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Cell wall modifications of two *Arabidopsis thaliana* ecotypes, Col and Sha, in response to sub-optimal growth conditions: An integrative study

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ABSTRACT

With the global temperature change, plant adaptations are predicted, but little is known about the molecular mechanisms underlying them. *Arabidopsis thaliana* is a model plant adapted to various environmental conditions, in particular able to develop along an altitudinal gradient. Two ecotypes, Columbia (Col) growing at low altitude, and Shahdara (Sha) growing at 3400 m, have been studied at optimal and sub-optimal growth temperature (22 °C vs 15 °C). Macro- and micro-phenotyping, cell wall monosaccharides analyses, cell wall proteomics, and transcriptomics have been performed in order to accomplish an integrative analysis. The analysis has been focused on cell walls (CWs) which are assumed to play roles in response to environmental changes. At 15 °C, both ecotypes presented characteristic morphological traits of low temperature growth acclimation such as reduced rosette diameter, increased number of leaves, modifications of their CW composition and cuticle reinforcement. Altogether, the integrative analysis has allowed identifying several candidate genes/proteins possibly involved in the cell wall modifications observed during the temperature acclimation response.

1. Introduction

In the global warming context, elevated temperature is considered as the most serious change and it is already observed. The seasons are often altered with changes in temperature and occurrence of freezing stress that can appear without any preceding chilling period [1]. A study has shown that in the main European mountains, climate changes are ongoing and gradually transforming mountain plant communities [2]. As a consequence of this process, the more cold-adapted species are declining whereas the warm-adapted ones are prospering. Global warming is also critical to maintain agricultural productivity in the future [3]. *Arabidopsis thaliana* (L.) Heyhn, a *Brassicaceae* originating from the Eurasian continent [4], is adapted to multiple environmental conditions. This annual self-fertilized plant is a very good model for phenotypic plasticity studies. Furthermore, the large accumulation of genomics, genetics and molecular data regarding this plant is very helpful for the understanding of stress responses at multiple levels [5,6]. Although the variability between *A. thaliana* populations is well-recognized and studied at the genomics level, the molecular mechanisms below are still poorly described [7]. Conversely, phenotypic predictions from genotypes are complex because of epistatic interactions between genes usually controlling responses to environment.

Despite the evidence that adaptation to local climate changes is common in plant populations, a lot of work remains to be done to understand the genetic evolution contributing to climate acclimation. As an example, plant ecotypes are able to specifically develop along an altitudinal gradient and modification of their cell walls (CWs) could be one trait of their adaptation and/or acclimation. Indeed, CWs represent dynamic external physical barriers, the composition and structure of which vary upon developmental and environmental changes [8,9]. They play critical roles in the control of growth, cell shape, and structural integrity [10] thanks to modifications in CW protein (CWP) content which lead to changes in cell wall architecture [11–13].

The large collection of *A. thaliana* ecotypes [14] living in contrasted

Abbreviations: CW, cell wall; CWP, cell wall protein; DIR, dirigent protein; GH, glycoside hydrolase; HG, homogalacturonan; HRGP, hydroxyproline-rich glycoprotein; LTP, lipid transfer protein; PCA, principal component analysis; PME, pectin methylesterase; Prx, class III peroxidase; RGI, rhamnogalacturonan I; RNA seq, RNA sequencing; XG, xyloglucan

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habitats differing by their climate is a material of choice to look at the physiological responses to environmental constraints [15]. Although there are numerous studies aiming at understanding the plant response to limited-exposure to abiotic stresses (for reviews, see [16,17]), data regarding the physiology of plants grown at sub-optimal temperatures is scarce. Two studies have recently been performed with three ecotypes from Sweden, Poland and Italy corresponding to three different growth temperature regimes [18,19]. Changes in photosynthetic capacities, leaf thickness and morphology of tracheary elements in correlation with transpirational water loss have been observed when these ecotypes were cultivated at low (day at 12.5 °C/night at 8 °C), moderate (25 °C/20 °C) or high temperature (35 °C/25 °C). The results of this phenotyping could be correlated with the latitude and the temperature of the habitat of origin. However, data are still lacking to deeply understand the acclimation mechanisms at the molecular level.

For our study, two ecotypes of *A. thaliana* originating from contrasted natural environments, Columbia (Col) and Shahdara (Sha) have been used [20]. Col initially originating from Poland was adapted to both low altitude and high temperature, whereas Sha is growing at 3400 m in a high valley of Tajikistan. Since, it was not possible to recreate the real ecological environment of Col and Sha, we have focused the study on the temperature effect as a first step towards the understanding of the environment acclimation response. Two different growth conditions have been studied: 22 °C (optimal growth condition for Col) and 15 °C (optimal growth condition for Sha). An integrative approach has been performed, combining macro- and micro-phenotyping, and CW monosaccharide, CW proteomics, and transcriptomics analyses. Statistical analysis of the data has allowed establishing correlations between the different datasets and identifying candidate genes/proteins possibly involved in the temperature acclimation response of Col and Sha ecotypes.

2. Materials and methods

2.1. Plant material

The Col and Sha ecotypes of *A. thaliana* (L.) Heyhn were used (Supplementary Fig. S1). Climatic variables were obtained from WorldClim [21] (www.worldclim.org) at the GPS geographic origins of the Col (52.745416, 15.235557) and Sha (39.250103, 68.249919) ecotypes (publiclines.versailles.inra.fr). Seeds were sown in Jiffy-7® peat pellets (Jiffy International, Kristiansand, Norway). After 48 h of stratification at 4 °C in darkness, plants were grown at a light intensity of 90 µmol.photons/m^2/s, a humidity of 70% and under a 16 h light/8 h dark photoperiod at two different temperatures: 22 °C and 15 °C. Four- or 6-week-old rosettes were collected at the bolting developmental stage after growth at 22 °C or 15 °C, respectively (Supplementary Fig. S1).

2.2. Macrophenotyping

The rosette diameter and mass were measured at the time of sampling, together with the count of leaves. Before storage at −80 °C, pictures were taken to measure rosette areas with the ImageJ software [22]. Leaf density was determined from the leaves of three representative plants per experiment.

2.3. Anthocyanin content

Ground rosette material (0.1 g) was mixed with 1 mL of 95% ethanol/1% HCl and stored at 5 °C for 24 h in darkness. The samples were centrifuged for 10 min at ×1000*g*. The absorbance of the supernatant was measured at 530 and 657 nm. Anthocyanin concentration (µg anthocyanin/mg fresh material) was calculated using the following formula: $(A_{530} - 0.25 \times A_{657})$/mg of fresh material [23].

2.4. Histological staining of cell walls

Whole rosettes were harvested by cutting above the crown and rapidly individually infiltrated under vaccum in 50 mL Falcon tubes with FAA (10% formalin (37% formaldehyde solution, Sigma-Aldrich, Saint-Quentin Fallavier, France); 50% ethyl alcohol; 5% acetic acid; 35% distilled water). They were fixed for 16 h at 4 °C. The dehydration and paraplast infiltration protocol was as previously described [24]. The whole rosettes were processed in individual tubes used as embedding molds in order to keep traces of the phyllotaxy. Twenty µm-thick serial sections were disposed on silane-coated microscope slides (2–4 rosettes per slide). In order to ensure comparison of labelling intensities among the rosettes, a 20 slide-plastic holder and 200 mL staining jars were used [24]. Intracellular material was removed by incubating sections in 2.6% bleach for 20 min [25] followed by thorough washes with distilled water. Sections were then incubated for 5 min in 0.5% safranin red solution (CI 50240; Kuhlmann, Paris, France), washed with distilled water to remove excess staining solution and then incubated for 90 min in 0.005% alcian blue solution (CI 74240, Sigma-Aldrich) [26,27]. Following extensive washing, slides were dried, mounted in Eukitt® (quick-hardening mounting medium, Sigma-Aldrich) and scanned using a NanoZoomer HT scanner (Hamamatsu, Hamamatsu City, Japan). The auto-fluorescence of aromatic compounds was observed using a DAPI filter set (excitation: 387 nm +/− 11 nm; dichroic mirror 405 nm; emission: 440 +/− 40 nm) using a NanoZoomer RS scanner (Hamamatsu).

2.5. Measurement of cuticle permeability

To quantify water-loss, rosettes were excised, gently wiped to remove excess water and put in an oven at 40 °C. The fresh masses were recorded, before and after different times at 40 °C, using a micro-balance. Data were expressed as percentage of fresh rosette mass reduction in reference to the initial fresh mass. Epidermal permeability was also assessed using chlorophyll efflux. Entire rosettes were collected and immersed in 40 mL 80% ethanol/0.7 g of fresh mass and gently shaken at room temperature. Aliquots of 2 µL were sampled at different times after immersion and chlorophyll content was determined by measuring absorption spectra at 664 and 647 nm using a spectrophotometer DS-11 FX (DeNovix, Wilmington, DE, USA). Chlorophyll content was calculated using the following formula: $(A_{663} \times 7.15 + A_{647} \times 18.71)$/mg of fresh material = µg chlorophyll/mg fresh material [28].

2.6. Extraction of proteins from purified cell walls

CW purification was performed using 20 rosettes (about 10 g) for each experiment as described [29]. The sequential extraction of proteins from purified CWs was performed as described [30]. Typically, 0.2 g of lyophilized CWs was used for one extraction and about 500 µg proteins were extracted. The final protein extract was lyophilized. Proteins were quantified with the CooAssay Protein Assay kit (Interchim, Montluçon, France).

2.7. Cell wall monosaccharide analyses

The sequential extraction of CW polysaccharides in four steps was adapted from [31]. One hundred mg of a deproteinized CW fraction (corresponding to 20 mg dry cell walls) were used. Four successive extractions were carried out to obtain extracts enriched in pectins (E1 and E2) and hemicelluloses (E3 and E4). Extractions were performed at room temperature as follows: (i) overnight-incubation with 300 µL of 50 mM diamino-cyclo-hexane-tetra-acetic acid (CDTA), pH 7.5, to obtain E1 extracts; (ii) 3 h-incubation with 300 µL of 50 mM Na_2CO_3, 20 mM $NaBH_4$, to obtain E2 extracts; (iii) 3 h-incubation with 300 µL 20 mM $NaBH_4$, 1 M NaOH and then 4 M NaOH, to obtain E3 and E4 extracts, respectively. The supernatants were recovered by