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BRIEF COMMUNICATION

Chloroplast ultrastructure of *Hypericum perforatum* plants regenerated *in vitro* after cryopreservation

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Abstract

The ultrastructure of leaf mesophyll cells of *in vitro* cultured *Hypericum perforatum* L. plants regenerated after cryopreservation was studied. Electron microscopy analysis revealed that the chloroplasts in plants pretreated with abscisic acid and regenerated after cryopreservation were round, with increased amount of starch, rather small volume of the thylakoid system, and destroyed envelope. Plants pretreated with 0.3 M mannitol and cooled at rates of 0.1 or 0.3 °C min^{-1} possessed chloroplasts with high starch content that resulted in a reduction of a membrane system. However, the pretreatment with 0.3 M mannitol and cooling at a rate of 0.2 °C min^{-1} was the best as chloroplast ultrastructure resembled the controls regenerated without cryopreservation.

Additional key words: ABA, mannitol, medicinal plant, micropropagation, St. John's wort.

Cryo-procedures are innovative technique which allows preservation of plant material with valuable qualities at ultra-low temperatures for theoretically unlimited period of time (Ozudogru *et al.* 2010). The key factor for successful cryopreservation is minimizing damages of the cell ultrastructure (Xu *et al*. 2006, Fki *et al*. 2013). Physiological, biochemical, and genetic properties of *Hypericum perforatum* L. plants regenerated after cryopreservation were tested previously (Urbanová *et al*. 2002, 2006, 2010, Skyba *et al*. 2010) but the structural organization of the plastid apparatus has not been thoroughly examined. According to some studies, a common reaction to low temperatures is a collapse of the photosynthetic apparatus (Danova *et al*. 2009, Ganeva *et al.* 2009). Studies of the leaf structure of *in vitro* cultured plants revealed that the examination on subcellular level is very important for evaluation of plant regeneration potential (Stefanova 2011, Stoyanova-Koleva *et al*. 2012).

The aim of this study was to establish the chloroplast ultrastructure of *H. perforatum* plants propagated *in vitro* without and after cryopreservation and to analyze the alterations of some variables during precryogenic/cryogenic treatments. The obtained results would be very useful in elaborating the protocol for successful cryopreservation.

The photosynthetic apparatus of *in vitro* cultured *Hypericum perforatum* L. plants regenerated after cryopreservation was investigated by transmission electron microscopy (TEM). After 14 - 21 d of *in vitro* culture on standard Murashige and Skoog (1962; MS) medium containing vitamins according to Gamborg *et al*. (1968), 30 g dm^{-3} sucrose, 2 mg dm^{-3} glycine, and 100 mg dm^{-3} myo-inositol (pH 5.6), 140 isolated shoot tips were pretreated with 0.076 μM abscisic acid (ABA) for 10 d or 0.3 M mannitol for 7 d. Cryopreservation was performed according to Skyba *et al*. (2011). The shoot tips were subjected to controlled cooling at different rates: 0.3, 0.2, and 0.1 °C min^{-1}. Survival was determined as percentage of explants capable of regrowth and regeneration from the initial number of frozen tips. The cryopreserved shoot tips regenerated on the MS medium supplemented with 0.5 mg dm^{-3} benzyladenine (BA). Shoot tips which did not undergo cryo-procedure and regenerated on MS medium without growth regulators served as control plants.

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Abbreviations: ABA - abscisic acid; BA - benzyladenine.
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Electron microscopy was performed approximately 2 months after thawing and regeneration. Leaf segments (1 mm^2) were cut from the middle part of the leaves for TEM observation. These segments were then fixed in 3 % (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C for 12 h and postfixed in 2 % (m/v) OsO_4 in the same buffer at room temperature for 4 h. After dehydration by increasing concentrations of ethyl-alcohol (from 25 to 100 %), the material was embedded in *Durcupan* (*Fluka*, Buchs, Switzerland). Ultra-thin sections were cut from palisade parenchyma using a *Reichert* (Wien, Austria) ultra-microtome, contrasted with lead citrate solution according to the method of Reynolds (1963), and examined using electron microscope (*JEOL 1200EX*, Tokyo, Japan).

Mesophyll cells of the control plants contained large vacuoles and peripherally situated protoplasmic compartments. The plastid apparatus comprised chloroplasts with maximum volume of the internal membrane system (Fig. 1*A*). The chloroplast thylakoid system consisted of many grana which differed in height (5 - 10 to 25 - 30 thylakoids) and were mostly connected with stromal thylakoids. Peculiar feature of these chloroplasts was lack of starch and plastoglobuli. TEM analysis of the photosynthetic tissue indicated very well developed structural contact between chloroplasts and mitochondria

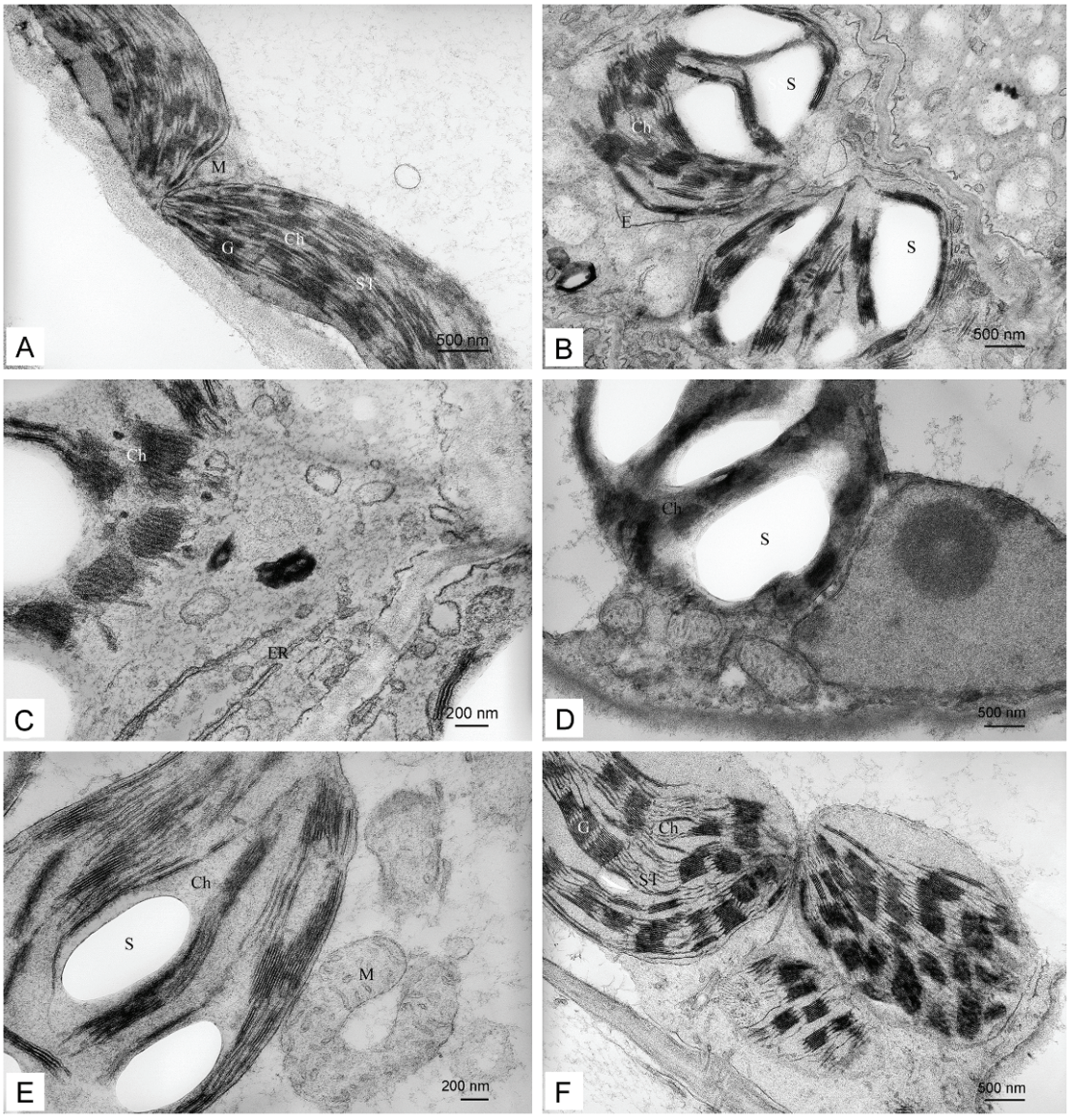

Fig. 1. Chloroplast ultrastructure of control plants (*A*), ABA pretreated plants and regenerated after cryopreservation (*B*, *C*), mannitol pretreated plants cooled at rate 0.1 °C min^{-1} and regenerated after cryopreservation (*D*), mannitol pretreated plants cooled at rate of 0.3 °C min^{-1} and regenerated after cryopreservation (*E*), and mannitol pretreated plants cooled at rate of 0.2 °C min^{-1} and regenerated after cryopreservation (*F*); Ch - chloroplast, ER - endoplasmic reticulum, G - granum, M - mitochondrion, S - starch, ST - stroma thylakoids.

by means of large associative areas. The topography of these compartments and well developed endoplasmic reticulum observed within the cells suggest for an active plastid, intracellular, and intercellular transport. It is well known that the low temperature causes functional changes of the photosynthetic apparatus which results in reduction of the photosynthetic activity. These functional diminutions can be explained by changes in the architecture of the chloroplasts along with structural reorganization of the mitochondria, endoplasmic reticulum, and vacuolar system. The recovery of chloroplast ultrastructure results in reorganization of the thylakoid system, thus affecting development of chloroplast peripheral reticulum and causes deviation in metabolite accumulation (Taylor and Craig 1971, Ristic and Ashworth 1993, Kratsch and Wise 2000, Deryabin *et al.* 2007, Popov e*t al.* 2007). The structure of endoplasmic reticulum is generally considered as susceptible to temperature stress whereas mitochondria and nucleus as more stable (Gamalei *et al.* 1994, Ishikawa 1996, Kratsch and Wise 2000). Many of the ultrastructural symptoms appearing during moderate stress resemble those seen in programmed cell death (Kratsch and Wise 2000). The destructive changes of the chloroplast envelope and protoplasmic membranes can be defined as symptoms of chilling injury. They cause deviations in normal intra- and inter-cellular sugar transport.

In the present experiment, ABA and mannitol were used as low temperature stress protectants to answer the question to what extent the cell compartments are structurally preserved under given conditions of cryopreservation. The plants regenerated after cryopreservation from the ABA-pretreated shoot tips exhibited round shape chloroplasts with reduced volume of the thylakoids but without destructive changes (Fig. 1*B*) and destroyed envelope (Fig. 1C). No destructive alterations in the internal membrane system indicate tolerance to low temperature. The endoplasmic reticulum was characterized by vesiculation and fragmentation. Although the protective role of ABA pretreatment in response of photosynthetic apparatus to water stress was confirmed (Haisel *et al.* 2006), according to some studies, the efficacy of ABA pretreatment depends on its concentration (Dallaire 1994). Plants pretreated with 0.3 M mannitol and cooled at rates of 0.1 and 0.3 °C min^{-1} manifested reduction of the membrane system and high starch content in the chloroplasts (Fig. 1*D*, *E*) in comparison with the control plants. Taylor and Craig (1971) showed that low temperature stress causes grana reduction and increase in stroma volume. However, in the current study, low temperature caused reduction of the whole membrane system and thylakoid membrane destructions (Fig. 1*D*). Such structural changes could block sugar transport leading to the untypically large starch grains occupying almost the entire chloroplast volume. Under stress, the transport of sugars out of chloroplasts is often interrupted leading to accumulation of large amount of starch in the organelles and decrease of sugar synthesis in cytosol (Pshybytko *et al.* 2003). In our study, TEM analysis did not determine thylakoid swelling which has been described as universal symptom of low temperature stress (Kratsch and Wise 2000). We can only presume whether observed chloroplast structure corresponds with the protective role of mannitol. Only pretreatment with 0.3 M mannitol and cooling at the rate of 0.3 °C min^{-1} resulted in reduced volume of the internal membrane system but without thylakoid destruction (Fig. 1*E*) and therefore could indicate protective role of mannitol. Furthermore, the chloroplasts accumulated large amounts of starch. The structure contact between chloroplasts and mitochondria was not disturbed despite the observed atypical mitochondrion shapes. Typical reaction to low temperature-stress is the formation of vesicles in the protoplast and fragmented reticulum (Gamalei *et al.* 1994). Electron microscopy analysis revealed that the plantlets pretreated with 0.3 M mannitol and cooled at the rate of 0.2 °C min^{-1} resembled the control ones in terms of ultrastructure of the photosynthetic apparatus (Fig. 1*F*). The mesophyll cells were characterized by normal structure of the chloroplasts and other organelles. The chloroplasts had typical thylakoid structure and absence of starch in the stroma.

Data obtained allowed us to confirm that pretreatment of *in vitro* cultured plants with 0.3 M mannitol and cooled at the rate of 0.2 °C min^{-1} was the most suitable way to control the degree of temperature stress effects on cell ultrastructure most likely by mechanisms which caused greater stability and by stimulation of recovery.

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