Long chain base changes triggered by a short exposure of Arabidopsis to low temperature are altered by AHb1 non-symbiotic haemoglobin overexpression

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Abbreviations: SL, sphingolipids; LCB, long chain bases; Cer, ceramides; NO, nitric oxide; t18:0, phytosphingosine; t18:0-P, phytosphingosine-phosphate; t18:1; d18:0, dihydrosphingosine; d18:1, sphingosine; cPTIO, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide;

Abstract

Long chain bases (LCB) are both precursors of complex sphingolipids (SL) and cellular signals in eukaryotic cells. Increasing evidence support a function for SL and/or LCBs in plant responses to environmental cues. In this study we analysed the impact of a short exposure to cold on the global LCB content and composition in Arabidopsis thaliana seedlings. We report that the total LCB amount significantly decreased after low temperature exposure. The decline was essentially due to reduction of t18:1 isomer content. On the other hand, chilling led to the increase of LCB content in a mutant over-expressing the non-symbiotic haemoglobin AHb1. Furthermore, this mutant was impaired in cold-dependent root growth inhibition and anthocyanin synthesis. As AHb1 is an element of nitric oxide turnover, our data suggest a possible link between nitric oxide, SL content and cold stress response.

1. Introduction

When exposed to low non-freezing temperature, tolerant plants have the ability to set up appropriate adaptative responses that allow them to maintain their cellular homeostasis and to progressively acquire tolerance towards freezing conditions. This is achieved by complex metabolic changes aimed to restrict plant growth and to protect cellular components (for review [1,2]). Upstream from these modifications lies a deep reprogramming of gene expression that is controlled by a set of transcription factors, the best-characterized being C-box Binding Factors (CBFs). Such transcription factors are integrated into complex signalling networks, together with other signals such as calcium, inositol phosphate or protein kinases, and regulate plant response to low temperature [3].

A series of studies has pointed out lipid signals as major elements of the cold stress transduction pathway. In particular, the involvement of phospholipid-derived signals has been investigated and is now well-documented [3–8]. More recently, a second class of lipids i.e. sphingolipids (SL) has been proposed as transducers of low temperature stress [9]. As phospholipids, complex SL are major constituents of cell membranes. Several studies have reported changes in plant membrane SL content/composition over long term exposure to cold, eventually correlated with plant cold tolerance [10–13]. Furthermore several mutants harbouring defects in membrane SL biosynthesis present impaired performance when subjected to cold [14]. Beside their structural function, a range of SL species are also crucial elements of signalling networks. In this view the precursors of complex SL i.e. long chain bases (LCB) and their acylated derivatives ceramides (Cer), together with their phosphorylated counterparts (LCB-P and Cer-P), regulate multiple aspects of plant and animal cell biology [15,16]. In good accordance with such a function during cold stress, a rapid and transient synthesis of two phosphorylated SL occurs in Arabidopsis exposed to low temperature [9,17]. One of these was identified as t18:0-P and participates in the regulation of cold-responsive MAP kinase activity and gene expression [9] Unexpectedly, cold-evoked t18:0-P formation was strongly enhanced in H7 mutant seedlings, which over-expressed the Arabidopsis non-symbiotic haemoglobin AHb1 [17]. A major function for AHb1 is the elimination of nitric oxide (NO) *via* its oxidation into nitrate [18]. NO formation was detected in plants exposed

to low temperature [17,19–21] and genetic and pharmacological experiments evidenced that the absence of cold-evoked NO in H7 plantlets was responsible for t18:0-P over-production [17].

In the present study, we report that a short exposure to cold affects Arabidopsis total LCB content with limited impact on LCB composition in term of molecular species. Interestingly, such modification was not observed in H7 seedlings. These data highlight a new facet of SL signalling during cold response.

2. Results

In order to determine the effect of a short exposure to cold on the total LCBs of WT Arabidopsis, two-week old seedlings were treated for 1 h at 4°C. Following extraction and derivatization with *o*-phtaldialdehyde, the HPLC profile of their total LCBs was compared with that of unstressed plantlets (Fig. 1). As previously reported by others [22], we could identify seven different LCB species in unstressed Arabidopsis. These are the two t18:1 isomers (8E and 8Z) and their glucosylated derivates (t18:1(8E)-Glc and t18:1(8Z)-Glc), t18:0, d18:0 and d18:1. Due to the extraction and purification procedures, only non-phosphorylated LCBs were detected. For the same reason, each peak eventually corresponds to the total amount of LCBs present in cells i.e. free LCB and LCB released from Cer and complex SL. Following cold-stress exposure we did not detect the formation nor disappearing of any specific molecular species (Fig 1A,B). Nevertheless the global LCB content was significantly reduced (by 28%) in chilled plants (Fig. 2A). To determine if cold exposure specifically affected certain LCB species, the content of each LCB was determined. As shown on Fig. 2A, the amount of all the t18:1 species was significantly decreased (by 26 to 53%) after cold exposure. To a lesser extend d18:1 content was also lowered (by 21%) upon cold stress. In contrast the amounts of t18:0 and d18:0 were not modified (Fig. 2A). This led to a slight enrichment in these two species in stressed plantlets at the expense of the major LCB species t18:1(8E) (Fig. 2B). Taken together, these data indicate that chilling rapidly affects the total LCB quantity, with only a limited impact on the relative abundance of the different LCB species.

We recently evidenced that cold-related SL signalling was altered in an Arabidopsis mutant line over-expressing the non-symbiotic haemoglobin AHb1 [17].

Indeed, the formation of phosphosphingolipid signals including t18:0-P that is triggered by low temperature, was strongly enhanced in this mutant designated H7 [17]. Our finding that cold exposure led to a global decrease of LCB content in WT plants brought us to investigate if such response was impaired in H7 seedlings. As shown on Fig. 2A, unstressed H7 plantlets present a total LCB content similar to that of WT seedlings (132.29 ± 7.7 compared 119.95 ± 4.84 nmol.g^{-1} FW, respectively). It is noteworthy that H7 seedlings exhibit twice as much t18:0 and d18:0 as WT (Fig 2A). It led to an enrichment in d18:0 and t18:0 in H7 plantlets (representing together 20.6% compared to 11% in WT) at the expense of t18:1(8E) (Fig. 2B). When exposed to cold, H7 plantlets exhibited a higher total LCB content than unstressed H7 (133%; Fig. 2A). In these conditions the LCB content of H7 raised up to 213% of that of cold-stressed WT seedlings (Fig. 2A). This increase affected all the species with the exception of d18:0 and d18:1. The greatest difference was observed for t18:0 content that is 3.1 fold higher in H7 than in WT after cold shock (Fig. 2A). On the other hand the relative abundance of the different LCB was remarkably conserved between H7 and WT following cold exposure. Only the equilibrium between d18:1 and t18:0 was modified. Although they represented together ~ 22% of the LCBs in H7 and WT, the t18:0/d18:1 ratio was higher in cold-stressed H7 seedlings (2.6 compared to 1.0 in WT). Taken together these data indicate that the total LCB amount is dramatically modified following exposure to cold, in an opposite way in WT and H7 plants. In contrast the ratio between the different LCB species only presents discrete differences principally affecting t18:0 and to a lesser extend d18:0 and d18:1.

We reported previously that mutant plants affected in cold-triggered SL signalling were less sensitive to cold-dependent root growth inhibition [9],. As H7 line presented higher formation of t18:1-P and an increased LCB content when exposed to cold, we analysed the impact of these alterations on the overall cold response of H7 mutant. We first examined H7 root growth in plantlets grown at 12°C. Although the root growth of both WT and H7 was reduced in cold-grown seedlings (by 75.2 and 65%, respectively), H7 plantlets were significantly less sensitive than WT ones (Fig. 3A). In addition, we compared the level of anthocyanins in WT and H7 plantlets unstressed or transferred to 4°C. As shown on Fig. 3B, unstressed H7 plantlets presented a significantly lower level of anthocyanins. When WT seedlings were

exposed to 4°C, anthocyanin levels rapidly increased (1.57 and 4.57 fold after 1 and 7 days of stress, respectively). Even though a slight increase was also observed for H7 seedlings, the anthocyanin content remained lower in 7 day-stressed H7 than in unstressed WT plantlets. Taken together these data highlight alterations of H7 phenotypic responses to low temperature correlated with modifications of SL signalling in this mutant.

3. Discussion

LCBs are the precursors of complex membrane sphingolipids; they also constitute *per se* major signalling molecules. We therefore analysed the modifications of LCB content and/or profile to get insight on the possible involvement of these molecules in the early response of Arabidopsis to chilling. Using a method developed by Markham and co-workers [22], we obtained similar LCB profiles as previously described by these authors in unstressed Arabidopsis plants. Interestingly, LCB patterns were not modified upon chilling, indicating that LCB species neither appeared nor disappeared after stress exposure. We also observed that the relative abundance between the different LCB species was only marginally affected in the total LCB extract from unstressed and cold-stress plantlets. These proportions are in good accordance with data previously reported for Arabidopsis SL analyses [22,23]. In contrast the amount of total LCBs was dramatically lowered by cold. This decrease particularly affected t18:1 isomers which are the most abundant LCB and the preferential LCB backbone for membrane SL [14]. On the other hand, neither d18:0 nor t18:0 which are the most abundant free LCB [14] were impacted by cold exposure. It is therefore likely that the overall decrease of LCB content in chilled plants mainly reflects changes in membrane SL content. Modifications of plant membrane SL after cold exposure has been reported in several species including Arabidopsis and rye [13,24]. In these studies SL remodelling was observed after several days of chilling and was associated with the setup of acclimation. Our data indicate that changes in LCB content not only occur over long-term chilling but are already detected after a 1 h exposure of Arabidopsis plants to 4°C. The mechanisms leading to LCB impoverishment are currently unknown. It might involve changes in SL metabolism leading to LCB degradation. As we analysed the content of total LCB, i.e.. free and

complexed LCBs, it would require the activation of a set of enzymes catabolising complex SL, ceramide and LCB [16]. To date, only two SL-catabolising enzymes (ceramidase and LCB lyase) have been characterized in plants and their regulation is currently unknown [25,26]. Alternatively it cannot be ruled out that cold exposure might lead to rapid changes in membrane structure having a direct impact on complex SL extractability. This might particularly affect glycosylinositol phosphorylceramides which are less extractable than other SL and mainly contain t18:1 LCB core [22].

The decline of LCB content upon cold exposure was not observed in the H7 Arabidopsis mutant that overexpressed the non-symbiotic haemoglobin AHb1. On the opposite H7 plantlets exhibited a high LCB content under low temperature stress. As observed for WT plants this increase was primarily the consequence of changes in t18:1 isomer content which reinforces the hypothesis of a major impact of a short-term chilling on membrane SL. Interestingly H7 plantlets were less sensitive to cold-dependent root growth inhibition. This response might be directly correlated to SL metabolism as it has been reported for several mutants of SL pathway exposed to abiotic stress [10,26,28]. Together with other data on anthocyanine formation or cold gene expression (this study; [17]), this phenotype suggests that H7 plantlets present an altered responsiveness to low temperatures. The mechanisms underlying H7 behaviour are currently unclear. AHb1 haemoglobin has been initially characterized as a key component of NO turnover [18]. In good agreement with this function H7 plantlets do not accumulate NO under cold stress [17]. One can therefore hypothesize that NO participates in the regulation of SL metabolism under low temperature. In animal cells NO can promote sphingomyelin degradation *via* the activation of sphingomyelinases [27]. Whether NO participates in the modification of LCB content triggered by cold through the regulation of SL degradative enzymes in plants is currently unknown. On the other hand, we evidenced that NO regulates the formation of particular cold-evoked SL species i.e. t18:0-P and Cer-P [17]. Our analysis of LCB compositions indicates that unstressed H7 plantlets present a dramatically higher t18:0 content that is even further accumulated following cold stress. This high t18:0 level could afford for the increased formation of t18:0-P observed in H7. As the modulation of NO levels using pharmacological approaches also regulated t18:0-P formation [17], it would be interesting to investigate the effect

of such treatments on the overall LCB content, and in particular on t18:0, to further establish the link between NO and cold-dependent SL remodelling.

High levels of SL in biological membranes have been associated with decreased membrane fluidity. Interestingly, it has been suggested that membrane fluidity constitutes an important factor for the priming of cold response [28]. Thereby changes in LCB content could not only constitute a fast adaptative response but also participate in transducing cold stress signal. Minami and collaborators recently evidenced that the decrease of SL abundance in the plasma membrane of cold-adapted plants was correlated with a decrease in raft microdomain amount [12][13]. Indeed, the raft lipid fraction is particularly enriched in SL. Rafts are considered as platforms for membrane-associated signalling events, but also contribute to membrane rigidity [29][30]. The fact that the LCB content decreased as fast as 1 h after cold exposure might therefore reflect a rapid modification of membrane structure involving rafts. In that sense it is in good accordance with the hypothesis raised by Minami and collaborators for a function of microdomains in plant cold response [13].

4. Methods

4.1. Plant growth and treatments

Experiments were performed on Arabidopsis thaliana L. Heynh. ecotype Columbia (Col-0) wild type (WT) or over-expressing AHb1 non-symbiotic haemoglobin (H7) [18]. Sphingolipid and anthocyanin analyses were carried out on 14 day-old plantlets grown in vitro as described in [17]. Plantlets were cold-stressed by transfer to growth chamber set to 4°C for the indicated duration. Following treatment, seedlings were harvested, deep-freezed in liquid nitrogen and kept at -80°C.

For root growth analysis, three day-old seedlings were transferred to basic half-strength Murashige & Skoog (MS) medium (M0221; Sigma-Aldrich, Lyon, France) (10 g l^{-1} sucrose, pH 5.7, 8 g l^{-1} agar). Plates were transferred vertically to

growth chambers set to 22°C or 12°C, under continuous light. Primary roots were measured after a 10 day-growth at the indicated temperature.

4.2. Long chain base extraction and analysis

Total LCBs were extracted from 50 mg liquid N_2-frozen fresh plantlets using Lester IIIB procedure [28]. To standardize extraction and quantitation procedure, C20-4-SPH (d20:1) was used as internal standard. Equivalent amounts of extracts were derivatized with *o*-phthaldialdehyde [31]. HPLC analyses were carried out using Beckman Coulter system Gold 128 series pumps by reverse phase HPLC on a 4.6 mm x 250 mm gemini- C18 column (Phenomenex, Le Pecq, France). Elution was performed at 0.7 $ml.min^{-1}$ with 30% solvent RA (5 mM potassium phosphate (pH 7)) and 70% solvent RB (100% methanol) for 2 min, increasing to 90% solvent RB by 15 min, followed by an isocratic flow for 10 min, before increasing to 100% solvent RB by 30 min, with a 3-min 100% solvent RB wash, before returning to 80% solvent RB and re-equilibrating for 2 min. Fluorescence was excited at 340 nm and detected at 455 nm (RF-10A XL; Shimadzu, Champs sur Marne, France). Results were analysed and integrated using 32 Karat software (Beckmann Coulter, Inc., Fullerton, CA, USA).

4.3. Anthocyanin measurement

For each condition 40 treated seedlings were collected in a 2 mL tube, weighted and anthocyanins were extracted for 18h at 4°C by the addition of 300 µL of methanol containing 1% HCl. After addition of 200 µL water and 500 µL chloroform, the extract was centrifuged (15 min, 18 000 g) and the upper phase was collected. Absorption was measured at 530 nm and anthocyanin content was calculated as OD unit per mg of fresh weight. Results are expressed as a mean ± SE of three independent biological experiments.

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Figure legends

Figure 1: Profiles of *o*-phtaldialdehyde derivatives of total LCBs from unstressed (A) and cold-stressed plants (B).

Total LCBs were extracted from seedlings kept at 22°C (A) or exposed for 1h at 4°C (B), labelled with *o*-phtaldialdehyde and separated by HPLC. Profiles are representative of at least three independent biological repeats. The identity of the different peaks was determined by co-elution with commercial standards or by reference to previous studies [22] and is as follow: 1, t18:1(8Z)-Glc; 2, t18:1(8E)-Glc; 3, t18:1(8Z); 4, t18:1(8E); 5, t18:0; 6, d18:1(8E/8Z); 7, d18:0; 8, d20:0 (internal standard).

Figure 2: Content (A) and relative abundance (B) of the different LCB in unstressed and cold-stressed WT and AHb1-overexpressing mutant (H7).

A- The content of each LCB was determined from the area of the integrated peaks presented in Figure 1. Data (Supplemental Table 1) are means of three independent biological repeats.. Asterisks represent statistical differences of the total LCB content compared to that of unstressed WT plantlets (Student's test; *, $P<0.05$)

B- The content of every LCB species detected is expressed as a percentage of the total LCB content (Supplemental Table 1) for each experimental condition.

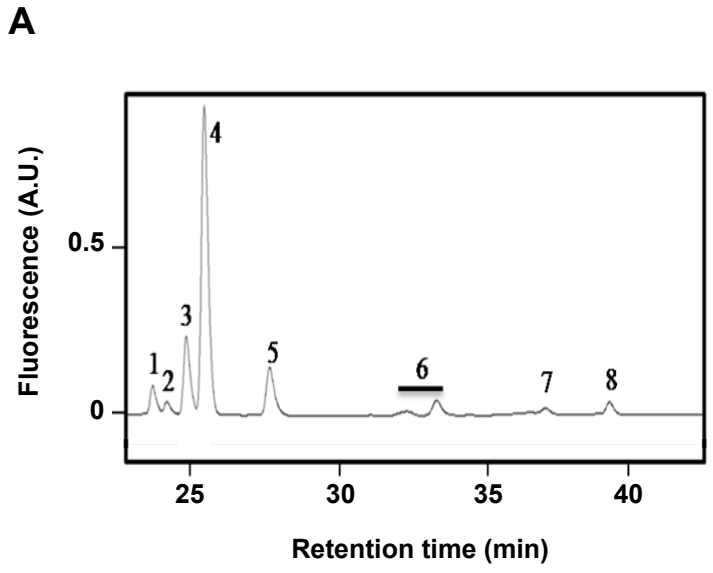
Figure 3: Root growth (A) and anthocyanin content (B) of WT and H7 mutant seedlings exposed to low temperature.

A- Root growth was analysed after 10 transfer of seedlings to 22°C and 12°C. Results are reported as the percentage of primary root growth at 12°C compared to that at 22°C (n=80-120). Asterisks represent statistical differences between WT and H7 plantlets (Student's test; ***, $P<0.001$).

B- Anthocyanin content was determined after exposing two week-old seedlings for 1 or 7 day at 4°C. Results are means ± SE of three independent biological repeats. Asterisks represent statistical differences compared to unstressed WT plantlets (Student's test; **, $P<0.01$; ***, $P<0.001$).

Supplemental Table 1: Contents and abundances of the major LCB species in unstressed and chilled WT and AHb1-overexpressing mutant (H7).

The content of individual LCB species was determined from the area of each peak identified in Figure 1, after integration. Molar contents were calculated by reference to a standard curve using t18:0 derivatized with *o*-phtaldialdehyde as a standard. Data are means ± SE of three independent biological repeats. Between brackets are indicated the percentage of each species in the whole LCB fraction of the corresponding condition.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Retention time (min)

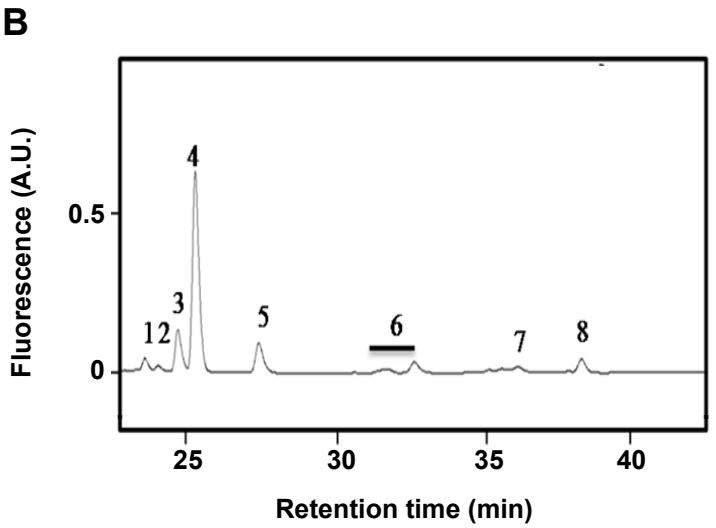
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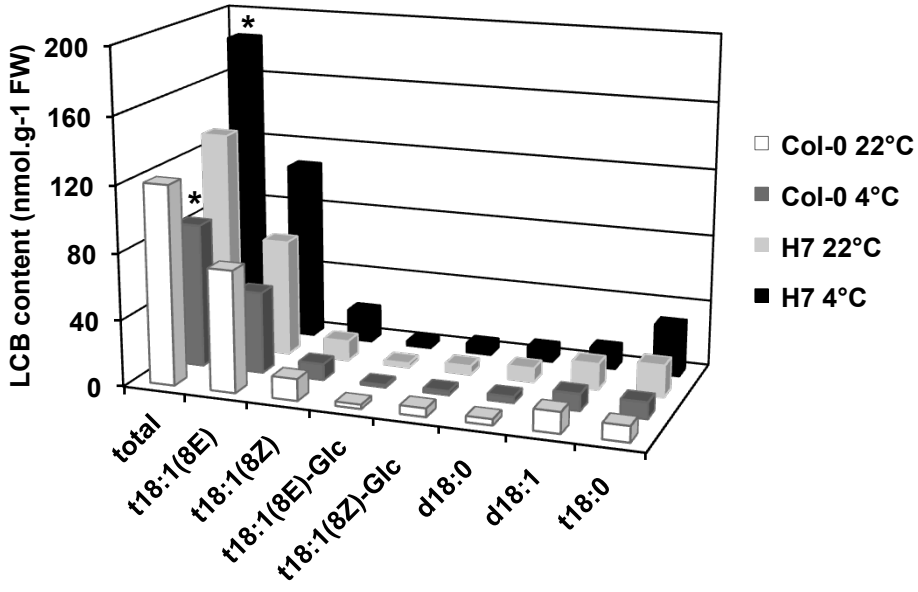
Figure 1

A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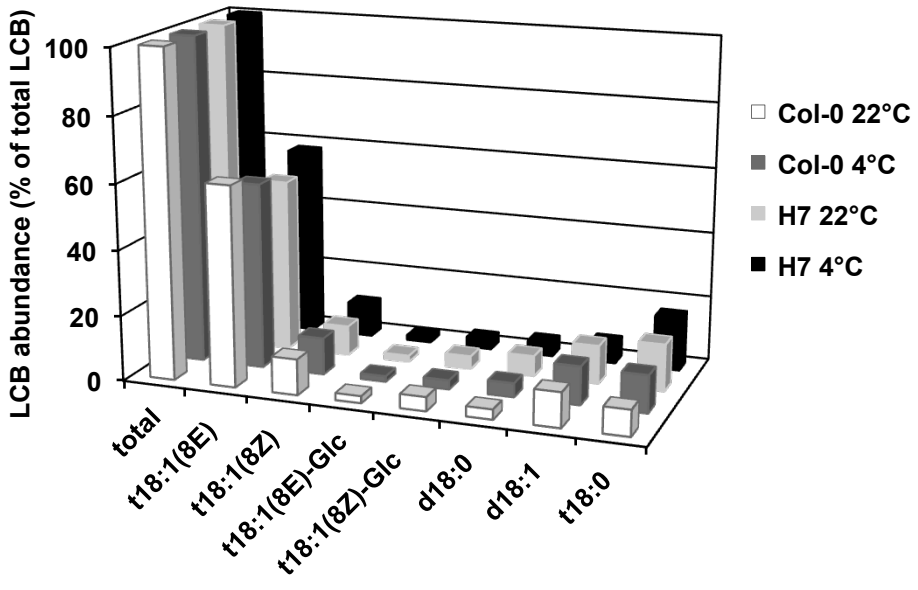
d18:0
d18:1
t18:0
Col-0 22°C
Col-0 4°C
H7 22°C
H7 4°C

B


LCB abundance (% of total LCB)
100
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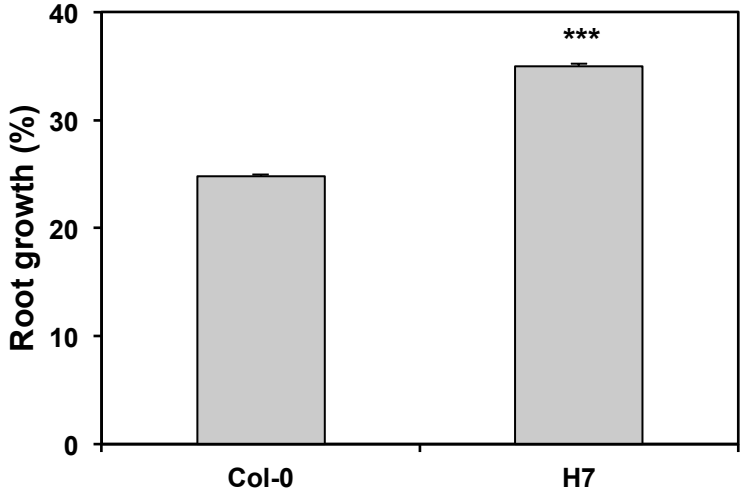
total
t18:1(8E)
t18:1(8Z)
t18:1(8E)-Glc
t18:1(8Z)-Glc
d18:0
d18:1
t18:0
Col-0 22°C
Col-0 4°C
H7 22°C
H7 4°C

Figure 2

A


40
30
20
10
0
Root growth (%)
***
Col-0
H7

B

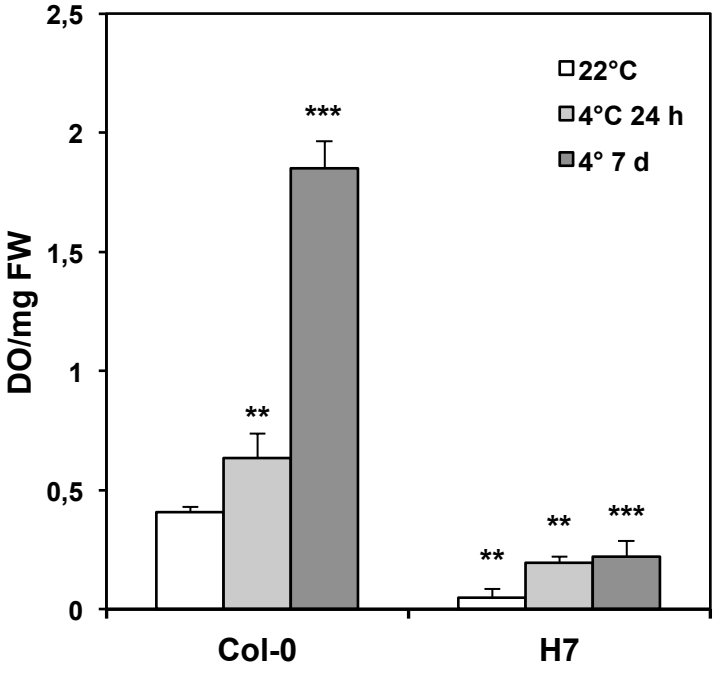

2,5
2
1,5
1
0,5
0
DO/mg FW
22°C
4°C 24 h
4° 7 d
***
**
**
**
***
Col-0
H7

Figure 3

LCB species	LCB content (nmol.g^{-1} FW)			
	Col-0		H7	
	22°C	4°C	22°C	4°C
t18:1(8Z)-Glc	5.15 ± 1.7 (4.30)	2.43 ± 0.51 (2.83)	5.22 ± 1.39 (3.94)	5.89 ± 0.63 (3.19)
t18:1(8E)-Glc	2.56 ± 0.67 (2.14)	1.48 ± 0.2 (1.73)	2.45 ± 0.43 (1.85)	2.90 ± 0.05 (1.57)
t18:1(8Z)	13.09 ± 2.68 (10.92)	9.72 ± 1.70 (11.25)	11.97 ± 1.91 (9.04)	18.91 ± 2.92 (10.24)
t18:1(8E)	73.18 ± 8.91 (61.06)	49.03 ± 6.5 (56.76)	69.92 ± 6.88 (52.86)	107.05 ± 6.09 (58)
t18:0	9.51 ± 1.62 (7.94)	9.90 ± 0.9 (11.45)	18.98 ± 1.32 (14.34)	30.32 ± 2.96 (16.42)
d18:1	12.68 ± 0.02 (10.58)	10.14 ± 0.77 (11.71)	15.47 ± 1.47 (11.70)	11.63 ± 0.03 (6.30)
d18:0	3.67 ± 0.28 (3.06)	3.69 ± 1.01 (4.27)	8.28 ± 1.82 (6.25)	7.87 ± 1.87 (4.26)
Total LCB	119.95 ± 4.84 (100)	86.39 ± 2.9 (100)	132.29 ± 7.7 (100)	184.58 ± 11.82 (100)

Supplementary Table 1