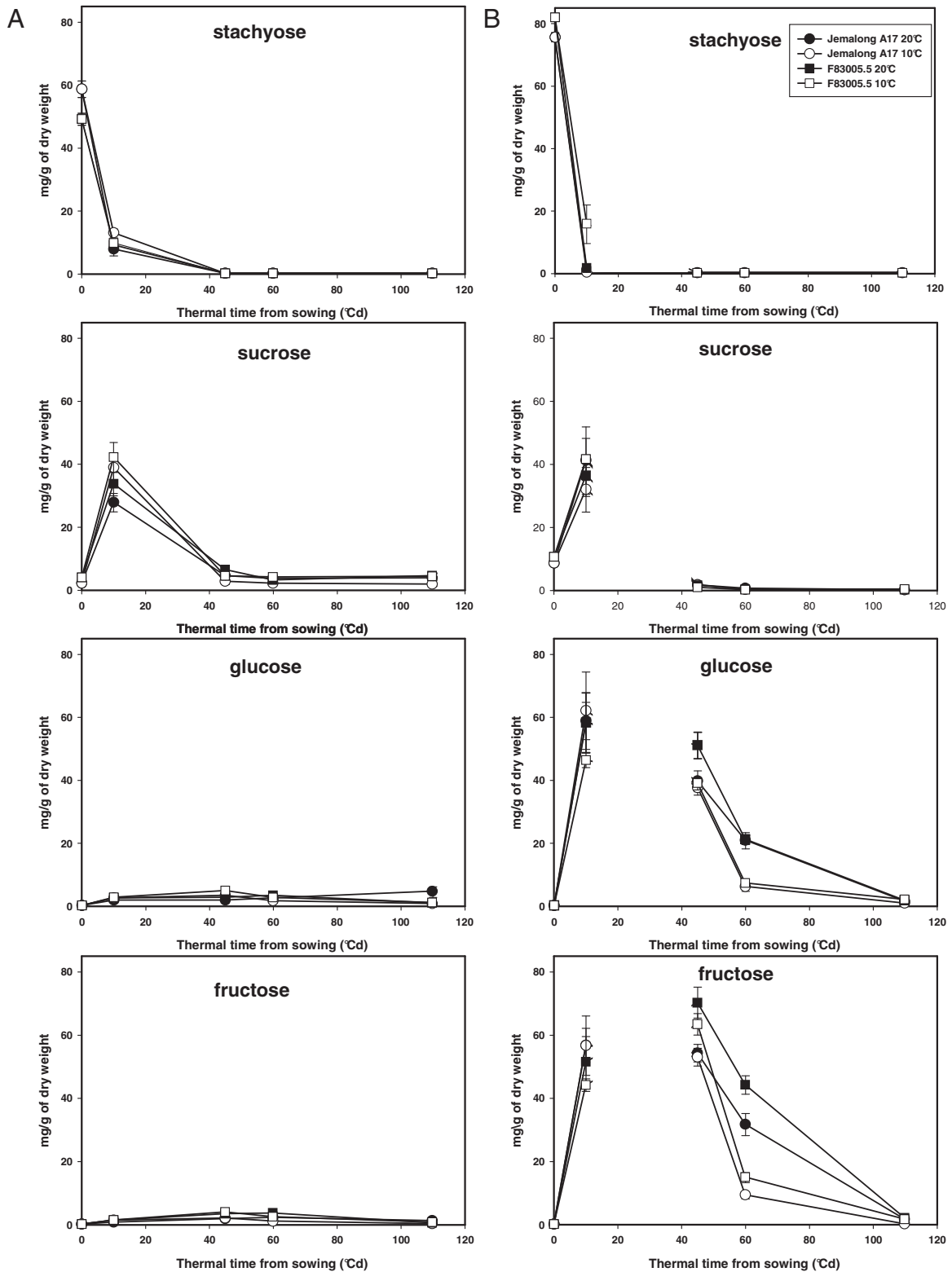


Fig. 4. Changes with time in soluble sugar contents at 20 °C and 10 °C during germination (two first points) and seedling heterotrophic growth in (A) cotyledons, and (B) embryo axis (two first points) and then in hypocotyls of the two *M. truncatula* genotypes (Jemalong A17 and F83005.5). Vertical bars are standard errors.

3.4. Glucose and fructose were abundant in the hypocotyl during heterotrophic growth but their contents were lower in cold conditions

Stachyose was the main soluble sugar stored in the embryo axis and cotyledons of dry seeds (Fig. 4). The initial stachyose

contents differed between the two genotypes, with significantly higher contents in cotyledons and lower contents in the embryo axis in Jemalong A17 than in F83005.5. The concentrations of other soluble sugars (sucrose, raffinose, verbascose; not all shown) were much lower, but were significantly higher in F83005.5 cotyledons and embryo axes. Neither glucose nor fructose was found.

Stachyose content decreased very rapidly during germination in cotyledons and in the embryo axis, and had disappeared in all parts by 45 °Cd (data not shown for radicles). At the same time, sucrose content reached a peak during germination and then decreased very rapidly in all seedling parts. Hexose contents, fructose and glucose, were always very low in the cotyledons, less than 5 mg g⁻¹ of dry weight. Hexose contents measured in the radicle after germination were almost zero. By contrast, fructose and glucose contents were very high in the embryo axis during germination and then in the hypocotyl at 45 °Cd. Subsequently, their contents decreased dramatically to reach almost zero at 110 °Cd. Differences were observed between the two temperature regimes. Glucose and fructose contents decreased more steeply between 45 and 60 °Cd at 10 °C than at 20 °C. At 60 °Cd, these two sugar contents were significantly lower at 10 °C in both genotypes. In addition, significant differences in fructose contents were found between genotypes, with more fructose in F83005.5 hypocotyls at 45 and 60 °Cd at both temperatures. To summarize, during germination, the raffinose family oligosaccharides stored in the dry seed disappeared rapidly and hexose contents increased considerably in the embryo axes. After germination, hexoses were abundant in the hypocotyl but they were less abundant at 10 °C after 45 °Cd.

4. Discussion

The hypocotyl, which pushes the seedling out of the soil, plays a major role in plant emergence. We analyzed cellular changes in *M. truncatula* hypocotyls grown at different temperatures using two contrasting genotypes to contribute to build an ecophysiological framework of seedling growth. Elongation of the hypocotyl can be reduced by a range of stresses such as water or heat stress, mechanical obstacles or plant protection treatments. Differences in elongation of the hypocotyl or epicotyl have also been observed among genotypes. Cell characterization of hypocotyl growth can help analyze all these variations and guide plant breeding.

4.1. Initial embryo cell number and variations in cell elongation in response to environmental conditions determine heterotrophic hypocotyl growth

In our study on *M. truncatula*, the number of epidermal cells in a cell file in the hypocotyl after 12 h of imbibition was between 150 and 200. The number of epidermal cells in the hypocotyl did not change during elongation. If the number of cells does not change during elongation, it is inevitably determined in the seed embryo and does not depend on environmental conditions during germination and heterotrophic seedling growth. The time for embryo formation is very short after pollination, and the environmental and trophic conditions during this period are therefore of utmost importance given their consequences for further seedling growth. In this study, differences between two genotypes were observed in the number of cells in the longitudinal axis of the embryo hypocotyl epidermis. The possible effects of environmental and trophic conditions on the number of these cells during seed formation need to be studied, along with the genetic diversity of this trait, as already done for grain or fruit storage parts [39,44,48]. Potential hypocotyl length may be associated with differences in the number of cells in the longitudinal axis of the hypocotyl in the embryo. Elongation of epidermal cells is thus assumed to drive the elongation of hypocotyls [31] and to depend on environmental conditions. The increase in the length of the cells was huge even at the lowest temperature we studied, and the epidermal cells reached as much as 1 mm in length in the upper part of the hypocotyl. This increase is similar to that of *A. thaliana* hypocotyls at their maximum length, which are consequently much shorter only because they initially have fewer cells

(about 10 times fewer than in *M. truncatula* [4]). Cell lengths were shorter at low temperature in Jemalong A17, which also showed the largest reduction in hypocotyl length. Finally, differences in hypocotyl potential growth between genotypes or species could be due mainly to differences in the number of cells in the embryo, whereas environmental conditions during hypocotyl growth only affect cell length.

4.2. Endoreduplication: a key determinant of variations in hypocotyl growth?

In our study, the variations we observed in ploidy levels were linked with variations in cell volume in two different ways: on one hand, differences in cell length during hypocotyl elongation with time, and on the other hand differences in cell diameter for differences between genotypes or temperatures. The present results show that in *M. truncatula*, ploidy levels begin to increase in the embryo axis during germination, as observed in other species. Radicle protrusion out of the seed coat (germination *sensu stricto*) has been shown to rely on cell expansion only [49]. Simultaneously, the cell nucleus DNA content in the root tips and the transient zone between the hypocotyl and the root has also been shown to increase [34,35,50]. Our results showed that after the end of germination, endoreduplication increased even more steeply at the beginning of hypocotyl elongation. High ploidy has also been observed with increased cell volume in other species and organs [4,39,51–53]. Although there is a clear relationship between endoreduplication and cell growth in plants, the exact role of endoreduplication has not yet been elucidated [34,51–53]. Some authors suggest that endoreduplication could be a more rapid and energy saving process than a complete division process due to the reduced number of syntheses needed to reach the same organ size [52,54]. Endoreduplication is regulated by both endogenous and environmental signals. Phytohormones have been shown to play an important role in the control of endoreduplication in hypocotyls [12,55,56]. Studies with mutants showed that gibberellins (GA) and ethylene hormones acted on endoreduplication in dark-grown hypocotyls. In *A. thaliana*, GA-deficient mutants that did not elongate in the dark, showed reduced endoreduplication, while normal endoreduplication was restored with an external supply of GA [55]. Ethylene was shown to inhibit longitudinal expansion but to promote radial expansion in dark-grown hypocotyls [57]. Thus, general hormonal control can be hypothesized for the variations in ploidy we observed during hypocotyl elongation. In the present study, a higher ploidy level was observed during hypocotyl growth in genotype F83005.5 with the shortest hypocotyls, and its ploidy level was even higher in cold conditions. An increase in ploidy level was seen to increase cell diameter when breeding sugar beet [58] or when describing genetic diversity in the size of tomato fruit [39]. The ecotype F83005.5 originates from a mountainous area and its higher ploidy level could be the result of adaptation to this geographical area. Several authors suggested relationships between high genome DNA contents and tolerance to extreme environmental conditions (e.g. cold, heat) as an ecological adaptation and as a potential for breeding [38,59–63].

4.3. Impact of soluble sugars on cellular changes during heterotrophic growth

The heterotrophic seedling functions on the basis of the embryo characteristics in the dry seed, with a given number of cells and a given amount of seed reserves as its source of carbohydrates. In such conditions, its sugar supply is very different from that of an autotrophic plant. In the present study, we specifically investigated changes in soluble sugar contents because the amount of sugars and their ratios play many roles in cell changes, and growth

could be regulated differently in non-photosynthetic seedlings than in photosynthesizing plants. We observed that fructose and glucose contents were high when endocycles occurred, and decreased sharply when C-mean values remained stable. We also observed that no cell division occurred in the growing hypocotyl. Moreover, during heterotrophic growth, no new organs appeared in addition to those already present in the embryo, the meristems produced no new organs until photosynthesis started. One possible hypothesis is that the heterotrophic seedling uses its resources just to elongate and reach the soil surface, and to start photosynthesis. Non-division in hypocotyl cells could be an energy saving process. Soluble sugars arriving in the cells can act as either a signal or as an energy support. Local variations in the amount of available carbohydrates have been shown to determine variations in the length of the cell cycle in proliferating cells. Non-dividing cells were assumed to lack carbohydrates [23,24]. Glucose or its ratio to other sugars may allow endoreduplication during heterotrophic growth but may be insufficient to allow complete cell division. In agreement with this hypothesis, in photosynthetic plants, a high ratio of glucose to other sugars was shown to increase the rate of cell division in leaf meristems [25] as well as during development of the seed embryo [64]. It has been also shown that exogenous sucrose or glucose supplied to the aerial parts (cotyledons and apical meristem) of dark-grown seedlings restores photomorphogenetic development in *A. thaliana* [65].

In addition to the effects of sugar contents on the cell cycle, high sucrose concentrations in the basal hypocotyl cortex have been assumed to act as osmotic solute [20]. In the present study, sucrose disappeared very rapidly after germination, and large amounts of glucose and fructose were found in the hypocotyls, as also observed by Kuo et al. [16] and Elamrani et al. [66] in other species. These hexoses may be present in the vacuoles and favor water entry, increasing osmotic pressure, and hence cell elongation. In our study, differences were observed in the rate at which glucose and fructose contents decreased between 20 °C and 10 °C. This trend was observed when time was calculated as thermal time. But elongation took many more days at 10 °C. The daily basal metabolism of the seedling consumes hexoses. Thus at 10 °C, the basic energy cost to reach the same hypocotyl length could be higher than at 20 °C. Furthermore, different sugar contents were observed in the two genotypes, suggesting a lower rate of hydrolysis of stachyose and sucrose, and of the appearance of hexoses in the genotype with the shorter hypocotyls, perhaps linked with the smaller number of cells that elongate in this genotype. Finally, marked changes in sugar contents occurred during germination and heterotrophic growth, during which carbon supply for seedling growth much differ from an autotrophic plant. These changes in sugar contents may be associated with many aspects of cell changes that were observed in our study (endoreduplication, non division) or with other changes that need to be studied in more detail (osmotic potential, respiration). In addition, the influence of seed composition needs to be better evaluated as it can be altered by breeding and by environmental conditions during seed filling.

4.4. Implications for seed quality and plant breeding

The present study addresses for the first time the analysis of heterotrophic hypocotyl growth taking into account one major environmental factor that impacts pre-emergence growth, and demonstrates the contribution of genotype to variations in hypocotyl growth. The number of cells in the embryo appears to be a key characteristic of potential variations in seedling growth. The present results should renew interest in the formation stages of the embryo and hence improve our understanding of seedling growth. Initial numbers of embryo cells and cell elongation during seedling growth should be further studied in different genotypes, together

with seed composition and seed reserve depletion, to determine if they are altered under different environmental conditions and what consequences are obtained on seedling heterotrophic growth. Post-genomic and QTL studies of these early stages are currently on the increase (e.g., Ref. [41,67,68]). The present study provides new information on where to look for possible genotypic differences and provides a framework to help molecular and genetic analyses for breeding purposes.

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