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UNDERSTANDING THE FUNCTIONAL ARCHITECTURE OF THE *Arabidopsis thaliana* CELL WALL UNDER COLD STRESS

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ABSTRACT

Cold is one of the critical environmental conditions that negatively affects plant growth and development and determines the geographic distribution of plants. Cold stress signaling is dynamic and interacts with many other signal transduction pathways to efficiently cope with adverse stress effects in plants. Cold response is highly complex process that involves an array of physiological and biochemical modifications. Furthermore, alterations of the expression patterns of many genes, proteins, metabolites and the cell wall in response to cold stress have been reported. Cell walls are the plant 'exoskeleton' that dictates the cell shape and collectively the plant form. Cell walls provide plants with strength and protection, and also represent the most abundant source of renewable biomass. The model plant *Arabidopsis thaliana* and the availability of its genome sequence have been invaluable for the identification and functional characterization of genes encoding enzymes involved in plant cell-wall biosynthesis. In the present study, *Arabidopsis thaliana* were used to study the effects of cold stress on cell wall and morphology of plants. Bright, confocal and scanning electron microscopy was used to measure wall thicknesses of different cell types in freeze-fractured stem sections of *Arabidopsis thaliana*. Apart from cell wall studies, cold also affected the yield and biomass of plants. Present study revealed that cold stress has a key role to plant in cell wall metabolism during cold assimilation.

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INTRODUCTION

Freezing or extremely low temperature constitutes a key factor influencing plant growth, development and crop productivity. Cold response is highly complex process that involves an array of physiological and biochemical modifications. Cold adversely affects crop productivity and determines the geographic distribution of plants. Plants in tropical and subtropical regions are generally killed by a slight freeze, whereas plants in temperate regions exhibit varying degreesof freezing tolerance through the process of cold acclimation(Sakai and Larcher, 1987).

Plant cell walls are composite structures, mostly made up of polysaccharides, proteins, and lignin's, the last group being found only in specific

cell types. Polysaccharides represent up to 95% of cell wall mass, whereas cell wall proteins(CWPs) only account for 5–10%. Models of cell wall structure describe the arrangement of their components into dense interwoven networks of polysaccharides and proteins (Carpitaans Gibeaut, 1993; Cosgrove, 2005). Cellulose micro fibrils and hemicelluloses constitute a network, another one is formed by structural proteins, *e.g.*, extensions, and both embedded in a pectin matrix. Plant cell walls are dynamic structures essential not only for cell division, enlargement, and differentiation, but also for response to environmental constraints (Roberts, 2001;Huckelhoven, 2007).By studying cell walls with altered compositions, either as a consequence of developmental regulation, environmental adaptation, or genetic modification, we can assess the effect of such modifications on cell wall properties and on plant development.

To address the structural and functional relationships of individual cell wall components, we need to better characterize a broad range of structural and architectural alterations in cell walls, appearing as a consequence of developmental regulation, environmental adaptation or genetic modification. Thus the present study was done to understand the architecture and morphology of plants under cold stress in *Arabidopsis thaliana* and a comparative study was done. It was observed that with the increase in the duration of cold stress conditions, plants showed change in the cell wall and shape indicating susceptibility of plants to cold stress.

MATERIAL AND METHODS

Plant material and stress treatment

Arabidopsis thaliana seeds were surface sterilized, rinsed with sterile water and stratified at4 °C for two days on half-strength Murashige and Skoog (½ MS; 1962) medium supplemented with 1 % agar, 1 %sucrose. The seedlings were transplanted to the soil mixture of vermiculite: peat moss: perlite (1:1:1) in the greenhouse under a 16 h light and 8 h dark cycle at 20 ±1 °C and light intensity of 60–70 µmol PPFD m^{-2} $s^{-1}$and irrigated with ½ MS salts, weekly. For stress treatment, 21 days old seedlings were transferred to cold room (4°C). Samples were collected 0 h, 6 h, 12 h, 24 h, 48 h (2d), 96 h (4d) and 192 h(8d) during cold treatment for the analyses.

Microscopy

For confocal microscopic analysis, inflorescence stems of WT and transgenic lines were harvested and fixed in formalin, glacial acetic acid and ethyl alcohol (FAA, 1:1:18)at room temperature. Sections of 8–10 µm thickness were cut and stained with 1 % safranin and 4 % fast green. These sections were mounted and examined using Confocal Laser Scanning Microscope (Zeiss LSM510 metaGmbh, Germany).

For SEM analysis, segments from the apical 1 cmo f stem cross-sections were fixed in a mixture of 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 mol/l cacodylae buffer, pH 7.4 for 1 h and then with 1 % OsO4 in0.1 mol/l cacodylate buffer, pH 7.4 for 30 min. After critical point drying, the samples were sputter-coated with gold, and the coated samples were viewed with a HitachiS-3400N field emission SEM using an accelerating voltage of 30 kV. For TEM study, stem slices were fixed in4 % (w/v) paraformaldehyde and 1 % (v/v) glutaraldehydein 0.1 mM phosphate buffer, pH 7.2, for 4 h at room temperature and then postfixes in 1.33 % OsO4 in cacodylate buffer, pH 7.2, and stained with 1.5 % uranyl acetate. All samples were dehydrated in acetone series, followed by propylene oxide. Embedment was in Araldite, Epon, and dodecenyl succinic anhydride in proportions 1:1:3. Polymerization was carried out at 80 °C, and micrographs were taken with a Tecnai G2 TF20 electron microscope (FEI, Netherlands).

In-situ ROS staining

In situ ROS staining was done in accordance with Beyer and Fridovich (1987), on the basis of the principle of NBT (nitrobluetetrazolium) reduction to blue Formosan by O_2^{-}. The intracellular concentration of ROS (O_2^{-}) was directly proportional to the development of intensity of blue color in the leaves. Briefly, leaf tissue was detached from the wild type and transgenic plants and vacuum infiltrated with 10 mM sodium azide (NaN3) in 10 mM potassium phosphate buffer for 1 min. The infiltrated leaf tissue was incubated in 0.1% NBT (nitrobluetetrazolium) in 10 mM potassium phosphate buffer; pH 7.8 for 30 min. The stained leaf tissue was boiled in acetic acid: glycerol: ethanol (1:1:3) solution to remove other pigments and the stain content was visually documented under Carl-Zeiss Stereo DiscoveryV12 with Axiovision software. This experiment was repeated three times from three biological replicates.

Cell wall Measurements

Measurements of cell wall thickness and shape were done with a HitachiS-3400N field emission SEM using an accelerating voltage of 30 kV.

Biomass and yield calculations

After completion of *Arabidopsis* lifecycle both under cold and stress conditions, the biomass calculations in terms of rosette diameter, number of leaves, root and shoot biomass was done. Also, yield measurements in terms of number of pods, seeds/pod and total number of seeds/plant were done.

Statistical analysis

All experiments were conducted with at least three independent repetitions in triplicates. All values are shown as the mean ± the standard deviation. The statistical analysis was performed using Statistical software (v.7). The statistical significance between the mean values was assessed by analysis of variance (ANOVA) applying Duncan's Multiple Range Test (DMRT). A probability level of $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

Effect of Cold Stress on plants

Plants exposure to low level salinity and cold activates an array of processes leading to an improvement of plant stress tolerance. This has already been demonstrated for different herbaceous species such as soybean, rice, sorghum and *Arabidopsis* (Umezawa et al., 2000; Djanaguiraman et al., 2006; Shafi et al., 2014; 2015a; 2015b; 2017). *Arabidopsis* plants were found tolerant when exposed to cold (4°C) stress till 24 h of stress (Fig.1). The morphological and vegetative growth of transgenic plants was more or less similar under normal growth conditions i.e. 20±1°C and 16 h/8 h light regime (Fig. 1) whereas, after 24 h, under cold stress the plants showed changes in growth (Fig. 1). Effects of cold stress were clearly observed in the growth of plants, where leaves started yellowing which shows the symptoms of chlorosis with stunted growth.

Anatomical and morphological changes under Cold stress

Plant cell walls also provide the first line of defense against invading micro-organisms and abiotic stresses. Since the main objective of this was to observe the anatomical changes in the plant, we have taken tissues at different time points of cold stress conditions. Sections were observed under different microscopic techniques such as bright field, confocal and electron microscopy. It was observed that under control conditions the *Arabidopsis* stem sections showed normal anatomy with distinct vascular system (Fig.2). Under cold stress conditions and with increase in time duration the vascular system collapsed and cell wall degradation was visible under electron microscopy, which indicates that plant vascular system and the cell wall is experiencing the cold stress. Thus in the present study, anatomical investigation of vascular structures using Confocal and electron microscopy clearly showed that disruption and distortion in the morphology of plants with cold stress.

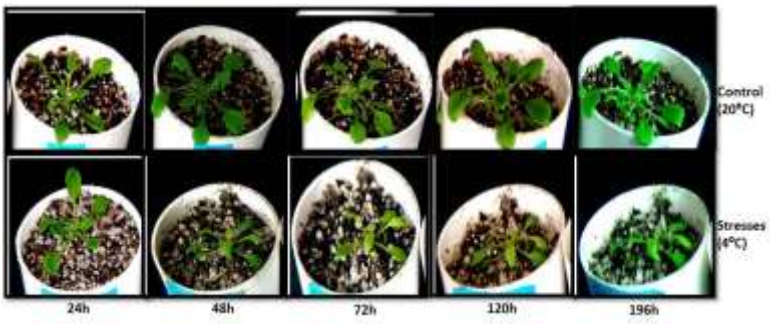

Fig.1. Phenotypic Changes in *Arabidopsis* under control (20° C) and stress conditions 4° C (24h, 48h and 196h duration).

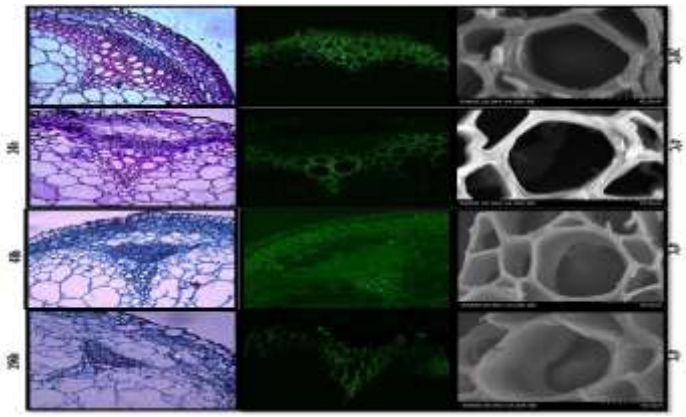

Fig.2. Stem sections of *Arabidopsis* stem observed with bright field, confocal and scanning microscope under control (20° C) and stress conditions 4° C(24h, 48h and 196h duration).

Effect of Cold stress on yield and Biomass

Root growth and development pattern was also studied during cold stress. After 192 h of cold stress plants showed decrease in root growth biomass (Table 1). In order to determine if this difference existed in young seedlings at the in vitro stage, root growth on normal medium without any stress was also measured. A marginal increase in root length was observed in under normal growth conditions (Table 1).Initially, plants exhibited similar root growth under cold stress, but with the increase in duration of cold stress, root growth rate was lower than that of transgenic, which had longer roots after a prolonged culture period (10 d). Some yield and biomass aspects such vegetative growth like rosette diameter, number of leaves; reproductive growth in terms of number of pods, length and number of seeds per silique, were also analysed under control and cold stress conditions (Table 1). The present results showed that the plants were 'chilling sensitive' with stunted growth, reduced metabolism and with low biomass accumulation during cold stress.

Table1.Biomass and yield attributes of *Arabidopsis* under control (20°C) and cold stress (4°C) conditions.

S.No.	Attributes	20°C	4°C (24h)	4°C (48h)	4°C (72h)	4°C (192h)
1.	Plant Height (cm)	28.3	27.2	23	23.4	22
2.	Root Length (mm)	9	6	5	4	3
3.	Rosette Area (cm^2)	5	3	2.5	2	2
4.	No. of Leaves	16	13	12	10	11
5.	No. of Pods	43.6	39.2	37	37.4	36.2
6.	Pod size (cm)	1.7	1.5	1.4	0.94	0.91
7.	Total seeds	914	822	768	661	621

Reduced accumulation of ROS contents under cold stress

Accumulation of ROS may cause damage to many biomolecules of the cells. We also measured the accumulation of ROS in their leaf samples at the end of the cold stress by staining with NBT. Histo-chemical staining of leaves from control and stressed plant with NBT revealed that it could also be stained ROS without cold stress also (Fig. 3). The figure showed that the control plants had slightly lower ROS accumulation than the cold stresses, and increase in the blue coloration with the time indicates that ROS levels has gone up in plants under stress conditions. Cold resulted in significantly higher levels of ROS accumulation in control leaves whereas the control plants had low levels accumulation as evidenced by the lower intensity of the blue colour (Fig. 3).In our study, WT plants induced the defensive system to protect against free radicals but longer the stress time, the more ROS molecules were produced which impaired antioxidant enzyme activity and as a result ROS could not be efficiently removed.

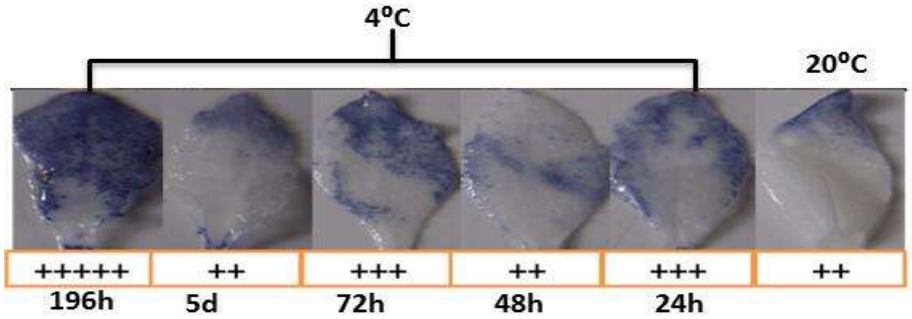

Fig.3.NBT staining of *Arabidopsis* leaves under cold stress (4^0C) and control (20^0C) conditions. (-) absence of stain indicating low ROS; (+) presence of stain indicates accumulation of ROS.

Changes in shape and size of Cell wall under cold stress

Measurements of cell wall (Table 2) and shape of the cell of plants under control and cold stress conditions was observed with the help of electron microscopy (Table 2). The thickness of the walls of vascular bundles were more or less the same under normal conditions (Table 2),whereas under cold stress the thickness of the cell wall drastically decreased, where thin line of thickness was observed at 192h of stress conditions. These results indicate that the deformation of vessels (Table 2), which leads to impaired water transport and wilting phenotypes. The wealth of information gained from the *Arabidopsis* genome sequence (*Arabidopsis* Genome Initiative 2000), coupled with the powerful

tools available to *Arabidopsis* researchers (Seki et al., 2002; Rhee et al.,2003), has facilitated much progress within the cell-wall research community in identification of genes encoding enzymes involved in cell-wall biosynthesis.

Table 2.Wall thickness ofvessels and fibers in stems ofthe WT and transgenic lines

S.No.	Sample	Interfascicularfibers (µm)	Vessels (µm)	Xylaryfibers (µm)
1.	**20°C**	2.62 ± 0.09	0.912 ± 0.02	0.298 ± 0.02
2.	**4°C (24h)**	2.31 ± 0.14	0.803 ± 0.03	0.181 ± 0.03
3.	**4°C (48h)**	2.10 ± 0.18	0.615 ± 0.02	0.176 ± 0.02
4.	**4°C (72h)**	1.68 ± 0.14	0.525 ± 0.05	0.145 ± 0.02
5.	**4°C (192h)**	1.23 ± 0.03	0.325 ± 0.02	0.125 ± 0.01

Wall thickness was measured from electron micrographs of fibers and vessels. Data are mean (µm) ±SE from cells. Means were compared using ANOVA.

CONCLUSION

In conclusion, the results of our study demonstrate that cold stress plays an important role in effecting cell wall architecture. This knowledge will not only contribute to the understanding of plant cell wall interaction with the environment but also provide new information about the direct and indirect mechanisms of stress on cell wall metabolism.

ACKNOWLEDGEMENT

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