БОТАНИЧЕСКИЙ ЖУРНАЛ, 2024, том 109, № 1, с. 31–43

ОРИГИНАЛЬНЫЕ СТАТЬИ

LOW TEMPERATURE-INDUCED CHLOROPLAST RELOCATION IN MESOPHYLL CELLS OF *PINUS SYLVESTRIS* (PINACEAE): SBF SEM 3D RECONSTRUCTION

© 2024 г. N. K. Koteyeva[a,#], A. N. Ivanova[a,b], T. A. Borisenko[a,b], M. S. Tarasova[a,b], O. E. Mirgorodskaya[a], E. V. Voznesenskaya[a]

[a]Komarov Botanical Institute RAS
Prof. Popova Str., 2, St. Petersburg, 197022, Russia
[b]St. Petersburg State University
Universitetskaya Emb., 7–9, St. Petersburg, 199034, Russia
[#]e-mail: nkoteyeva@binran.ru

Received 27.11.2023
Revised 04.12.2023
Accepted 05.12.2023

Evergreen species of temperate zone acclimate to seasonal climates by reorganizations of mesophyll cell structure including chloroplast movement as a photoprotective reaction. However the exact factor inducing structural changes is still unexplored. To reveal the specific pattern of chloroplast arrangement during the annual cycle and the effect of temperature on their movement, the mesophyll cell structure in *Pinus sylvestris* grown out- and indoors was studied. The serial block-face scanning electron microscopy (SBF SEM) was used for the 3D imaging of mesophyll cells to show the spatial position and shape modification of chloroplasts. It has been shown that during the growing season, chloroplasts have a well-developed thylakoid system, are located along the cell wall and occupy predominantly the part of the cell wall faced the intercellular airspace. Chloroplast movement starts in October-November, and during the winter they aggregate in the cell lobes clumping together. At that time, the thylakoid system is reorganised and consists mainly of long doubled thylakoids and small grana. The 3D reconstruction shows that the chloroplasts are irregularly oriented, swollen, and develop multiple protrusions filled by stroma that can be recognized as stromules. In indoor plants, seasonal reorganization of the mesophyll ultrastructure does not occur suggesting low temperatures but not photoperiod and light quality induce seasonal chloroplast movement in *P. sylvestris* mesophyll. Finally, we indicate 3D reconstruction is a powerful tool in study of low-temperature-induced change of chloroplast positioning.

Key words: *Pinus sylvestris*, mesophyll, chloroplast movement, seasonal changes, low temperatures, 3D reconstruction

DOI: , **EDN:**

Overwintering plants of temperate zone acclimate to low temperatures during transition period by development of freezing tolerance. Cold acclimation includes accumulation of cryoprotectors (Gusta, Wisniewski, 2013) and antifreeze compounds (Duman, Wisniewski, 2014), membrane stabilization (Steponkus, 1984; Uemura et al., 2006), modifications of photosynthetic machinery (Demmig-Adams et al., 2012; Ensminger et al., 2012), all associated with change in gene expression (Thomashow, 1999; Xin, Browse, 2000). Evergreen woody plants are subject to seasonal changes and develop physiological mechanisms that lead to a strict periodicity in the development of freezing tolerance (Bigras et al., 2001). Freezing tolerance in woody plants is mainly induced by low temperatures, or may be a response to a short photoperiod (Guy, 1990) or a combination thereof (Li et al., 2004; Arora, Taulavuori, 2016; Chang et al., 2021). It is closely but not necessarily associated with dormancy development combined with growth cessation (Bigras et al., 2001; Wisniewski et al., 2018) for which photoperiod is an induction signal (Lloyd et al., 1996; Howe et al., 1997; Jian et al., 1997; Jian et al., 2000; Maurya, Bhalerao, 2017). This suggests that

variable elements of cold acclimation may be induced differently as a response to different ecological factors or their combination (Chang et al., 2021).

Seasonal structural changes in woody evergreens were studied in relation to winter freezing tolerance in conifer needles (Chabot, Chabot, 1975; Jokela et al., 1998; Miroslavov, Koteyeva, 2002; Tanaka, 2007; Ovsyannikov, Koteyeva, 2020), stem cortical cells (Wisniewski, Ashworth, 1986; Sagisaka, Kuroda, 1991), ray parenchyma cells (Sauter et al., 1996), secondary phloem parenchyma (Pomeroy, Siminovitch, 1971). Most structural changes in differentiated cells are related to decrease of subcellular ice crystallization and subsequent dehydration; these include vacuole and endomembrane system restructuralization (Wisniewski, Ashworth, 1986). Photoprotective strategies in evergreen conifers involve seasonal chloroplast movement and thylakoid system reorganization decreasing damage by photoinhibition (Chabot, Chabot, 1975; Martin, Oquist, 1979; Miroslavov, Koteyeva, 2002; Ovsyannikov, Koteyeva, 2020). Seasonal chloroplast relocation was shown for *Picea* (Senser et al., 1975; Soikkeli, 1978), *Pinus* (Martin, Oquist, 1979; Soikkeli, 1980; Koteyeva, 2002), *Abies balsamea* (Chabot, Chabot, 1975), and *Taxus* (Miroslavov, Koteyeva, 2002; Tanaka, 2007). Specific winter arrangement of organelles differs significantly depending on species.

Most studies of plant cells are based on two-dimensional (2D) imaging, which provides incomplete or sometimes misleading information about the shape and position of organelles. This is especially important for cells that have an irregular shape. *Pinus* species have so-called lobed (also called armed or plicate) mesophyll cells; the function of lobes is thought to increase the surface area of the mesophyll and therefore facilitate the conduction of CO_2 to the chloroplasts (Wiebe, Al-Saadi, 1976). Only three-dimensional (3D) imaging can provide complete information on chloroplast distribution throughout the volume of a cell with a complex shape. This information is crucial for understanding of CO_2 and H_2O exchange that is limited by 3D network of resistance within leaf (Earles et al., 2019). In particular, spatial position of chloroplasts in relation to airspaces affects the mesophyll conductance and consequently CO_2 assimilation efficiency (Evans, 2021). Additionally, relocation of chloroplasts is a known photoprotective strategy documented as a reaction to high light (Kagawa, Wada, 1999) or cold acclimation (Ovsyannikov, Koteyeva, 2020). The shape of chloroplasts is also subject to change including stromule formation and divisions that cannot be studied using 2D images (Yamane et al., 2020). This led us to investigate and reconsider dynamics of the pine chloroplast positioning, shape and ultrastructure using 3D methodologies.

In current study, a comparison was made on young Scotch pine plants grown out- and indoors under natural light and photoperiod to exclude the effect of low temperature. This allowed us to identify a main factor that contributes to structural responses of mesophyll cells during cold acclimation. The serial block-face scanning electron microscopy (SBF SEM) was applied to reveal the specific organelle positioning and their shape change at the three-dimensional level.

MATERIALS AND METHODS

8-year-old *Pinus sylvestris* L. plants were grown in the arboretum and greenhouse of Komarov Botanical Institute, St. Petersburg, Russia (59°94'51" N, 30°37'17" W, 1–5 m above sea level). For each accession, 50 individual plants were grown in soil outdoor and in 4 L pots with the same soil indoor. Greenhouse plants were watered every 2–3 days. Plants were grown during one year, with ~25/18°C day/night temperatures and a maximum mid-day PPFD of 1000 μmol photosynthetic quanta m^{-2} s^{-1} at sunny days. Material was collected monthly from February 1996 to February 1998, additionally samples were fixed during 2015–2017, 2021–2022 growth cycles from outdoors plants.

For light microscopy (LM) and transmission electron microscopy (TEM) middle parts of current- and 1-year-old needles (6 samples from 5 different plants per date) were fixed at 4°C in 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.2) and post fixed in 2% (w/v) OsO_4 at 4°C overnight. After dehydration in ethanol and acetone series samples were gradually infiltrated by a mixture of Araldite-Epon epoxy resins (Fluka, Buchs, Switzerland). Cross-sections were made using a Leica EM UC7 (Germany) ultramicrotome. For LM, semi-thin leaf sections (1 μm thick) were stained with 1% (w/v) Toluidine blue O in 1% (w/v) $Na_2B_4O_7$, and studied under the light microscope AxioScop.A1 (Zeiss, Germany). For TEM, ultrathin sections (~70 nm)

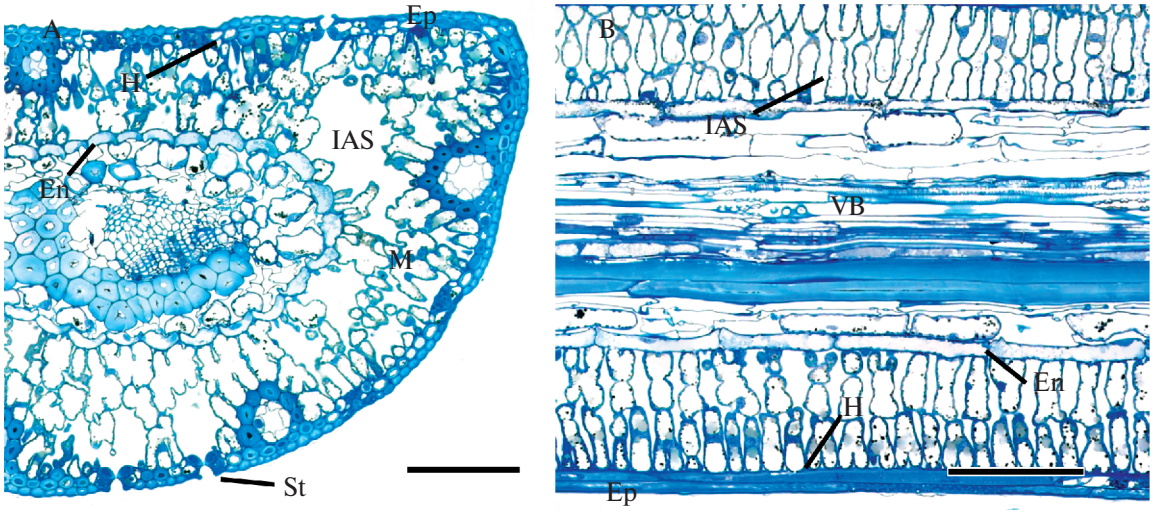

Fig. 1. General anatomy of *Pinus sylvestris* needles on cross (A) and longitudinal (B) sections, August. En – endodermis; Ep – epidermis; H – hypodermis; IAS – intercellular airspace; M – mesophyll; St – stomata; VB – vascular bundle. Scales: A, B, 150 μm.

were stained with 2% (w/v) uranyl acetate followed by 2% (w/v) lead citrate. Zeiss Libra 120 transmission electron microscope (Oberkochen, Germany) was used for observation and photography.

For serial block-face scanning electron microscopy (SBF SEM) middle parts of needles were fixed at 4°C in 2.5% (v/v) glutaraldehyde in 150 mM Cacodylate buffer (pH 7.2) and post fixed in 2% (w/v) OsO_4 containing 1.5% potassium ferrocyanide (w/v) in 150 mM cacodylate buffer for 1 h on ice, followed by 1% thiocarbohydrazide solution in water for 20 min at room temperature (RT), then in 2% OsO_4 for 30 min at RT, after it in 1% uranyl acetate overnight at 4°C, and Walton's lead aspartate solution for 30 min at 60°C. After each step, the samples were rinsed in buffer or water 3 times for 5 min at RT. The samples were then dehydrated with graded series of ethanol and acetone, and were embedded in Epon epoxy resin (hard modification). Embedded samples were trimmed and mounted on aluminum specimen pin stubs using carbon tape and silver conductive epoxy resin H20E Epo-Tek, then, the samples were sputtered with 20 nm thick layer of gold or platinum. Samples were sectioned (100 nm thick) and imaged under a scanning electron microscope Volumescope2 (ThermoFisher, the Netherlands) with an accelerating voltage of 2.49 kV. Serial sections and image arrays for subsequent 3D reconstruction were acquired using Maps 3.9 and Amira 2020 3.1 software (ThermoFisher, The Netherlands). To specify the details of the position and shape of chloroplasts, each chloroplast was highlighted with a different color during reconstruction. The methodology required pick out the outline of each chloroplast on each serial section using about 500 sections, each 100 nm thick. The curved lines in the images represent the outline of one section, giving them a stepped appearance.

RESULTS

General mesophyll anatomy

The mesophyll of *P. sylvestris* needles consist of two to three layers of chlorenchymatous cells when viewed in two-dimensional (2D) cross sections (Fig. 1, *A*). Mesophyll cells are usually lobed at the proximal and distal ends with deeper invaginations at the distal end; lateral sides of the cells are less invaginated. On longitudinal sections mesophyll cells often have a palisade-like outline with rare lobes (Fig. 1, *B*), suggesting that the lobes are oriented preferentially in one direction, perpendicular to the central axis of the needle (oriented radially to epidermis). Intercellular airspaces are well developed, but mesophyll cells are packed more loosely on longitudinal section creating files of cells (Fig. 1, *A*, *B*). The 3D reconstruction and longitudinal sections show that the cells are more irregularly shaped in the longitudinal plane than would be expected from the cross sections. Some single mesophyll cells extend from the hypodermis to the endodermis, indicating that the mesophyll is not strictly bilayered (Fig. 2). Such cells are bent in a tangential plane so that in cross sections only a

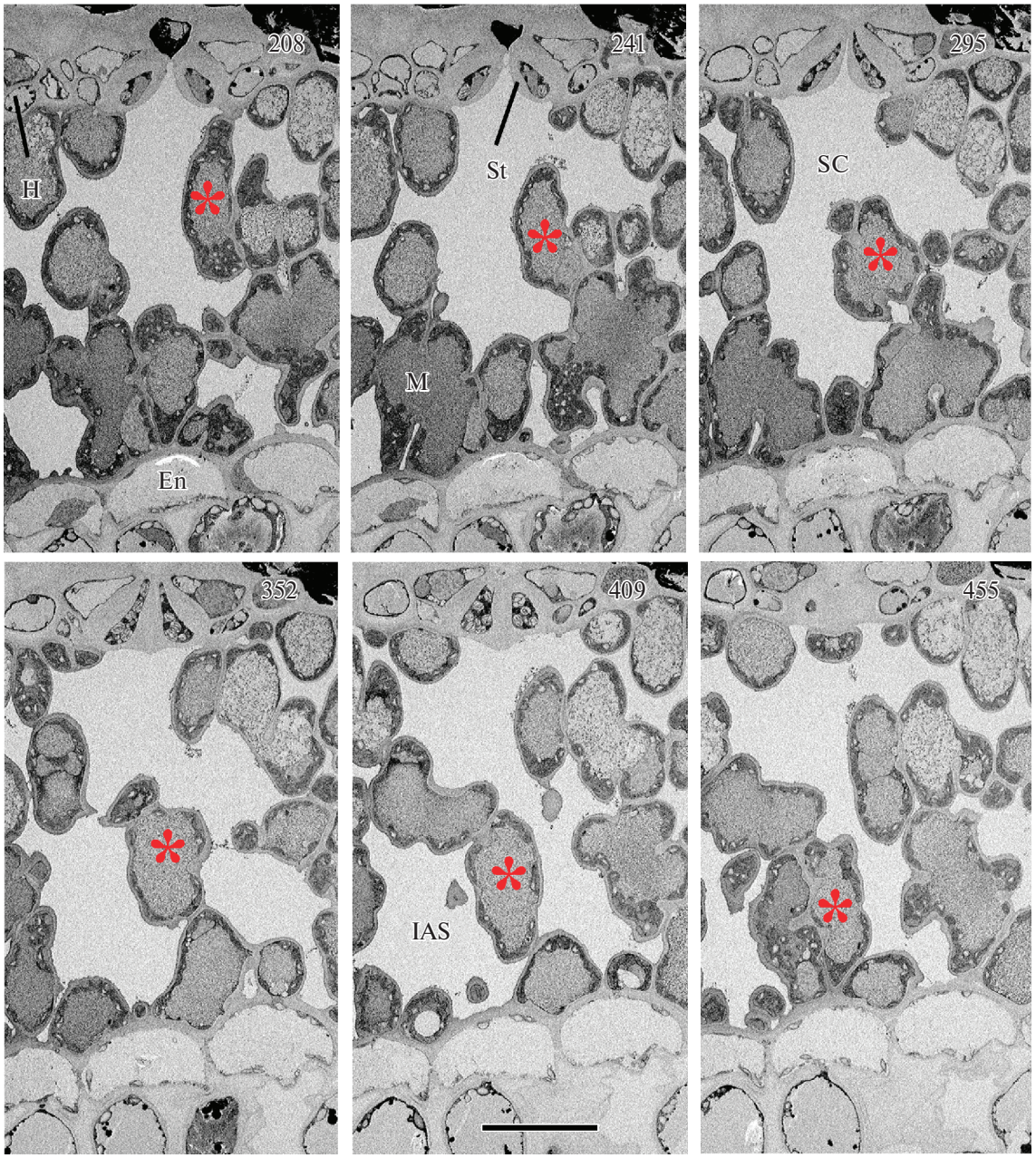

Fig. 2. Serial cross sections through mesophyll of *Pinus sylvestris* showing mesophyll cell extended from hypodermis to endodermis (labelled by red asterisk). The total number of sections is 351. The serial number of individual section is in the right corner of the image. En – endodermis; H – hypodermis; IAS – intercellular airspace; M – mesophyll cell; St – stomata. Scale: 50 μm.

part of the cell is visible. This is especially true for mesophyll cells around the substomatal cavity.

Mesophyll cell structure during growing season

In St. Petersburg, growing season lasts from April to November. At that time, 2D and 3D images show that mesophyll cells contain a large central vacuole with cytoplasm located parietally and nucleus located in central part of the cell inside of cytosolic strand (Fig. 3, *A*). Vacuole contains lipid droplets and globular deposits (tannins). Chloroplasts are positioned along the cell walls covering the inner wall areas faced to airspaces more densely than those adjacent to other neighboring cells or folds of the cell wall. 3D images visualize chloroplasts as arranged tightly and having classical flatten-lens shape without protrusions (Fig. 4, *B*–*D*). Chloroplasts have well developed thylakoid system consisting of big grana and stromal thylakoids (Fig. 3, *C*). Mitochondria

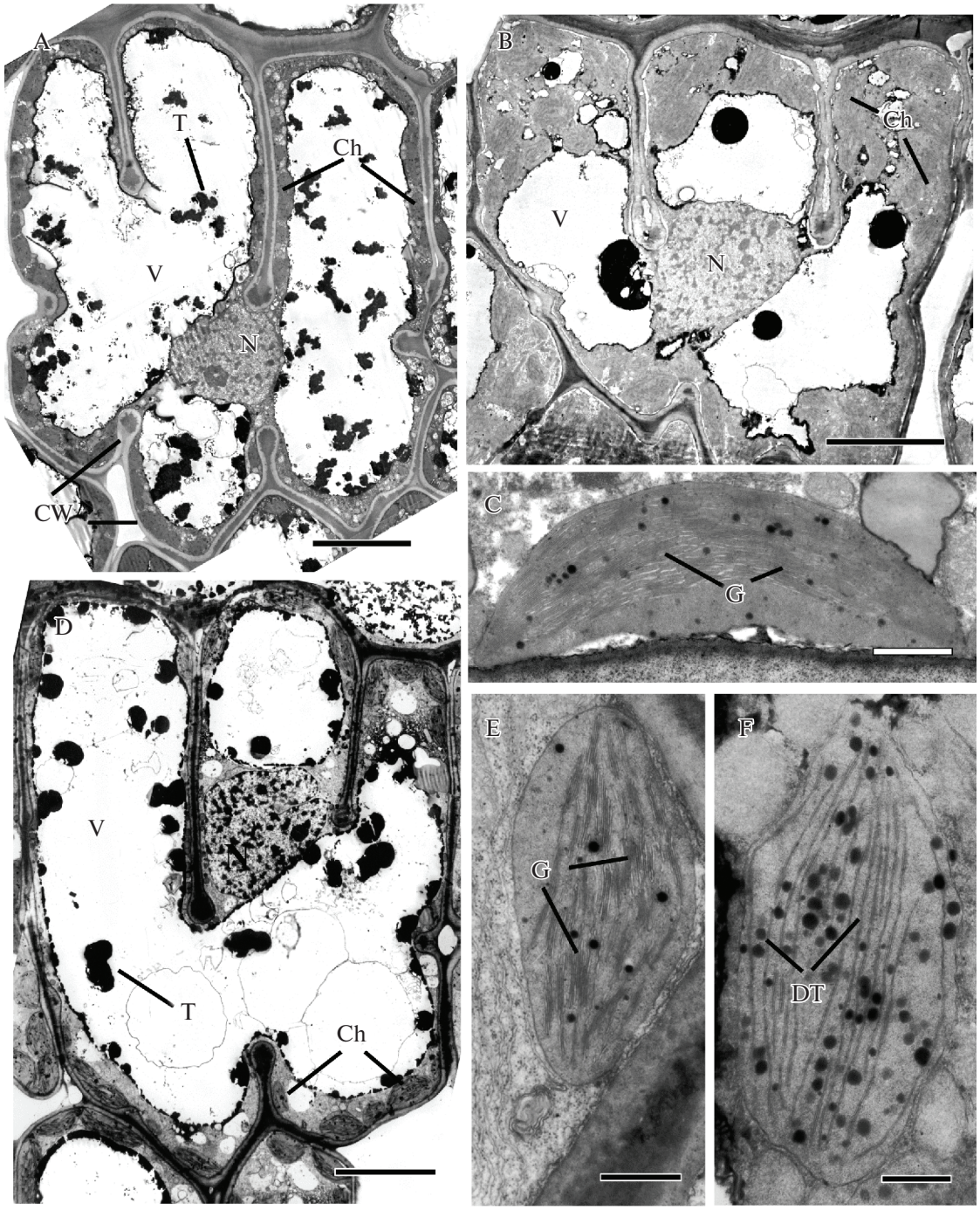

Fig. 3. Transmission electron microscopy of mesophyll cells in outdoor- (A–C, F) and indoor-grown (D, E) *Pinus sylvestris*. A, B, D. Cross sections in the midplane of a mesophyll cell in July (A) and February (B, D). C, E, F. Cross sections of chloroplasts in July (C) and February (E, F). Ch – chloroplasts; CW – cell wall; DT – doubled thylakoids; G – grana; N – nucleus; T – tannins; V – vacuole. Scales: A, B, D, 10 μm; C, E, 1 μm; F, 0.5 μm.

usually but not necessarily are associated with chloroplasts. Mesophyll cell ultrastructure is similar in plants grown out- and indoors.

Mesophyll cell structure during winter

Outdoors, the transition period of structural changes begins in October-November with a change in the orientation of chloroplasts and their movement towards the distal and proximal ends of cells along the cell walls.

The typical "winter" cellular structure is observed in November-December and continues until March-April, depending on weather conditions. At this time,

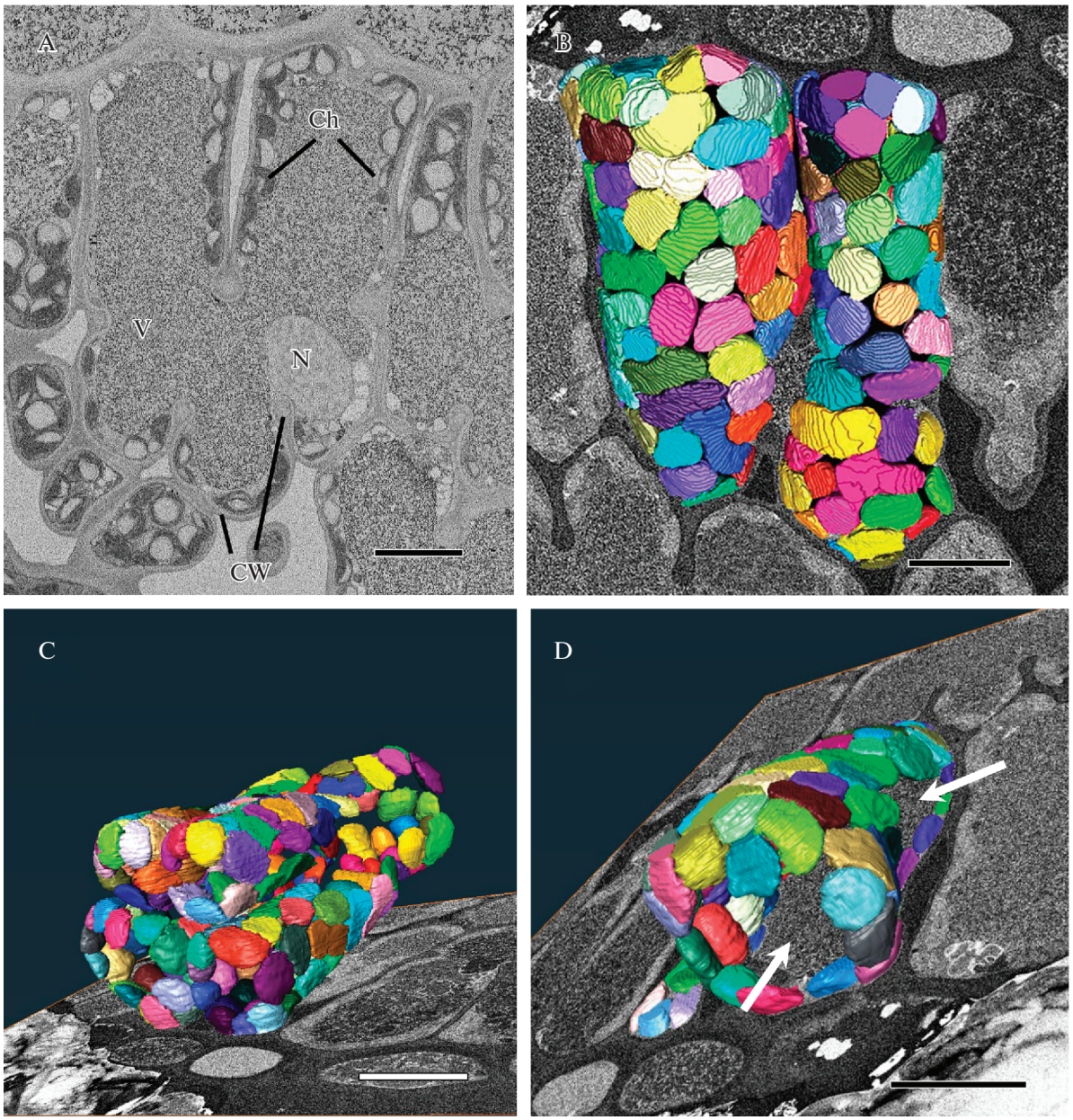

Fig. 4. 3D reconstruction of mesophyll cells of *Pinus sylvestris* during vegetation (May–October) period. A. Cross section in the midplane of the cell. B–D. 3D reconstruction of chloroplasts located along the cell wall showing "summer" positioning (cell wall is not presented on the reconstruction), view from the lateral side of the cell (B) and view from the hypodermis (C) showing absence of chloroplasts along the adjacent cell walls (arrows) (D). Ch – chloroplasts; CW – cell wall; N – nucleus; V – vacuole. Scales: A–D, 10 μm.

the central vacuole decreases in size with increase of cytoplasm volume (Fig. 3, *B*, 5, *A*). The cisterns of rough endoplasmic reticulum disappear, but tubular elements of smooth endoplasmic reticulum appear. The number and size of lipid droplets located in the cytosol along the plasma membrane and in the vacuole increase (not shown). The general topography of organelles in the cell changes; they are randomly grouped in folds formed by cell wall invaginations (lobes) (Fig. 3, *B*, 5, *A*). Chloroplasts are usually clumped close to each other contacting by envelopes. The volume of chloroplasts increase due to the increase of stroma. The thylakoid system maintains its integrity while it is reorganized with drastic reduction in the size of grana up to 2–3 thylakoids per grana (Fig. 3, *F*). Analysis of serial sections showed that doubled thylakoids have a large diameter (often extending through the entire chloroplast) and can be recognized as a round plates lying parallel to each other. Starch disappears.

On 2D sections, the chloroplasts are swollen with slightly undulated envelope. Some chloroplasts have thylakoid-free protrusions filled with stroma containing plastoglobuli. Roundish bodies, comparable in size to mitochondria and surrounded by a double-membrane envelope, are cross sections of these protrusions. 3D reconstructions confirm that most chloroplasts have extremely irregular shape with short (up to 3 μm) protrusions measuring 0.3–0.6 μm in diameter (Fig. 5, *B–D*). Outgrowths have similar diameter and usually tightly contacts with neighboring chloroplasts. Additionally, 3D view shows that mitochondria are not associated with individual chloroplasts and grouped together near the chloroplast conglomerates or in chloroplasts-free areas of cells (often near the nucleus).

In April, the restoration of the summer structure of mesophyll cells begins and the cells acquire a structure characteristic to the growing season to the end of May.

Indoors, no reorganization of mesophyll cell ultrastructure is detected. The general topography of the organelles in the cell remains unchanged: chloroplasts do not move into the folds formed by the outgrowths of the cell wall (Fig. 3, *D*). The endoplasmic reticulum is of a granular type. In contrast to natural conditions, the granal structure of chloroplasts remains practically unchanged, and starch grains do not disappear completely (Fig. 3, *E*).

DISCUSSION

Induction of seasonal structural changes

A comparative analysis of mesophyll cell structure in pine trees growing out- and indoors showed that low temperatures stimulate structural reorganization during autumn cold acclimation. It is low temperature that triggers a decrease in the volume of central vacuole, the development of tubular smooth endoplasmic reticulum, the reorganization of chloroplast thylakoid system, and the relocation of chloroplasts into the cell lobes. All changes in the structure are reversible and the summer structure is restored by the next growing season together with the increase of outdoor temperature. Using different experimental models, the contribution of temperatures to structural reorganization was shown for *Taxus cuspidata* mesophyll cells (Miroslavov, Koteyeva, 2002; Tanaka, 2007).

Low temperatures and short photoperiod with light quality shift are the main environmental signals that induce cold acclimation and cold hardiness including a range of physiological, structural and molecular responses (Öquist, Huner, 2003; Welling, Palva, 2006; Chang et al., 2021). However, cold acclimation events vary in timing, suggesting different contributions of climatic factors. It is believed that the short day characteristic to early autumn leads to cessation of growth and induces an initial dormancy, which is controlled by internal factors (Lloyd et al., 1996; Lee et al., 2017). The annual regularity in the day length change makes photoperiod the most reliable for initiating dormancy among the abiotic factors. In contrast, downregulation of photosynthesis, characteristic of evergreen trees, occurs later in autumn and is caused primarily by low temperatures. It was shown that onset of low temperatures triggers the decrease of the fluidity of the thylakoid membrane, inactivation of PSII reaction centers, reorganization of light harvesting complexes, inhibition of the regeneration of ribulose bisphosphate and decreasing the efficiency of Rubisco carboxylation (Ottander et al., 1995; Vogg et al., 1998; Savitch et al., 2002; Crosatti et al., 2013; Brunkard et al., 2015; Fréchette et al., 2016). Here, we show that low temperatures induce structural reorganization of chloroplasts that are closely associated with decrease of photosynthetic activity and photoprotection.

Of course, the photosynthetic apparatus responds to seasonal changes in light intensity and decrease in day length (Fréchette et al., 2016). In particular, the pigment system is reorganized under short day (Vogg et al., 1998). However, retained chlorophyll captures light throughout the winter due to the structural rearrangements that increase the assimilative capacity of each photosynthetic unit (Vogg et al., 1998; Ensminger et al., 2006). The photosynthetic capacity is maintained even under low temperatures (Vogg et al., 1998). Maintaining the functioning of the energy donor tissues and continue photosynthesis to maximize the carbon gain over the longer period (as long as the temperature is favorable) seems to be beneficial for evergreen species in temperate zone.

Spatial structural organization of mesophyll cells and cold acclimation

Significant inaccuracy in 2D studies causes misunderstanding of organelle arrangement, especially when irregularly shaped chloroplasts overlap during

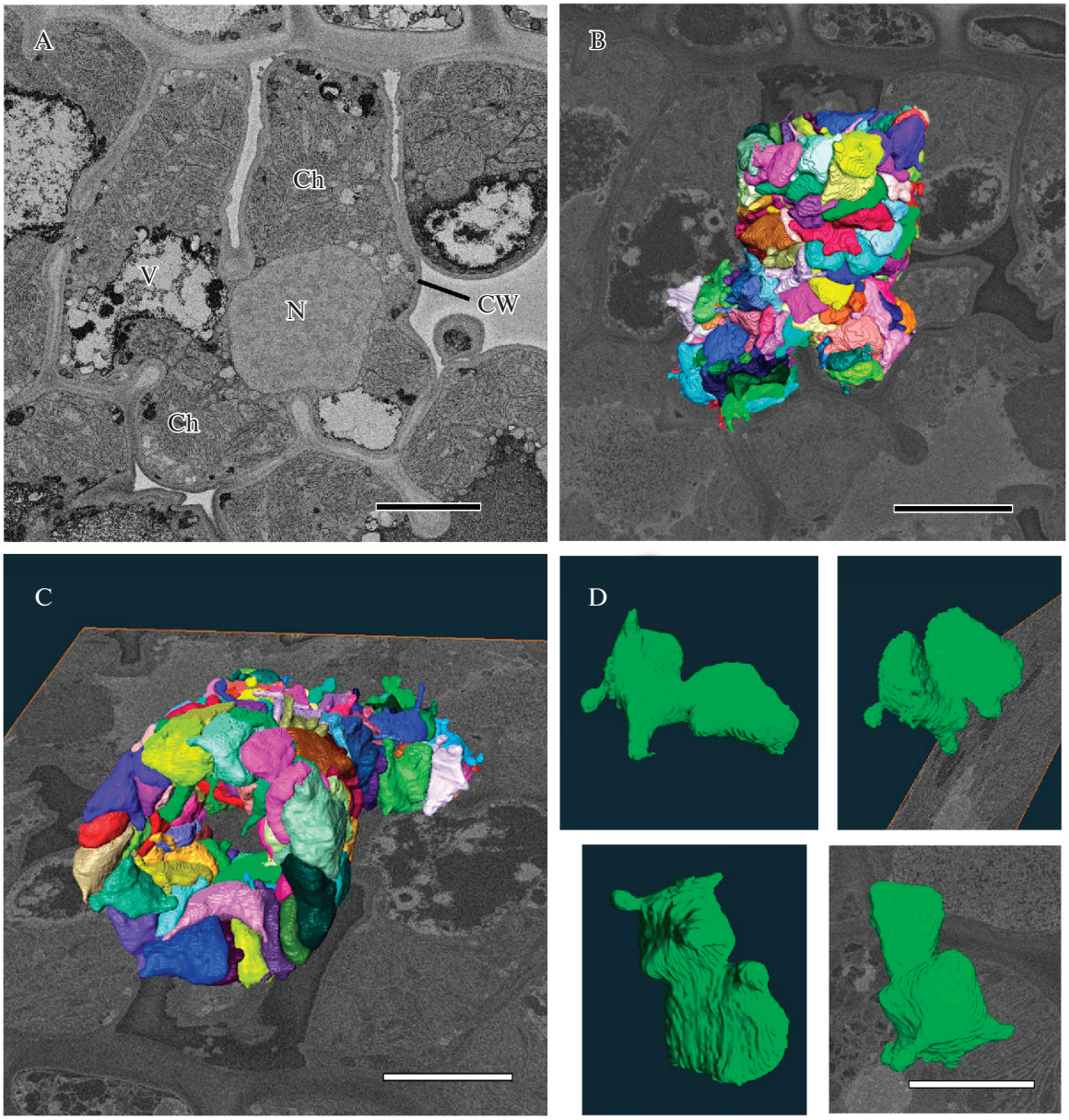

Fig. 5. 3D reconstruction of chloroplasts in mesophyll cells of *Pinus sylvestris* during winter (November–April). A. Cross section in the midplane of the cell. B, C. 3D reconstruction of chloroplasts grouped in one of the mesophyll cell lobe (cell wall is not presented on the reconstruction), view from the lateral side of the cell (B) and view from the hypodermis (C). D. 3D reconstruction of a single chloroplast viewed from different sides showing irregular shape and several protrusions. Ch – chloroplasts; CW – cell wall; N – nucleus; V – vacuole. Scales: A–C, 10 μm; D, 5 μm.

aggregations (Yamakawa et al., 2023) or there is special selective pattern of cellular organization (Ovsyannikov, Koteyeva, 2020). For example, in mesophyll cells of *Picea* species chloroplasts are located along two opposite radial cell walls facing the airspaces and are almost absent on the lateral sides. This mesophyll architecture results in the visual appearance of only a few chloroplasts in 2D cross section. Study of seasonal changes in mesophyll on 2D plane showed that chloroplasts are grouped together during winter (Senser et al., 1975; Soikkeli, 1978). However, 3D reconstruction using light microscopic (LM) serial sections revealed more complex pattern of mesophyll winter structure in *Picea* species (Ovsyannikov, Koteyeva, 2020). The chloroplasts during cold acclimation moved towards the newly formed cytoplasmic strand that penetrated through the central vacuole in *P. pungens* or was attached to the radial cell wall in *P. obovata*, connecting two opposite sides of the cell. 3D view allowed also to reveal multiple vesicles involved in strand construction.

However, the cellular machinery of targeted chloroplast movement is still unknown.

To overcome the limitations of 2D TEM imaging, we applied SBF SEM techniques for 3D reconstruction of the pine mesophyll cell. In contrast to serial LM sections, SBF SEM allows to reveal intracellular organization at the ultrastructural level. Compared to basic TEM, sample preparation protocol for the SBF SEM includes additional heavy metal impregnation to increase the contrast of cell components. Conifers are considered difficult for sample preparation, even for TEM, due to high levels of tannins and thick cell walls (Soikkeli, 1980; Ebel et al., 1990). Protocol for the SBF SEM used in current study showed excellent conservation of the intracellular structure of pine mesophyll.

In general chloroplasts have a conservative lens-shaped shape, which is the most effective for light absorption, and this is exactly the shape that is characteristic of pine mesophyll in summer. 3D reconstructions of chloroplasts using SBF-SEM revealed for the first time the complex shape with outgrowths of chloroplast envelope during winter time. Irregular plastid shape was described previously for the leucoplasts and was related to the synthesis and transport of secondary metabolites (Muravnik, 2021). It was suggested that the increase of surface area facilitates transmembrane exchange through the plastid envelope. For chloroplasts, as for most other types of plastids, dynamic stroma-filled tubular protrusions have been described (Hanson, Conklin, 2020). Chloroplast protrusions called stromules vary in length (up to 20 μm) and width (0.3–0.8 μm), and change their frequency in response to internal (Brunkard et al., 2015) and external signals including temperature (Holzinger et al., 2007). The exact function of stromules is unclear but it is suggested that they may be involved in transmitting signals from the chloroplast to other subcellular compartments (Brunkard et al., 2015). Since stromules were defined as stroma-filled tubes less than 0.8 μm in diameter to distinguish them from irregularly shaped plastids (Köhler, Hanson, 2000), chloroplast protrusions in *P. sylvestris* during the winter can be identified as stromules.

There are several reports of stromule involvement in chloroplast clustering around the nucleus, where chloroplast movement is directed by stromule/microtubule interactions (Hanson, Conklin, 2020). In the present study, chloroplast movement is accompanied by a change in shape, but multiple protrusions develop only after chloroplast aggregation is complete, and they have close contacts with neighboring chloroplasts, anchoring them to each other. It has also been shown in tobacco that stromules do not affect plastid motility (Kwok, Hanson, 2003). Although our results suggest the involvement of stromules in chloroplast interactions but not in chloroplast movement, further research is needed on the function of stromules in cold acclimation.

The architecture of thylakoid membranes is a highly dynamic system that quickly responds to changes in the environment, especially the light intensity (Kirchhoff, 2019). The combination of low temperature and high irradiation causes photoinhibition leading to the damage of photosynthetic machinery in winter (Öquist, Huner, 2003). Reorganization of thylakoid system with drastic decrease of the number of appressed membranes in grana found in pine chloroplasts during winter is related to the photoprotective strategy. Unstacking of granal thylakoids reduces photosystem II (PSII) that is located in grana, is more sensitive to photodamage (Andersson, Anderson, 1980) and facilitates protein turnover and photoprotective thermal dissipation (Öquist, Huner, 2003; Demmig-Adams et al., 2015). However, remaining PSIIs are photochemically active during the winter (Ottander et al., 1995) and can also dissipate absorbed energy, which may be important during unexpected winter thawing (Öquist, Huner, 2003). Thus, reorganization of chloroplast ultrastructure contributes to the protection and to the maintenance of the functional integrity of photosynthetic machinery during winter.

Chloroplast movement is a well known light-dependent reaction that provides more effective light absorption or protection from photodamage (Wada et al., 2003). Temperature-dependent chloroplast movement differs by the intracellular pattern of organelle arrangement during movement and after aggregation, as well as by reduction of leaf photosynthetic capacity (Ovsyannikov, Koteyeva, 2020). Seasonal change of chloroplast position has been reported for evergreen conifers suggesting similar photoprotective strategy of winter survival (Chabot, Chabot, 1975; Senser et al., 1975; Soikkeli, 1978; Martin, Oquist, 1979; Soikkeli, 1980; Koteyeva, 2002; Miroslavov, Koteyeva, 2002; Tanaka, 2007).

Using *chloroplast unusual positioning 1* (*chup1*) mutant of *Arabidopsis thaliana* it was shown that chloroplast positioning is crucial for photosynthetic and metabolic acclimation to low temperature during cold acclimation (Kitashova et al., 2021). It was suggested that chloroplast grouping minimizes photooxidation by limiting light energy absorption (Tanaka, 2007). However, the precise physiological role of chloroplast aggregation in winter remains unclear and requires further experimental studies.

In summary, we provide the first 3D reconstruction of conifer mesophyll cell to show chloroplast shape and relocation during change of seasons using SBF SEM. We have shown that aggregation of chloroplasts is accompanied by the formation of protrusions of envelopes (stromules) involved in chloroplast interactions and anchoring them to each other. We definitely confirm that low temperature, rather than short photoperiod, is the trigger for chloroplast relocation and shape modification in mesophyll cells of *P. sylvestris*.

ACKNOWLEDGMENTS

Research was supported by the Russian Science Foundation (grant No. 22-24-01124). We are grateful to the Core Facility Center "Cell and Molecular Technologies in Plant Science" of Komarov Botanical Institute and Centre for Molecular and Cell Technologies of the Research Park of St. Petersburg State University for use of their facilities.

REFERENCES

Andersson B., Anderson J.M. 1980. Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. – Biochimica et Biophysica Acta (BBA). – Bioenergetics. 593 (2): 427–440. https://doi.org/10.1016/0005-2728(80)90078-X

Arora R., Taulavuori K. 2016. Increased risk of freeze damage in woody perennials VIS-À-VIS climate change: Importance of deacclimation and dormancy response. – Frontiers in Environmental Science. 4 (44). https://doi.org/10.3389/fenvs.2016.00044

Bigras F., Ryyppö A., Lindström A., Stattin E. 2001. Cold acclimation and deacclimation of shoots and roots of conifer seedlings. – In: Conifer Cold Hardiness. Tree Physiology. P. 57–88. https://doi.org/10.1007/978-94-015-9650-3_3

Brunkard J.O., Runkel A.M., Zambryski P.C. 2015. Chloroplasts extend stromules independently and in response to internal redox signals. – Proc. Natl. Acad. Sci. 112 (32): 10044–10049. https://doi.org/10.1073/pnas.1511570112

Chabot J., Chabot B. 1975. Developmental and seasonal patterns of mesophyll ultrastructure in *Abies balsamea*. – Can. J. Bot. 53 (3): 259–304. https://doi.org/10.1139/b75-037

Chang C.Y.-Y., Bräutigam K., Hüner N.P.A., Ensminger I. 2021. Champions of winter survival: cold acclimation and molecular regulation of cold hardiness in evergreen conifers. – New Phytol. 229 (2): 675–691. https://doi.org/10.1111/nph.16904

Crosatti C., Rizza F., Badeck F.W., Mazzucotelli E., Cattivelli L. 2013. Harden the chloroplast to protect the plant. – Physiol. Plant. 147 (1): 55–63. https://doi.org/10.1111/j.1399-3054.2012.01689.x

Demmig-Adams B., Cohu C.M., Muller O., Adams W.W. 2012. Modulation of photosynthetic energy conversion efficiency in nature: from seconds to seasons. – Photosynth. Res. 113 (1): 75–88. https://doi.org/10.1007/s11120-012-9761-6

Demmig-Adams B., Muller O., Stewart J.J., Cohu C.M., Adams W.W. 2015. Chloroplast thylakoid structure in evergreen leaves employing strong thermal energy dissipation. – Journal of Photochemistry and Photobiology B: Biology. 152: 357–366. https://doi.org/10.1016/j.jphotobiol.2015.03.014

Duman J.G., Wisniewski M.J. 2014. The use of antifreeze proteins for frost protection in sensitive crop plants. – Environ. Exp. Bot. 106: 60–69. https://doi.org/10.1016/j.envexpbot.2014.01.001

Earles J.M., Buckley T.N., Brodersen C.R., Busch F.A., Cano F.J., Choat B., Evans J.R., Farquhar G.D., Harwood R., Huynh M., John G.P., Miller M.L., Rockwell F.E., Sack L., Scoffoni C., Struik P.C., Wu A., Yin X., Barbour M.M. 2019. Embracing 3D complexity in leaf carbon–water exchange. – Trends Plant Sci. 24 (1): 15–24. https://doi.org/10.1016/j.tplants.2018.09.005

Ebel B., Hamelmann U., Nieß C. 1990. A rapid preparation method for ultrastructural investigations of conifer needles. – J. Microsc. 160 (1): 67–74. https://doi.org/10.1111/j.1365-2818.1990.tb03048.x

Ensminger I., Busch F., Huner N.P.A. 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. – Physiol. Plant. 126 (1): 28–44. https://doi.org/10.1111/j.1399-3054.2006.00627.x

Ensminger I., Berninger F., Streb P. 2012. Response of photosynthesis to low temperature. – In: Terrestrial Photosynthesis in a Changing Environment: A Molecular, Physiological, and Ecological Approach. Cambridge. P. 272–289. https://doi.org/10.1017/CBO9781139051477.022

Evans J.R. 2021. Mesophyll conductance: walls, membranes and spatial complexity. – New Phytol. 229 (4): 1864–1876. https://doi.org/10.1111/nph.16968

Fréchette E., Chang C.Y., Ensminger I. 2016. Photoperiod and temperature constraints on the relationship

between the photochemical reflectance index and the light use efficiency of photosynthesis in Pinus strobus. – Tree Physiol. 36 (3): 311–324. https://doi.org/10.1093/treephys/tpv143

Gusta L.V., Wisniewski M. 2013. Understanding plant cold hardiness: an opinion. – Physiologia Plantarum. 147 (1): 4–14. https://doi.org/10.1111/j.1399-3054.2012.01611.x

Guy C.L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. – Annu. Rev. Plant Physiol. Plant Mol. Biol. 41: 187–223. https://doi.org/10.1146/annurev.pp.41.060190.001155

Hanson M.R., Conklin P.L. 2020. Stromules, functional extensions of plastids within the plant cell. – Curr. Opin. Plant Biol. 58: 25–32. https://doi.org/10.1016/j.pbi.2020.10.005

Holzinger A., Buchner O., Lütz C., Hanson M.R. 2007. Temperature-sensitive formation of chloroplast protrusions and stromules in mesophyll cells of *Arabidopsis thaliana*. – Protoplasma. 230 (1): 23–30. https://doi.org/10.1007/s00709-006-0222-y

Howe G.T., Davis J., Jeknic Z., Chen T.H.H., Frewen B., Bradshaw H.D.J., Saruul P. 1997. Physiological and genetic approaches to studying endodormancy-related traits in *Populus*. – Hortscience. 34 (7): 1174–1184. https://doi.org/10.21273/HORTSCI.34.7.1174b

Jian L.C., Li J.H., Li P.H. 2000. Seasonal alteration in amount of Ca^{2+} in apical bud cells of mulberry (*Morus bombciz* Koidz): an electron microscopy cytochemical study. – Tree Physiol. 20: 623–628. https://doi.org/10.1093/treephys/20.9.623

Jian L.C., Li P.H., Sun L.H., Chen T.H.H. 1997. Alterations in ultrastructure and subcellular localization of Ca^{2+} in poplar apical bud cells during the induction of dormancy. – J. Exp. Bot. 48 (311): 1195–1207. https://doi.org/10.1093/jxb/48.6.1195

Jokela A., Sarjala T., Huttunen S. 1998. The structure and hardening status of Scots pine needles at different potassium availability levels. – Trees – Structure and Function. 12: 490–498. https://doi.org/10.1007/s004680050179

Kagawa T., Wada M. 1999. Chloroplast-avoidance response induced by high-fluence blue light in prothallial cells of the fern *Adiantum capillus-veneris* as analyzed by microbeam irradiation. – Plant Physiol. 119 (3): 917–924. https://doi.org/10.1104/pp.119.3.917

Kirchhoff H. 2019. Chloroplast ultrastructure in plants. – New Phytol. 223 (2): 565–574. https://doi.org/10.1111/nph.15730

Kitashova A., Schneider K., Fürtauer L., Schröder L., Scheibenbogen T., Fürtauer S., Nägele T. 2021. Impaired chloroplast positioning affects photosynthetic capacity and regulation of the central carbohydrate metabolism during cold acclimation. – Photosynth. Res. 147 (1): 49–60. https://doi.org/10.1007/s11120-020-00795-y

Köhler R.H., Hanson M.R. 2000. Plastid tubules of higher plants are tissue-specific and developmentally regulated. – J. Cell Sci. 113 (Pt1): 81–89. https://doi.org/10.1242/jcs.113.1.81

Koteyeva N.K. 2002. Patterns of seasonal rhythmics in ultrastructure of shoot apical meristem and mesophyll cells in *Pinus sylvestris* (Pinaceae). – Bot. Zhurn. 87 (11): 50–60.

Kwok E.Y., Hanson M.R. 2003. Microfilaments and microtubules control the morphology and movement of non-green plastids and stromules in *Nicotiana tabacum*. – Plant J. 35 (1): 16–26. https://doi.org/10.1046/j.1365-313X.2003.01777.x

Lee Y., Karunakaran C., Lahlali R., Liu X., Tanino K.K., Olsen J.E. 2017. Photoperiodic regulation of growth-dormancy cycling through induction of multiple bud–shoot barriers preventing water transport into the winter buds of Norway spruce. – Frontiers in Plant Science. 8. https://doi.org/10.3389/fpls.2017.02109

Li C., Junttila O., Palva E.T. 2004. Environmental regulation and physiological basis of freezing tolerance in woody plants. – Acta Physiol. Plant. 26 (2): 213–222. https://doi.org/10.1007/s11738-004-0010-2

Lloyd A.D., Mellerowicz E.J., Riding R.T., Little C.H.A. 1996. Changes in nuclear genome size and relative ribosomal RNA gene content in cambial region cells of *Abies balsamea* shoots during the development of dormancy. – Can. J. Bot. 74 (2): 290–298. https://doi.org/10.1139/b96-035

Martin B., Oquist G. 1979. Seasonal and experimentally induced changes in the ultrastructure of chloroplasts of *Pinus silvestris*. – Physiol. Plant. 46 (1): 42–49. https://doi.org/10.1111/j.1399-3054.1979.tb03183.x

Maurya J.P., Bhalerao R.P. 2017. Photoperiod- and temperature-mediated control of growth cessation and dormancy in trees: a molecular perspective. – Ann. Bot. 120 (3): 351–360. https://doi.org/10.1093/aob/mcx061

Miroslavov E., Koteyeva N. 2002. Characteristic of seasonal dynamics of mesophyll cell ultrastructure in *Taxus cuspidata* (Taxaceae) grown out- and indoors. – Bot. Zhurn. 87: 40–49.

Muravnik L.E. 2021. The structural seculiarities of the seaf slandular srichomes: a review. – In: Plant Cell and Tissue Differentiation and Secondary Metabolites: Fundamentals and Applications. Cham. P. 63–97. https://doi.org/10.1007/978-3-030-30185-9_3

Öquist G., Huner N.P.A. 2003. Photosynthesis of overwintering evergreen plants. – Annu. Rev. Plant Biol. 54 (1): 329–355. https://doi.org/10.1146/annurev.arplant.54.072402.115741

Ottander C., Campbell D., Öquist G. 1995. Seasonal changes in photosystem II organisation and pigment composition in *Pinus sylvestris*. – Planta. 197 (1): 176–183. https://doi.org/10.1007/BF0023995

Ovsyannikov A.Y., Koteyeva N.K. 2020. Seasonal movement of chloroplasts in mesophyll cells of two *Picea* species. – Protoplasma. 257 (1): 183–195. https://doi.org/10.1007/s00709-019-01427-6

Pomeroy M.K., Siminovitch D. 1971. Seasonal cytological changes in secondary phloem parenchyma cells in *Robinia pseudoacacia* in relation to cold hardiness. – Can. J. Bot. 49 (5): 787–795. https://doi.org/10.1139/b71-118

Sagisaka S., Kuroda H. 1991. Changes in the ultrastructure of plastids after breaking of dormancy in perennials. – Agric. Biol. Chem. 55 (6): 1671–1673. https://doi.org/10.1080/00021369.1991.10870811

Sauter J.J., Wisniewski M.E., Witt W. 1996. Interrelationships between ultrastructure, sugar levels, and frost hardiness of ray parenchyma cells during frost acclimation and deacclimation in poplar (*Populus* x *canadensis* Moench [Robusta]) wood. – J. Plant Physiol. 149: 451–461. https://doi.org/10.1016/S0176-1617(00)80239-4

Savitch L.V., Leonardos E.D., Krol M., Jansson S., Grodzinski B., Huner N.P.A., Öquist G. 2002. Two different strategies for light utilization in photosynthesis in relation to growth and cold acclimation. – Plant Cell Environ. 25 (6): 761–771. https://doi.org/10.1046/j.1365-3040.2002.00861.x

Senser M., Schötz F., Beck E. 1975. Seasonal changes in structure and function of spruce chloroplasts. – Planta. 126 (1): 1–10. https://doi.org/10.1007/BF00389354

Soikkeli S. 1978. Seasonal changes in mesophyll ultrastructure of needles of Norway spruce (*Picea abies*). – Can. J. Bot. 56 (16): 1932–1940. https://doi.org/10.1139/b78-231

Soikkeli S. 1980. Ultrastructure of the mesophyll in scots pine and norway spruce seasonal variation and molarity of the fixative buffer. – Protoplasma. 103 (3): 241–252. https://doi.org/10.1007/BF01276270

Steponkus P.L. 1984. Role of plasma membrane in freezing injury and cold acclimation. – Annu. Rev. Plant Physiol. 35: 543–584. https://doi.org/10.1146/annurev.pp.35.060184.002551

Tanaka A. 2007. Photosynthetic activity in winter needles of the evergreen tree *Taxus cuspidata* at low temperatures. – Tree Physiol. 27 (5): 641–648. https://doi.org/10.1093/treephys/27.5.641

Thomashow M.F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. – Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 571–599. https://doi.org/10.1146/annurev.arplant.50.1.571

Uemura M., Tominaga Y., Nakagawara C., Shigematsu S., Minami A., Kawamura Y. 2006. Responses of the plasma membrane to low temperatures. – Physiol. Plant. 126 (1): 81–89. https://doi.org/10.1111/j.1399-3054.2005.00594.x

Vogg G., Heim R., Gotschy B., Beck E., Hansen J. 1998. Frost hardening and photosynthetic performance of Scots pine (*Pinus sylvestris* L.). II. Seasonal changes in the fluidity of thylakoid membranes. – Planta. 204: 201–206. https://doi.org/10.1007/s004250050246

Wada M., Kagawa T., Sato Y. 2003. Chloroplast movement. – Annu. Rev. Plant Biol. 54: 455–468. https://doi.org/10.1146/annurev.arplant.54.031902.135023

Welling A., Palva E.T. 2006. Molecular control of cold acclimation in trees. – Physiol. Plant. 127 (2): 167–181. https://doi.org/10.1111/j.1399-3054.2006.00672.x

Wiebe H.H., Al-Saadi H.A. 1976. The role of invaginations in armed mesophyll cells of pine needles. – New Phytol. 77 (3): 773–775. https://doi.org/10.1111/j.1469-8137.1976.tb04673.x

Wisniewski M., Ashworth E.N. 1986. A comparison of seasonal ultrastructural changes in stem tissues of peach (*Prunus persica*) that exhibit contrasting mechanisms of cold hardiness. – Botanical Gazette. 147 (4): 407–417. https://doi.org/10.1086/337608

Wisniewski M., Nassuth A., Arora R. 2018. Cold hardiness in trees: A mini-review. – Frontiers in Plant Science. 9 (1394). https://doi.org/10.3389/fpls.2018.01394

Xin Z., Browse J. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. – Plant Cell Environ. 23 (9): 893–902. https://doi.org/10.1046/j.1365-3040.2000.00611.x

Yamakawa S., Kato Y., Taniguchi M., Oi T. 2023. Intracellular positioning of mesophyll chloroplasts following to aggregative movement in *Setaria viridis* analysed three-dimensionally with a confocal laser scanning microscope. – Flora. 306: 152364. https://doi.org/10.1016/j.flora.2023.152364

Yamane K., Oi T., Taniguchi M. 2020. Three-dimensional analysis of chloroplast protrusion formed under osmotic stress by polyethylene glycol in rice leaves. – Plant Prod. Sci. 23 (2): 160–171. https://doi.org/10.1080/1343943X.2019.1709513

ДВИЖЕНИЕ ХЛОРОПЛАСТОВ В КЛЕТКАХ МЕЗОФИЛЛА *PINUS SYLVESTRIS* (PINACEAE) ИНИЦИИРУЕТСЯ НИЗКОЙ ТЕМПЕРАТУРОЙ: SBF SEM 3D-РЕКОНСТРУКЦИЯ

Н. К. Котеева[1,*], А. Н. Иванова[1,2], Т. А. Борисенко[1,2], М. С. Тарасова[1,2], О. Е. Миргородская[1], Е. В. Вознесенская[1]

[1]Ботанический институт им. В.Л. Комарова РАН
ул. Проф. Попова, 2, Санкт-Петербург, 197376, Россия
[2]Санкт-Петербургский государственный университет
Университетская наб., 7–9, Санкт-Петербург, 199034, Россия
**e-mail: nkoteyeva@binran.ru*

Вечнозеленые виды умеренной зоны приспосабливаются к сезонному климату за счет реорганизации структуры клеток мезофилла, включая передвижение хлоропластов как фотозащитную реакцию. Однако фактор, вызывающий структурные изменения, до сих пор остается неизученным. С целью выявления особенностей расположения хлоропластов в течение годового цикла и влияния температуры на их движение было изучено строение клеток мезофилла *Pinus sylvestris*, выращиваемых в открытом и закрытом грунте. Серийная блочная сканирующая электронная микроскопия (SBF SEM) использовалась для трехмерной реконструкции клеток мезофилла, чтобы показать пространственное положение и изменение формы хлоропластов. Выявлено, что в течение вегетационного периода хлоропласты имеют хорошо развитую тилакоидную систему, располагаются вдоль клеточной стенки и занимают преимущественно ту часть клеточной стенки, которая обращена к межклетникам. Движение хлоропластов начинается в октябре-ноябре, а зимой они группируются в складках клеток мезофилла. В это время тилакоидная система перестраивается и состоит преимущественно из длинных удвоенных тилакоидов и мелких гран. 3D-реконструкция показывает, что хлоропласты ориентированы в случайном порядке, увеличивают объем стромы и образуют множественные выпячивания, заполненные стромой, которые можно распознать как стромулы. У растений, выращенных в условиях оранжереи, сезонная реорганизация ультраструктуры мезофилла не происходит, что позволяет предположить, что именно низкие температуры, но не фотопериод и качество света индуцируют сезонное движение хлоропластов в мезофилле *P. sylvestris*. Мы подтверждаем, что 3D-реконструкция является мощным инструментом в изучении изменений положения и формы хлоропластов, вызванных низкой температурой.

Ключевые слова: *Pinus sylvestris*, мезофилл, движение хлоропластов, сезонные изменения, низкие температуры, 3D-реконструкция